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Breeding in a changing Arctic

Physiology and behaviour of barnacle geese

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Breeding in a changing Arctic

Physiology and behaviour of barnacle geese

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Chapter 1.

General introduction

1.1. Aims and objectives

This PhD thesis combines four years of research and long-term data on Arctic-breeding barnacle geese (*Branta leucopsis*). The overall aim of this PhD was to gain understanding on how barnacle geese are affected by and cope with several changes in their Arctic breeding area. These environmental changes are linked to human-induced changes to a greater or lesser extent. To address this aim, the thesis had four objectives:

- To quantify the effects of nest fleas (*Ceratophyllus vagabundus vagabundus*), a parasite whose role might become more prominent under climate warming in Arctic regions, on the behaviour and reproductive success of Svalbard barnacle geese (**Chapter 2**).
- To investigate the rate of advancement in the timing of reproduction in three Arctic barnacle goose populations (in Russia and Svalbard) with earlier springs and what the possible effects are of climate warming on some aspects of their reproductive success (**Chapter 3**).
- To examine the effects of exposure to contaminants from a historic coal mine area on physiology and behaviour in Svalbard barnacle goslings (**Chapters 4, 5 & 6**).
- To study the potential for behavioural change in Svalbard barnacle geese by investigating individual variation in nest defence behaviour over season and age (**Chapter 7**).

1.2. Research description

I have performed several individual-based and experimental studies to achieve the aforementioned objectives, using the barnacle goose population breeding in Kongsfjorden (Svalbard) as a case-study.

This work is represented in the thesis by six chapters divided into three different parts. All chapters consist of articles which have already been accepted and published in peer-review journals. I have had a major contribution in designing the studies, in the field- and lab-work, in the statistical analyses and the writing of all chapters (detailed author contribution statements can be found at the end of this thesis).

In this introductory chapter (**Chapter 1**), I will first set the stage by giving a short introduction for the general academic audience on environmental change and how populations can deal with changes that exceed the capacity of the mechanisms they have. I will then highlight several important changes in the Arctic environment, with a focus on the terrestrial ecosystem and zoom in on geese. Next, I will detail more about my study system, which is used as a case-study in this thesis: the Kongsfjorden barnacle goose population. At the end of this chapter, I will give the outline for the rest of this thesis.

1.3. Mechanisms by which populations respond to change

Change is a normal part of many ecosystems. Population numbers may go up and down, temperatures may vary from incredibly cold to scorching hot, and precipitation may fluctuate from raging blizzards to periods of drought. Species are resilient and have adaptations that allow them to cope with such changes in their environment. However, they are only able to cope to a certain point; a high speed of change, a decreased predictability and new factors such as contamination and emerging parasites and diseases may exceed the capacity of the mechanisms that species have evolved to cope with changes in their environment (e.g. Chevin, Lande, & Mace, 2010; Davidson et al., 2011; Garant, 2020).

Generally, there are three main

processes through which populations can deal with change (Chevin et al., 2010; Davis, Shaw, & Etterson, 2005; Jump & Peñuelas, 2005). The first one is dispersal or range shift, which allows a population to track its preferred environment in space (Pease, Lande, & Bull, 1989; Thompson & Fronhofer, 2019). However, if a population occupies a habitat from which dispersal is not possible or dispersal is slow relative to the rate of environmental change, individuals have to adapt to the changing local circumstances (Polechová, Barton, & Marion, 2009). A second process is, therefore, the ‘matching’ of the individual’s phenotype to the changing conditions through phenotypic plasticity, i.e. the ability of an individual genotype to generate different phenotypes over different environments (Stearns, 1989). This can typically occur in the short-term (e.g. Nussey, Wilson, & Brommer, 2007). The third process, on the mid- and long-term, is adaptive evolution to the new conditions through natural selection (Garant, 2020; Merilä & Hendry, 2014). The adaptive capacity of a species is strongly linked to these last two processes; species that show low evolutionary potential or decreased plasticity have a lower chance to persist in a changing environment (Lande & Shannon, 1996; Merilä & Hendry, 2014). Empirical field-based research is essential to investigate if and how individuals are affected by changes in their environment and what processes play a role.

1.4. Changes in the Arctic environment

Some relatively recent and upcoming changes in the Arctic can pose challenges for organisms that make use of this region, both resident and migrant species. The main new rapid changes are human-induced such as climate warming (through increased greenhouse gases) and industrial pollution and contamination

(through e.g. mining and shipping) (e.g. Huntington, 2009). Environmental changes are often called stressors (Schulte, 2014), but even though the effects on species might be negative as the word stressor implicitly implies for many people, this does not always need to be the case. An environmental change that is stressful in one situation does not have to be stressful in another (Schulte, 2014). Therefore, in this thesis, I have explicitly chosen to use the more neutral, unambiguous term “change” when I simply refer to changes in the environment. An exception is the use of the words stress and stressors (i.e. the environmental factors that cause stress) in Part II of this thesis, where we explicitly expect that performance or fitness might be reduced because of chronic elevation of glucocorticoids due to trace metal contamination (*sensu* Schulte 2014). Below, I will highlight a few environmental changes in the Arctic that are important in the light of this thesis: parasites, changes in phenology and contamination.

1.4.1. Parasites

One hypothesis for why animals undertake energetically costly migrations from their wintering grounds to the Arctic is the avoidance of disease (Altizer, Bartel, & Han, 2011; Piersma, 1997). The idea is that the Arctic has a relatively low parasite and pathogen abundance in comparison to the low latitudes, which allows animals to allocate more energy to reproduction instead of needing to use this energy for costly immune responses (Piersma, 1997).

Also, their offspring should encounter fewer parasites or pathogens, which may improve their survival. However, it is too simple to say that the Arctic is totally disease-free; we actually have a limited understanding of pathogen diversity, biology and epidemiology in the Arctic (Kutz, Hoberg, & Polley, 2001). The limited knowledge of

parasite diversity has been emphasised by recent findings of new hosts (e.g. Harriman, Alisauskas, & Wobeser, 2008), new species (e.g. Gwiazdowicz, Coulson, Grytnes, & Pilskog, 2012; Faltýnková, Pantoja, Skírnisson, & Kudlai, 2020) and new distributions (e.g. Kutz et al., 2013). More work on host-parasite interactions in the Arctic is therefore needed (e.g. Kutz et al., 2001). What makes it more complicated is that increasing temperatures in the Arctic can benefit the spread of new diseases or parasites or increase the prevalence of existing ones (Davidson et al., 2011). Migratory bird species can play a role in the spread of parasites and pathogens to the Arctic (e.g. Sandström et al., 2013; Van Hemert, Pearce, & Handel, 2014). In general, emerging diseases may expose potentially naïve Arctic species to new diseases with often increased severity of clinical disease and a higher disease prevalence (Bradley, Kutz, Jenkins, & O'Hara, 2005).

1.4.2. Changes in phenology

The Arctic is one of the places that is warming the fastest as the global increase in temperatures is not the same across the globe (ACIA, 2005). This higher temperature trend in the Arctic together with a higher temperature variability, in comparison with the Northern hemisphere or the entire globe, is called Arctic amplification (Serreze & Barry, 2011). Several processes, which operate on different spatial and temporal scales, trigger Arctic amplification. Albedo feedback is probably the most well-known; with increasing temperatures spring sea ice melt and snowmelt on land is earlier and leads to an earlier exposure of dark ice- and snow-free surfaces, which absorb solar radiation more strongly and lead to further melt of ice and snow. The expectation is that Arctic amplification will become stronger over the coming decades (e.g. Serreze and Barry, 2011).

Changes linked to climate warming

are noted all over the Arctic and one good example is the Svalbard archipelago. This area has seen the strongest air temperature rise of Europe in the last three decades (Nordli, Przybylak, Ogilvie, & Isaksen, 2014). As a result snow melts earlier in spring and falls later in summer, shortening the long snow-covered season and increasing the short growing season (Piao et al., 2019). One of the effects of warming on the Arctic terrestrial system is an increase in plant productivity (i.e. greening), but the extend of greening varies over the Arctic (e.g. van der Wal & Stien, 2014). In Svalbard, it is predicted that vegetation communities will change from moss-dominated to graminoid-dominated swards or bare ground (Ravolainen et al., 2020). Moss insulates the soil and, therefore, keeps it cool with a shallow active layer. With the loss of the moss layer, it is expected that grounds will have warmer soils and deeper active layers potentially increasing productivity (Ravolainen et al., 2020; van der Wal & Stien, 2014). Changes in vegetation can enhance the nutritional landscape for Arctic herbivores such as reindeer and geese (Loe et al., 2020; Ravolainen et al., 2020).

Predation is another factor that can strongly influence some on Arctic herbivore populations (e.g. Loonen, Tombre, & Mehlum, 1998). Arctic terrestrial and marine ecosystems are strongly interconnected through both physical and biological processes (Descamps et al., 2017). Processes such as sea ice loss can change predator-prey interactions (e.g. Hanssen, Moe, Bårdsen, Hanssen, & Gabrielsen, 2013). One interesting example, is that the summer occurrence of polar bears (*Ursus maritimus*) on land has increased with decreasing sea-ice, which can severely affect the reproductive success of ground-nesting bird species, such as the barnacle goose, common eider (*Somateria mollissima*) and glaucous gull (*Larus hyperboreus*) (Prop et al., 2015).

1.4.3. Contamination

More changes are, however, occurring in the Arctic than the effects of rising temperatures alone. The common perception is that the Arctic is a remote area far away from major industrial centres and human settlements and that, therefore, there is hardly any contamination from anthropomorphic sources. This is nevertheless far from true (Kruse, 2016). In general, the Arctic is subject to contamination through two pathways; global sources (nonpoint-source) and local sources (point source) (Thomas, Tracey, Marshall, & Norstrom, 1992). Contaminants can be transported from remote sources to the Arctic through the atmosphere, ocean currents and rivers (Macdonald & Bewers, 1996). Nevertheless, the Arctic is not free of industrialization itself, as mining has been taken place for centuries in some places such as on Svalbard and in northern Russia and Alaska (AMAP, 2009; Kruse, 2016). These local sources can lead to contamination of the Arctic environment in the form of heavy metals (e.g. mercury), polyaromatic hydrocarbons (PAHs) and polychlorinated biphenyls (PCBs).

It has been long known that exposure to bioaccumulative contaminants, such as mercury, can be a health risk for Arctic marine and terrestrial wildlife (AMAP, 2018). Trace metal bioaccumulation in animals has been associated with negative effects on fitness (e.g. Brasso and Cristol, 2008). For example, both effects on the hypothalamus-pituitary-adrenal (HPA) axis (i.e. dysregulation of adrenocortical function) and/or the immune system (i.e. 'unnecessary' immune suppression or enhancement) are seen as possible underlying causes (e.g. Bichet et al., 2013; Hallinger, Cornell, Brasso, & Cristol, 2011). It is expected that global contamination and possibly also local industrial activity in the Arctic will increase (AMAP, 2009). In addition, climate warming might worsen the

impacts of contaminants directly by changing chemical patterns and/or behaviour, or indirectly by e.g. changes in agriculture and industries (Kallenborn, Halsall, Dellong, & Carlsson, 2012). Considering this, there is an ongoing need to investigate contamination of Arctic marine and terrestrial habitats and its effects on wildlife.

1.5. Environmental changes and long-distance migrants

Long-distance migrants, such as many bird species that breed in the Arctic, might especially be sensitive to changes in their environment such as those described in the above section. In comparison to species that stay in the same area year-round, species that migrate over long distances between breeding and wintering sites have the disadvantage that they are not familiar with the local conditions on the breeding grounds and possible changes that have occurred here in their absence (e.g. Pulido and Berthold 2010). For example, climatic changes might affect local food abundance and climatic conditions that are optimal for breeding. Predicting these conditions on breeding or stopover sites is difficult for migrating species and the predictability seems to differ with the migration strategy that is used (short or long distances between stopover sites; Piersma 1987. Tombre et al. 2008, Clausen and Clausen, 2013, Kölzsch et al. 2015). In addition, different migration strategies can expose long-distance migrants to dissimilar pollutant levels (Hitchcock et al. 2019). Long-distance migrants might also have a higher chance to fetch parasites or diseases underway, especially when habitat loss decreases the number of suitable stopover sites resulting in higher densities of animals at these sites (Altizer et al., 2011).

1.6. Environmental changes and Arctic geese

One very abundant group of long-distance migrants that make use of the Arctic for breeding are geese. Millions of them make the long migration to the Arctic regions every year to breed on tundra, islands and rocky outcrops. Many wild goose species that winter in Western Europe and breed in the Arctic have come back from the brink of extinction and seem to have recovered well even though they are in a very much changing environment (Fox & Madsen, 2017; Fox & Abraham, 2017). Such species are intriguing as we can learn more about adaptive capacity, and therefore resilience, to changes in their environment.

An example is the flexible nature of geese such as recently observed changes in winter and spring migration behaviours of some species, which are contrary to the general notion that geese are highly philopatric (aka traditional or site-faithful) (Clausen, Madsen, Cottaar, Kuijken, & Verschuere, 2018; Eichhorn, Drent, Stahl, Leito, & Alerstam, 2009; Jonker et al., 2013; Oudman et al., 2020; Tombre, Oudman, Shimmings, Griffin, & Prop, 2019). Geese seem to alter their migration strategies by plastically responding to environmental change in the form of developments in agricultural practices and, therefore, food availability. Thus, climate warming makes new sites usable for goose species and competition between and within species can change in response (Clausen et al., 2018; Oudman et al., 2020; Tombre et al., 2019). By switching to new habitats some goose species seem to be able to escape density dependence (Fox et al., 2005; Tombre et al., 2019). Reading this, one might be inclined to reason that, on a population level, geese can adapt to the ever-changing world (Black, Prop, & Larsson, 2014; Fox & Abraham, 2017). Yet, their group-living lifestyles may make them sensitive to parasites and diseases, pollution, and radical changes

in their environment such as future changes in agricultural practises (through interaction between climate change, the innovation of new crops and market demands), climate change and natural catastrophes (Black et al., 2014; Fox & Abraham, 2017; Lameris et al., 2018).

1.7. The study system

This thesis focuses on one goose species: the barnacle goose. There are five barnacle goose populations in the Western Palaearctic; two temperate breeding and three Arctic breeding populations. Their names are based on the breeding area locations (Black et al., 2014). The Baltic, North Sea and Russian populations winter mostly on the North Sea coast of the Netherlands and Germany, the Greenland population winters in Ireland and Western Scotland and the Svalbard population mostly winters on the west coast of the UK on the border between England and Scotland on the Solway Firth estuary (Black et al., 2014).

Svalbard barnacle geese were first mentioned in the scientific literature in the 19th century, but were seen as quite a rare species by the 20th century (Løvenskiold, 1963). After intense exploitation in both their wintering areas and during the breeding season, concern was raised in 1948 when only about 300 individuals were counted on the Solway Firth. Following full protection in mainland Britain in 1954, in Svalbard in 1955 and the establishment of a national nature reserve at the main wintering area at Caerlaverock in 1957, numbers increased to 3000-4000 by the 1960s (Owen & Norderhaug, 1977). The protection of the Svalbard barnacle goose population turned out to be a conservation success story as their numbers kept on increasing and reached the conservation target of 25,000 individuals for a five-year period in 2007 (Black et al., 2014). The most recent estimate of the population

size for the 2019/2020 season was 36,000 (Wildfowl & Wetlands Trust (WWT), 2020).

In this PhD thesis I will focus on the Kongsfjorden population of Svalbard barnacle geese. This population has been closely monitored on the islets Storholmen (ca. 30 ha) and Prins Heinrichøya (ca. 3 ha) in Kongsfjorden, near the village of Ny-Ålesund (78°55'N, 11°56'E). In the early 1980s a barnacle goose colony established on the islands in Kongsfjorden and there was a strong increase in nest numbers over the years (I. Tombre, Mehlum, & Loonen, 1998). From 1990 to 2017 the local population size was estimated to have increased with a mean asymptotic population growth rate (λ) of 1.05 (0.92, 1.17), with density-dependent and direct and indirect climate change effects apparently stabilizing population size (Layton-Matthews, Hansen, Grøtan, Fuglei, & Loonen, 2019).

1.8. Thesis outline

This introductory chapter is followed by three different parts of the PhD thesis in which I will dive more deeply into the abovementioned objectives and the accompanying literature.

Part I of the thesis concentrates on nest parasites, changes in breeding phenology and their effects on reproductive success. We know little about parasites in the Arctic, how they affect Arctic animals, and how endemic parasites can become more important and/or new parasites can be introduced under climate warming. Fleas in goose nests are parasites which in some cases seem to be newly emerging in the Arctic, but in other cases are already endemic. In **Chapter 2**, my co-authors and I contribute to the scarce knowledge on the effects of nest fleas on precocial bird species (such as geese), where the young only stay a short period in the nest after hatch. First, we investigate the question if flea abundance negatively correlates with

reproductive success in Svalbard barnacle geese, as was detected earlier for geese in the Canadian Arctic. Second, we study if fleas change female incubation and anti-parasite behaviours by experimentally decreasing flea abundance by heat treatment of nests. I then dive into a different topic in **Chapter 3**, by investigating the change in breeding phenology due to climate warming of three different populations of barnacle geese breeding in Arctic Russia (low Arctic) and Svalbard (high Arctic). We expect that geese in the high Arctic are more time constrained in general and, therefore, lay their eggs earlier to the relative onset of spring and are able to adjust their laying dates at a lower rate than the variation in snowmelt date in comparison to low Arctic geese.

Part II of the thesis consists of three chapters on an experiment with human-raised Svalbard barnacle goslings in which I investigate if and how physiology and behaviour are affected by a contaminated grazing environment. One group of goslings grazed daily on the contaminated area near an abandoned coal mine in Ny-Ålesund, Svalbard ('mine goslings'). The second group of goslings, with the social siblings of the mine area group, grazed daily on clean tundra ('control goslings'). In **Chapter 6**, we assess the total mercury concentration of soil and vegetation in the mine and control area, as well as the concentrations in faeces and livers of the goslings. Furthermore, we examine the relationship between the mercury levels and two brain neuroreceptors, since mercury is often found to be neurotoxic. Many studies that investigate effects of contaminants on animal physiology, do not consider that free-living animals often have to deal with various stressors at the same time. In **Chapter 4**, therefore, we experimentally examine effects of exposure to contaminants on plasma corticosterone levels and on four innate immune parameters (haemolysis, haemagglutination, haptoglobin-like activity

and nitric oxide) before and after social isolation. Experimental social isolation mimics a survival threat. In **Chapter 5**, we compare stress-related behaviours and excreted immuno-reactive corticosterone metabolites (in droppings) during three experimental stress tests in the same goslings.

Behavioural plasticity is one mechanism by which populations can cope with changes in their environments. In the last part of the thesis (**Part III**), I therefore investigate one aspect of behavioural plasticity in Svalbard barnacle geese. In **Chapter 7**, we examine individual variation in nest defence behaviour over season and with increasing age in female Svalbard barnacle geese by making use of a long-term dataset on this behaviour.

The thesis concludes with a short synthesis (**Chapter 8**) that integrates the results from the different papers, returns to and reflects on the aims and objectives and makes suggestions for future research to build on the results presented here and to close knowledge gaps.

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Part I.
Nest parasites, changes in
breeding phenology and
reproduction

Chapter 2.
Effects of fleas on nest success
of Arctic Barnacle geese:
experimentally testing
the mechanism

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Article

Effects of fleas on nest success of Arctic barnacle geese: experimentally testing the mechanism

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Parasites have detrimental effects on their hosts' fitness. Therefore, behavioural adaptations have evolved to avoid parasites or, when an individual is already in contact with a parasite, prevent or minimize infections. Such anti-parasite behaviours can be very effective, but can also be costly for the host. Specifically, ectoparasites can elicit strong host anti-parasite behaviours and interactions between fleas (Siphonaptera) and their hosts are one of the best studied. In altricial bird species, nest fleas can negatively affect both parent and offspring fitness components. However, knowledge on the effects of fleas on precocial bird species is scarce. Research on geese in the Canadian Arctic indicated that fleas have a negative impact on reproductive success. One possible hypothesis is that fleas may affect female incubation behaviour. Breeding females with many fleas in their nest may increase the frequency and/or duration of incubation breaks and could even totally desert their nest. The aim of our study was to 1) determine if a similar negative relationship existed between flea abundance and reproductive success in our study colony of Arctic breeding barnacle geese *Branta leucopsis* and 2) experimentally quantify if such effects could be explained by a negative effect of nest fleas on female behaviour. We compared host anti-parasite and incubation behaviour between experimentally flea-reduced and control nests using wildlife cameras and temperature loggers. We found that flea abundance was negatively associated with hatching success. We found little experimental support, however, for changes in behaviour of the breeding female as a possible mechanism to explain this effect.

Keywords: Arctic goose colony, insect harassment, parasite–host interaction

Introduction

Parasites generally have detrimental effects on their hosts' fitness. Therefore, hosts have evolved a wide range of physiological and behavioural responses to reduce parasitic costs (Norris 2000, Clayton et al. 2010, Owen et al. 2010). Behavioural adaptations aid to avoid parasitic infection or, when already in contact with a parasite, prevent or minimize infections (Hart 1992, 1994). As a result, anti-parasite behaviours can be roughly divided into pre-infection and post-infection behaviours (Schmid-Hempel 2011). Pre-infection behaviours include temporal and spatial avoidance of parasite

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prone environments, foods and individuals or hosts altering their niche to discourage parasites (e.g. prophylactic self-medication: Castella et al. 2008; see review by Curtis 2014), while post-infection behaviours consist of for example parasite removal (e.g. grooming/preening: Hart 1992, Clayton et al. 2010), and therapeutic self-medication (Clayton and Wolfe 1993, de Roode et al. 2013).

Anti-parasite behaviours can be very effective in minimizing infection risk as is exemplified by an experimental study by Daly and Johnson (2011). They showed that Pacific chorus frog larvae *Pseudacris regilla* that were anesthetized and therefore behaviourally impaired were more likely to become infected and had higher infection intensities with pathogenic trematodes (*Ribeiroia* and *Echinostoma*) than frog larvae that could display their natural avoidance behaviour. Also, when parasite removal behaviours were restrained in different animal species, ectoparasite infection increased in comparison to control animals that could perform their natural parasite removal behaviours (e.g. birds: Brown 1972, Clayton et al. 2005, Waite et al. 2012; mammals: Bennett 1969, Mooring et al. 1996).

On the other hand, such behavioural adaptations can also present fitness costs themselves. Avoidance behaviours can impose trade-offs for the host in terms of 1) decreased time spend feeding and resting, while increasing active behaviours to avoid parasites (reindeer *Rangifer tarandus tarandus*: Hagemoen and Reimers 2002, Weladji et al. 2006), 2) selection of lower quality forage over high quality parasite infested forage (sheep: Hutchings et al. 2000, 2002) and 3) increased nest desertion in the face of high nest parasite presence (e.g. cliff swallow *Hirundo pyrrhonota*: Emlen 1986; great tits *Parus major*: Oppliger et al. 1993). Furthermore, a post-infection behaviour such as parasite removal can be costly as well: it can e.g. increase energy expenditure (greater mouse-eared bats *Myotis myotis*: Giorgi et al. 2001), damage fur (moose *Alces alces*: Samuel 1991, Mooring and Samuel 1998) and decrease the available time for other behaviours such as vigilance (impala *Aepyceros melampus*: Mooring and Hart 1995).

The interactions between fleas (order Siphonaptera) and their hosts are one of the best studied (Rothschild and Clay 1952, Proctor and Owens 2000, Krasnov 2008). Fleas are typical ectoparasites of higher vertebrates and are obligatory blood-feeding insects. For the majority of flea species, adults live in a close, but temporary association with their hosts (Krasnov 2008). Their behaviour, morphology and physiology are adapted as such that they can make optimal use of their hosts temporary visits to lair, nest, dwelling or burrow (Wall and Shearer 1997). In birds, most studies on interactions between fleas and their hosts have investigated altricial species where fleas can affect both parent and offspring simultaneously and thereby decrease fitness (Richner et al. 1993, Nilsson 2003). However, knowledge on effects of nest fleas on precocial bird species, where the young only stay a short period in the nest after hatch, is scarce. Nevertheless, Harriman and Alisauskas (2010) found

that nest flea abundance was negatively correlated with nesting success in precocial Ross's *Anser rossii* and lesser snow geese *Anser caerulescens caerulescens*. A possible hypothesis explaining this finding is that nest fleas affect female behaviour (Harriman and Alisauskas 2010). Alterations in female behaviour can become visible in the form of increased grooming and nest sanitation behaviours in parasite infested nests (Cantarero et al. 2013). Increased irritation might lead to higher nest desertion (Fitze et al. 2004) or possibly increases the frequency and/or duration of incubation breaks (Cantarero et al. 2013). The latter can decrease nest success when eggs get too cold and the embryo dies (Webb 1987) or when eggs are eaten by a predator during female absence (Prop et al. 1984, Samelius and Alisauskas 2001). To investigate if fleas indeed affect behaviour, experimental studies are vital to independently quantify effects of flea infestation from potential differences in the quality of the breeding individuals, which can influence their suitability and attractiveness for parasites (Krasnov et al. 2005). However, experimental studies investigating the possible mechanisms behind the failure of nests of precocial species with high ectoparasite infestation are lacking.

Here, we aim to experimentally quantify the effect of nest fleas on the behaviour and reproductive success of a precocial species; Arctic breeding barnacle geese. In barnacle goose nests on Spitsbergen (Svalbard), fleas *Ceratophyllus vagabundus vagabundus* are the only ectoparasites detected and they can be present in high numbers (Pilskog et al. 2014). In our study, we examined whether flea abundance also negatively correlates with reproductive success in this Arctic goose species. Furthermore, to investigate the hypothesis raised by Harriman and Alisauskas (2010) that fleas change female incubation and anti-parasite behaviours, we experimentally decreased flea abundance by heat treatment of nests. We compared behaviour of hosts with control and flea-reduced nests using automatically triggered wildlife cameras and temperature loggers (Cantarero et al. 2013). Our aims were to explore the changes in the frequency of anti-parasite behaviours and the frequency and length of incubation breaks as a consequence of experimentally changed nest flea abundance, and examine the possible effects of these changes on reproductive success. We expected that female geese with flea infested control nests would show increased anti-parasite behaviours, an increased frequency and/or length of incubation breaks and a lower reproductive success.

Methods

Study site and study species

We conducted this study on the islands Storholmen (ca 30 ha) and Prins Heinrichøya (ca 3 ha) in Kongsfjorden, near the village of Ny-Ålesund (78°55'N, 11°56'E), Spitsbergen (Svalbard). Since the first barnacle goose nest was detected in Kongsfjorden in the early 1980s, there has been a strong

increase in number of nests on all islands in the fjord (Tombre et al. 1998).

Observational data on flea abundance and nest success

Egg blood coverage and flea abundance

In the years 2012–2016 we took photographs of eggs in barnacle goose nests on the islands Storholmen and Prins Heinrichøya to estimate flea abundance by investigating the blood coverage on the eggs. We took one photograph per nest and from this photograph we visually estimated blood coverage of eggs on a scale from 0 to 4, with 0 being no blood at all and 4 being fully covered in blood (method adapted from Harriman et al. 2008). As eggs in the same clutch usually had similar blood coverage, the score was averaged over the nest (Harriman et al. 2008). Scoring was done by one person (MDJ) and reference photographs were used to aid in scoring.

To assess flea abundance, we collected nest material from incubating geese by quickly reaching down in the nest next to the nest cup with a hand lined by a plastic bag, to prevent fleas from escaping. We reached right up to the bottom of the nest and collected about a similar handful of nest material from all nests. The plastic bag with the nest material was closed and stored in a refrigerator at ca +5°C until extraction (for comparable methods see Pilskog et al. 2014). Extraction was done using a Tullgren funnel setup, comprised of stainless steel funnels, light fixtures consisting out of aluminum hoods (Heat reflector OLBA complete) and 25–60 W lamps (due to lamp breakages and therefore shortages Wattages differed between funnels, but were compensated for by hanging the lamp lower or higher above the sample to create a similar amount of heat). Adult fleas and larvae were forced down in the funnel because of the heat gradient and were collected in 96% ethanol in tubes at the bottom end of the funnel. Samples were extracted for approximately 48 h until dry and no fleas or larvae were seen in the material. Nest material samples were collected after extraction and dry mass of the samples was measured.

Harriman et al. (2008) found that flea numbers in the nests were positively related to blood coverage on eggs. To check whether this was also the case in our study population, we collected nest material from a subset of 109 goose nests during the breeding seasons of 2015 and 2016 to extract adult fleas and flea larvae. We also detected a positive correlation between blood coverage and adult flea numbers (generalized linear model: intercept = 3.732 ± 0.249 , β blood coverage = 0.383 ± 0.148 , $F_{1,107} = 6.707$, $p = 0.01$) and flea larvae numbers (generalized linear model: intercept = 4.212 ± 0.622 , β blood coverage = 0.397 ± 0.193 , $F_{1,107} = 4.395$, $p = 0.038$) when corrected for year variation.

Standard measurements of nest success

In the years 2012–2016, we checked all nests approximately every other day during incubation and around hatch to determine clutch size and number of hatchlings when present,

and to estimate hatch date and hatching success. Clutch size was determined as the maximum number of eggs in the nest during at least two subsequent visits. When present, we defined the number of hatchlings as all eggs that showed signs of hatching and/or all successfully hatched goslings, with the assumption that all hatching eggs would become hatchlings. The number of hatchlings was counted when 1) at least half of the eggs in the nest were hatching (cracks, hatching or hatched goslings), or 2) less than half of the eggs were successfully hatched (goslings are present) while other eggs were not yet in the process of hatching. Hatching success (0 = no hatch, 1 = successful hatch) was estimated by the observation of hatching eggs, goslings or the presence of eggshells in combination with egg membranes (Davis et al. 1998). A nest was considered as successfully hatched when at least one egg had hatched. Hatch dates were estimated on the basis of signs of hatching. When at least 1 egg showed cracks the hatch date was assumed to be the day of observation plus 1 (the nest would hatch the following day). When at least 1 egg with holes, hatching goslings or still wet goslings were observed, the hatch date was the day of observation. When goslings where dry and fluffy or when we found an empty nests with eggshells and egg membranes, hatching date was assumed to be the day of observation minus 1 (the nest hatched the previous day). In the event that the islands could not be reached for multiple days in a row, because of e.g. adverse weather conditions or polar bear presence, hatch dates could not be accurately estimated and were not used. When we found an empty nest with cold eggs, only eggshells or a totally empty nest without any eggshells and membranes, this indicated that the nest was abandoned in the first case or predated in the latter two cases. Total nest predation was not taken into account as in these cases we could not distinguish whether eggs were predated because of temporal goose absence or total nest abandonment. Therefore, these nests were all grouped as unsuccessful. Partial egg predation during incubation was noted when eggs went missing in between checks or when egg predation was observed.

Experiment to reduce flea numbers

Experimental setup

In the breeding season of 2016, we aimed to reduce flea numbers by a method that has been used often to study the interaction between nest fleas and passerine birds, namely by microwaving the nest (Richner et al. 1993, Gallizzi et al. 2008). Goose nests were selected for the experiment from nests on the island of Storholmen and paired on the basis of clutch size and blood coverage (see above: blood score 1: $n = 7$ pairs, blood score 2: $n = 7$ pairs, blood score 3: $n = 11$ pairs, blood score 4: $n = 5$ pairs). Nests within a pair were randomly assigned to either a control group ($n = 30$) or a flea-reduced group ($n = 30$). On a single day (12 June 2016) all these nests were visited. Firstly, a nest material sample was taken to estimate pre-experimental flea abundance, after which the eggs were taken out of the nest and placed into a padded box. Then, all nest material was taken from the ground and placed

into a plastic bag. Nest debris from underneath the nest was also taken out as much as possible and put into a separate plastic bag. The padded box with the eggs was left near the nest location and the nest material was carried away to be treated. Fleas were eliminated from the flea-reduced nests to be by microwaving the nest material and nest debris in the plastic bag for 3 min at 900 W. Afterwards, the nest material needed to cool down before the nest was restored. The microwave appliance was fed by a relatively silent portable 230-V generator. Control nests were not treated in the microwave, but were disturbed in a similar way. All nests were regularly checked after the experiment (see above). For two nests, the eggs had been placed back in nests that turned out later to have been too warm after returning from the microwave (this was visible from burn marks on the eggs during later checks). Both nests were taken out of all analyses of data gathered after the experiment.

As nest mass and flea numbers can be positively correlated (Eeva et al. 1994, Heeb et al. 1996), we determined fresh mass of the entire nest before the experimental procedure using a digital kitchen scale positioned on a flat surface. Fresh mass of the entire nest did not differ between the experimental groups (ANOVA: $F_{1,59}=0.089$, $p=0.765$) and was on average 261.6 g (SD=76.4). Also, the mass of the nest material samples did not differ between the experimental groups ($F_{1,179}=0.123$, $p=0.726$) and was on average 39.5 g dry mass (SD=16.5, $n=180$) or 54.5 g fresh mass (SD=28.3, $n=165$). Nest material samples were taken just before, 2 d after the experiment and 24 d after the experiment, when the eggs hatched and the geese had left.

Our experiment was effective in reducing adult flea numbers in the heat-treated nests (treatment $\times$ moment of sampling: $\chi^2_{2,8}=13.995$, $p<0.001$; Fig. 1A shows numbers 100 g⁻¹ nest material. See below for detailed statistical methods). Before the experiment, there were slightly more fleas in control nests but this difference was not significant (post-hoc comparison; contrast control – flea-reduced: $\beta=0.646 \pm 0.347$, z ratio = 1.857, $p=0.063$). Two days after the experiment however, the heat-treated group had significantly less fleas than the control group (post-hoc comparison; contrast control – flea-reduced: $\beta=2.164 \pm 0.416$, z ratio = 5.205, $p<0.001$) and this difference was more pronounced 24 d after the experiment (post-hoc comparison; contrast control – flea-reduced: $\beta=2.195 \pm 0.332$, z ratio = 6.616, $p<0.001$). Heat-treatment of nests also decreased flea larvae numbers (treatment $\times$ moment of sampling: $\chi^2_{2,8}=19.285$, $p<0.001$; Fig. 1B shows numbers 100 g⁻¹ nest material). Before the experiment, there was no difference in the number of flea larvae between the groups (post-hoc comparison; contrast [control – flea-reduced]: $\beta=0.035 \pm 0.405$, z ratio = 0.086, $p=0.932$). Two days after the experiment, larvae numbers decreased in both groups, but there were significantly less larvae in the heat-treated group (post-hoc comparison; contrast [control – flea-reduced]: $\beta=2.548 \pm 0.428$, z ratio = 5.945, $p<0.001$). This difference was still visible 24 d after the experiment

Figure 1. Differences between control nests (control, $n=30$) and heat-treated nests (flea-reduced, $n=30$) in (A) the number adult fleas 100 g⁻¹ nest material and (B) the number of flea larvae 100 g⁻¹ nest material sampled before the experiment (0 d, sample before experiment on the same day) and after the experiment (2 d and 24 d after). Error bars depict standard error.

(post-hoc comparison; contrast [control – flea-reduced]: $\beta=0.999 \pm 0.408$, z ratio = 2.446, $p=0.014$).

Nest temperature data

We placed temperature loggers (DS1921G-F5 thermochron iButton device, Maxim Integrated) in a subset of nests ($n=56$) to investigate the effects of fleas on nest temperature and nest temperature fluctuations. The iButton loggers were glued on top of 70 mm golf tees and pushed into the nest material in the centre of the nest in such a way that the logger rested on top of the nest material and was in contact with the eggs (Ringelman and Stupaczuk 2013). The iButton loggers recorded nest temperature on three dates for which a full day was recorded with a temperature measurement every minute (before the experiment on 10 June 2016, and after the experiment on 15 June 2016 and 21 June 2016). Unfortunately, the iButtons were apparently too low and too insulated in the nest and we were therefore unable to extract accurate data on incubation recesses as in Ringelman and Stupaczuk (2013). So the data were not suitable for determination of fine-scale presence/absence, but we deemed it possible to gain information on crude scale absence/presence by investigating overall nest temperature and nest temperature fluctuations. We expected and observed on basis of visual inspection of the data that when a goose left the nest for a longer time period, nest temperature would still drop, but more slowly. We therefore calculated daily average temperature per nest and, as a measure of daily temperature fluctuation, we calculated the standard variation per nest. We compared these measures between the experimental groups to gain insight in

whether fleas affect goose incubation behaviour. To investigate the frequency of absence (goose was not sitting on nest) and absence time, we used data collected by the wildlife cameras (see below).

Wildlife camera observations

We placed in total 20 wildlife cameras (Magainon WK3 HD) at a distance of 1 m from a random selection of flea-reduced ($n = 10$) and control nests ($n = 10$) to study female incubation and anti-parasite behaviours. The camera was set to take a photograph every time the goose moved with a minimum interval of 5 min in between photos. We scored goose behaviour from photos taken on days when 1) geese were not disturbed by researchers, 2) this day was at least one day before hatching (seen from goose posture, presence of eggshells/goslings) and 3) all cameras were working properly (i.e. on 1, 2 d before and 1, 3, 4, 5, 7, 10, 12 d after the experiment). On average, 80.3 (SD = 56.9) photos were taken of a nest per day. We used an ethogram to aid in scoring of behaviour and scoring was done by three observers who were blind to treatment group. Photos that were of such bad quality that nothing could be seen (e.g. sun glare), were taken out of the analyses. We scored all photos where a goose was sitting on her nest (assuming incubation: presence) as 1 and when a

goose was not incubating (i.e. standing on the nest/leaving nest/arriving at nest/absent from nest: absence) as 0. First, we were interested in the frequency and time of absence and therefore we used the time stamp of the photos to calculate the time between all consecutive photos of nests. We then selected all photos on which the geese were absent and, using the photo ID, we identified consecutive photos or single photos on which geese were absent. From this we obtained on the number of absences per nest per day and the summed length of these absences. Furthermore, we scored female preening (female is preening her feathers, bill in feathers) and when the female was busy with her nest (bill in nest material, often also her entire head is in the nest). Here, we will use the term nest maintenance to describe the latter behaviour in the remainder of the article. When a female has her head in the nest it might be that she is turning her eggs, but in passerine species an active search with the head dug into the nest material has been described and linked to nest sanitation against ectoparasites (Christe et al. 1996a). See Figure 2 for examples of photos that were taken.

During the experimental treatment, the geese stayed around the nest location and all birds returned after the nest was restored. Based on the wildlife camera pictures (see below, $n = 20$) the average time for females to resume incubation

Figure 2. Examples of photographs from the wildlife cameras used for monitoring female behaviour showing (A) presence of the female on the nest, (B) absence of the female, (C) preening (female present) and (D) nest maintenance (female present). See Methods and Table 1 for an explanation of definitions.

after initial disturbance was 31 min, the minimum amount of time was 9 min and the maximum 1 h and 24 min.

The use of these data is based on some assumptions. We assumed that the cameras were able to detect when geese were leaving the nest and arriving back at the nest, and that the detection probability did not differ between the experimental groups. However, as the minimum interval of the camera to take a photo was set at 5 min, this might have hindered the accurate estimation of females leaving and arriving back at the nest (e.g. females might have moved in front of the camera, sat on the nest within 5 min and sat very still for a long time, escaping accurate detection of when she was present again on her nest). Therefore, we expected that the calculated time when females were absent, would be an overestimation. We compared our measurement with measurements of daily recess time (calculated from the average recess length and the average number of recesses per female) of Arctic barnacle geese performed by two studies (Eichhorn and Karagicheva 2008, Tombre et al. 2012). Based on our data, we calculated that the time a goose was absent was on average 157.1 min (SE = 15.9) per nest per day. Tombre et al. (2012) studied incubation behaviour in the same population and observed, during four 24-h cycles of 18 nests, an average recess length of 19.8 (SE = 1.2) min and a daily average number of recesses of 4.9 (SE = 0.5), which would on average give a daily recess time of 97.02 min. Eichhorn and Karagicheva (2008) observed 42 barnacle goose nests in Arctic Russia during bouts of 6–48 h in all periods of the day and incubation stages and found a daily recess time of 157 min. Thus, our measurement falls within the measurement of Eichhorn and Karagicheva (2008) but not within the measurement of Tombre et al. (2012). This is possible due to differences in methodology, measurement period and/or year differences. Overall, we judge that we can use our data to make a comparison between our experimental groups.

Statistics

All analyses were done in R ver. 3.4.2. We present model intercept $\pm$ standard error (SE), estimate (β) $\pm$ SE, F- or χ^2 -test statistics with degrees of freedom (df) and group means and standard deviations (SD) in text or table where appropriate.

Blood coverage and nest parameters

We examined the association between blood coverage and two breeding parameters, clutch size and hatching success, using generalized linear mixed effects models (GLMMs) with a Poisson error distribution for the first and a binomial error distribution for the latter (glmer function in the R package lme4: Bates et al. 2015). We investigated the association between egg blood coverage and the number of hatchlings seen using GLMMs, with the number of eggs not seen as hatchlings (failures) and the number of hatchlings (successes) combined in a two-vector response variable, and a binomial error structure (Crawley 2007). We used a similar model for the analysis of the association between egg blood coverage

and partial egg predation, with the number of eggs predated (failures) and the number of eggs not predated (successes) combined in a two-vector response variable. We added blood coverage as a continuous predictor in these models as we were interested in the direction of the effect. We added island and year as random effects to account for possible differences between the two islands and between the years. Binomial error bars for the graph on hatching success were calculated using the binom.confint function with the Wilson method in R (Dorai-Raj 2014).

Experiment to reduce flea numbers

We investigated the effect of microwaving nests on adult flea and flea larvae numbers using GLMMs with a Poisson error distribution. The logarithm of the dry mass of the nest sample was included as an offset in the models. Including a logarithm of exposure in this model (here dry mass of the nest sample) was useful as we were interested in the rate of flea and flea larvae numbers per dry nest mass. An offset is similar to including a regression predictor, but its coefficient is fixed to the value 1 (Crawley 2007, Gelman and Hill 2007). Furthermore, the interaction between treatment and the moment when the sample was taken (before, after (2 d) and longer after (24 d) the experiment) were added as factors. As a random effect we added nest identity, as nests were repeatedly measured. We checked for overdispersion, and when this was the case we added an individual-level random effect to the model to account for this (Agresti 2002). We made post-hoc comparisons by computing estimated marginal means (predicted marginal means) for the final models using the R package emmeans (Lenth 2018). To illustrate the experimental effects on the numbers of adult fleas and flea larvae per nest material sample graphically, we calculated the numbers per 100 g nest material to give a more straightforward measure.

Experimental effects on nest parameters

Hatching success data showed clear separation as all control nests hatched, but 3 flea-reduced nests did not (also referred to as the Hauck–Donner effect: Hauck and Donner 1977). Therefore, to investigate whether hatching success was significantly different between the experimental groups, we examined the effects of the experiment on hatching success using the Fisher's exact test. We investigated the experimental effect on the number of hatchlings seen using generalized linear models (GLMs) with a binomial error structure. We therefore combined the number of eggs not seen as hatchlings (failures) and the number of hatchlings seen (successes) in a two-vector response variable. We used a similar model to investigate the experimental effect on partial egg predation using by combining the number eggs predated (failures) and the number of eggs not predated (successes) in a two-vector response variable. We added the factor treatment as a predictor in these models. In case of overdispersion we fitted a quasibinomial error structure (Crawley 2007).

Experimental effects on female behaviour

First, to investigate whether the experimental groups differed in the total number of movements made by the geese as an indication for irritation, we analysed whether there was a difference in the total number of photos taken of control and flea-reduced nests per day (irrespective of photo quality). Therefore, we used a GLMM with a Poisson error distribution and we analysed the number of photos taken when the geese were present on their nest (Table 1 for an overview of the measurements). Second, we wanted to find out whether the experimental groups differed in the number of absences per day and the absence time per day. We analysed this data with a GLMM with a Poisson error distribution for the first and a linear mixed-effects model (LMM: lmer function in the R package lme4; Bates et al. 2015) for the latter. We used log-transformed absence time to meet model assumptions. Third, to investigate whether there was a difference between the experimental groups in anti-parasite related behaviours we investigated the probability that the detected behaviour was preening or nest maintenance. We analysed this data using GLMMs with a binomial error distribution. For the analyses of the number of photos, preening and nest maintenance, we selected photos when the female was present on her nest as this was when all geese were visible (some geese might have stood up preening in front of the camera, but other geese might have done so out of sight of the camera). In all these models, we added an interaction between the factor treatment and the time in days since the experiment as fixed effects. We added a random slope to the model when the random slope model was significantly better than a model with only a random intercept model. This was the case for almost all models. The random part in these allowed individuals to differ in the intercept as well as the slope of their reaction over time, thereby preventing overconfident estimates (Schielzeth and Forstmeier 2009). We checked for overdispersion, and when this was the case we added an individual-level random effect to the model to account for this (Agresti 2002). For all measures we checked whether there were differences between the groups before the experiment (−1 and −2 d before) and, in separate models, we investigated whether there were experimental effects by using the data with day −1 as a starting point.

Effects on nest temperature

To investigate the effects of treatment on daily average nest temperature and nest temperature fluctuations, we fitted linear mixed effects models. We added the interaction between the factors treatment and measurement day as fixed effects and nest identity as a random effect.

Data deposition

Data available from the Dryad Digital Repository: <<https://doi.org/10.5061/dryad.80n9608>> (de Jong and Loonen 2019).

Results

Observational data on flea abundance and nest success

Correlations between egg blood coverage and nest parameters

We did not detect any relationship between egg blood coverage and clutch size (Table 2). Of 846 nests of which blood coverage was determined from 2012 to 2016, 86 nests had lost one or more eggs during incubation. There was also no correlation between egg blood coverage and partial egg predation. However, with increasing blood coverage on eggs, hatching success decreased significantly from 95% of nests hatched with no blood coverage to 83% of nests hatched with eggs fully covered in blood (Fig. 3, Table 2). We did not detect any correlation between egg blood coverage and the number of hatchlings in the nest (Table 2). We encountered on average three hatchlings per nest in 380 nests in total.

Experimental data

Experimental effects on nest parameters

We detected partial predation during incubation for six nests of both groups. The number of eggs predated versus the number of eggs not predated did not differ between the groups (average % of eggs predated (95% binomial confidence interval); control nests: 7.5% (4.1–13.2), flea-reduced nests: 8.9% (5.0–15.2). Intercept = 2.518 ± 0.478 , β flea-reduced = -0.188 ± 0.662 , $F_{1,57} = -0.171$, $p = 0.776$). Flea-reduced nests hatched on average 12.4 d (SD = 2.9) and control nests 11.4 d (SD = 2.7) after the experiment, which

Table 1. Overview of measurements taken from the wildlife camera data, their definitions, the data type and what models were used to analyse the data.

Measurement	Definition	Type and model
Number of photos	All photos that were taken during the experiment when geese were present on their nest, on days when they were not disturbed by researchers and when this day was at least one day before hatching.	Poisson: GLMM
Number of absences	The summed number of absences per nest per day extracted from single or consecutive photos when geese were absent.	Poisson: GLMM
Time absent	The summed time the female was absent in seconds per day extracted from time between consecutive photos when geese were absent.	Log-transformed: LMM
Preening	Female is on her nest preening her feathers (bill in feathers). Absence or presence of this behaviour.	Binomial: GLMM
Nest maintenance	Female is on her nest with her bill in the nest material, often also her entire head is in the nest. Absence or presence of this behaviour.	Binomial: GLMM

Table 2. ANOVA table for GLMMs on the correlation between egg blood coverage and nest parameters. Variables in bold font were kept in the final model. Values for non-significant predictors represent values just before removal in backward elimination.

Measurement	Variable	β	SE	χ^2	df	p
Clutch size	Intercept	1.280	0.054	—	—	—
	Blood coverage	0.020	0.018	1.253	1, 4	0.263
Partial egg predation	Intercept	3.689	0.472	—	—	—
	Blood coverage	-0.032	0.102	0.099	1, 4	0.753
Hatching success	Intercept	3.189	0.246	—	—	—
	Blood coverage	-0.341	0.131	6.540	1, 4	0.011
Number of hatchlings	Intercept	3.689	0.472	—	—	—
	Blood coverage	-0.033	0.102	1.593	1, 4	0.207

was not significantly different (intercept = 11.367 ± 0.510 , β flea-reduced nests = 1.073 ± 0.756 , $F_{1,54} = 2.015$, $p = 0.162$). We found no significant difference between control and flea-reduced nests in hatching success, although flea-reduced nests hatched slightly less well than control nests (control: 30/30 hatched, flea-reduced: 25/28 hatched; $p = 0.106$). In 19 control and 21 flea-reduced nests, we encountered hatchlings. The number of hatchlings seen in these nests was not different between the groups (average % of eggs seen as hatchlings (95% binomial confidence interval): control nests = 50.7% (42.4–59.1), flea-reduced nests = 62.9% (54.1–70.9). Intercept = 1.386 ± 0.332 , β flea-reduced nests = 0.331 ± 0.486 , $F_{1,39} = 0.468$, $p = 0.494$).

Experimental effects on female behaviour

For all measurements, we did not detect a difference between the experimental groups before the experiment was started. The number of photos taken when the goose was present

decreased for flea-reduced nests over time in comparison to control nests (Table 3, Fig. 4). However, the model containing this interaction was not significantly better than the null model ($\chi^2_{3,8} = 7.63$, $p = 0.054$), indicating that this effect was not very strong. A median number of 68 photos were taken of control nests and 59 of flea-reduced nests per day. Both females with control and flea-reduced nests were an equally number of times per day absent from the nest (Table 3; control: median = 5, flea-reduced: median = 6). The number of absences decreased over time irrespective of treatment. Control females and flea-reduced females did not differ in the time they were absent from the nest (back calculated from log; control: mean = 118.3 min d^{-1} , flea-reduced: mean = 119.9 min d^{-1}). When on the nest, control females were seen preening on average 14.8% of the photos (binomial 95% confidence interval: 13.8–15.8) while flea-reduced females preened less, as they were seen preening on average 12.0% of the photos (binomial 95% confidence interval: 11.0–13.0). However, we did not detect this difference in the analysis of the data over time (Table 3). Detection of nest maintenance did not differ between the groups (Table 3; control: 10.8% of the photos (binomial 95% confidence interval: 10.0–11.7), flea-reduced: 10.7% of the photos (binomial 95% confidence interval: 9.7–11.7), but decreased significantly over time irrespective of treatment.

Experimental effects on nest temperature

We did not detect differences between the control and flea-reduced group in average nest temperature ($\chi^2_{1,6} = 0.047$, $p = 0.828$) or nest temperature fluctuations ($\chi^2_{1,6} = 0.435$, $p = 0.510$) before or after the experiment (Table 4). Daily average nest temperature did increase over time ($\chi^2_{2,5} = 29.851$, $p < 0.001$).

Discussion

In this study we investigated the effects of nest fleas on the behaviour and reproductive success of a precocial species; Arctic breeding barnacle geese. First, we examined whether there was an association between flea abundance (estimated by egg blood coverage) and several parameters of reproductive success. Second, we experimentally reduced nest fleas in a subset of nests by using a heat-treatment and

Figure 3. The correlation between egg blood coverage (from 0 no blood, to 4 fully covered in blood) and hatching success of Arctic breeding barnacle geese. Sample size is indicated by symbol size and the numbers next to the error bars. Error bars depict 95% binomial confidence intervals.

Table 3. ANOVA table for the GLMMs on the total number of photos, the total number of absences, female preening and nest maintenance and for the LMM on the total time absent for barnacle goose females with control and flea-reduced nests. Values for non-significant predictors represent values just before removal in backward elimination. Intercepts are given from minimal adequate model. Variables in bold stayed in the final model.

Measurement	Variable	β	SE	$\Delta\chi^2$	df	p
Number of photos	Intercept	4.066	0.201	—	—	—
	Treatment			0.151	1, 7	0.697
	Flea-reduced	0.217	0.285			
	Time since experiment	0.008	0.033	2.801	1, 6	0.094
	Treatment flea-reduced × Time since experiment	-0.108	0.048	7.63	1, 8	0.031
Number of absences	Intercept	1.820	0.087	—	—	—
	Treatment			0.096	1, 4	0.757
	Flea-reduced	-0.049	0.156			
	Time since experiment	-0.035	0.011	10.168	1, 3	0.001
	Treatment flea-reduced × Time since experiment	-0.013	0.022	0.320	1, 5	0.572
Time absent	Intercept	8.889	0.095	—	—	—
	Treatment			0.007	1, 7	0.935
	Flea-reduced	-0.018	0.195			
	Time since experiment	-0.038	0.026	2.136	1, 6	0.144
	Treatment flea-reduced × Time since experiment	0.045	0.053	0.809	1, 8	0.369
Female preening	Intercept	-1.966	0.142	—	—	—
	Treatment			0.507	1, 6	0.477
	Flea-reduced	-0.196	0.274			
	Time since experiment	-0.026	0.027	0.857	1, 5	0.355
	Treatment flea-reduced × Time since experiment	-0.078	0.051	2.191	1, 7	0.139
Nest maintenance	Intercept	-2.16	0.218	—	—	—
	Treatment			0.828	1, 6	0.363
	Flea-reduced	-0.281	0.307			
	Time since experiment	-0.154	0.041	11.531	1, 5	0.001
	Treatment flea-reduced × Time since experiment	-0.028	0.08	0.125	1, 7	0.724

compared anti-parasite and incubation behaviours of female geese with control and flea-reduced nests. We expected that female geese with natural levels of flea-infestation in their nests would show increased anti-parasite behaviours, an increased frequency and/or length of incubation breaks and a lower nest success relative to the females with flea-reduced nests. In line with previous work on Ross's and lesser snow geese (Harriman and Alisauskas 2010), we detected a negative association between egg blood coverage, used as a proxy for the level of flea infestation, and hatching success in the Spitsbergen barnacle geese. We found little support, however, for changes in incubation behaviour as a possible mechanism to explain this effect. The number of photos taken by the wildlife camera upon movement, decreased over time for females with flea-reduced nests, while the number of photos stayed similar for females with control, flea-infested, nests. Females incubating on flea-reduced nests thus seemed to move relatively less while on the nest. This difference was however not significant. Also, females with flea-reduced nests seemed to preen slightly less, but this was not apparent in the analyses of the overall dataset. Females with control or flea-reduced nests further did not differ in the number of absences, their time absent, the proportion of photos on which nest maintenance was observed or their nest temperature. Thus, though some suggestions were found for differences in female behaviour as a consequence of flea infestation, we found no clear effects.

One possibility is that there were effects of fleas on female anti-parasite and incubation behaviour, but we were unable to detect these as the effect size was too small given our current sample size. We have some indications for small effects of fleas on the number of movements and preening and if our sample size would have been larger we may have been able to better estimate whether these small effects can be significant (Forstmeier et al. 2017). Below, we discuss other options for why we found no clear evidence to support our hypotheses.

Is it likely that fleas can exert effects?

While predators kill their prey and consume them completely, parasites, though often ubiquitous, may not always have obvious effects on their host (Newton 1998). Bird nests are an ideal environment for a range of ectoparasites such as ticks, mites, lice, flies, bugs and fleas, as they have a relatively stable micro-environment and provide a predictable food source. This predictable food source first consists of the incubating bird, that is exposed to the nest-dwelling parasites through close contact with the nest material during the lengthy incubation period (López-Rull and Macías García 2015). As most avian species also develop a bare, highly vascularized, brood patch on the ventral abdominal area to aid in heat-transfer to the eggs (Lea and Klandorf 2002), they provide an 'easy target' for ectoparasites (López-Rull and Macías García 2015). After hatching, nestlings are available

Figure 4. Difference between the number of photos taken of females with control and flea-reduced nests over time. Points indicate values of individual nests. Boxes show medians as well as 25% and 75% quartiles. Whiskers indicate the range between the 10th and 90th percentiles.

as a food source for the nest parasites (López-Rull and Macías García 2015). Especially nestlings of altricial birds are relatively helpless as they are not very mobile, cannot leave the nest to escape parasites until they fledge, are not able to remove parasites from their body and their physiological defences are not yet entirely developed (Starck and Ricklefs 1998, Adelman et al. 2013). Several studies have detected quite substantial negative effects of ectoparasite abundance on fitness measures in altricial bird species (see López-Rull and Macías García, 2015 and references below). Parents may be directly affected by ectoparasites when attending to the brood through possibly e.g. body mass loss, tissue/blood loss, feather loss, infection with pathogens and an increase in time spent on parasite removal, resulting in a decrease in survival (Brown et al. 1995). Furthermore, also, indirect effects on parental fitness components can play an important role.

For instance, several studies have found that young beg more when they are in infested nests, parents in response increase their food supply accordingly (Christe et al. 1996b, Tripet and Richner 1997, Hurtrez-Boussès et al. 1998) and, as a consequence, can have a reduced future reproductive success (Richner and Tripet 1999). Furthermore, experimentally increased nest ectoparasite abundance has been shown to decrease e.g. nestling growth and survival (Richner et al. 1993, Møller 1994, Cantarero et al. 2013). In precocial birds, it is to be expected that direct effects on offspring are limited, but that there could be substantial negative effects of ectoparasites on attending parents (Harriman and Alisauskas 2010). Interestingly, López-Rull and Macías García (2015) propose that susceptibility to nest-dwelling ectoparasites may be a major driver promoting and/or maintaining precociality. As far as we know, only three previous studies have investigated effects of ectoparasites on reproductive success in precocial bird species. Hoodless et al. (2003) and Baines and Taylor (2016) detected positive effects of treatments against ticks on clutch survival in pheasants *Phasianus colchicus* and chick survival in red grouse *Lagopus lagopus scotica* respectively. Harriman and Alisauskas (2010) detected a negative association between nest fleas and reproductive success in a population of Ross's and lesser snow geese in Arctic Canada. Similarly, in our study in a Spitsbergen barnacle goose population we also detected such an association. The above discussed scientific evidence thus indicates that ectoparasite presence on precocial birds can be associated with negative reproductive success. It must be noted however that experimental work investigating the effects of fleas in precocial birds has up to now not been done. In our experiment we did not find any effects of nest fleas on female behaviour or hatching success. What can explain this apparent discrepancy between the observational data, showing a negative relationship between fleas and hatching success, and our experiment in which we did not find any effects? Below we discuss several possibilities.

Did our experiment work?

Experiments have often been used to separate possible effects of the individual, and the resources it is able to obtain, on individual fitness (Both and Visser 2000). We adapted a method previously used to effectively eliminate nest parasites from nests of nest-hole breeders, namely heat-treatment of nests (Richner et al. 1993). Using this method, we were able to successfully decrease the adult flea numbers that take

Table 4. Daily average nest temperature (average T in degrees Celsius) and fluctuations in nest temperature (average T fluctuation) measured on two days before and three and nine days after the experiment in control and flea-reduced nests. Sample size is indicated between brackets.

Days before/after experiment	Control		Flea-reduced	
	Average T $\pm$ SD (n)	Average T fluctuation $\pm$ SD	Average T $\pm$ SD (n)	Average T fluctuation $\pm$ SD
-2	26.3 $\pm$ 3.6 (29)	1.2 $\pm$ 0.8	26.5 $\pm$ 3.2 (26)	1.0 $\pm$ 0.4
3	27.8 $\pm$ 3.7 (26)	1.4 $\pm$ 1.3	27.9 $\pm$ 3.6 (23)	1.4 $\pm$ 0.8
9	30.2 $\pm$ 2.1 (17)	1.2 $\pm$ 0.3	30.6 $\pm$ 2.3 (9)	1.2 $\pm$ 0.3

blood from the incubating bird. When measured 2 d after the experiment heat-treated nests had significantly less fleas and flea larvae than control nests and this difference still there after 24 d. Yet, as larvae stay deep in the nest and do not take blood themselves (Rothschild and Clay 1952) they are likely not directly irritating for the incubating birds. Despite that the experimental treatment was successful in decreasing nest flea numbers, we detected no large effects on female behaviour or nest parameters. A possible reason for this lack of effects is that the experiment started relatively late; nests hatched approximately 12 d after the heat-treatment, while the entire period of nest attendance in barnacle geese from the first egg is approximately 29 d. This late removal of fleas might have been unable to affect nest parameters such as hatching success, that could already have been influenced by flea infestation. Overall, hatching success of experimental nests was actually very high. While effects on hatching success may have not been apparent due to the short period the experiment lasted, we expect that this would not have been a problem for detecting possible effects on female behaviour. Flea numbers were nearly absent in flea-reduced nests and abundant in control nests. We expect that females would have been able to notice this (Hawlena et al. 2008) and if indeed female behaviour is affected by nests fleas, we expect we would have picked up on this. Repeating the experiment would provide a stronger basis to the knowledge on effects of ectoparasites on precocial birds.

Are alternative mechanisms at play?

So if our experiment worked, why then did we detect a relationship between flea abundance and hatching success in our breeding colony, but have no experimental evidence for effects on female breeding behaviour underlying this effect? It is possible that some other mechanism has caused the observed negative association between egg blood coverage and hatching success. One other proposed mechanism is that egg pores are blocked by dried-up blood in heavily flea-infested nests and thereby reduce effective gas exchange and subsequently lower embryo survival (Harriman and Alisauskas 2010). Reduced gas exchange, induced by partially wrapping eggs with an impermeable film after 14–18 d of incubation, negatively affected hatch rate and caused cognitive deficits in chickens (Rodricks et al. 2004). In our study area we have no data to judge whether effects of blood coverage on the gas-exchange of eggs were at play, future work should focus on testing this hypothesis. Another possibility is that nests with blood-covered eggs are more likely to attract predators due to the scent of blood (Harriman and Alisauskas 2010). In our study area foxes have not been able to enter the breeding islands since 1995 due to early breakup or non-existence of a sea-ice connection to the mainland (Hübner et al. 2002). However, the number of polar bears *Ursus maritimus* that have been sighted in Kongsfjorden on the breeding islands have increased and, when they are present, often cause the destruction of complete clutches (Prop et al. 2015). It could be a possibility that polar bears are attracted to blood scent.

Other egg predators that are present on the island are glaucous gulls *Larus hyperboreus*, Arctic skua *Stercorarius parasiticus* and great skua *Stercorarius skua* (Hübner et al. 2002). These aerial predators can rapidly take eggs when a nest is left unattended (Prop et al. 1984). As it is difficult to separate nest desertion from nest predation, we investigated partial egg predation during incubation. We did not find an association between egg blood coverage and partial egg predation, nor were there differences in partial egg predation between the experimental groups. This indicates that at least partial egg predation, which most likely is caused by aerial predators, is not affected by egg blood coverage.

Can quality differences between parents play a role?

Next to fleas exerting negative effects on goose hatching success via the above mentioned alternative mechanisms, it could also be that other factors associated with flea infestation are the actual cause of the effect (third variable problem). One possibility is that flea infestation is correlated with parental quality. It could be that lower quality parents have more nest fleas and the negative effect of nest fleas on hatching success is actually an effect of parental quality rather than directly of the fleas. Harriman and Alisauskas (2010) also discussed the possibility that hosts differ in quality in the light of their finding of a negative association between high flea abundance and reproductive success in two goose species. They argued that there were no indications in their study that fleas aggregate on low quality hosts, as fleas were more abundant in areas of the colony with a longer history of nesting and in nests with more eggs and there was no correlation between female mass and egg blood coverage (Harriman et al. 2008, Harriman and Alisauskas 2010). We did not detect a significant correlation between egg blood coverage and the number of eggs in the nest in our study, but there was a similar trend that nests with bloodier eggs contained more eggs. Geese that arrive in good condition at the breeding grounds can start nesting earlier and lay larger clutches (Béty et al. 2003). Therefore, if even, higher quality birds might acquire more nest fleas when they return to certain good areas in the colony with a longer history of nesting. Another option is that fleas are doing better when feeding on high quality birds (Dawson and Bortolotti 1997, Christe et al. 2003). Thus, in our colony, if anything, flea abundance may have a positive association with parental quality, which would not serve as an explanation for the negative effect of blood coverage on hatching success. Specific experiments in which both the effect of parental quality and the effect of nest fleas are independently estimated are needed to solve this conundrum.

Conclusion

Our findings provide new important evidence that nest fleas and reproductive success of a precocial species are negatively associated. The mechanism, however, remains unknown. The outcome of our experiment indicated that negative effects of nest fleas on hatching success are likely not mediated through

effects of nest fleas on female incubation behaviour. Perhaps other mechanisms such as a decrease in gas exchange or total clutch predation play a role. However, it is also possible that a third variable, such as quality differences between goose parents, may be an important mediator. Further experimental work is needed on precocial species to understand the mechanism behind the negative effect of nest fleas on hatching success. Knowledge of the exact mechanism is vital to understand how parasites can affect individual fitness. Especially, in a changing Arctic, parasite abundance may increase and become a bigger force to reckon with in understanding species population dynamics.

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Chapter 3.
**Climate warming may affect
the optimal timing of reproduction
for migratory geese differently in
the low and high Arctic**

Climate warming may affect the optimal timing of reproduction for migratory geese differently in the low and high Arctic

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Abstract

Rapid climate warming is driving organisms to advance timing of reproduction with earlier springs, but the rate of advancement shows large variation, even among populations of the same species. In this study, we investigated how the rate of advancement in timing of reproduction with a warming climate varies for barnacle goose (*Branta leucopsis*) populations breeding at different latitudes in the Arctic. We hypothesized that populations breeding further North are generally more time constrained and, therefore, produce clutches earlier relative to the onset of spring than southern populations. Therefore, with increasing temperatures and a progressive relief of time constraint, we expected latitudinal differences to decrease. For the years 2000–2016, we determined the onset of spring from snow cover data derived from satellite images, and compiled data on egg laying date and reproductive performance in one low-Arctic and two high-Arctic sites. As expected, high-Arctic geese laid their eggs earlier relative to snowmelt than low-Arctic geese. Contrary to expectations, advancement in laying dates was similar in high- and low-Arctic colonies, at a rate of 27% of the advance in date of snowmelt. Although advancement of egg laying did not fully compensate for the advancement of snowmelt, geese laying eggs at intermediate dates in the low Arctic were the most successful breeders. In the high Arctic, however, early nesting geese were the most successful breeders, suggesting that high-Arctic geese have not advanced their laying dates sufficiently to earlier springs. This indicates that high-Arctic geese especially are vulnerable to negative effects of climate warming.

Keywords Barnacle goose · *Branta leucopsis* · Trade-off · Fitness · Phenology

Introduction

The earth's climate has warmed rapidly in the past decades, resulting in warmer and earlier springs (Schwartz et al. 2006; Stocker et al. 2013). In response, many migratory bird

species have advanced their arrival on the breeding grounds (Jonzén et al. 2006; Gunnarsson and Tómasson 2011; Gill et al. 2014) as well as the dates at which they lay their eggs (Crick et al. 1997; Pearce-Higgins et al. 2005; Gill et al. 2014). A general finding is that this advance does not fully compensate for any forward shifts of seasonal peaks in food abundance (Thackeray et al. 2010; Gienapp et al. 2014). This inability to advance timing of reproduction may lead to a so-called phenological mismatch between the peak in food availability and the moment of high energy requirements of

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the growing young (Both and Visser 2005), with potential negative consequences for fitness (Both et al. 2006; Visser et al. 2012; Doiron et al. 2015). At the same time, an advancement of the breeding season may diminish nutritional stress for the parents (Boyd and Madsen 1997) and relieve seasonal time constraints on the birds' reproductive cycle (Tomotani et al. 2016) with potential positive effects on reproductive output (Dickey et al. 2008; Van Oudenhove et al. 2014; Lameris et al. 2017b).

To what extent birds should advance their laying dates under climate warming revolves around a trade-off between the most favourable conditions for the parent bird versus the most favourable conditions for its offspring (Lack 1968; Trivers 1974). This trade-off is especially important in strongly seasonal environments with a short annual breeding season (Tomotani et al. 2016, 2018) such as the Arctic, where migratory birds are on a tight schedule to raise offspring and prepare for their return journey to the wintering grounds. While benefitting from favourable conditions for the hatched offspring requires early laying dates to avoid a phenological mismatch, parents may also benefit from laying their eggs later (Perrins 1970; Drent 2006). For example, for Arctic-nesting geese, it is known that postponing egg laying allows for more time to obtain body stores, which enables birds to lay more eggs (Rowe et al. 1994), and leads to more favourable conditions for foraging during the incubation period (Prop and de Vries 1993; Eichhorn et al. 2010). As larger body stores and better foraging conditions enable birds to have shorter incubation recesses (Aldrich and Raveling 1983; Tombre et al. 2012) and thereby reduce the chance of nest predation (Prop et al. 1984), postponing egg laying adds to the probability of successfully hatching the clutch (Prop and de Vries 1993). The outcome of the decision when to produce a clutch may depend on the factor that is most strongly limiting reproductive output, and may thus vary depending on the environmental conditions, including climate.

Since the Arctic breeding season is restricted to the short snow-free summer, fitness is most likely limited by the time available for the offspring to become full-fledged (Owen 1980), and birds should start laying eggs as soon as body stores allow (Prop and de Vries 1993). Within the Arctic, this will hold even stronger for birds breeding in the high Arctic, where snow cover is more prolonged than in the low Arctic. A warming climate may relieve a time constraint (Gaston et al. 2005), as food becomes available earlier (Lameris et al. 2017a) and birds are able to collect more local food resources prior to laying (Hupp et al. 2018). In this way, earlier springs can have positive effects on reproductive output via increased nesting propensity (Syroechkovskiy et al. 1991; Madsen et al. 2007; Dickey et al. 2008), clutch size (Rowe et al. 1994; Van Oudenhove et al. 2014), and nesting success (Prop and de Vries 1993). However, birds may

only be able to tune laying dates to an earlier phenology of the food if they are not constrained by the timing of arrival on the breeding grounds (Both and Visser 2001; Lameris et al. 2018). This may form a particular important constraint for birds breeding at higher latitudes in the Arctic, given their longer migratory journeys (Drent and Piersma 1990) and inability to predict climatic conditions on the breeding grounds (Tombre et al. 2008; Kölzsch et al. 2015).

A graphical model serves to illustrate how climatic conditions impinge on laying dates in the low and the high Arctic (Fig. 1). This is exemplified by Arctic-nesting geese, which adjust their laying dates to date of snowmelt (Prop and de Vries 1993; Bëty et al. 2003; Madsen et al. 2007). The date of snowmelt can be used as a measure of food phenology on the breeding grounds, since the peak in food availability is linked to the date of snowmelt (Tulp and Schekkerman 2008; Lameris et al. 2018), and is a main driver of fitness (Barry 1962; Lameris et al. 2018). Decisions on when to produce a clutch then result from a trade-off between laying early, i.e. before the date of snowmelt, to leave prime conditions for the hatched offspring, and postponing laying to after the date of snowmelt to benefit from prime conditions to increase clutch size and enhance clutch survival.

Fig. 1 Graphical model exploring how laying dates could vary with climatic conditions at lower and higher latitudes. Earlier date of snowmelt (as a proxy for food phenology; white–green transition) advance the phenology of required conditions for clutch survival (upper dotted lines) and for the hatched offspring (lower dotted lines), which together drive the timing of egg laying. Laying dates are thought to vary by latitude within the Arctic. Birds at in the low Arctic (orange line) lay their eggs close to the date of snowmelt and advance laying dates in synchrony with earlier dates of snowmelt. Birds in the high Arctic (blue line) face shorter breeding seasons and, therefore, lay their eggs earlier relative to the date of snowmelt. Constrained by conditions during migration birds adjust laying dates at a lower rate than the advance in date of snowmelt (note the difference in slope between the orange and blue line) (color figure online)

Given that breeding seasons towards the North are shorter (Owen 1980; Klaassen et al. 2006) and moreover, birds are likely more constrained by conditions encountered en route, while migrating towards their breeding grounds, we propose the following three hypotheses. (1) In the low Arctic, birds will lay their eggs close to the date of snowmelt, and adjust laying dates with a changing date of snowmelt to achieve optimal conditions for their offspring (orange line with a slope of 1 in Fig. 1). (2) In the high Arctic, birds must lay their eggs earlier relative to the date of snowmelt due to the shorter breeding season (blue solid line in Fig. 1). (3) In the high Arctic, birds adjust laying dates at a lower rate than the variation in date of snowmelt as they are likely constrained by arrival on the breeding grounds, which results in a slope of the relationship between dates of laying and snowmelt of less than 1 (blue solid line in Fig. 1).

Here, we ask whether a long-distance migratory bird species breeding in the low and the high Arctic adjusts timing of reproduction optimally to climate warming, by testing the above-described hypotheses. For this purpose, we collated

data from the years 2000–2016 on dates of egg laying and reproductive output in barnacle geese (*Branta leucopsis*) from three different study populations at low- and high-Arctic sites. To understand whether the observed laying dates were optimal or resulted from a constraint (following hypothesis 3), we explored whether reproductive output peaked at intermediate laying dates, or whether geese that produced their eggs earlier gained a higher reproductive success. We determined the optimal timing of breeding by examining laying-date-specific reproductive output, focusing on the period between arrival at the breeding grounds and the moment of gosling hatch.

Methods

Study sites

Arctic-nesting barnacle geese are divided into three flyway populations, with geese breeding in Eastern Greenland,

Fig. 2 The flyways of barnacle geese breeding on Svalbard and along the Barents Sea coast (a) with location of study colonies (stars) and staging sites (white circles) in the high Arctic on Svalbard (b) and in the low Arctic at the Barents Sea coast (c). Dotted arrows sketch

migration routes. Staging site names are abbreviated: Barents Sea (ND Neruta river delta, MD Molotsnii river delta); Svalbard (HN Hornsundneset, RS Ralstrånda, LF Lognedalsflya, VB Vårsolbukta, DØ Daudmannsøyra, SØ Sarsøyra)

Svalbard and along the Barents Sea coast; wintering in Ireland, the UK and the Netherlands/Germany, respectively (Madsen et al. 1999, Fig. 2a). Between 2000 and 2016, barnacle geese were studied in three breeding colonies, of which two are located in the high Arctic (Svalbard) and one in the low Arctic (at the Russian coast of the Barents Sea). (1) On the islet Storholmen in Kongsfjorden (KF), Svalbard (78°55'N, 12°12'E, Fig. 2b); (2) on the islet Diabasøya and adjacent tundra at Nordenskiöldkysten (NSK), Svalbard (77°46'N, 13°42'E, Fig. 2b) and (3) surrounding the abandoned village of Tobseda at the Kolokolkova Bay (KB), Russia (68°35'N, 52°20'E, Fig. 2c). While these sites vastly differ in geographical position, they are all lowland sites in close proximity to the coast, which facilitates comparison. Data collection in the colonies took place in different years: Kongsfjorden (2000, 2001, 2003, 2005–2016); Nordenskiöldkysten (2004, 2010–2016), Kolokolkova Bay (2003–2009, 2014, 2015). From geographic positions of geese equipped with tracking devices (Tombre et al. 2017; Lameris et al. 2018), we determined staging sites close to the breeding grounds, which geese use prior to moving to the breeding colonies. Geese forage at these staging sites until conditions become suitable for laying in the breeding colonies (Hübner 2006; Lameris et al. 2018). We identified three proximate staging sites, which were closest to the breeding colonies (Hübner 2006), on Svalbard: Lognedalsflya (LF), Vårsolbukta (VB), Sarsøya (SØ), (Fig. 2b); and two sites around the Kolokolkova Bay: Neruta river delta (ND) and Molotsnii river delta (MD) (Fig. 2c). We further identified another three southern staging sites on Svalbard, which were at a larger distance from the colonies on Svalbard: Hornsundneset (HN), Ralstrånda (RS), Daudmannsøya (DØ).

Snow cover

We used the period between the moment of snowmelt (spring) and snowfall (autumn) as a measure of the length of the Arctic plant growth season. Snowmelt is an important driver of the timing of reproduction (Madsen et al. 2007) through its effect on the phenology of Arctic plants (Prop and de Vries 1993; Livensperger et al. 2016). Snowfall in autumn, on the other hand, puts a rigid end to feeding opportunities and thus to the breeding season. We estimated daily snow cover (as percentage cover over total area) for all study sites and years (see Table S1 for an overview of sites) for the period with sufficient daylight (26 February–30 September) using satellite images of the MODIS snow cover product (MOD10a2 version 6, Hall et al. 2006). As the areas for the breeding colonies KF and NSK were too small to determine snow cover, we instead chose the nearest coastal tundra which geese used for foraging during egg laying (Prop and de Vries 1993). To limit the effects of clouds obscuring the image, composite satellite images were generated over

8 days. Any composite images with a cloud cover exceeding 25% were excluded. A pixel (500 m resolution) was assigned as snow when classified as snow at least once during an 8-day period. With a spatial overlay of the breeding areas with the MODIS images, the number of pixels classified as snow within the study site could be retrieved. From the number of snow pixels and the total number of pixels in the breeding area, we calculated the percentage of snow cover. We linearly interpolated between values from composite images to attain a daily percentage of snow cover. From the snow cover data, we extracted the date of snowmelt, which we defined as the first day of the season at which snow cover was less than 50% (a measure which correlates with date of peak food quality; Lameris et al. 2018). Similarly, we calculated the moment of snowfall as the last day of the season at which snow cover was less than 30%. We chose this cut-off value for snowfall as higher levels of snow cover were not always reached before 30 September. We calculated season length at the breeding colony as the interval between dates of snowmelt and snowfall.

Nesting parameters

Per nest, we determined egg-laying date (date when first egg was laid), clutch size (number of eggs laid), number of hatchlings (number of eggs which successfully hatched) and nest success (whether a nest produced hatchlings) for as many nests as possible (Table S2). The parameters and the precise methodologies varied among breeding colonies. We collected data on egg-laying dates, number of hatchlings and nest fate in all colonies, and data on clutch size in KB and KF colonies. In addition, we recorded the number of nests in the colonies.

In the KB colony, we systematically searched for nests and checked nests every 2–3 days during the laying and early incubation period (late May–late June). Eggs were marked and the number of eggs was recorded at every visit. In the early and mid-incubation period, we determined clutch size as the total number of eggs in a nest when encountered with the same number of eggs during two subsequent visits. We excluded nests in which egg dumping was evident (more eggs per interval than expected or additional eggs after clutch completion). We visited nests during hatch (mid-June–late July) every 2 days to estimate date of hatch and record nesting success and number of hatchlings. We recorded nest fate as successful, predated, flooded or abandoned. A nest was considered successful when at least one chick had hatched, which we determined either by presence of hatchlings at the nest or presence of egg membranes and trampled nest rim (Davis et al. 1998). Empty nests and nests containing eggshells without membranes were considered as predated, or considered flooded when the nest was partly under water. Nests encountered after the laying

period containing cold eggs and without nest owners present were considered abandoned. We recorded the number of hatchlings when (1) at least 50% of the eggs were in the process of hatching (cracks, hatching or hatched chicks), or (2) less than 50% of the eggs had successfully hatched (thus goslings present) and other eggs did not show signs of hatching. For the number of hatchlings, we assumed all eggs with signs of hatching to produce hatchlings. Hatching success was calculated as the number of eggs hatched divided by clutch size. To minimize disturbance, not all successful nests were visited at hatch. The total number of nests found in the colony during the study period was recorded as a measure of nesting propensity.

In the KF colony, the same methods were applied, except that nests were only visited from the early incubation period onwards, and not in the laying period.

At the NSK colony, we observed the goose colony on an offshore island from an observation tower on the mainland, 200 m away from the colony. Nests were monitored 6–16 h/day during the period that nesting geese were present. Nests in view of the tower were mapped on high-resolution images of the island, which enabled us to assess the breeding history of individual birds by visual observation from laying until hatching. 30–60% of the pairs was recognizable by coded leg rings (either one or both partners carrying a ring). As we did not find a difference in any of the parameters estimated between marked and unmarked pairs, all pairs were used in subsequent analyses. To avoid disturbance, the island was not visited during the breeding period and, therefore, clutch sizes were not determined. Nest fate was established from direct observations, and rated as successful (at least one gosling was seen at the nest and no predation of eggs or goslings was observed), predated (eggs or chicks were taken by a predator, most often polar bears *Ursus maritimus*), or abandoned (nest owners abandoned the nest territory before the eggs hatched and prior to any predation event taking place—after which the eggs were usually taken by glaucous gull *Larus hyperboreus*). The number of hatchlings was recorded by visual observation of nests that successfully hatched. The first day that goslings were seen at the nest rim was taken as the date of hatch. The total number of nests was recorded for every year.

For KB and NSK, we used nest fate to calculate the nesting success as the proportion of initiated nests that successfully hatched per year. As nests were not observed during the entire incubation period in the KF colony, we did not calculate nesting success for KF. To combine nesting success and number of hatchlings into a single measure of reproductive success, we calculated the total number of expected hatchlings per nest, per laying date, year and colony, as the product of (1) nesting success and (2) average number of hatchlings in successful nests.

Laying dates

Methods to determine the date of egg laying differed among study sites. In KB, laying date was estimated by back calculation for clutches found during egg laying, assuming a laying interval of 33 h, as follows: day of discovery when one egg was found; day of discovery minus 1 at two eggs; day of discovery minus 3 at three eggs; day of discovery minus 4 at four eggs (van der Jeugd et al. 2009). Both in KB and KF, laying dates were also back calculated from hatch date. Hatch date was estimated for clutches found in the process of hatching as follows: date of observation was taken when the nest contained at least one egg with holes, a hatching chick or a wet chick; 1 day was subtracted from the date of observation when all chicks were fluffy and dry; 1 day was added to the date when the nest contained only eggs with cracks. For back calculation, we assumed a period of 29 days between laying date and hatch date (as derived from 573 nests in the KB colony between 2005 and 2015 for which both lay and hatch date were determined), which is similar to results from NSK (30 days between laying date and hatch date, derived from 99 nests for 2010–2016). In NSK, laying date was estimated as the first day during which a pair occupied a territory. Territories that were occupied for only 1 day were not considered in analyses.

Site-specific approaches in collecting data might affect the potential to make comparisons between study sites. In KB and NSK, where we used back-calculated as well as observed laying dates, the close proximity of the period between laying dates and hatch dates (see above) gives us reason to believe that these methods are comparable. By back calculating laying dates from hatching dates as done for the KF colony, we did not take into account the laying dates of nests which did not survive until hatch. However, we found no reason to suspect that this affected estimates of laying dates considerably, as extensive nest searches in the colony throughout the incubation period indicated that only few nests were lost (7.5% on average).

Statistics

We tested relationships between date of snowmelt, laying dates and reproductive success by linear models in R 3.5.1 (R Development Core Team 2018), using the package “lme4” (Bates et al. 2018). We added year and/or study site (all sites where we measured snow cover) as random factors to account for either the different years during which data were collected when a trend over years was not of interest, or to account for data from different study sites when the specific sites were not of interest. Candidate models were constructed from all possible combinations of predictor variables, including interactions which were considered ecologically meaningful. All models were compared using

Akaike's information criterion corrected for small sample sizes (AICc; Burnham and Anderson 2004) and we chose the model with the lowest AICc value as our final model. Models within 2 Δ AICc of the final model were considered as competitive as long as these did not contain extra, potentially uninformative, parameters in comparison to the final model (Arnold 2010). Model-averaged parameter estimates were obtained by the package MuMIn (Bartoń 2018). Support of the selected model (or models) relative to next best model was calculated from the ratio of model weights (Burnham et al. 2011). Besides predictor variables relating to snow cover, study year and the fitness components (clutch size, number of hatchlings and nesting success), we used predictor variables which separated high- and low-Arctic sites and staging and breeding sites, including 'area' (high or low Arctic), 'site' (all sites from which we gathered data on snow cover), 'site type' (southern staging sites/proximate staging sites/breeding colonies) and 'colonies' (the three study colonies).

First, to analyse if the snow-free period differed between the high and the low Arctic and among years, we ran linear mixed effect models (LMMs) with date of snowmelt/snowfall/season length as a response variable, year as fixed factor and area as fixed covariate, with site as a random factor. To test whether the snow-free period differed between breeding and staging sites, we ran LMMs with date of snowmelt as a response variable and site type and area as fixed factors, and with site and year as random factors.

Second, to analyse whether laying dates differed among years and between colonies, we used a linear regression model (LM) with average yearly laying dates as a response variable, and year and colony as fixed factors. To analyse how laying dates were affected by date of snowmelt, we ran LMMs with yearly average laying date as a response variable, date of snowmelt (in colonies and at proximate staging sites) and colony as fixed effects, and year as random factor. We tested whether the difference between laying dates and date of snowmelt at proximate staging sites differed between colonies by running an LMM with the difference in days as a response variable, colony as a fixed factor, and year as random factor.

Third, we aimed to analyse the association between laying date and date of snowmelt with fitness components. We ran generalized linear regression models (GLMs) with a Poisson distribution for clutch size and number of hatchlings as response variables, and GLMs with a binomial distribution with a logit link function for hatching success as a response variable. In these GLMs, we included colony as fixed factor and either date of snowmelt or laying date as fixed covariates. We tested the effects of these variables in separate models as the variables were highly correlated. A year effect was accounted for by date of snowmelt, and therefore, year was not included in the analyses as an additional covariate. We

tested the association between clutch size and number of hatchlings in an LMM with year and site as random factors. Furthermore, we ran GLMs with a binomial distribution and a logit link function with nesting success as response variable. We included either laying date and laying date squared or date of snowmelt as predictor variables. For NSK, we excluded the years 2012 and 2014, when nest success was 0. We ran LMMs with total expected number of hatchlings as response variable, included laying date and laying date squared as response variables. We ran a similar analysis for total expected number of hatchlings in a GLM per year to retrieve slopes per year per site. We tested the association between number of nests and date of snowmelt in an LM, including colony as a fixed factor.

Results

Snowmelt and snowfall

In the high Arctic, snow melted 16 ± 2 (SD) days later than in the low Arctic (19 June and 3 June in high and low Arctic, respectively, Fig. 3), and between 2000 and 2016 the date of snowmelt advanced at similar rates in the high and low Arctic by on average 0.66 ± 0.12 days/year (Table S7A; model without interaction term year and area is 3.0 times more likely than model with interaction term, Table S4A). Snow in colonies melted 4.37 ± 1.66 days later compared to the date of melt at proximate staging sites, similar in the high and low Arctic (Table S7B; model with interaction term area and site type contains more parameters, and model without is 1.4 times more likely than model with interaction term, Table S4A). As a result of earlier spring snowmelt and later autumn snowfall, the snow-free period became longer by on average 1.06 ± 1.20 days per year (Fig. 3 and Table S7D; S4D), and was 13–16 days longer in the low Arctic as compared to the high Arctic (Table S3).

Timing of reproduction

Laying date was inversely related to latitude, with barnacle geese in the high-Arctic sites laying the earliest, and geese in the low Arctic laying up to 12 days later (3 June in KF, 5 June in NSK, 10 June in KB, Figs. 3, 4). Geese advanced their egg-laying dates at a rate of 0.43 ± 0.12 days per year which did not differ between colonies (model without interaction between colony and year is 5.6 times more likely than model without interaction term, Table S5A). Laying dates were positively related to date of snowmelt at proximate staging sites (regression coefficient 0.27 ± 0.05 , Fig. 4a and Table S8B) and in colonies (regression coefficient 0.26 ± 0.05 , Fig. 4b and Table S8B). This relationship did not differ between colonies (model with

Fig. 3 Annual snow-free periods (indicated by green area) for barnacle geese in the high Arctic (**a** Kongsfjorden and **b** Nordenskiöldkysten) and in the low Arctic (**c** Kolokolkova Bay) during the years 2000–2016. Linear trends over the years of dates of snowmelt and snowfall are indicated by dashed black lines. Dates of snowmelt and

snowfall at the staging sites are indicated by green lines. Average laying dates are indicated by symbols and associated error bars (showing standard deviations), and linear trends are indicated by solid black lines (color figure online)

Fig. 4 Relationship between yearly average laying dates (shown with standard deviations) and date of snowmelt at proximate staging sites (**a**) and in breeding colonies (**b**). Lines show linear regressions, symbols and line types correspond to the three study sites: Kongsfjorden (black dots, solid lines), Nordenskiöldkysten (grey squares, dotted lines) and Kolokolkova Bay (white triangles, dashed lines)

interaction term snowmelt at proximate staging sites and year contains more parameters than model without interaction term and model without is 1.8 times more likely than model with interaction term, Table S5B). Models containing date of snowmelt at proximate staging sites gained higher support than models containing date of snowmelt in colonies (models 1 is 70.1 times more likely than model 5, Table S5B). Therefore, we performed subsequent analyses with snowmelt data of proximate staging sites. Geese in the high Arctic produced eggs on average before the date of snowmelt, while low-Arctic geese laid their eggs after the date of snowmelt (KF: 16 ± 8 days before date of snowmelt, NSK: 3 ± 7 days before date of snowmelt, KB:

10 ± 6 days after date of snowmelt, Fig. 4a, Table S8B). The number of nests was not associated with date of snowmelt (model without date of snowmelt is 4.3 times more likely than model with, Table S6A).

Clutch size

Clutch size declined with laying date with a steeper seasonal decline in the low Arctic than in the high Arctic (KF: regression coefficient = -0.037 ± 0.008 egg per day; KB: regression coefficient = -0.084 ± 0.020 egg per day, Fig. 5a and Table S9B; model with interaction term colony and laying date is 99.0 times more likely than model without interaction

Fig. 5 Clutch size (a, b), number of hatchlings (c, d) and nesting success (e, f) in relation to laying date (a, c, e) and date of snowmelt at proximate staging sites (b, d, f). Estimates are the averages by laying date across years (a, c, e), and by year (b, d, f), with error bars depicting standard errors. Lines show linear regressions resulting from model averaging, symbols correspond to the three study sites: Kongsfjorden (black dots, solid lines), Nordenskiöldkysten (grey squares, dotted lines) and Kolokolkova Bay (light grey triangles, dashed lines). In E, nesting success is depicted for KB for all years, and for NSK, only for years when nesting success was higher than 0

term, Table S6B). Clutch size also declined with date of snowmelt, at similar rates in the high and low Arctic (regression coefficient = -0.034 ± 0.004 egg per day, Fig. 5b and Table S9C; model with interaction term colony and date of snowmelt contains more parameters than model without interaction term and model without is 2.0 times more likely than model with interaction term, Table S6C).

Number of hatchlings

Hatching success showed a slight increase with laying date (regression coefficient = 0.022 ± 0.005 increase in hatching success per day, Table S9F, Table S6F) and date of snowmelt (regression coefficient = 0.021 ± 0.003 increase in hatching success per day, Table S9G, Table S6G). The number of hatchlings was positively related to clutch size

(regression coefficient = 0.24 ± 0.01), and at the same rate in high and low Arctic sites, declined with laying date (regression coefficient = -0.040 ± 0.008 hatchling per day, Fig. 5c and Table S9D; model with interaction term colony and laying date contains more parameters than model without and model without is 1.1 times more likely than model with interaction term, Table S6D) and date of snowmelt (regression coefficient = -0.015 ± 0.004 hatchling per day, Fig. 5d and Table S9E; model without interaction term colony and date of snowmelt is 3.1 times more likely than model with interaction term, Table S6E).

Nesting success

Nesting success decreased with laying date, but the precise shape of the relationship varied between colonies. In the

high Arctic (NSK), nesting success was close to zero in 2 years when all nests were predated by polar bears (2012 and 2014). When excluding these years (see “Methods”), nesting success was higher for nests initiated on earlier dates and differed between the low and the high Arctic (NSK: regression coefficient for laying date = -0.86 ± 0.92 ; for laying date squared = 0.002 ± 0.003 ; KB: regression coefficient for laying date = 0.74 ± 1.99 ; for laying date squared = -0.002 ± 0.006 , Fig. 5e and Table S9H, model with interaction term colony and laying date is 99.0 times more likely than model without interaction term, Table S6H). Nesting success was affected by date of snowmelt (NSK: regression coefficient = -0.028 ± 0.013 decrease in nesting success per day; KB: regression coefficient = 0.011 ± 0.040 , Table S9I, model with date of snowmelt is 1.7 times more likely than model without, Table S6I). However, this effect appeared to be largely caused by differences between colonies (Fig. 5f) and was no longer present in the best performing models when standardizing variables by subtracting the site-specific means of nesting success (model without date of snowmelt is 10.1 times more likely than model with, Table S6J). When comparing peak date of expected number of hatchlings with average laying dates in the high-Arctic colony NSK, geese nested later than the date of peak expected success. In the low-Arctic colony, geese nested in synchrony with the date of peak expected success (Figure S1).

Discussion

Advance of egg laying

Congruent with our hypothesis, we found that in the high Arctic geese started laying eggs well before the date of snowmelt, while geese in the low Arctic produced their eggs close to the date of snowmelt. This confirms the idea that the short summer in the high Arctic causes geese to produce eggs relatively early. Surprisingly, while in the high Arctic the snow melted on average 16 days later compared to the low Arctic, average dates of laying in the three study colonies were inversely related to latitude, with geese in the most northern colony laying eggs earlier than in the southern colony. This is in contrast to the finding that the onset of the birds' breeding season is later at higher latitudes (Owen 1980). Apparently, in our study species individuals are able to acquire body stores for breeding earlier in the high Arctic than in the low Arctic. This contra-intuitive result may arise from high-Arctic geese drawing more from endogenous body stores for egg production (Hahn et al. 2011), and from benefitting from mosses and woody plants available at the very first start of snowmelt (Prop and de Vries 1993; de Fouw et al. 2016). Such an early surge of food, albeit

low-quality, is lacking in the low Arctic, where geese depend entirely on graminoids which appear later in the season (van der Graaf et al. 2004, 2006).

We found that both high- and low-Arctic-breeding barnacle geese advanced egg laying at a lower rate than the advance in date of snowmelt (0.27 days advance in laying date per earlier day of snowmelt). This is in line with our hypothesis on laying dates by geese in the high Arctic, where advancements in breeding are likely constrained by a timely arrival at the breeding grounds (Both and Visser 2001; Lameris et al. 2017b). However, we expected that low-Arctic geese would be able to synchronize egg laying with earlier snowmelt as their migration distance is shorter and the continental migration route might enable the geese to track the recession of snow. Our results suggest that low-Arctic geese may be experiencing similar constraints during migration as high-Arctic geese due to a low correlation between climatic conditions along their route (Kölzsch et al. 2015). Alternatively, the observed slow advancement in laying dates in the low Arctic might follow from a set of optimal decision rules (Visser et al. 2012) with highest fitness benefits associated with the observed laying dates.

Laying dates, timing of snowmelt and fitness components

We did not find a relation between the date of snowmelt and the number of nests, which we use as a proxy of breeding propensity. While several species of geese show a lower breeding propensity in years with a late onset of spring (Reed et al. 2004; Madsen et al. 2007), this appears not to be the case for barnacle geese. This can potentially be explained as barnacle geese primarily breed in coastal areas, where snow-free patches can be found even in years with a late date of snowmelt.

We found a seasonal decline in clutch size and number of hatchlings in both high- and low-Arctic colonies, meaning that early-laying birds produced more eggs and hatchlings. We also found that high- and low-Arctic geese produced larger clutches in earlier springs (a difference of on average 0.6–1.2 eggs between the earliest and latest snowmelt years) and produced more hatchlings (a difference of on average 0.5–0.6 hatchlings between the earliest and latest snowmelt years). A seasonal decline in clutch size is common in geese and birds in general (Drent and Daan 1980; Crick et al. 1993; Rowe et al. 1994; Dalhaug et al. 1996), just as smaller clutches in later springs (Barry 1962; Dalhaug et al. 1996; Bêty et al. 2003; Van Oudenhove et al. 2014).

We found nesting success to be strongly associated with laying date, with a higher probability of hatching at intermediate laying dates in the low Arctic and at early laying dates in the high Arctic, without an additional effect of date of snowmelt. Nest failure in Arctic geese is usually attributed

to depletion of body stores by the incubating females (Prop et al. 1984), or to nest predation, such as by glaucous gulls (van der Jeugd et al. 2003), Arctic foxes *Vulpes lagopus* (Jensen et al. 2014) and polar bears (Prop et al. 2015). Geese settling early and well before the peak in laying dates may experience intense predation pressure, as predators are focusing on the few early nests (Findlay and Cooke 1982). Our finding that early-initiated nests in the high-Arctic are the most successful is in contrast with previous studies, which showed that early breeding geese experienced low nesting success compared to geese starting at intermediate dates (Prop and de Vries 1993; Spaans et al. 2007). This shift is likely caused by polar bears recently moving towards land under climate warming (Iverson et al. 2014; Prop et al. 2015) and predating substantial numbers of nests in bird colonies (Rockwell et al. 2011; Prop et al. 2013, 2015). Our observations indicate that early-initiated nests have a chance to hatch before polar bear arrival and thus may escape predation. This implies that climate warming does not only directly drive reproduction phenology by timing of snowmelt, but also indirectly via changes in predator community composition (Descamps et al. 2017).

Considering our measures of reproductive output, low-Arctic geese lay their eggs at the time of the peak in expected reproductive output (see also Figure S1, van der Jeugd et al. 2009). This suggests that the observed slow advancement in laying dates of low-Arctic geese does not reflect a constraint, but is in line with maximum reproduction output, at least up to the period of hatching. If this is the case, the observed laying dates of low-Arctic geese may be explained as the importance of beneficial conditions for the clutch outweighing those for hatched offspring in years with earlier snowmelt. In contrast, most high-Arctic geese lay their eggs after the expected peak in reproductive output. This suggests that in line with our expectations, high-Arctic geese face a constraint that limits a stronger advancement of laying dates or changes have been too rapid for them to adjust their migration phenology.

Conclusions

From the moment onwards that the heterogeneity of climate warming effects has been recognized (Gill et al. 2012), high-Arctic communities are supposed to be especially vulnerable to climate warming (Høye et al. 2007; Post et al. 2009) and are expected to show strong advancements in phenology to counter any negative impacts. We show that both in the high- and low-Arctic, barnacle geese do not advance date of egg laying in pace with earlier dates of snowmelt. However, only in the high Arctic, this advancement appears to be insufficient to reach the level of reproductive success associated with earlier laying dates. For high-Arctic geese,

an advance in laying dates may be particularly constrained by the timing of migration, which is thought to be tuned to climate conditions en route rather than to the weather in the Arctic (Tombre et al. 2008; Kölzsch et al. 2015). Given the potential risks of fitness reductions due to phenological mismatches under relatively slow advancement of laying dates (Lameris et al. 2018), high-Arctic bird populations in particular may be prone to negative effects of climate warming.

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Author contribution statement TKL, MEJ, MJEL and JP conceptualized the study, TKL, MEJ, MJEL, HPJ, KEL, BAN and JP conducted the fieldwork, MPB provided data, TKL analysed the data, TKL wrote the initial manuscript and all authors contributed to the final manuscript.

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Compliance with ethical standards

Conflict of interest The authors declare to have no conflicts of interest.

Ethical approval All applicable institutional and national guidelines for the care and use of animals were followed during this study.

Data storage Data is stored online at Mendeley data at <https://doi.org/10.17632/r77sr2t5hz.1>.

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Part II.

Environmental contamination

Chapter 4.
Mercury associated neurochemical
response in Arctic barnacle goslings
(*Branta leucopsis*)

Mercury associated neurochemical response in Arctic barnacle goslings (*Branta leucopsis*)

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HIGHLIGHTS

- Arctic coal mine impacted site showed elevated Hg concentrations.
- Differences in soil Hg were reflected in hepatic concentrations of goslings.
- Brain levels of D2-receptors in Arctic birds were related to hepatic Hg levels.
- The use of siblings increased the statistical resolution of the experiment.

GRAPHICAL ABSTRACT

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ABSTRACT

There remains great concern over mercury pollution in the Arctic, though relatively little is known about impacts on biota that inhabit Arctic terrestrial systems. To help address this, the current study was performed with barnacle goslings (*Branta leucopsis*) from a coal mine-impacted site and a control site near Ny-Ålesund, Spitsbergen (Svalbard). The works focused mainly on mercury, as coal contains trace levels of this element. Total mercury concentrations were quantified in soil and vegetation from the two sites, as well as feces and liver from the goslings. Next, the mercury exposures were related to dopamine 2 (D2)- and NMDA-receptors in the brain, given that mercury is a proven neurotoxicant. Soil and vegetation in the mining area contained mercury levels that were approximately 3- and 2.2-times higher than in the control site. Despite a significant difference between the sites, the soil and vegetation mercury levels were within ranges found at other Arctic locations. Goslings grazing in the mine-impacted area contained significantly higher hepatic mercury levels than those sampled from the control site. Compared to other species, the hepatic concentrations were relatively low possibly due to dilution of the mercury in growing goslings (growth dilution) and deposition of mercury in the growing feathers. Hepatic mercury concentrations were positively related to D2-neuroreceptor levels but not to NMDA-receptor levels thus suggesting a possible subtle neurological effect. To our knowledge, this is among the first studies on mercury exposure in Arctic terrestrial organisms, and one of the first to document potential subtle neurological responses associated with exposure to low, environmentally relevant mercury levels, which also can be found at other locations in the Arctic. However, as a pilot effort, the results here need to be examined in

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1. Introduction

Mercury (Hg) is a trace metal found throughout all parts of the world (Driscoll et al., 2013). Depending on its speciation, mercury has the tendency to accumulate in food webs (Douglas et al., 2012), resulting in elevated levels in organisms at higher trophic levels and potentially impacting exposed individuals (Kobiela et al., 2015). Due to biochemical processes, mercury is subject to long-range atmospheric transport and can thus deposit in remote areas such as the Arctic (Douglas et al., 2012). In addition to long-range transport, human activities in the Arctic like coal mining have resulted in locally elevated concentrations of mercury (Poissant et al., 2008). After deposition, mercury can be transformed into organic methylmercury (Gamberg et al., 2015) which is more bioavailable (Poissant et al., 2008) and generally more toxic (Boening, 2000). Over 90% of mercury in Arctic organisms can be attributed to anthropogenic sources (Dietz et al., 2009), ranging from e.g. non-ferrous metal, iron and steel production, cement production, waste incineration and coal fired power generation to artisanal gold production (Muntean et al., 2014). Temporal trends in mercury concentrations vary among Arctic sites, but it has been shown that on average concentrations in Arctic biota have increased 0.5% annually (Riget et al., 2011).

In the Arctic, exposure to mercury and associated responses have been illustrated in a number of marine mammals birds, and fish species (Dietz et al., 2011). However, although recent data on mercury concentrations in terrestrial Arctic ecosystems are available on e.g. Arctic soils (Choy et al., 2010; Krajcarová et al., 2016; Wojtun et al., 2013), vegetation (Choy et al., 2010; Krajcarová et al., 2016; Poissant et al., 2008; Wojtun et al., 2013), caribou (*Rangifer tarandus*) (Braune et al., 1999; Gamberg et al., 2015; Riget et al., 2004), Arctic fox (*Vulpes lagopus*) (Bocharova et al., 2013; Dehn et al., 2006; Treu et al., 2017), wolf (*Canis lupus*) (McGrew et al., 2014) and arctic hare (*Lepus arcticus*) (Pedersen and Lierhagen, 2006), we are not aware of studies that have aimed to link responses of Arctic terrestrial species to exposure to environmental mercury. Exposure to mercury may lead to a range of adverse health outcomes in wildlife even at low chronic exposures (Becker et al., 2017; Hawley et al., 2009; Scheuhammer et al., 2012; Spalding et al., 2000), such as embryo toxicity in birds (Yu et al., 2016), immune modulation in birds (Fallacara et al., 2011; Hawley et al., 2009) and mammals (Becker et al., 2017; Kim et al., 2003) and neurochemical and morphological effects in the brains of different species (Arini et al., 2016; Basu et al., 2007b; Nam et al., 2012; Yu et al., 2017). Effects of mercury on levels of neuroreceptors may be induced via different pathways. For examples mercury may affect the stimulation of the *N*-methyl *D*-aspartate receptor (NMDA receptor) via interaction with the uptake of glutamate in synapses (Basu et al., 2007b). Mercury may lower the activity of monoamine oxidase (Bernstsen et al., 2003), which is involved in the metabolism of dopamine, a pathway along which mercury may induce dopamine receptor-mediated effects. Several studies have focused on the effects of modulation of D2 receptors on the behavior of animals. For example, female chickens (*Gallus gallus domesticus*) injected with a D2-receptor antagonist showed decreased pecking behavior (Kjaer et al., 2004) and, reduced aggression (Dennis and Cheng, 2011), while exposure to a D2-receptor antagonist suppressed head movements and foraging behavior (Moe et al., 2014). In turkeys (*Meleagris gallopavo*), injection with a D2-receptor antagonist decreased brooding behavior (Thayananuphat et al., 2011), while D2 receptor expression in starlings (*Sturnus vulgaris*) was negatively related

to vocal activation (DeVries et al., 2015). Although this overview on effects of D2-receptor levels is not exhaustive, it illustrates that changes in D2 receptor levels, potentially induced by exposure to mercury, may affect organismal behavior.

To address the knowledge gap on neurotoxic effects that mercury may have on Arctic terrestrial species, an exposure experiment was conducted with barnacle goslings (*Branta leucopsis*) from the Arctic tundra. Barnacle goslings were selected because at this age, they are not exposed to other than local sources of mercury (apart from maternal transfer), they ingest (contaminated) grid and vegetation, and they can be imprinted on humans allowing to guide them to specific locations, and as such their exposure can be manipulated. In this experiment, human-raised goslings were exposed to locally deposited mercury, related to historic coal mining activities, over the course of their development. Two groups of goslings were led systematically to either a mercury contaminated site or a control area, under environmental relevant conditions. Detailed analyses of exposure, internal mercury concentrations and specific levels of neuroreceptors, D2 and NMDA receptors in the brains, were performed to gain a comprehensive insight in the relationships in species specific exposure and effects of mercury. It was expected that tissue concentrations of mercury in goslings are elevated in individuals feeding in the mining areas, and that brain D2 receptor levels are positively, but NMDA receptor negatively related to mercury levels.

2. Materials and methods

2.1. Sites

The experiment was conducted in the vicinity of Ny-Ålesund, Spitsbergen (Svalbard, 78.55°N, 11.55°E) (de Jong et al., 2017). Near Ny-Ålesund, different coal mines were in operation from 1916 to 1963, approximately 1 km SE of the village, with intermittent periods of inactivity. In 1967, the activities terminated after a fatal incident and the mine was abandoned. In the mining area, however, remains of the mining activities are still clearly visible. Large piles of coal and abandoned installations and equipment are littered in the area. Since the area was deserted, vegetation has re-established to a certain extent, which is available for grazing and geese are known to utilize the area (pers. obs). The area was expected to be contaminated by coal-associated chemicals, among which mercury (Hylander and Goodsite, 2006). The control area is a vegetated tundra, 2 km WNW of the village, which is also used by geese to graze (pers. obs.).

2.2. Goslings and experimental design

Barnacle goslings were collected from Indre Brøygane, an uncontaminated island near Ny-Ålesund on June 30th 2014, as described in de Jong et al. (2017). Pipping eggs were marked at June 29th in order to ensure that goslings hatched on the same day. From 8 nests, two siblings each were collected, immediately marked with web-tags as well as a unique color ring, and randomly assigned to either the "control" or "mine" group. Eight goslings per group was the maximum that could be handled, and mimics the high end of natural goose families. Goslings were raised by humans as foster parents, and from day 5 onwards were herded to their respective areas for grazing (Martin and Forsyth, 1998). At the beginning of the experiment, goslings spent about 160 min per day in their respective grazing areas and

this increased to about 360 min per day later on. This gradual shift mainly depended on age of the goslings and weather conditions. On rare occasions, and particularly at the beginning of the experiment, all goslings were provided with measured amounts of supplemental feed (Anseres I waterfowl starter pellets, Kasper Faunafood, Woerden, The Netherlands) during their walks to ensure they were not nutritionally deprived and kept healthy during periods of bad weather.

The goslings were 23 days old when they were sacrificed through decapitation and immediately dissected (de Jong et al., 2017). Liver tissue was collected for mercury analyses and stored in polyethylene blue cap tubes at -20°C . Because concentrations were likely to be relatively low in most tissues, liver was selected since concentrations were expected to be highest in this tissue (Tsipoura et al., 2011). Brains were snap frozen in liquid nitrogen and stored in blue cap tubes at -80°C for analysis of neuroreceptor levels.

2.3. Soil, vegetation and dropping sampling

Soil was collected during the experiment from both sites randomly ($n = 7$ for mining area, $n = 6$ for control) from the surface (upper 5 cm) with a large PVC spoon. Plant material and stones were removed from the soil samples. Samples were stored in polyethylene bags at -20°C , and shipped frozen to the Netherlands. Vegetation from the same locations of the soil was clipped with scissors to collect above-ground plant parts from the two sites ($n = 4$ each site). Plant material was also stored in polyethylene bags at -20°C . As much as possible, single floral type samples were collected from moss, as well as *Carex* spp., and *Saxifraga* spp.

As an indication of actual exposure, droppings from goslings were collected after they had foraged for a minimum of three hours in their respective areas. Gut passage time in adult barnacle geese is approximately 2–4 h (Prop and Vulink, 1992), and droppings produced after being at a site for such period may be used as a proxy of actual mercury exposure. Within this time frame, droppings of goslings that foraged in the mining area turned dark likely because of the occurrence of coal particles in their droppings. Droppings from birds feeding on the additional feed were also collected at night, after having foraged on the additional feed for a minimum of 4 h, in order to assess potential exposure to mercury related to this feed source. No additional feed was available at the time of mercury analysis, but in this indirect way it is possible to assess the actual exposure of the goslings to the additional feed, relative to the vegetation from the two sites. Droppings were stored at -20°C in polyethylene bags ($n = 6$ for control site; $n = 5$ for mining site; $n = 6$ for additional feed).

2.4. Chemical analyses

Soil, vegetation droppings and liver tissues (concentrations in other tissues like e.g. brains, were expected to be too low for analyses) were analyzed for total mercury with cold vapor/atomic fluorescence spectrometry (Hoogenboom et al., 2015). Approximately one gram of dried sample digested in 10 mL nitric acid (70%) heated in a microwave. After digestion, samples were filled up to 50 mL with Milli-Q ultrapure water. All mercury species were reduced to metallic mercury with Sn(II)Cl_2 released from solution and quantified in their gaseous phase by fluorescence at 253.7 nm. All concentrations given are on total mercury, based on dry-weight (70°C , 48 h). A certified reference material was always included in the analyses. Samples were analyzed under ISO9001 accreditation and ISO 17025:2005 standard. The laboratory participates in inter-laboratory performance studies, including those organized by QUASIMEME (www.quasimeme.org). The results on certified reference materials (fish and fish liver) have always been labelled as “good”, according to the evaluation criteria.

2.5. Biochemical analyses

Membranes were prepared from gosling brains following Arini et al. (2016), with slight modifications. Of each sample, 1 g of cerebrum was homogenized in 10 mL (i.e. 1:10 average weight) of 50 mM Tris buffer (50 mM Tris HCl, 50 mM Tris Base, pH 7.4). Membranes were isolated by centrifugation of the homogenates at 48,000g for 15 min at 4°C . The resulting pellets were resuspended in 10 mL of Tris buffer. This operation was repeated twice for a total of three centrifugations per sample, after which each final pellet was resuspended in 3 mL of Tris and aliquoted. Aliquots were immediately frozen at -80°C until further analysis for neuroendocrine receptor-binding assays.

Radioligand binding to the NMDA and D2 receptors were performed using cellular membranes following Arini et al. and Basu et al., respectively (Arini et al., 2016; Basu et al., 2009). For NMDA, 300 $\mu\text{g/mL}$ of membrane preparation was incubated with $[3\text{H}]\text{-MK-801}$ (5 nM, 22.5 Ci/mmol; Perkin Elmer), and slowly vortexed for 120 min at room temperature. Non-specific binding was determined by incubating samples with 100 μM unlabeled MK-801. To minimize non-specific binding, plates for D2 were pre-wetted with a polyethyleneimine buffer (0.1%). Samples (300 $\mu\text{g/mL}$ of membrane preparation) were incubated first with 50 μM ketanserin (to block serotonin receptor binding) and next with $[3\text{H}]\text{-Spiperone}$ (3.2 nM, 15.3 Ci/mmol; Perkin Elmer) and slowly vortexed for 90 min at room temperature. Non-specific binding was determined by incubating samples with 100 μM unlabeled Butaclamol. All samples were assayed in quadruplicate and pooled control samples (chicken brain) were used to monitor variability between plates. Specific binding was defined as the difference between radioligand bound in the presence or absence of the respective displacers.

2.6. Statistical analyses

Analyses of Variance (ANOVA) were performed to assess differences in mercury concentrations in soil and vegetation between site (control/mine as a factor) and to analyze differences in weight, mercury concentrations and receptor levels among siblings (“Sibling” as factor). Least-significant-differences were used as post-hoc test. As mercury levels were not normally distributed (Shapiro-Wilk-test), we log-transformed the residue data prior to an ANOVA. To exclude the effect of sibling on further statistical analyses, receptor levels and hepatic mercury were normalized for the effect of sibling by subtracting the average of each sibling pair from the two corresponding individual sibling observations. The sibling-normalized observations were also analyzed for factorial effect of “Site” by performing ANOVA. To correlate potential relationships between receptor levels (both D2 and NMDA receptor) and log-transformed mercury concentrations, linear regressions were used. All tests are given two-tailed with an α of 0.05. Statistical analyses were performed with GENSTAT version 18.1 (VSN International Ltd., Hemel Hempstead, UK).

3. Results and discussion

3.1. Gosling development

On average, control and mine goslings did not differ in mass at the end of the experiment (de Jong et al., 2017), but the average mass of the siblings marked “Black” was significantly higher than the others (Table S11, ANOVA: $F = 3.63$; $n = 16$; residual d.f. = 8; $p = 0.046$). The lack of differences between sites was likely due to the fact that the goslings received additional feed, which was provided not to initiate growth limitation, potentially affecting the toxicokinetics/dynamics of mercury in the goslings. No additional significant differences in somatic indices e.g. relative organ weights, could be detected between or within the two groups of goslings (Table S11).

3.2. Mercury concentrations

3.2.1. Soil and vegetation

Mercury concentrations were significantly higher in soils from the mining area in comparison to the control area (Fig. 1A; ANOVA: $F = 7.68$; $n = 13$; residual d.f. = 11; $p = 0.018$). The mercury soil concentrations of both areas are approximately an order of magnitude lower than in soils from more industrialized areas in e.g. the Netherlands (Roodbergen et al., 2008) or Slovenia (Gnamuš et al., 2000), and reaching the lower soil concentrations in Switzerland (Ernst et al., 2008). Mercury levels in soil peat in the north of Norway as well as in peat soils from sub-Arctic locations at the Faroe Islands were approximately 5–10 times higher than the ones of the current study (Riget et al., 2000; Shotyik et al., 2005). Mercury soil concentrations from East Greenland were below detection limits (<0.01 mg/kg dry), while concentrations ranged from 0.01 to 0.03 mg/kg dry weight in soils from three locations from West Greenland (Riget et al., 2000). The latter concentrations are similar to the levels found in our control site. In a more recent study at the northwestern side of the Hornsund fjord, Svalbard (77.00°N, 15.33°E), without local contamination, soil mercury concentrations ranged from 0.01 to 0.25 mg/kg dry weight, depending on the type of tundra (Wojtun et al., 2013). In a study near the town of Pyramiden, Svalbard (78.39°N, 16.20°E), with known coal mining activities in the past, mercury soil concentrations ranged from 0.004 to 0.736 mg/kg (Krajcarová et al., 2016). The concentrations detected in soils from the control site of the current study are in the lower range of the Pyramiden study, while the soils in the mine area contained mercury in the higher ranges of those found in Hornsund and the lower range of Pyramiden. This indicates that the concentrations detected in soil and vegetation from both our sites may be indicative for larger areas in the Arctic. Although differences between sites were relatively small (though significant), and a more contaminated site may have provided better options to detect toxic effects, it was decided to perform the study at the selected sites, as these are representative for true Arctic conditions.

The difference in soil concentrations between sites is reflected in the mercury concentrations in vegetation, albeit to a smaller degree (ratio between sites: 2.9 in soil versus 2.3 in vegetation, all samples combined). No significant differences could be detected among species (*Carex* spp. versus *Saxifraga* spp. versus moss; ANOVA: $F = 3.7$; $n = 8$; residual d.f. = 5; $p = 0.103$), hence all species are pooled to assess differences between sites. Concentrations in the vegetation (log-transformed) from the mining area were significantly higher (Fig. 1B, 0.060 versus 0.026 mg/kg dry weight; ANOVA: $F = 8.58$; $n = 8$; residual d.f. = 6; $p = 0.026$). Biota to Soil Accumulation Factors (BSAFs) with spatially matched soil and vegetation samples were significantly

higher in the control versus the mining vegetation (0.89 versus 0.40; ANOVA: $F = 25.16$; $n = 8$; residual d.f. = 6; $p = 0.002$). Mercury levels in moss samples ($n = 2$), which averaged 0.08 mg/kg, were in the same range as found in mosses from the US (Landers et al., 1995) and Canadian Arctic (Choy et al., 2010) and also from locations north of the Arctic circle in Finland (Poikolainen et al., 2004). Concentrations in vascular plants from the Hornsund fjord (Svalbard) ranged from 0.01 to 0.09 mg/kg dry weight (Wojtun et al., 2013), again similar our findings (0.02 to 0.06 mg/kg dry weight).

To summarize, concentrations from both soils and vegetation indicate that the mercury levels from both sites of the current study fell well into the range of previously reported mercury concentrations in soil and vegetation from various Arctic locations. Still, there were significant differences in concentrations in soil and vegetation between the mine contaminated versus control site in our study.

3.2.2. Droppings

Mercury concentrations (log-transformed) in droppings from goslings that fed in the mining area were significantly higher than in droppings collected from control goslings (Fig. 2, 0.086 versus 0.048 mg/kg dry weight; ANOVA: $F = 8.42$; $n = 16$; residual d.f. = 14; $p = 0.004$). The slightly higher mercury concentrations in droppings relative to the concentrations in vegetation indicate that the uptake rate of organic matter from the vegetation exceeds the uptake rate of mercury. Also the droppings of goslings that foraged longer than 4 h on supplemental feed, which both groups received overnight, contained mercury, at levels similar to the droppings from the control site (Fig. 2). This implies that both groups were exposed to mercury as a result of the supplemental feed they received, which might have (partially) decreased potential differences between the groups.

3.2.3. Liver tissue

Overall, hepatic mercury levels in goslings were relatively low, i.e. 0.02–0.04 mg/kg dry weight. Still, mercury levels in goslings which foraged in the mining area were significantly higher than in the ones from the control group (Fig. 1C, 0.030 versus 0.022 mg/kg dry weight; ANOVA: $F = 5.16$; $n = 16$; residual d.f. = 14; $p = 0.039$). After normalization for sibling, the significance of the difference between sites increased considerably (ANOVA: $F = 11.84$; $n = 16$; residual d.f. = 14; $p = 0.004$). Hepatic mercury concentration (log-transformed) did not correlate significantly with gosling mass, however (linear regression: variance ratio = 2.42; $n = 16$; residual d.f. = 14; $p = 0.142$).

Biomagnification Factors (BMFs: Hg ratio between concentrations in liver versus vegetation, based on dry weight) differed between the two sites, i.e. 1.2 for the control and 0.4 for the mining site. The lower BMF for the mining site may be due to goslings receiving supplemental

Fig. 1. A. Average mercury concentrations in soils from control and mining area. B. Average mercury concentrations in vegetation from control and mining area. C. Average mercury concentrations in livers of gosling herded in control and mining area (all: mg/kg dry matter (DM); mean + standard error). All concentrations differ significantly between sites, for (statistical) details see text.

Fig. 2. Mercury concentrations in droppings from goslings that foraged at least three hours on respectively additional feed, in the control or mining area (mg/kg dry matter (DM); mean $\pm$ standard error). For (statistical) details see text.

feed, which contained low mercury levels, similar to vegetation in the control area (see above). Since all goslings received additional feed, the resulting mercury hepatic concentrations were likely affected, thus lessening any potential difference among the two treatment groups. The BMFs indicate only limited accumulation at both sites. Terrestrial breeding snow buntings (*Plectrophenax nivalis*) from Devon Island Canada showed whole body mercury concentrations of approximately 0.18 mg/kg (Choy et al., 2010), which is considerably higher than concentrations found in the current study. However, in the former study mercury may have been marine derived, as birds bred adjacent to a seabird colony (Choy et al., 2010). Concentrations in livers of Arctic seabirds, which generally feed at a much higher trophic level than geese, ranged from 0.9–9.7 mg/kg dry weight, one or two orders of magnitude higher than concentrations in the current study (Provencher et al., 2014). Threshold levels for seabirds are in the order of >30 mg/kg wet weight in liver (Fisk et al., 2005), but adult seabirds are thought to be less sensitive to mercury exposure than terrestrial species (Thompson, 1996). For non-marine birds, an effect threshold for adult birds has been derived at approx. 2 m/kg wet weight for reproduction (Shore et al., 2011), which is not applicable for growing goslings, however. As far as we know, there are no threshold levels available for developing chicks. This renders interpretation of our results difficult. Furthermore, mercury concentrations at the moment at which the goslings were sacrificed were presumably considerably lower than concentrations at a younger age. This is due to dilution by increase of somatic mass prior to sacrifice, together with the development of feathers, for which mercury has a high affinity. At the time of sacrifice, feather development was well on the way, but not completed, and therefore it is likely that mercury concentrations would have been higher when goslings were sampled at an earlier age. This idea is corroborated by Ackerman et al. (2011), who found mercury concentrations in chicks of Forster's tern (*Sterna forsteri*), black-necked stilt (*Himantopus mexicanus*) and American avocet (*Recurvirostra americana*) to be highest shortly after hatching, followed by a rapid decline when chicks aged.

3.3. Neurochemical receptors

Levels of D2 and NMDA receptors in the gosling brains were 404 ± 47 and 752 ± 48 fmol/mg protein (mean $\pm$ standard error), respectively. Overall, D2 and NMDA receptor levels did not differ significantly between sites (Table S12). However, when corrected for sibling pair, D2 levels, but not NMDA levels, had a tendency to be higher in goslings from the mining versus the control site (Table S12, D2: ANOVA: $F = 4.37$; $n = 16$; residual d.f. = 14; $p = 0.055$; NMDA: ANOVA: $F = 0.27$; $n = 16$; residual d.f. = 14; $p = 0.609$). There was a significant effect of sibling group on the levels of D2 in the brains of the goslings: D2 levels of both siblings from one pair (marked 'black') were significantly lower, whereas levels of another pair (marked 'red') were significantly

higher in comparison to the other six sibling groups (Table S11, ANOVA: $F = 6.56$; $n = 16$; residual d.f. = 14; $p = 0.008$). We found no such effect on NMDA levels (ANOVA: $F = 1.97$; $n = 16$; residual d.f. = 8; $p = 0.181$). D2 and NMDA levels were negatively correlated with body mass (linear regression: D2: variance ratio = 17.91; $n = 16$; residual d.f. = 14; $p < 0.001$; NMDA: variance ratio = 7.54; $n = 16$; residual d.f. = 14; $p = 0.016$). Moreover, D2 levels in brains correlated positively with hepatic mercury concentrations, (Fig. 3, linear regression: variance ratio = 4.71; $n = 16$; residual d.f. = 14; $p = 0.048$), even more in case of sibling normalized D2 and mercury data (linear regression: variance ratio = 8.15; $n = 16$; residual d.f. = 14; $p = 0.013$). NMDA levels, on the other hand, did not correlate with hepatic mercury levels (linear regression: variance ratio = 0.00; $n = 16$; residual d.f. = 14; $p = 0.985$).

Mercury-associated changes in D2 receptors have been observed in previous studies, although the mode of toxicity is somewhat equivocal. For example, exposure of Sprague–Dawley rat dams (*Rattus norvegicus domesticus*) to methylmercury resulted in a significant increase in D2-receptor densities in male offspring (Cocchini et al., 2011). Similarly, the density of hypothalamic D2 receptors was positively correlated with Hg concentrations in yellow perch (*Perca flavescens*) exposed in the laboratory to dietary methylmercury (0.5 to 50 ppm) (Arini et al., 2016). In contrast, D2-receptor densities in field-exposed mink (*Mustela vison*) were negatively correlated with mercury exposure (Basu et al., 2005). None-unidirectional effects of mercury on dopaminergic endpoints have been illustrated in the literature before. For instance, in yellow perch, exposure from 0 to 5 PPM of MeHg in the feed resulted in increased D2-receptor levels, which however at 50 PPM decreased again (Arini et al., 2016). In Atlantic salmon (*Salmo salar*), superoxide dismutase levels in brain increased at low exposure levels mercury, however at higher exposure this was decreased, which was accompanied by decreased activity of monoamine oxidase (a neural enzyme) (Berntssen et al., 2003). Such decrease in monoamine oxidase activity was also evident in river otters (*Lontra canadensis*) exposed to mercury (Basu et al., 2007a). These literature data indicate that, although there is a relationship between mercury exposure and D2 densities, the direction of the association may vary according to species or exposure scenarios, which makes it difficult to speculate on specific further impacts on e.g. behavior of the goslings.

In contrast to the D2 levels, NMDA receptor levels were not correlated with hepatic mercury concentrations in goslings. *In ovo* exposure of thick-billed murre (*Uria lomvia*) and Arctic terns (*Sterna paradisaea*) to mercury did show any changes in NMDA levels in the chicks' brain (Braune et al., 2012). Furthermore, in herring gulls (*Larus argentatus*), there was no relationship between mercury levels and NMDA receptors (Rutkiewicz et al., 2010). In contrast, however, mercury concentrations

Fig. 3. Relationship between hepatic mercury concentrations (mg/kg dry matter (DM)) and levels of D2 in the brain of barnacle goslings (fmol/mg protein; all observations from control and mining sites combined). For (statistical) details see text.

correlated negatively with NMDA levels in discrete regions of the brain of mink (Basu et al., 2007b). At much higher hepatic mercury concentrations (average 8 mg/kg dry weight), NMDA receptor levels were negatively correlated with mercury concentrations in bald eagles (*Haliaeetus leucocephalus*) (Rutkiewicz et al., 2011). Polar bears (*Ursus maritimus*) containing relatively low levels of brain mercury, i.e. 0.3 mg/kg dry weight, also showed a decrease in NMDA levels at elevated mercury concentrations (Basu et al., 2009). Hence, the absence of a relationship between NMDA receptor levels and total hepatic mercury concentrations in the current study may be due to a number of reasons. Foremost is that the mercury levels are just too low to elicit any response. In addition, here we did not measure in the brain (hepatic mercury was used) while NMDA was measured in the whole brain versus discrete regions.

Our data show that contaminants related to historic mining activities may be linked to decreased levels of D2 receptor levels in gosling brains. In this first study we focused on mercury, as this is one of the major contaminants in coal that has neurotoxic properties. Other contaminants, such as polycyclic aromatic hydrocarbons (PAHs), may also affect organisms which feed in coal contaminated sites. PAHs, however, express toxic modes of action other than neurotoxicity. The hepatic mercury levels at which D2 receptor levels showed declines were lower than found in other studies. In our study, birds were systematically exposed to elevated, although environmentally relevant, levels of mercury. The assignment of siblings from the same nest to each treatment group allowed to include this source of variation in the statistical analyses. The results indicate that such background may need to be considered when interpreting (neurotoxic) effects of chemicals under field conditions. Nevertheless, some issues warrant further investigations, e.g. the limited design of the current (pilot) study, the potential influence of e.g. selenium or other compounds on the toxicokinetics/dynamics of mercury in developing goslings and further potential impacts of mercury associated decreases in D2 levels on e.g. behavior of goslings. Notwithstanding these limitations, we conclude that in this Arctic breeding goose species, relatively low mercury exposures may be associated with subtle changes in D2 receptor levels. As the effect concentrations were low and effects subtle, there is a need for larger, more detailed field experiments for further assessment of actual risks. Classical monitoring studies, missing a systematic inclusion of individual background of the organisms involved, may lack the power for such assessment and may therefore overlook potential impacts.

Author contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

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Appendix A. Supplementary data

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Chapter 5.

Indices of stress and immune function in Arctic barnacle goslings (*Branta leucopsis*) were impacted by social isolation but not a contaminated grazing environment

Indices of stress and immune function in Arctic barnacle goslings (*Branta leucopsis*) were impacted by social isolation but not a contaminated grazing environment

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HIGHLIGHTS

- Effects of coal mine exposure were experimentally studied in barnacle goslings.
- Baseline and stress-induced corticosterone and immune parameters were measured.
- Mine goslings tended to show decreased haemagglutination after social isolation.
- Social isolation increased corticosterone and decreased haptoglobin in all goslings.
- Exposure to mine contamination had little impact on immunology and corticosterone.

GRAPHICAL ABSTRACT

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ABSTRACT

In many areas around the Arctic remains and spoil heaps of old mines can be found, which have been abandoned after their heydays. Runoff from tailings of these abandoned mines can directly contaminate the local environment with elevated concentrations of trace metals. Few studies have investigated the possible negative effects of contaminants on Arctic terrestrial animals that use these areas. Trace metals can accumulate in animals and this accumulation has been linked to negative effects on fitness. Both, the hypothalamus-pituitary-adrenal (HPA) axis and/or the immune system have been named as possible underlying causes for these observations. Free-living animals are often exposed to multiple stressors simultaneously, however, and this is often not considered in studies on the effects of contaminants on animal physiology. Here, we performed a study on Spitsbergen (Svalbard) taking both potential effects of trace metal contamination and social stress into account. We investigated experimentally effects of exposure to contaminants from a historic coal mine area on plasma corticosterone levels and on four innate immune parameters (haemolysis, haemagglutination, haptoglobin-like activity and nitric oxide) before and after social isolation in human-raised barnacle goslings (*Branta leucopsis*). Baseline corticosterone and immune parameters were not affected by mine-exposure. After social isolation, mine goslings tended to show decreased haemagglutination in comparison with control goslings, but we detected no difference

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1. Introduction

The Arctic is commonly considered as an untouched wilderness due to its remoteness. However, for many areas, such as Spitsbergen (Svalbard, 79°N/12°E), this is historically incorrect (Kruse, 2016). Here, the commercial exploitation of natural resources has taken place for four centuries and coal mining has been the main industry since 1904 (Avango et al., 2011; Kruse, 2013). After coal prices declined on the world market in the 1920's, many mines were abandoned with only a few active ones remaining (Hacquebord and Avango, 2009). Abandoned mines can still be a source of environmental contamination, even though they closed down decades ago (Amuno et al., 2016; Perner et al., 2010). Runoff from tailings of abandoned Arctic coal mines may contaminate the local environment through the generation of acid mine drainage, which can contain trace metals and other contaminants (Søndergaard et al., 2007; Sullivan and Yelton, 1988). In the coal mining town of Pyramiden on Spitsbergen, which was abandoned in 1998, sites inside the town were shown to be contaminated with cadmium and molybdenum. Furthermore, a site near an abandoned coal dump remains contaminated with mercury, copper and cobalt (Krajcarová et al., 2016). There have been very few studies on the possible negative effects of contaminants on terrestrial mammals and birds that inhabit such contaminated areas in the Arctic. One study by Amuno et al. (2016) compared the accumulation of trace metals and histopathological alterations in Arctic hares (*Lepus arcticus*) living close to a former lead-zinc mine and those inhabiting an area further away. They discovered that hares inhabiting the mining area accumulated more lead and cadmium in their liver, which however did not correlate with increased lesions in liver or kidneys.

In many animal species, trace metals can accumulate and have been linked to negative effects on fitness (e.g. birds: Brasso and Cristol, 2008; insects: Morón et al., 2014; invertebrates: Notten et al., 2006). In birds, fitness effects of trace metals have been linked to negative effects on physiological mechanisms, such as the hypothalamus-pituitary-adrenal (HPA) axis and/or the immune system (e.g. Bichet et al., 2013; Hallinger et al., 2011). The HPA axis is one of the two major stress response systems in vertebrates, which are turned on to restore homeostasis after unpredictable environmental events. By doing so, it integrates behavioural and physiological responses to help the individual to survive and cope with this acute allostatic overload in the short-term (Wingfield, 2013; Wingfield and Kitaysky, 2002). When the HPA axis is activated for a prolonged period of time, chronically elevated levels of glucocorticoids (e.g. corticosterone as the main glucocorticoid in birds) influence e.g. growth, metabolism, immune function, neurobiological functions and reproductive physiology (Sapolsky et al., 2000). Trace metals not only dysregulate adrenocortical function (Franceschini et al., 2009), they may also modulate mechanisms of immune cell regulation, which, in turn, enhances or suppresses either the innate and/or acquired immune system (Krocova et al., 2000; Lawrence and McCabe, 2002). Immune suppression can affect fitness negatively, as the immune system is vital in protecting the organism against parasites and pathogens (Norris and Evans, 2000; Sheldon and Verhulst, 1996). On the other hand, unnecessary stimulation of the immune system can be damaging to an organism, as mounting an immune response is costly (Verhulst et al., 2005) and may eventually lead to hypersensitivity and autoimmune diseases (Schiraldi and Monestier, 2009; Schwenk et al., 2009).

Deleterious effects of trace metal contamination are thought to be more pronounced in young, developing animals (Scheuhammer, 1987; Varian-Ramos et al., 2014). During this life stage, the HPA axis and the immune system are still developing (Fair and Ricklefs, 2002; Wada et al., 2009b). Disturbances during development can have acute effects and/or can lead to long-lasting fitness effects (Gebhardt-Henrich and Richner, 1998; Lindström, 1999).

Physiological effects of contaminants have been studied mostly by investigating effects of single contaminants on captive animals or in descriptive field studies by comparing animals from contaminated and clean areas (e.g. Amuno et al., 2016; Lewis et al., 2013; Vermeulen et al., 2014). To our knowledge, only one experimental field study has investigated the effects of trace metals in a terrestrial system. In this experiment, free-living great tit nestlings (*Parus major*) were experimentally dosed with a single contaminant (lead) and effects were assessed on physiological biomarkers, growth, plumage coloration and survival (Eeva et al., 2014). However, the results of experimental studies on single contaminants in free-living or captive animals do not always match with descriptive field studies that compare animals in exposed and non-exposed areas (Caudill et al., 2015; Eeva et al., 2009; Eeva and Lehtikoinen, 1996). One reason for this may be that contaminated areas harbour a mix of different contaminants to which animals are exposed (Yang, 1994). These mixtures can have additive, synergistic, or antagonistic effects (Boyd, 2010; Vijver et al., 2011). Furthermore, contaminants may not be available for uptake due to e.g. soil properties (van den Brink et al., 2010). While studies into the negative effects of single contaminants on captive or free-living animals provide an important insight, they are not able to capture the complexity associated with the exposure of free-living animals to contaminated areas (Fair and Ricklefs, 2002). Descriptive field studies comparing exposed and non-exposed areas can, on the other hand, not be used as a test of causality. At the moment, experimental work, in which animals are exposed to contaminated and non-contaminated areas, is scarce.

Another point, which is often not considered in studies on the effect of contaminants on animal physiology, is that free-living animals in their natural environment are exposed to multiple stressors simultaneously (e.g. food shortage, cold exposure, social stress, immune challenges; Caudill et al., 2015; Fair and Ricklefs, 2002; Varian-Ramos et al., 2014). Animals, which are concurrently exposed to contaminants and other major stressors, might be more affected than when they are exposed to few stressors. Therefore, knowledge on such interactions is crucial (Baos et al., 2006; Fair and Ricklefs, 2002; Munns, 2006). In social species, such as geese, social isolation is an ecologically relevant and potent stressor (Hawkey et al., 2012; Kralj-Fišer et al., 2013; Ludwig et al., 2017), which can be useful to study such interactions. Living in a structured group is a key element of social species and a proper social embedding is crucial for an individual's survival and well-being (see Hawkey et al., 2012). Especially goslings younger than approximately six weeks of age are vulnerable to predation from gulls and foxes when they get isolated from their families and lone goslings have, therefore, little chance to survive (Black et al., 2014). Goslings are particularly prone to get lost in the first days after hatch, as there are many circumstances, in which they may wander from their families (Black et al., 2014). Experimental social isolation can, therefore, mimic a survival threat. Many studies have found increased levels of glucocorticoids, mainly in response to acute periods of social isolation and when already socially isolated animals had to deal with additive acute stressors (reviewed by Hawkey et al., 2012).

In the current study we performed an experiment taking the potential effects of a social stressor into account. We explored the effects of trace metal exposure on corticosterone level and immune parameters before and after social isolation in two groups of human-raised barnacle goslings (*Branta leucopsis*). Barnacle geese were selected as they are obligatory terrestrial grazers on Spitsbergen (Løvenskiold, 1964), and potentially exposed to soil contaminants near mines. A group of goslings was walked daily from hatch until three weeks old to the contaminated area near an abandoned coal mine in Ny-Ålesund, Spitsbergen. A second group of goslings, composed of social siblings of the mine area group, grazed daily on clean tundra. Instead of focusing on the influence of specific contaminants, the aim of this study was to investigate whether the goslings were affected by the overall mixture of contaminants. Therefore, we compared corticosterone levels and immune parameters of goslings exposed to the historic mining area and a control area, which does not have a mining history following Fair et al. (2003).

2. Materials & methods

2.1. Study site and study species

We conducted this study in Ny-Ålesund, Spitsbergen (78°55'N, 11°56'E). On islands in the Kongsfjord near the village of Ny-Ålesund, a barnacle goose colony was established in the early 1980s and has experienced rapid growth ever since (Loonen et al., 1998, MJEL pers. obs.). On June 30th 2014, we removed a total of 16 eggs, which were in the process of hatching (0 days old, down not fully dried) from 8 different nests on the contamination-free island Indre Breøyane, which does not have a mining history. We collected two hatchlings per nest from clutches with a minimum of four, in order to ensure that goose parents were left with a minimum of two young to raise. Immediately, we randomly assigned one of the two goslings per nest to either the control or mine group to diminish potential genetic effects and any possible effects of *in ovo* exposure to contaminants (Bunn et al., 2000; de Franciscis and Boccalatte, 1962). Goslings from the same nest were likely to be genetic siblings, as we collected hatchlings that were similar in appearance. As soon as they were taken from the nest, we marked goslings with web tags, small identification tags which are clipped through the foot web (Alliston, 1975; Seguin and Cooke, 1985). In addition, goslings received unique colour bands for easy identification in the field without handling. In order to control for possible effects of parenting styles of human foster parents, the goslings were hand-reared by four humans (AB, MEDJ, NvdB, IBRS) who took turns in providing care. Socially involved hand-raising was applied in order to produce animals that are trustful, calm and cooperative (Hemetsberger et al., 2010). This was crucial for a companion study, in which we investigated the long-term effects of contamination on gosling behaviour and stress coping abilities (Scheiber et al., in prep.). Until they were four days old, we kept all 16 goslings as a large group in the village and they were led around the meadows in the village by their foster parent for feeding, brooding and predator protection. Overnight, they were housed inside a pen with an infrared lamp and provided with a commercial diet for young waterfowl (Anseres I food, starter pellet, Kasper Faunafood, Woerden, The Netherlands), a supply of fresh vegetation, and water *ad libitum*.

2.2. Experiment: exposure of goslings to mine or control areas

When five days old, the mine group was walked approximately 1.5 km to the southeast of the village to be exposed to contamination in a former coal mining area. The coal mine near Ny-Ålesund was in operation from 1916 until closure in 1963, with periods of inactivity in between (Reymert, 2016). The control group was walked approximately 1.9 km in the opposite direction to the northwest of the village to a grazing area on clean tundra. Both groups were allowed to graze during the walk and at the final destination *ad libitum*. Both areas are also utilized by the wild geese for foraging (pers. obs. MEDJ, IBRS, MJEL).

To ensure that goslings were exposed to the mine and control area and ingested vegetation from these two areas on a daily basis, walks lasted at least 5 h. We attempted to standardize the time and distance of the walks of both groups. When the goslings were very young (age 1 to 8 days old), supplemental food was sometimes required during the walks, since grass availability was limited in the beginning of the season. Supplemental food was provided to avoid potential effects of malnutrition, but was kept to a minimum so that goslings maximally foraged on natural vegetation. The respective gosling groups were fed separately when on the walks, but received supplemental food together when residing in the village. Both groups ate a similar amount of supplemental food every day throughout the study, under the assumption that both groups took a similar amount of food when they were feeding together (paired *t*-test: matched pair comparison: $t = -0.835$, $df = 22$, $p = 0.4126$). We trust this assumption to be correct on the basis of no detected difference in mass gain between the gosling groups (see Results). In the field, goslings were regularly artificially brooded when they needed warmth.

2.3. Area mercury contamination status

In 2012, pre-experimental soil samples were collected in both the mine and control area and analysed for mercury contamination (nine samples mine area, eight samples control area). Samples were collected with a PVC-spoon, which was cleaned after each sampling to avoid cross-contamination. Organic material was removed from the soil as much as possible, as well as stones. After collection, the samples were stored at -20°C in poly-ethylene bags and shipped to The Netherlands. Mercury was analysed using cold vapour/atomic fluorescence spectrometry (Hoogenboom et al., 2015). In short, dried soil (1 g, 70°C , 48 h) was digested in a microwave in 10 ml nitric acid (70%) after which samples were made up to 50 ml with ultrapure Milli-Q water. All different forms of mercury were reduced to total mercury with Sn(II)Cl , then released from solution and quantified in gaseous phase by fluorescence at 254 nm. All concentrations are based on dry-weight. Vegetation was also collected on both respective sites (*Poa* sp.) and analysed for mercury, but as this were only two samples per site no statistics are given.

2.4. Social isolation as an acute stressor and blood sampling

We sampled gosling sibling pairs in random order when they were 23 days old. During the sampling procedure, all goslings that were not sampled yet remained outside in their home enclosure. After one pair of goslings was completed, we continued with the next randomly chosen pair. Within pairs, one of the two siblings was caught, brought inside, and sampled (ca. 1 ml of blood collected on heparin from the brachial vein) at (near) baseline, i.e. less than three minutes after capture (Chastel et al., 2005; Romero and Reed, 2005). Immediately afterwards, we caught and sampled the second sibling, i.e. within 10 min of the first. Twenty minutes after each baseline sample, we collected a follow-up sample from the brachial vein of its other wing. Between collection of baseline and follow-up blood samples, goslings were placed individually in closed boxes in different rooms, which were out of visual and auditory contact. Blood samples were centrifuged to separate cellular and plasma fractions, which were separately stored at -20°C until further analyses. Directly after the stress and blood collection protocols, goslings were euthanized by decapitation.

2.5. Body measurements

To determine mass and size gain, we weighed (in grams) goslings daily (from 1 to 22 days old) by having them step on a digital scale. Every other day (from age 2 to 14 days old) we measured their total tarsus length with analogous callipers to the nearest 0.1 mm following Dzubin and Cooch (1992).

2.6. Corticosterone

Corticosterone is the main glucocorticoid in birds. It is released after a cascade of events which starts with the detection of a stimulus that is expected to be harmful by the higher brain centres (Romero and Reed, 2005). Depending on where a young bird falls within the altricial-precocial range (Starck and Ricklefs, 1998), the degree of increase in corticosterone at baseline and after a stressor differs. In nest-bound altricial species, the difference between baseline and stressor-induced corticosterone increases throughout development. On the other hand, precocial species, which hatch covered in down and leave the nest shortly after hatch, show a well-developed stress-response from an early age. A possible explanation for this difference is that, as precocial chicks are more mobile than altricial chicks, they may have to deal with more stressors at an early age (see Chin et al., 2013). Plasma concentration of corticosterone was determined at the University of Vienna using a commercial radioimmunoassay kit (catalogue no. 07-120102; ICN Bio-medicals/MP Biomedicals, Solon, OH, USA). We performed the assay as described by Washburn et al. (2002) with the modifications made by Soldatini et al. (2015). The radioimmunoassay determines the concentration of the hormone by using a radioactive label that quantifies the amount of hormone on the basis of the extent to which it binds to its antibody. All samples were analysed in duplicate with an intra-assay coefficient of variation of 15%. This inter-assay coefficient was comparable to other studies (D'Alba et al., 2011; Soldatini et al., 2015). As all samples were analysed on a single plate there was no inter-assay coefficient of variation. The detection limit was below 2 ng/ml. Unexpectedly, we were unable to quantify samples from two control and one mine gosling after the social isolation, because corticosterone concentrations were above the reliable detection range (max. 1000 ng/ml). The two control goslings had the highest corticosterone concentration of all the goslings at baseline (164.03 and 146.43 ng/ml) already, but the mine gosling did not (48.45 ng/ml). We speculate that these very high concentrations could have been caused by cross bindings or contamination, but as the assay was done at the same time with another species presumably it was not an assay problem (pers. obs. V. Canoine). Regardless of the causes, we excluded these three values from further analyses.

2.7. Immune assays

We assessed several components of the innate immune system, which forms the first line of defence against invading pathogens (Davison et al., 2008).

2.7.1. Haemolysis-haemagglutination assay

Natural antibodies (NAbs) and complement are both humoral components of the constitutive innate immunity (Matson et al., 2005; Schmid-Hempel and Ebert, 2003). NAbs are special among immunoglobulins in that they do not require previous exposure to an antigen and are, therefore, also present in naïve individuals. NAbs have several functions, one of which is that they are the initiators of the complement cascade, which ends in cell lysis by opsonizing invading pathogens (Boes, 2001; Carroll and Prodeus, 1998; Ochsenbein and Zinkernagel, 2000; Shishido et al., 2012). NAbs and complement can be assessed using a haemolysis-haemagglutination assay, with lysis titres reflecting the interaction of NAb and complement, while agglutination reflects only NAb activity (Matson et al., 2005). We performed the assay as described by Matson et al. (2005) with the modifications suggested by Mauck et al. (2005). In short, gosling plasma was serially diluted in 96-well round (U) bottom assay plates using Dulbecco's PBS after which 1% rabbit blood cell suspension was added to all wells. Plates were then sealed, shortly vortexed, whereupon they were floated in a 37 °C water bath for 90 min. Plates were then tilted to a 45° angle at room temperature for 20 min to facilitate haemagglutination scoring. Plates were scanned using a flatbed scanner and, after another 70 min, were scanned again for haemolysis scoring. After assay results were

digitized, we scored haemagglutination and haemolysis titres blindly to plate and sample identity from the digital images. We scored all samples twice, assigning half scores to wells that had partial agglutination or lysis (Matson et al., 2005; Mauck et al., 2005). When the same sample was scored ≥ 1 titre apart between scoring repeats, we scored it a 3rd and 4th time and used the median score in analyses. All samples were re-scored on different days, by the same person (MEDJ).

2.7.2. Haptoglobin assay

Haptoglobin is an acute-phase protein that is normally present at low concentrations in plasma but increases rapidly in response to inflammation, infection, and trauma (e.g. Coon et al., 2011; Cray et al., 2009; Matson et al., 2012; Millet et al., 2007). One of the primary functions of haptoglobin is to bind free haemoglobin after haemolysis or normal red blood cell turnover (functions reviewed by Quaye (2008)). Removal of free haemoglobin is vital as haem has oxidative and toxic properties. Haptoglobin is also indicated to minimize the access to free haem for extracellular and intracellular pathogens (Parrow et al., 2013). Plasma samples from the goslings were functionally assayed for haptoglobin-like activity by following the instructions of a commercially available assay (TP801; Tri-Delta Development Limited). The assay is based on the principle that haptoglobin preserves the peroxidase activity of haemoglobin at a low pH when they are bound to each other. From the preservation of the peroxidase activity of haemoglobin at low pH the amount of haptoglobin can be measured using this colorimetric assay. Concentrations (mg/ml) were calculated following minor modifications concerning the assay wavelengths (Matson et al., 2012). We used absorbances measured at 450 nm before adding the assay chromogen to correct statistically for differences in plasma sample redness (see Statistics), an indication of haemolysis, which can affect the assay (Matson et al., 2012).

2.7.3. Nitric oxide assay

We also measured nitric oxide, a small multifunctional messenger and effector molecule with many widespread physiological functions. The most important function of nitric oxide in the immune system is to modulate inflammation and inhibit or kill various pathogens through peroxynitrite formation. The latter initiates free radical damage (reviewed by Vajdovich, 2008). Nitric oxide quantification can be used as a proxy for the activation of the innate immune system. We used the spectrophotometric assay as described by Sild and Hörak (2009) to measure the concentration of nitric oxide ($\mu\text{mol/l}$) in gosling plasma samples. We slightly modified the assay by using 20 μl plasma (instead of 10 μl) because nitric oxide concentrations in our samples were too low to be detected in the smaller plasma amount. The quantification of nitric oxide is based on deproteinization of plasma followed by nitrite reduction by copper-coated cadmium granules and a Griess reaction (Sild and Hörak, 2009). One gosling of the control group lacked a baseline nitric oxide measurement due to insufficient plasma volume and was therefore treated as non-observed.

2.8. Molecular sexing

We accounted for the sex of the goslings, as sexes may differ in trace metal accumulation and its physiological consequences (Bunn et al., 2000; Burger, 2007; Thompson et al., 2014; Vahter et al., 2007). Goslings were sexed genetically at the University of Groningen following the method by Griffiths et al. (1998). Using DNA extracted from erythrocytes, polymerase chain reaction (PCR) was employed to amplify conserved CHD genes on the avian sex chromosome. The PCR products were separated on 3% agarose gel. For males one band appears on a gel; and for females, two bands. Sexing revealed that, in total, we had 9 males 7 females, with the following distribution (female/male): control group (4/4) and the mine group (5/3), and across sibling pairs (1 pair females only, 2 pairs males only, 2 pairs; male control, female mine, 3 pairs; female control, male mine).

2.9. Statistical analyses

All analyses were done in R version 3.2.3. First, to investigate whether there were differences between the mine and control groups in mass and total tarsus measurements during the experiment, we analysed these data using linear mixed effects-models (lmer function in the R package lme4, Bates et al., 2015). Exposure to the mine or control area and sex were added in the model as fixed effects and gosling identity was added as a random effect to account for the multiple measurements per gosling. First we tested whether the growth was best described by linear, quadratic or cubic relationships by adding gosling age, age² or age³ in the model. These models were compared, and we further analysed the model that fit the data best (see below).

Next, we analysed the effects of mine exposure on soil mercury concentrations, gosling baseline corticosterone, haptoglobin-like activity and nitric oxide concentrations using linear models (LMs). Soil mercury concentrations were log-transformed to meet model assumptions of normality of model residuals, which was assessed visually. Haptoglobin-like activity and nitric oxide measurements were square root transformed to meet model assumptions. Data transformation of haptoglobin-like activity and nitric oxide did not change model outcome when compared with analyses of the raw data, so the latter is reported in the tables. We analysed effects of mine-exposure on baseline haemagglutination and haemolysis by fitting cumulative link models with the clm function from R package ordinal, because these response variables were ordinal rather than continuous (Christensen, 2015). In the model analysing soil mercury concentrations, area (control or mine) was included as a factor. For all other models, area and sex were included as factors. In the model for haptoglobin-like activity, we also added plasma redness (450_{total}) as a co-variate (Matson et al., 2012).

To investigate effects of individual isolation as an acute stressor on corticosterone and immune parameters, we calculated the change (Δ) in each measure from before to after the isolation (i.e., follow-up measure minus baseline measure). We analysed Δ corticosterone, Δ haptoglobin-like activity and Δ nitric oxide using LMs (Bates et al., 2015) and Δ haemagglutination and Δ haemolysis by fitting cumulative link models (Christensen, 2015). We used the same predictors in the model as in the analyses of baseline measures (see above). As baseline values did not differ between the mine and control group (see Results), we did not take these into account as a covariate in our analyses.

Lastly, to gain insight in the effects of social isolation on the measured parameters, we analysed raw data from before and after social isolation. We used similar models as in the analyses of baseline measures (see above), but added social isolation (0: before social isolation, or 1: after social isolation) as a fixed effect.

We reached our final models by following the approach of Zuur et al. (2009). We first started with the full model and used reverse stepwise likelihood ratio tests ($p < 0.05$) to determine the optimal fixed structure. For the analyses of mass and total tarsus, we used the Kenward-Roger approximation for denominator degrees of freedom (KRmodcomp function of the Pbktest package) in the model selection, as the procedure gives more reliable results when sample size is small (Halekoh and Højsgaard, 2014). For all other analyses we used the ANOVA method and likelihood tests (Christensen, 2015). We plotted data using the package ggplot2 (Wickham, 2009).

The nature of this field experiment meant that study size was limited. This was a direct consequence of the experimental method used to raise a naturalistic number of goslings, which could subsequently be led to the mine-exposed and control area. We decided not to test the interactions between exposure and sex in all our models, as it is known that exploring these effects in small samples increases rates of false-positive and false-negative findings (Schmidt et al., 2014).

3. Results

3.1. Influence of exposure to the contaminated mining area on body measurements

Gosling mass was not influenced by exposure to the mining or control area (means of last measurement when 22 days old: mean mine: 997.5 g [95% CI: 85.0], mean control: 1002.5 g [95% CI: 123.9], Table 1). Total tarsus length of the goslings was also similar among the control and mine exposure groups (means of last measurement when 14 days old: mean mine: 81.2 mm [95% CI: 5.2], mean control: 82.6 mm [95% CI: 3.5], Table 1). Males, however, grew bigger than females.

3.2. Area contamination status

Mercury levels were approximately 4 times higher in the mine area than in the control area (mean mine area: 0.086 mg/kg dry weight [95% CI: 0.039], mean control area: 0.021 mg/kg dry weight [95% CI: 0.006], linear model on log transformed data: area effect: $F_{1,16} = 32.9$, $p < 0.001$). Mercury concentration from vegetation collected in both areas (two samples per location, *Poa* sp.) indicated that mercury content was higher in the mine area (0.044 ± 0.022 mg/kg dry weight [average $\pm$ SD]) than in the control area (0.005 ± 0.0004 mg/kg dry weight [average $\pm$ SD]).

3.3. Influence of exposure to the contaminated mining area on baseline and isolation stress induced corticosterone levels

Goslings that were exposed to the mining area had higher baseline corticosterone concentrations than control goslings (mean mine: 70.22 ng/ml [95% CI: 50.51], mean control: 40.31 ng/ml [95% CI: 28.23]), but this difference was not statistically significant (Table 2). Δ Corticosterone showed a similar trend (mean Δ mine: 91.52 ng/ml [95% CI: 60.91], mean Δ control: 61.10 ng/ml [95% CI: 35.21], Table 3). After social isolation, corticosterone levels increased significantly in all goslings irrespective of sex or exposure to the mining or control area (mean mine: 130.67 ng/ml [95% CI: 53.44], mean control: 102.99 ng/ml [95% CI: 63.07], lmer: effect of social isolation: $F = 20.07$, $df = 13$, $p < 0.001$).

3.4. Influence of exposure to the contaminated mining area on immune measures

3.4.1. Haemagglutination

We detected no effect of exposure to the mining area on baseline haemagglutination titre (Table 2, mean mine: 8.19 titres [95% CI: 1.99], mean control: 8.28 titres [95% CI: 2.26]). Mine goslings tended

Table 1

Summary of the outcome of linear mixed effect models describing the effects of experimental exposure to foraging in a former coal mining area versus a clean control area on mass and total tarsus measurements (following Dzubín and Cooch, 1992) of barnacle goslings on Spitsbergen. Mass was measured daily from 1 to 22 days old and total tarsus was measured every other day from age 2 to 14 days old. Sex was added in the model to correct for possible sex differences and gosling identity was added as a random variable.

Measurement	Variable	Estimate (β)	SE	F	ddf	p
Mass	Intercept	-18.918	16.0	-	-	-
	Exposure			0.23	13	0.637
	Mine	-8.773	16.391			
	Sex			16.10	14	0.001
	Male	72.047	16.521			
Total tarsus	Age	19.602	1.567	155.52	334	<0.001
	Age ²	1.233	0.066	345.43	334	<0.001
	Intercept	27.604	0.977			
	Exposure			1.621	101.38	0.206
	Mine	-0.693	0.544			
	Sex			23.95	108.54	<0.001
	Male	3.404	0.661			
	Age	3.710	0.067	3027.05	102.01	<0.001

Table 2

Summary of the outcome of linear models or cumulative link models (*) describing the effects of experimental exposure to a former coal mining area versus a clean control area on baseline plasma corticosterone, haemagglutination, haemolysis, haptoglobin and nitric oxide concentrations. Plasma redness had no effect on haptoglobin-like activity ($\beta \pm \text{SE}$: -1.175 ± 1.938 , F : 0.367, $df = 1, 13$, $p = 0.555$). χ^2 statistics are given for the analyses of haemagglutination and haemolysis (*), while F statistics are given for all other analyses.

Variable	N (mine/control)	Intercept ($\beta \pm \text{SE}$)	Exposure				Sex			
			Effect: exposure to mine ($\beta \pm \text{SE}$)	F/χ^2	df	p	Effect: being male ($\beta \pm \text{SE}$)	F/χ^2	df	p
Corticosterone	16 (8/8)	84.04 ± 21.11	-26.46 ± 24.44	1.172	1, 14	0.299	-27.63 ± 24.63	1.589	1, 15	0.228
Haemagglutination*	16 (8/8)	–	-0.15 ± 0.88	0.030	1, 13	0.862	-1.22 ± 0.94	1.730	1, 12	0.189
Haemolysis*	16 (8/8)	–	1.43 ± 1.07	1.934	1, 6	0.164	2.39 ± 1.11	5.085	1, 5	0.024
Haptoglobin-like activity	16 (8/8)	0.412 ± 0.188	0.094 ± 0.068	1.637	1, 14	0.223	0.0820 ± 0.064	2.308	1, 15	0.151
Nitric oxide	15 (8/7)	0.001 ± 0.0003	-0.0003 ± 0.0004	0.618	1, 13	0.446	-0.0008 ± 0.0004	4.146	1, 14	0.063

to show lower Δ haemagglutination compared with the control group when controlling for sex (Fig. 1, Table 3, mean Δ mine: -2.88 titres [95% CI: 2.48], mean Δ control: -0.28 titres [95% CI: 3.85]). Social isolation on itself did not affect haemagglutination (mean mine: 5.31 titres [95% CI: 1.12], mean control: 8 titres [95% CI: 2.89], clmm2: effect of social isolation: $\chi^2_{1,16} = 2.477$, $p = 0.115$).

3.4.2. Haemolysis

We detected no difference in baseline haemolysis between mine-exposed and control groups, when controlled for sex effects (mean mine: 3.69 titres [95% CI: 0.38], mean control: 3.34 titres [95% CI: 0.56], Table 2). Mine-exposed goslings had lower haemolysis titres after social isolation in comparison with control goslings (mean Δ mine: 0.09 titres [95% CI: 0.61], mean Δ control: 0.16 titres [95% CI: 0.80]), but this difference was not significant (Table 3). Social isolation did not affect haemolysis in any way (mean mine: 3.78 titres [95% CI: 0.34], mean control: 3.5 titres [95% CI: 0.5], clmm2: effect of social isolation: $\chi^2_{1,23} = 0.524$, $p = 0.468$).

3.4.3. Haptoglobin-like activity

Baseline haptoglobin-like activity was not affected by mine-exposure (Table 2, mean mine: 0.44 mg/ml [95% CI: 0.13], mean control: 0.35 mg/ml [95% CI: 0.07]). The control and mine-exposed groups did also not differ in Δ haptoglobin-like activity (Table 3, mean Δ mine: -0.14 mg/ml [95% CI: 0.17], mean Δ control: -0.12 [95% CI: 0.13]). Haptoglobin-like activity decreased significantly after isolation irrespective of mine or control area exposure (mean mine: 0.30 mg/ml [95% CI: 0.12], mean control: 0.22 mg/ml [95% CI: 0.15], lmer: effect of social isolation: $F = 8.596$, $ddf = 15$, $p = 0.010$).

3.4.4. Nitric oxide

Baseline nitric oxide was lower in mine-exposed goslings than in the control goslings (mean mine: 0.35 $\mu\text{mol/l}$ [95% CI: 0.37], mean control: 0.69 $\mu\text{mol/l}$ [95% CI: 0.99]), but this did not reach significance (Table 2). Exposure to the mining area also did not significantly affect Δ nitric oxide (Table 3, mean Δ mine: -0.21 $\mu\text{mol/l}$ [95% CI: 0.47], mean Δ control: -0.23 $\mu\text{mol/l}$ [95% CI: 1.10]). Social isolation did not influence

nitric oxide in any way (mean mine: 0.30 $\mu\text{mol/l}$ [95% CI: 0.35], mean control: 0.36 $\mu\text{mol/l}$ [95% CI: 0.26], lmer; effect of social isolation: $F = 0.853$, $ddf = 15$, $p = 0.370$).

4. Discussion

The aim of this study was to explore combined effects of contamination and social isolation on the physiology of barnacle goslings. We investigated experimentally these effects on corticosterone and plasma-based immune indices when exposed to a short-term stressor, i.e. individual isolation. We show that exposure to a coal mine contaminated area had little impact on the immunology and plasma corticosterone levels of barnacle goslings up to 3 weeks of age.

4.1. No effects of mine exposure on baseline values

In the current study, we did not detect an effect of mine-exposure on baseline plasma corticosterone levels. Previous studies have frequently investigated the relationship between mercury, as a single contaminant, and corticosterone in contaminated areas. These studies often show negative relationships between mercury exposure and baseline corticosterone in e.g. juvenile Forster's tern (*Sterna forsteri*, Herring et al., 2012), adult tree swallows and nestlings (*Tachycineta bicolor*, Franceschini et al., 2009; Wada et al., 2009a, 2009b) and female common eiders (*Somateria mollissima*, Provencher et al., 2016). However, two experimental studies have contrasting results. Adams et al. (2009) performed a study, in which they experimentally dosed captive juvenile white ibises (*Eudocimus albus*) with different concentrations of methylmercury, and investigated faecal corticosterone metabolite levels. The authors observed complex nonlinear responses over time with high methylmercury exposure resulting in increased baseline corticosterone values. In lifelong experimentally dosed zebra finches (*Taeniopygia guttata*), there was no difference in plasma corticosterone between differently dosed methylmercury exposure groups (Moore et al., 2014). One difference between these studies was that dosage was much higher in the zebra finch study (Moore et al., 2014), but also the timing and method of measuring corticosterone was very different.

Table 3

Outcome of the linear models or cumulative link models (*) describing the effects of experimental exposure of barnacle goslings to an abandoned coal mining area versus a clean control area on the change in plasma-based indices (Δ) after 20 min of social isolation. Plasma redness had no effect on haptoglobin-like activity ($\beta \pm \text{SE}$: -0.263 ± 2.951 , F : 0.008, $df = 1, 13$, $p = 0.930$). χ^2 statistics are given for the analyses of haemagglutination and haemolysis (*), while F statistics are given for all other analyses.

Variable	N (mine/control)	Intercept ($\beta \pm \text{SE}$)	Exposure				Sex			
			Effect: exposure to mine ($\beta \pm \text{SE}$)	F/χ^2	df	p	Effect: being male ($\beta \pm \text{SE}$)	F/χ^2	df	p
Δ Corticosterone	13 (7/6)	58.17 ± 28.20	29.15 ± 32.03	1.038	1, 12	0.330	5.86 ± 32.83	0.032	1, 11	0.862
Δ Haemagglutination*	16 (8/8)	–	-1.71 ± 1.01	3.089	1, 14	0.080	2.38 ± 1.01	4.942	1, 13	0.026
Δ Haemolysis*	16 (8/8)	–	0.393 ± 0.923	0.182	1, 8	0.670	-1.657 ± 1.017	2.656	1, 7	0.103
Δ Haptoglobin-like activity	16 (8/8)	-0.139 ± 0.287	-0.018 ± 0.104	0.052	1, 14	0.822	0.079 ± 0.098	0.742	1, 15	0.403
Δ Nitric oxide	15 (7/8)	0.0001 ± 0.0004	0.00006 ± 0.0005	0.012	1, 13	0.914	-0.0007 ± 0.0005	1.701	1, 14	0.215

Fig. 1. Barnacle goslings that were exposed to the coal mining area tended to show a decrease in haemagglutination titres (Δ haemagglutination) after social isolation in comparison with control goslings, when controlling for sex effects (F = female, black circles; M = male, grey circles). Boxes show medians as well as 25% and 75% quartiles. Whiskers indicate the range between the 10th and 90th percentiles. Detailed statistics results are given in Table 3.

Varian-Ramos et al. (2014) found that lifelong exposed zebra finches, which were also offspring of dosed parents, had a higher reproductive success at their highest treatment level (2.4 ppm mercury) in comparison with birds that were only exposed as adults. This indicates that fast adaptation to mercury exposure may occur, as the lifelong exposed birds were necessarily offspring from successful pairs only and they had to survive exposure as nestlings. Investigating a wider arrange of heavy metals (zinc, lead, copper, cadmium) and arsenic levels in free-living nestling white stork (*Ciconia ciconia*), Baos et al. (2006) did not detect any effects on baseline corticosterone.

In the late second half of the twentieth century, interest in the possible immuno-toxic effects of trace metal contamination in birds increased after reports of high lead exposure in combination with infectious diseases in several bird species (Locke and Bagley, 1967; Rocke and Samuel, 1991). Many studies under controlled laboratory conditions have since described negative effects of lead and other trace metals on bird immune components (e.g. Kenow et al., 2007; Nain and Smits, 2011; Snoeijs et al., 2005). Nowadays, more and more studies investigate the relationship between trace metals and immune indices in free-living birds. Many different assays are being used to assess effects on the immune system, but studies investigating a wide range of immune measures are still rare (Provencher et al., 2016). In our study, we did not find an effect of foraging in the coal mine contaminated area on our range of baseline measurements of the innate immune system (haptoglobin-like activity, nitric oxide, haemolysis and haemagglutination). Vermeulen et al. (2014) investigated the same range of immune indices to estimate the effects of a contamination

gradient near a non-ferrous smelter on great tit (*Parus major*) nestlings. They detected a gradient in trace metal concentrations in red blood cells of nestlings from different populations, with highest concentrations near the smelter. Nevertheless, they did not detect a correlation between distance to the smelter and immune parameters.

4.2. Effects of mine exposure and social isolation

Mine goslings tended to show a decrease in haemagglutination titres (Δ haemagglutination) after social isolation in comparison with control goslings, thus a negative effect of exposure to contaminants on NAb activity seemingly only becomes apparent after goslings experience an acute stressor. Often studies focus on the relationship between trace metal exposure and the corticosterone stress-response, while in the same studies only baseline immune indices are investigated (e.g. Beck et al., 2014; Eeva et al., 2005; but see Bartlett and Smith, 2003). Yet capture stress can affect immune function (Matson et al., 2006; Millet et al., 2007). As free-living birds are often exposed to multiple stressors, assessing effects of trace metals on immune indices in relation with additional stressors may provide new biological insights.

In the current study, we could not identify effects of trace metal contamination in combination with social isolation on other immune parameters (haptoglobin-like activity, nitric oxide, and haemolysis) or corticosterone. Effects of trace metal contamination on the corticosterone stress response have been equivocal in previous studies. In observational studies, exposure to mercury and selenium decreased stress-induced blood corticosterone (Wada et al., 2009a; Wayland et al., 2002), whereas cadmium and lead increased stress-induced corticosterone (Baos et al., 2006; Wayland et al., 2002). However, these and other studies also contradict each other in the effects of measured trace metals on adrenocortical function (Baos et al., 2006; Beck et al., 2014; Franceschini et al., 2009; Wayland et al., 2002, 2003). While most studies on the topic have been observational, Moore et al. (2014) performed an experimental laboratory study to investigate the effect of exposure to mercury on the ability of zebra finches to mount an adrenocortical stress-response. They concluded that zebra finches that were lifelong exposed to methylmercury had a decreased ability to increase blood corticosterone levels after 30 min of handling stress. Thus, trace metal contaminants may affect corticosterone in free-living birds, but predictable patterns are hard to derive (Franceschini et al., 2009). Therefore, more experimental field studies are needed to disentangle the effects of concentration, mixture, environmental influence and rate of trace metal contaminant uptake.

4.3. Effects of social isolation

Irrespective of mine-exposure, goslings had on average two-times-higher blood corticosterone concentrations after isolation. Our study thereby adds to the known effects of social isolation on glucocorticoids in social mammals (reviewed by Hawkey et al., 2012) and especially to the less extensive literature in social bird species (but see Apfelbeck and Raess, 2008; Banerjee and Adkins-Regan, 2011; Ludwig et al., 2017; Perez et al., 2012; Remage-Healey et al., 2003). In free-living adult grey-lag geese (*Anser anser*), socially isolated males excreted increased levels of corticosterone metabolites compared to baseline, while their mates, who were not taken out of their familiar environment, did not show increased levels of corticosterone (Ludwig et al., 2017). Circulating corticosterone increased in one-year-old starlings (*Sturnus vulgaris*) when they were visually isolated from their social group for approximately 20 h (Apfelbeck and Raess, 2008). Social isolation also increased the circulating level of corticosterone in zebra finches (Perez et al., 2012), and this was higher than after 10 min of handling stress (Banerjee and Adkins-Regan, 2011). Although we cannot totally rule out that the elevated corticosterone levels were due to handling, this might be negligible, as goslings were handled regularly when being measured, so they were used to the procedure.

Haptoglobin-like activity decreased about 1.5 times after social isolation in both gosling groups similarly. In humans, a growing body of research associates social isolation with increased morbidity and mortality from a range of diseases (Hawkey and Cacioppo, 2003). Changes in immune function have been particularly connected with these observations as a probable underlying mechanism (e.g. Uchino, 2006). In prairie voles (*Microtus ochrogaster*), a species that forms strong social bonds, social isolation reduced complement activity in both males and females and plasma bacterial killing ability in males. These results suggest that socially isolated voles are less able to destroy foreign cells (Scotti et al., 2015). In birds, fewer investigations have been performed on the effects of social isolation on immune function. However, zebra finches, that were housed in isolation or in a colony and subsequently challenged with lipopolysaccharide, did not differ in haptoglobin-like activity or bacterial killing capacity (Lopes et al., 2014). Therefore, effects of social isolation on immune function can be ambiguous; stress is not only immunosuppressive, but can also have no effect or can even be immunoenhancing (Gao et al., 2017; Martin, 2009). Nevertheless, in barnacle goslings, the negative effect of social isolation on haptoglobin-like activity may expose the growing birds to higher levels of oxidative stress, particularly when they become infected (Parrow et al., 2013).

4.4. Area and gosling contamination status

Pre-experimental mercury measurements indicated that mercury concentrations in soil and vegetation were higher in the mine than in the control area. This is in line with preliminary results from summer 2014 that show that the vegetation in the mining area contained approximately twice as much mercury than the control site. Moreover, the goslings that had foraged in the mining area had a 30% higher concentration of total mercury in their liver compared to control goslings (van den Brink et al. in prep.). This proxy of contamination thus indicates that the mine goslings were indeed exposed to higher levels of contamination. Interestingly, during the experiment, goslings were observed eating coal "grit" in the mine area as well as vegetation, as geese need grit in their gizzard to grind their food (see Amat and Varo, 2008). By doing so, the goslings possibly ingested more contaminants than they would have from vegetation alone. Furthermore, results from a study by Krajcarová et al. (2016), which investigated various trace metals in the soil in the abandoned coal mining town of Pyramiden, Spitsbergen (78°40'N, 16°23'E), showed that mercury levels here ranged from 0.04–0.736 mg/kg (median: 0.025 mg/kg), which is comparable to our pre-experimental data. Other trace metals, which were consistently higher than the world soil average, included cadmium (range: 3.34–10.6 mg/kg, median: 6.04 mg/kg) and molybdenum (range: 10.8–32.9 mg/kg, median: 17.7 mg/kg). Also copper seemed to be more abundant in this area than the world soil average, but this result was influenced by a very high measurement from a soil sample that was taken close to a coal dump (range: 24.5–659 mg/kg, median: 37.3 mg/kg). The study concludes that coal mining and 90 years of human activity have greatly contaminated all investigated areas (Krajcarová et al., 2016).

4.5. Conclusions

Overall, our results suggest that foraging in a contaminated coal mining area had little impact on baseline immune indices and plasma corticosterone levels of barnacle goslings. However, when goslings were subsequently exposed to an additional stressor, social isolation, mine goslings tended to show a decrease in haemagglutination titres, which indicates decreased NAB activity. This result highlights the biological relevance of taking into account multiple stressors in contamination studies. Social isolation significantly increased plasma corticosterone levels, indicating that this type of isolation is a potent stressor in barnacle goslings. At the same time, social isolation decreased haptoglobin-like

activity, an acute-phase protein with antimicrobial and antioxidant functions.

Our work is the first experimental investigation of the effects of a former coal mining area on a small terrestrial grazer and thus provides a basis for future studies. Additional experimental research is needed to fully understand the effects of trace metal contamination on terrestrial wildlife and especially grazers, which is a group that has been overlooked in previous research.

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Chapter 6.
Stress behaviour and physiology
of developing Arctic barnacle goslings
(*Branta leucopsis*) is affected by legacy
trace contaminants

Research

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Stress behaviour and physiology of developing Arctic barnacle goslings (*Branta leucopsis*) is affected by legacy trace contaminants

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Natural populations are persistently exposed to environmental pollution, which may adversely impact animal physiology and behaviour and even compromise survival. Responding appropriately to any stressor ultimately might tip the scales for survival, as mistimed behaviour and inadequate physiological responses may be detrimental. Yet effects of legacy contamination on immediate physiological and behavioural stress coping abilities during acute stress are virtually unknown. Here, we assessed these effects in barnacle goslings (*Branta leucopsis*) at a historical coal mine site in the Arctic. For three weeks we led human-imprinted goslings, collected from nests in unpolluted areas, to feed in an abandoned coal mining area, where they were exposed to trace metals. As control we led their siblings to feed on clean grounds. After submitting both groups to three well-established stress tests (group isolation, individual isolation, on-back restraint), control goslings behaved calmer and excreted lower levels of corticosterone metabolites. Thus, legacy contamination may decisively change stress physiology and behaviour in long-lived vertebrates exposed at a young age.

1. Introduction

One concern that challenges the health and well-being of natural populations is that they are persistently exposed to environmental pollution of anthropogenic sources [1,2]. It is becoming increasingly clear that this affects animal physiology and behaviour in a range of contexts (reviewed in [3]) and may ultimately result in reduced reproductive success and population declines [4–7]. Particularly polar regions, affected most strongly by global climate change [8,9], are fragile and vulnerable to ecological degradation, as they are less capable of self-regeneration and recovery due to their overall low temperatures [10].

An appropriate response to acute stress calls into action physiological and behavioural changes to maximize immediate survival [11]. The short-term activation of the hypothalamic–pituitary–adrenal (HPA) axis leads to a rise in glucocorticoids (i.e. cortisol, corticosterone) and facilitates adaptive physiological and behavioural reactions. Long-term activation, however, results in chronically elevated glucocorticoid levels, which may impair launching of the stress response in

case of an acute challenge [12]. Chronic stress may then affect the immune system, behaviour and reproductive performance [13], and even cause death (reviewed in [14]).

Pollutants can target various parts of the endocrine system (e.g. [15]), including the HPA stress axis ([7,14] for reviews). Elevated trace metal exposure may occur near active and abandoned mines due to emission and spreading of mine tailings [16] or calamities [17]. Free-ranging white storks (*Ciconia ciconia*), exposed to an industrial accident known as the Doñana disaster (Aznaicóllar, Spain), showed higher levels of corticosterone during handling and restraint than did birds from a reference site [18], with maximum levels of corticosterone being positively related to lead (Pb) [18]. Likewise, exposure to mercury (Hg) has been related to changes in glucocorticoid production [19], although in some studies corticosterone seemed to increase [20], decrease [21,22] or remain unchanged [23,24] (for a recent review see [25]). Adult male, but not female, common loons (*Gavia immer*), for example, showed a positive relationship between (Hg) levels and circulating corticosterone levels during handling and restraint, whereas other steroid hormones (testosterone, oestradiol) remained unaffected [19]. To some extent this variation might be related to the time of exposure, as individuals exposed continuously and from an early age may be particularly affected [21].

Effects of contaminants on physiological systems may translate into behavioural changes and reduced fitness. For instance, disruption of prolactin secretion as a result of (Hg) exposure lowered reproductive success in black-legged kittiwakes (*Rissa tridactyla*) through reduced paternal care [26]. Likewise, nesting near a long-term exposed (Hg) contaminated river correlated with smaller clutches and lower fledging success in female blue birds (*Sialia sialis*), possibly due to lower provisioning rates of males [27]. To the best of our knowledge, however, no studies measured effects of trace metal pollution on behaviour in response to acute stressors, neither in the wild nor in the laboratory. In particular, behaviours such as vigilance (e.g. looking up), escape (e.g. movement, pecking) and, in social species, behaviours facilitating group cohesion (e.g. vocalizations, re-establishing spatial proximity) are likely to be beneficial in the short term, but might cause a negative energy budget or call the attention of a potential predator when performed excessively [28].

The available literature provides important indications for an effect of trace metal pollution on physiology and behaviour, but as stated above, the link between pollution, physiology and behaviour in response to acute stressors is currently lacking. Earlier studies either experimentally added various amounts of (Hg) to the food of captive individuals or were non-experimental field studies that could not control whether observed effects were due to pollutants or potential differences in individual quality. Therefore, experimental field studies considering potential differences in individual quality are needed to understand the effects of environmental pollution on stress physiology and behaviour in natural environments. Hence, here we experimentally quantified how exposure to pollutants from a historic coal mine affected stress-related behaviours and excreted immuno-reactive corticosterone metabolites (CORTm) in developing barnacle goslings raised in their natural Arctic environment by human foster parents.

Coal mining began on the High Arctic Svalbard archipelago in the early 1900s and two mines are still in operation. One prominent coal mine implosion, 'the Kings Bay Affair', occurred in 1962 near Ny-Ålesund and resulted in immediate

termination of mining in 1963 [29]. Coal was remediated only close to the village, while the mine area itself was left alone. (Hg) is present in measurable amounts in soil and vegetation at relatively low levels compared to other Arctic sites, but still significantly different between the former mining area and previously not exposed areas. Furthermore, we showed in a complementary study that feeding in the former mining area resulted in higher levels of (Hg) in liver and concentration-related variations in D2-receptors in the brains of barnacle goslings [30].

For this study we submitted barnacle goslings raised either on polluted or clean grounds to three experimental stress tests: a group isolation, an open field individual isolation, and a back test, at an age of 13 to 23 days. We quantified stress-related behaviours during the tests and collected dropping samples for determination of immuno-reactive corticosterone metabolites (CORTm) prior to and after tests to (i) determine baseline levels over development as well as (ii) an acute stress response immediately after the tests. We predicted exposed goslings to show (1) stress-related behaviours during stress tests to a higher extent than their control siblings, (2) disrupted group cohesion during the group test as a result of increased stress-related behaviours and (3) an elevated absolute CORTm baseline over time as well as a stronger increase in their adrenocortical response after the tests than controls.

2. Methods

(a) Study population

We studied barnacle geese from a breeding population in Kongsfjord, Svalbard, as described in detail in [31]. For this study we used the same goslings as in [30] which were human-raised in two groups ($n = 8$ per group). For this purpose, we removed two goslings per nest ('sibling groups') during hatching on the contamination-free island Indre Brøyanne (Svalbard Archipelago, 79°00' N, 12°06' E [31]), approximately 9 km offshore from the village of Ny-Ålesund (78°55' N, 11°56' E) and marked them individually with coloured leg bands. From each pair of siblings, we randomly sorted one into the exposed group, the other one into the control group. This renders initial differences in physiology or pollution levels between experimental groups highly unlikely. Sex was determined genetically from blood samples after termination of the experiment. The exposed group consisted of five males and three females, the control group of four males and four females, respectively. Until they were 4 days old, we kept all 16 goslings as a large group which was allowed to feed in meadows in and in close proximity to the village. Once they were 5 days old, the exposed group was led daily for a minimum of 5 h to feed in a trace metal exposed mine area, sporting large coal heaps, 1.5 km to the southeast of the village, while the control group was raised in the opposite direction (1.9 km northwest) on clean locations around Ny-Ålesund ([31] for details). Both groups of goslings were allowed to graze freely during the walk and at the final destination. Since the desertion of the mine, typical Svalbard tundra vegetation (*Carex* spp., *Saxifraga* spp., mosses) regrew and now also wild geese use this area for feeding ([30] for details). Besides their separated daily walks in the assigned groups, all 16 goslings were kept together in one big group for the rest of the day. To avoid potential differences in parental effects of human foster parents between groups, goslings were accompanied by four humans (A.B., M.Ed.J., N.W.v.d.B., I.B.R.S.) in a round-robin fashion on a daily basis. The foster parents ensured the well-being of the animals by checking that goslings fed properly and had access to water, and by providing shelter and predator protection throughout daytime (i.e. continuously from 06.00 to 23.00). Overnight, goslings were

housed inside a predator-safe pen with an infrared lamp and were provided with a commercial diet for young waterfowl (Anseres I food, starter pellet, Kasper Faunafood, Woerden, The Netherlands) *ad libitum*, a supply of fresh vegetation from the clean area, and water ([31] for details). Goslings were checked daily for health and well-being, and we found no differences in various immune parameters between the groups ([31] for details). Furthermore, their body mass was taken on a daily basis. In order to minimize handling, goslings were trained to voluntarily step on a digital balance. Goslings of the two groups did not differ in their growth rates (electronic supplementary material, results, figure S1).

At the end of the experiment (i.e. when goslings were 23 days old) they were sacrificed through decapitation [30,31]. On account of this, single goslings were removed from the group and carried to a laboratory by one of their human foster parents in order to reduced stress levels before they were sacrificed. Immediately after decapitation, goslings were dissected to collect liver and brain tissue samples for determination of mercury and neuro-receptor levels, respectively [30].

(b) Behaviour during stress tests

When goslings were 13, 18 and 23 days old, goslings entered three well-established stress tests (see below). All tests were video recorded and, in all but one case (individual behaviour during the group isolation), analysed by two observers naive to the group background of the individuals, but familiar with goose behaviour.

Both the control and exposed group received a *group isolation* first (i.e. when 13 days old), in which one group was left in a novel fenced area of $2 \times 2 \times 1$ m (length $\times$ width $\times$ height) with a heat lamp as well as food and water *ad libitum* for one hour. From the video recordings we scored behaviours indicative of stress *per individual*: (a) number of 'look ups' as a measure for vigilance, (b) movement patterns, generally shown as stereo-typed pacing in the confined area, and (c) the number of pecks against the fence per 4-min interval. We further quantified on a *group-level* group density and group cohesion (i.e. the number of subgroups) every minute (see electronic supplementary material methods for details).

Furthermore, we performed an *open field individual isolation*, where we separated one individual gosling at a time for 20 min in a wooden box (length $\times$ width: 0.50×0.76 m). To control for habituation and age effects, one half of the sibling groups received the test when they were 18 days old, the other half when they were 23 days old. From video recordings, we again measured (a) number of jumps, (b) number of look ups, (c) 'border crosses', (d) number of pecks against the box and (e) the number of distress calls [32] in 4 min intervals (see electronic supplementary material methods for details).

Finally, we placed goslings in an 'on-back' position ('back test') and measured the time until the gosling righted itself. Again, one half of the sibling groups received the back test when they were 18 days old, the other half when they were 23 days old (see electronic supplementary material methods for details).

(c) Immuno-reactive corticosterone metabolites (CORTm)

When goslings were 3, 9, 12, 17 and 22 days old, we collected a minimum of three dropping samples [33] of all individuals in their respective feeding areas over a 3 h period to determine baseline corticosterone metabolites. To determine the acute physiological stress response, we collected droppings for 3 h [33] immediately after the stress tests (see electronic supplementary material methods for details). All droppings were frozen within 1 h, and later on analysed using an enzyme immuno assay (EIA, see electronic supplementary material methods for

details). We computed the average change (Δ CORTm) between baselines collected one day before a stress test and after the respective stress test as $\text{CORTm} (\bar{O}_{\text{samples stress test}}) - \text{CORTm} (\bar{O}_{\text{samples baseline}})$ per individual per test.

(d) Statistical analyses

We computed generalized linear mixed models (GLMMs) and linear mixed models (LMMs) in R version 3.2.3 [34]. To investigate the impact of the raising condition on *individual behaviour* we fitted separate models for each stress-related behaviour per 4 min time bin per individual as response variables. For individual behaviours during the group isolation we fitted the raising condition (exposed versus control) and its interaction with time (i.e. the time bin) as fixed effects test predictors, and time and sex as fixed effects control predictors. To investigate effects of the raising condition on *group behaviour*, we fitted group density and the number of subgroups (as a proxy for group cohesion) as response variables with raising condition and its interaction with time as fixed effects predictors. For behaviours during the individual isolation we fitted the raising condition and its interaction with time as fixed effects test predictors and further included the interaction of raising condition with age as test predictor. Age, time and sex were fitted as fixed effects control predictors. In all models we assessed if autocorrelation was an issue and, if necessary, included an autocorrelation term as an additional fixed effects control (see electronic supplementary material methods for details).

We analysed the impact of raising condition on stress hormones by fitting mean individual baseline CORTm and Δ CORTm after the stress tests as response variables and raising condition and its two-way interactions with age and test type (group isolation, individual isolation, back test) as fixed effects test predictors. We further fitted age, test type and sex as fixed effects control predictors and, in the model on Δ CORTm, the corresponding baseline CORTm value as an additional fixed effects control predictor (see electronic supplementary material methods for details on model formulation as well as computation of *p*-values and checks of model assumptions).

Finally, we conducted a Student's paired *t*-test to investigate if exposed and control siblings differed in the time they need to right themselves during the physical restraint in a forced 'on-back' position by using the paired *t*-test online calculator (<http://www.sthda.com/english/rsthda/paired-t-test.php>). All tests were conducted two-tailed.

3. Results

(a) Effects of exposure on behaviour in various stress tests

(i) Group isolation

Raising condition (exposed versus control) influenced *individual* stress related-behaviours during the 1 h group isolation. The two groups differed in individual levels of vigilance, i.e. the number of look-ups (likelihood ratio test (LRT): $\chi^2 = 7.865$, d.f. = 2, $p = 0.02$). Exposed goslings looked up more often than control goslings (median: exposed 59.5, control 34 times per hour; table 1). Furthermore, movement patterns differed between the two groups in a time-dependent manner (LRT: $\chi^2 = 30.513$, d.f. = 2, $p < 0.001$; table 1). As time progressed, all goslings moved around less, but this decrease in movements was far less pronounced in exposed than in control goslings (figure 1). There was no difference between the groups, however, in the number of stereo-typed pecks against the enclosure's fence throughout

Table 1. Effects of exposure to contaminants on individual and group behaviour. Results were obtained with GLMMs, test statistics were derived from likelihood ratio tests. Reference levels for factorial predictors were 'control' (condition), '18 days' (age) and 'female' (sex). Estimates depict effects of levels in parentheses relative to reference levels. Interactions are denoted by an asterisk. Non-significant interactions were removed from the models, values of non-significant interactions represent results of terms before removal from the model. All other results stem from models excluding non-significant interactions. estim. = model estimate, CI lo = CI 2.5%, CI hi = CI 97.5%, a.c.term = autocorrelation term, exp. = exposed. Significant terms are marked in bold, trends in italics. d.f. for all tests results is 1.

model	predictor	group isolation					individual isolation						
		estim.	s.e.	CI lo	CI hi	χ^2	p	estim.	s.e.	CI lo	CI hi	χ^2	p
jumps	intercept							-1.42	0.93	-4.38	0.44	a	a
	condition (exp.)							1.18	0.18	0.84	1.80	12.48	<0.001
	age (23 d)							2.65	1.23	0.06	6.29	3.98	0.046
	time ^b							-0.02	0.15	-0.36	0.31	0.02	0.882
	sex (male)							-1.06	0.22	-1.95	-0.65	11.38	0.001
	condition*age							-0.17	0.50	-1.19	0.89	0.12	0.734
look ups	condition*time							-0.39	0.28	-0.98	0.22	1.76	0.184
	intercept	0.89	0.19	0.54	1.26	a	a	54.35	10.91	30.77	78.87	a	a
	condition (exp.)	0.58	0.18	0.25	0.74	7.43	0.006	19.01	6.55	4.54	33.76	5.81	0.016
	age (23 d)							6.32	13.95	-24.96	37.25	0.20	0.653
	time ^b	0.04	0.06	-0.08	0.17	0.45	0.504	-6.35	1.84	-10.41	-2.30	7.37	0.007
	sex (male)	-0.21	0.15	-0.53	0.13	1.61	0.204	-2.09	7.83	-27.96	14.54	0.07	0.79
border crosses	condition*age							11.01	12.52	-16.69	39.53	0.74	0.39
	condition*time	-0.08	0.12	-0.33	0.18	0.44	0.507	4.45	3.37	-2.82	11.72	1.58	0.209
	intercept	3.59	0.10	3.79	3.82	a	a	3.49	0.31	2.82	4.15	a	a
	condition (exp.)	0.33	0.21	-0.11	0.79	a	a	0.48	0.23	-0.02	1.00	3.54	0.06
	age (23 d)							-0.51	0.38	-1.35	0.33	1.64	0.201
	time ^b	-0.41	0.03	-0.47	-0.34	a	a	-0.28	0.13	-0.58	0.00	3.78	0.052
	sex (male)	-0.49	0.13	-0.78	0.39	1.37	0.242	-0.21	0.27	-0.82	0.35	0.61	0.435
	a.c.term	0.12	0.02	0.07	0.16	25.53	<0.001						
	condition*age							0.47	0.43	-0.48	1.45	1.11	0.292
	condition*time	0.28	0.05	0.19	0.37	28.08	<0.001	0.22	0.14	-0.09	0.57	2.12	0.145

(Continued.)

Table 1. (Continued.)

model	predictor	group isolation					individual isolation						
		estim.	s.e.	CI lo	CI hi	χ^2	p	estim.	s.e.	CI lo	CI hi	χ^2	p
group area	intercept	9.05	0.13	8.79	9.31	a	a						
	condition (exp.)	-1.17	0.19	-1.53	-0.80	a	a						
	time ^c	-0.02	0.00	-0.03	-0.01	a	a						
	a.c.term	0.21	0.05	0.12	0.31	18.62	<0.001						
	condition*time	0.03	0.01	0.01	0.04	20.19	<0.001						
n sub-groups	intercept	0.88	0.17	0.54	1.20	a	a						
	condition (exp.)	-0.68	0.27	-1.22	-0.16	a	a						
	time ^c	-0.01	0.01	-0.02	0.00	a	a						
	a.c.term	0.06	0.06	-0.06	0.18	0.9	0.343						
	condition*time	0.02	0.01	0.00	0.03	4.37	0.037						

^avalues not presented because of having a very limited interpretation for intercepts and terms comprised in significant interactions.

^bz-transformed, the original values were 8.00 ± 4.33 (mean ± s.d.) for the group isolation and 2.50 ± 1.13 (mean ± s.d.) for the individual isolation.

^cz-transformed, the original values were 30.00 ± 17.68 (mean ± s.d.).

Figure 1. Number of border crosses (log-transformed) of exposed (black diamonds) and control (white circles) goslings over the 60 min group isolation. Points depict the raw values binned into 4 min intervals, lines show the fitted model conditional on all other predictors being at their average (dotted: exposed goslings, $n = 8$; dashed: control goslings, $n = 8$).

the whole duration of the test (median: exposed 79.5, control 63 times per hour; LRT: $\chi^2 = 0$, d.f. = 2, $p = 1$).

The raising condition also significantly affected group density (LRT: $\chi^2 = 35.465$, d.f. = 2, $p < 0.001$), whereby this effect interacted with time (table 1). In particular, the group area decreased in the control group as the isolation progressed (i.e. goslings gradually moved closer together). In the exposed group, on the other hand, the group area marginally increased over time (figure 2a). Group cohesion also differed between the exposed and control groups in a time-dependent manner (LRT: $\chi^2 = 6.522$, d.f. = 2, $p = 0.038$; table 1). At the start of the experiment the exposed group consisted of fewer subgroups, i.e. was more cohesive, but split into more subgroups as time progressed. The opposite was true for the control group: here the number of subgroups decreased marginally (figure 2b). This subgrouping pattern remained similar but became non-significant when excluding a potential outlier (electronic supplementary material, results, figure S2).

(ii) Individual isolation

Similar to the group isolation, stress-related behaviours differed between the exposed and control group when the goslings were isolated individually in a cage for 20 min. They differed significantly in the number of attempts to jump out of the cage (LRT: $\chi^2 = 14.316$, d.f. = 3, $p = 0.003$). Exposed goslings attempted to escape more often than did the controls irrespective of the goslings' age or the time progression during the test (median: exposed 18 times, control 1.5 times; table 1). Yet, overall, 23-day-old goslings jumped more often than 18 day old goslings and males jumped less than females, regardless of raising condition (median: 18 days 0 times, 23 days 28 times; males 2 times, females 15 times; table 1).

Vigilance also differed between raising conditions during the individual isolation (LRT: $\chi^2 = 8.125$, d.f. = 3, $p = 0.044$). Exposed goslings looked up significantly more often than did controls irrespective of the goslings' age or the time progression during the test (median: exposed 291.5 times, control 192 times; table 1). Regardless of the raising condition both groups looked up less as time went on (table 1). Furthermore, movement patterns of goslings during the individual isolation tended to differ between the groups (LRT: $\chi^2 =$

Figure 2. Group density and cohesion of exposed (black diamonds) and control (white circles) goslings over the course of the group isolation. Group density (a) is expressed as the area (in cm^2 , log-transformed) taken up by the group, i.e. low values indicate high density and vice versa. Group cohesion (b) is expressed as number of subgroups, i.e. low values indicate high cohesion and vice versa. Points depict the raw values binned into 4 min intervals, lines show the fitted model conditional on all other predictors being at their average (dotted: exposed goslings, $n = 8$; dashed: control goslings, $n = 8$).

6.778, d.f. = 3, $p = 0.079$). Although both groups generally tended to move less over time, exposed goslings tended to move around more than did controls (median: exposed 122.5 times, control 119.5 times; table 1). There was no interaction between raising condition and the age of the goslings or the time progression in the test (table 1). Raising condition neither had effects on the number of stereo-typed pecks against the cage (median: exposed 11 times, control 23 times; LRT: $\chi^2 = 2.162$, d.f. = 3, $p = 0.54$) nor the number of distress calls (median: exposed 438 times, control 471 times; LRT: $\chi^2 = 3.293$, d.f. = 3, $p = 0.349$).

(iii) Back test

Goslings of the exposed and control group did not differ in the time they needed to turn over after being physically constrained in the forced 'on-back' position (mean $\pm$ s.e.: exposed: $9.6 \text{ s} \pm 1.1$, control $12.1 \text{ s} \pm 3.5$; paired t -test: $t = 0.717$, d.f. = 7, $p = 0.497$).

(b) Effects of exposure on baseline and stress-induced corticosterone metabolites (CORTm)

Whereas raising condition had no effect on baseline CORTm throughout ontogeny (LRT: $\chi^2 = 2.542$, d.f. = 2, $p = 0.281$), it

Table 2. Effects of exposure to contaminants on the rise in excreted corticosterone metabolites (Δ CORTm) after stress tests. Results were obtained with an LMM, test statistics were derived from likelihood ratio tests. Reference levels for factorial predictors were 'control' (condition), 'group isolation' (test type) and 'female' (sex). Estimates depict effects of levels in parentheses relative to these reference levels. Non-significant interactions were removed from the models, values of non-significant interactions represent results of terms before removal from the model. All other results stem from models excluding the non-significant interaction. CI low = CI 2.5%, CI high = CI 97.5%. Significant terms are marked in bold, trends in *italics*. d.f. for all test results is 1.

predictor	estimate	s.e.	CI low	CI high	χ^2	<i>p</i>
intercept	17.486	0.794	15.88	19.16	<i>a</i>	<i>a</i>
condition (exposed)	2.283	0.746	0.73	3.95	<i>a</i>	<i>a</i>
test type					<i>a</i>	<i>a</i>
(indiv. isolation)	−5.068	0.996	−7.19	−3.03		
(back test)	−4.060	1.090	−6.40	−1.80		
age ^b	1.936	0.478	0.94	2.96	10.816	0.001
sex (male)	−0.793	0.394	−1.63	0.06	3.38	0.066
baseline CORTm	−0.061	0.006	−0.06	−0.05	27.039	<0.001
condition*test type					6.339	0.042
(exposed:indiv. isolation)	0.171	1.086	0.10	0.51		
(exposed:back test)	−2.099	1.037	−2.10	−1.16		
condition*age	1.705	1.189	−1.20	4.50	1.33	0.249

^avalues not presented because of having a very limited interpretation.

^bz-transformed, the original values were 18.00 ± 4.13 (mean $\pm$ s.d.).

significantly contributed to the observed variation in Δ CORTm after the stress tests (LRT: $\chi^2 = 18.033$, d.f. = 4, $p = 0.001$). The effect of raising condition was modulated by test type (table 2, figure 3), with exposed goslings showing a stronger increase in CORTm after the group isolation (LRT: $\chi^2 = 4.475$, d.f. = 1, $p = 0.034$) and individual isolation (LRT: $\chi^2 = 14.536$, d.f. = 1, $p < 0.001$) but not after the back test (LRT: $\chi^2 = 0.135$, d.f. = 1, $p = 0.714$). Irrespective of the raising condition, Δ CORTm was higher in older goslings and tended to be lower in males than in females (table 2).

4. Discussion

In this experimental field study, we show that feeding at a site polluted decades ago affects behavioural and physiological responses to acute stressors in developing barnacle goslings. This is remarkable because the contamination levels in the abandoned mine area are on the lower end relative to other, more recently polluted, sites in the Arctic [30]. Furthermore, exposure time of contamination to goslings was extremely short, only spanning a total of 19 days. Yet this is a potentially sensitive time window of development in this long-lived species, which is supported by effects that mining exposure had on neuroreceptor levels in these goslings [30].

(a) Effects of exposure on stress-related behaviours

All results combined reveal that control goslings were behaviourally either less stressed from the start and/or were able to calm down as time in the tests progressed, indicating more efficient acute stress coping compared to exposed individuals. Control goslings responded with a reduction of stress-related behaviours over the course of the individual and group isolation, particularly in attempts to escape, vigilance and movements.

Figure 3. Δ CORTm (ng CORTm/g droppings) of exposed (grey bars, $n = 8$) and control (white bars, $n = 8$) goslings after three stress tests (group isolation, individual isolation, back test). Positive values indicate an increase in CORTm compared to baseline levels, while negative values indicate a decrease. Boxplots show medians and first and third quartiles. Lower (upper) whiskers are located at the larger (smaller) value of the minimum (maximum) $\times$ value or the first (third) quartile $\pm 1.5 \times$ interquartile range. Dash-dotted lines show the fitted model conditional on the average age of the respective test type and all other predictors being at their average.

The acute stress response is flexible, and does not only depend on the type of stressor, but more importantly on how an individual perceives it [35]. Launching an appropriate stress response following an acute stressor is adaptive and crucial for health and survival [35], but chronic elevation of glucocorticoids imbalances the momentary beneficial components of HPA activation and results in a state where individuals no longer respond appropriately to life-threatening stimuli (reviewed in [35]). The elevated numbers of stress-related behaviours might be advantageous in the short term, for example, when being more vigilant results in fleeing faster from a potential predator, but detrimental in the long

term, if higher responses of exposed goslings repeatedly lead to dispensable, energetically costly, actions. Unnecessary escape attempts of single young make them easier prey, as parents can no longer protect them, and reduce time spent feeding, as those behaviours cannot be performed concurrently. After being exposed to the major bioavailable form of (Hg) methylmercury (MeHg), zebra finches reacted more strongly to a perceived threat of predation and risked starvation, as exposed birds began to feed later, resulting in lower body masses [28]. Here, an inspection of body mass data collected over development provided no indications for different body mass gain in the two groups (electronic supplementary material, results, figure S1), probably because both groups received supplemental food when outside their respective grazing areas. This could have allowed exposed goslings to replenish potential food shortages resulting from either inefficient feeding or an inability to use nutrients aptly.

Intriguingly, we also found differences in the area the groups used and the number of subgroups formed, a potential proxy for group density and cohesion, respectively. In social species, one effective mechanism of stress reduction is social support, where the presence of social allies reduces stress [36]. Not only during the individual isolation, but more importantly also during the group isolation, exposed goslings moved around more than control goslings. This did not appear to disrupt group cohesion: at the beginning of the group isolation we found fewer subgroups in the exposed group, although this difference became non-significant after excluding a potential outlier in the control group. Yet, the erratic movements displayed by the exposed goslings over the course of the test could potentially cause social disruption when a stressor continues, as they eventually split into more subgroups. Particularly relevant for precocial species, such as geese [37], movements away from the family in the wild cause a higher predation risk and demand more energy already in very young individuals, reinforcing the inappropriate stress behaviours under continuous stress described above. Such inappropriate stress responses during early development may be a mechanistic explanation for effects of early-life exposure to contaminants on reproduction as observed in white storks [38] (but see [39]).

(b) Effects of exposure on CORTm

This study provides further support that contamination modulates endocrine systems, specifically functioning of the HPA axis, because we found substantial variation in Δ CORTm after the stress tests among exposed versus control goslings. This is particularly relevant, as the range of change in the stress response, and not the exposure to stressors *per se*, ultimately determines fitness [38]. On average, all control goslings responded in a predicted manner by showing relatively stable Δ CORTm levels over all three tests, with only slight variations in Δ CORTm between individuals. In contrast, the values of exposed individuals were significantly higher in the group and individual isolation. Δ CORTm levels did not differ between exposed and control goslings in the back test, presumably a mild stressor, where we also found no differences in behaviour. In all three tests, however, variation of Δ CORTm levels in exposed individuals was much larger relative to control goslings. Notably, the range of responses in exposed goslings did not only comprise an upregulation of CORTm but in some instances an

actual downregulation, which is indicative of a more erratic, and potentially dysfunctional, stress response [7,14,35].

Chick age may play an important role as mercury accumulates with age [40], although growth dilution may actually result in (temporary) lower concentrations in developing chicks [30]. As already low concentrations of pollutants can impair the HPA system, the duration of exposure and/or nestling age are important in determining long-term negative effects [18,19,22,41]. In mercury contaminated areas, for example, baseline suppression occurred in adrenal corticosterone in juvenile tree swallows (*Tachycineta bicolor*) [21,22] at the end of the nestling period. The authors suggested that those effects are most evident once the endocrine systems are fully developed, as effects were more prominent in older nestlings [21].

We also found that age of goslings influenced Δ CORTm patterns, but it did so irrespective of the raising condition. In fact, Δ CORTm values in both groups were highest after the group isolation, when all goslings were youngest (i.e. 13 days old) and exposure time was shortest. Yet this may result from the group isolation being the first test for all goslings. Goslings therefore may have perceived this test as the strongest stressor overall. In the later tests, where goslings were tested individually and the age at the tests was randomized, goslings of both groups responded more when older (i.e. 23 versus 18 days old). A detailed analysis of how test type, order and age interact with raising condition to affect the HPA system warrants further studies.

Whereas stress CORTm differed between the two groups, this variation was absent in baseline CORTm, which illustrates that exposure does not necessarily lead to more stressed animals overall but rather to altered responses under stressful situations. This corroborates findings in a companion study involving the same goslings, where baseline plasma corticosterone levels (representing a single point in time rather than an integrated measure over time) did not differ between exposed and control goslings, but levels increased in both groups after the goslings were individually isolated [31]. Hence, both studies strengthen the fact that baseline glucocorticoid level were not (yet) altered by the past contamination. In the arctic summer, barnacle goslings were shown to excrete CORTm in a phase-shifted diel pattern, which might be indicative of a pre-maturely developed HPA system subject to change in older goslings [42]. It is possible that a suppressive effect of (Hg) on baseline CORTm levels might only become evident once the HPA system shows the characteristic adult corticosterone secretion pattern.

Generally, studying trace metal contamination is confounded by the fact that they tend to occur combined, either with other metals and/or with organic pollutants [43]. In the present study we cannot ascertain which metals and/or pollutants are responsible for our findings, but van den Brink *et al.* [30] found increased levels of Hg in droppings (mean $\pm$ s.e.: exposed 0.08 ± 0.02 , control 0.048 ± 0.01 mg kg⁻¹ dry weight [30]) and hepatic Hg (mean $\pm$ s.e.: exposed 0.030 ± 0.003 versus 0.022 ± 0.002 mg kg⁻¹ dry weight [30]) in the same exposed goslings, which further correlated positively with D2-neuroreceptors in the brain [30]. Blocking D2 receptors in the brain is known to reduce stress-related behaviours [44] and to affect the contribution of those receptors to HPA functioning [45].

By combining behavioural and physiological approaches, this study adds important insights into effects of environmental contamination on stress coping abilities in a highly

vulnerable ecosystem. Our study shows that past contamination persists in the long term at levels sufficient to elicit behavioural and physiological responses to acute stressors in developing animals already after a few days of exposure. Investigating these consequences is necessary to fill yet another gap in our understanding of the impacts of trace metals on threats to birds, wildlife in general and humans.

Ethics. The Governor of Svalbard and the Norwegian Animal Research Authority (FOTS 6331) approved the study. The well-being of the animals was ensured as described in the methods section.

Data accessibility. All relevant data are provided in the paper and its supporting information files (see electronic supplementary material, data table).

Authors' contributions. I.B.R.S., B.M.W. and J.K. devised the study, I.B.R.S., M.E.d.J., A.B., N.W.v.d.B. and M.J.J.E.L. participated in

data collection, J.K., M.J.J.E.L. and I.B.R.S. received funding, B.M.W. performed statistical analyses and E.M. supervised laboratory analyses. All authors contributed to the writing of the manuscript and gave final approval for publication.

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Part III.
Behavioural consistency
and plasticity

Chapter 7.
State-dependence explains
individual variation in nest defence
behaviour in a long-lived bird

State dependence explains individual variation in nest defence behaviour in a long-lived bird

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Abstract

1. Parental care, such as nest or offspring defence, is crucial for offspring survival in many species. Yet, despite its obvious fitness benefits, the level of defence can consistently vary between individuals of the same species. One prominent adaptive explanation for consistent individual differences in behaviours involves state dependency: relatively stable differences in individual state should lead to the emergence of repeatable behavioural variation whereas changes in state should lead to a readjustment of behaviour. Therefore, empirical testing of adaptive state dependence requires longitudinal data where behaviour and state of individuals of the same population are repeatedly measured.
2. Here, we test if variation in states predicts nest defence behaviour (a 'risky' behaviour) in a long-lived species, the barnacle goose *Branta leucopsis*. Adaptive models have predicted that an individual's residual reproductive value or 'asset' is an important state variable underlying variation in risk-taking behaviour. Hence, we investigate how nest defence varies as a function of time of the season and individual age, two state variables that can vary between and within individuals and determine asset.
3. Repeated measures of nest defence towards a human intruder (flight initiation distance or FID) of females of known age were collected during 15 breeding seasons. Increasing values of FID represent increasing shyness.
4. We found that females strongly and consistently differed in FID within- and between-years. As predicted by theory, females adjusted their behaviour to state by decreasing their FID with season and age. Decomposing these population patterns into within- and between-individual effects showed that the state-dependent change in FID was driven by individual plasticity in FID and that bolder females were more plastic than shyer females.
5. This study shows that nest defence behaviour differs consistently among individuals and is adjusted to individual state in a direction predicted by adaptive personality theory.

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KEYWORDS

behavioural reaction norms, boldness, nest defence, personality, phenotypic plasticity, risk-taking, selection, senescence

1 | INTRODUCTION

Parental care is known to be crucial for both offspring and parental fitness (Clutton-Brock, 1991; Klug & Bonsall, 2014; Royle et al., 2012). Yet, despite its obvious fitness benefits, there is huge variation in this behaviour among species, ranging from limited short term to extensive long-term parental care (Royle et al., 2012). Intriguingly, the amount of parental care can also vary among individuals of the same species. For example, many studies have reported, for a wide range of taxa, that individuals or pair members differ consistently in their nest or offspring defence behaviour over time or across contexts (e.g. mammals: Bubac et al., 2018, fish: Stein & Bell, 2015, birds: Burtka & Grindstaff, 2013; Clermont et al., 2019; Kontiainen et al., 2009; Patrick et al., 2013; Thys et al., 2019). Such consistent individual differences in behaviour (also referred to as 'animal personality') are intriguing because it implies that individual parents express limited plasticity in offspring care; that is, the range of plasticity an individual can display is smaller than the behavioural diversity that exists in the entire population (Sih et al., 2004). This raises the question why variation in offspring care arises and is maintained within populations.

Parental care in the form of nest and offspring defence directed against predators can be viewed as a 'risky' behaviour as it involves benefits by increasing offspring survival, but also comes with the risk of injury and possibly even death for the parents themselves when they, for example, attack or try to distract a predator (Alonso-Alvarez & Velando, 2012; Montgomerie & Weatherhead, 1988). Most adaptive explanations for consistent individual differences in 'risky' behaviour involve differences in stable state (Dingemanse & Wolf, 2010; Wolf & Weissing, 2010). State of an animal represents '*all those features that are strategically relevant, i.e. features that should be taken into consideration in the behavioural decisions in order to increase fitness*' (Wolf & Weissing, 2010). Consequently, when 'state' changes, individuals should adjust their behaviour accordingly (state-dependent behaviour, phenotypic plasticity; e.g. Wolf & Weissing, 2010). For example, one widely recognized state variable that can give rise to consistent individual differences in risky behaviours is an individual's residual reproductive value (RRV) or 'asset' (Wolf et al., 2007a, 2007b). The 'asset protection' principle states that individuals with a high asset, that is, high RRV, should behave risk-averse in order live long enough to harvest this asset, while individuals with a low asset, that is, low RRV, should take more risks as they have less future fitness to lose (Clark, 1994). Theoretical studies have confirmed that if there are stable differences in individual asset and/or there is a positive feedback between asset and behaviour, individuals exhibit long-lasting consistent differences in risky behaviour (Wolf et al., 2007a, 2007b; Wolf & Weissing, 2010). However, if

individual asset is changing, adaptive plasticity of the trait is expected (Nicolaus et al., 2012).

Empirically, studies across taxa report evidence for relationships between asset and risk-taking behaviour that are in line with theory (e.g. bird: Hall et al., 2015; mammal: Dammhahn, 2012; insect: Moschilla et al., 2018; crustacean: Ory et al., 2015). Specifically, differences in offspring defence behaviours of parents have been attributed to variation in state variables such as breeding experience, age, body condition, reproductive stage and timing, reproductive value and food abundance (e.g. Bubac et al., 2018; Clermont et al., 2019; Kontiainen et al., 2009; Selmann et al., 2012; Thys et al., 2019). Yet, to disentangle whether such behavioural variation originates from within- or between-individual effects in state, we need sophisticated statistical methods that are notoriously data hungry and require large datasets with many individual repeats (Brommer, 2013; van de Pol, 2012). Such longitudinal datasets are not easily obtained in the wild and can be insufficient for short lived species. Longitudinal studies on long-lived animals are ideally suited to fill this gap.

This study aims to test whether variation in individual 'state' predicts nest defence behaviour in free-living female barnacle geese *Branta leucopsis*. For 15 years, repeated measures of female nest defence towards a human intruder (flight initiation distance or FID) were collected during the breeding season. Increasing values of FID represent increasing cautiousness (also called shyness: Blumstein et al., 2016; Réale et al., 2007). We predict that FID should vary as a function of time of the season and individual age, two state variables that can vary within and between females and can determine asset: (a) net benefits of parental investment in nest defence may increase over a season through a decrease in parental re-nesting ability (Barash, 1975; Curio et al., 1984) and/or through an increase in current offspring value, as the replacements costs of these offspring increase when they get older (parental investment theory: Trivers, 1972; but see Dawkins & Carlisle, 1976; Boucher, 1977; for review see Montgomerie & Weatherhead, 1988; Caro, 2005), and (b) older individuals that are less likely to survive and reproduce in the future are predicted to take more risk to defend their current offspring than younger individuals with higher future fitness expectations (e.g. Class & Brommer, 2016). To test these predictions we used a behavioural reaction norm approach to study individual variation in plasticity and mean 'risky' behaviour (individual slope and elevation of the reaction norms respectively) over an environmental gradient (change in state; de Pol & Wright, 2009; van Dingemanse et al., 2010). We generally hypothesize that population mean FID should decrease with time of the season or population mean age but highlight three non-exclusive scenarios that can explain this pattern: (a) The pattern is explained by *behavioural plasticity* (within-individual effect). In this case, females differ in asset and mean FID (differences in elevation) but reduce FID with season or age either in a similar (individual slopes are

FIGURE 1 Examples of three behavioural reaction norms (BRN) that depict a decline in population mean flight initiation distance (FID, dashed black line) with decreasing asset but involve different types of individual responses (grey lines): (a) Population decline is caused by state dependence of FID in absence of individual variation in plasticity. Females differ in asset and mean FID (elevations of BRN) and reduce FID with decreasing asset in a similar way (slopes of BRN; I, E). (b) Population decline is caused by state dependence of FID in presence of individual variation in plasticity (I, E and I $\times$ E). (c) Population decline is caused by selection, for example, the selective disappearance of shy individuals (between-individual effect; I, no E)

similar; Figure 1a) or dissimilar manner (presence of individual by environment interaction I $\times$ E, individual slopes are dissimilar; Nussey et al., 2007; Figure 1b). (b) The pattern is caused by *selection*, that is, the selective (dis)appearance of certain individuals; here for example the selective disappearance of shy individuals (between-individual effect; Figure 1c). In this case, females differ in asset and mean FID, but do not exhibit plastic behavioural change (the slopes are null). Within a breeding season, this could indicate brood failure of shy individuals, while over age this could indicate that shy individuals suffer higher mortality. (c) The pattern is caused by a *combination of plasticity and selection* (not shown in Figure 1).

2 | MATERIALS AND METHODS

2.1 | Study system

We performed this study using a 15-year dataset (2001, 2003, 2005–2017) of a barnacle goose population nesting on the islets Storholmen (c. 30 ha) and Prins Heinrichøya (c. 3 ha) in Kongsfjorden, near the village of Ny-Ålesund (78°55'N, 11°56'E), Spitsbergen (Svalbard). The geese arrive at the breeding ground late May/early June from their wintering area in the United Kingdom. Barnacle geese have a high breeding site fidelity (Black, 1998). Geese usually start nesting a few days after arrival, although some pairs postpone laying for up to 2 weeks (Dalhaug et al., 1996). The period between laying and hatch takes approximately 29 days, and goslings fledge at about 6 weeks of age (Lameris et al., 2019; Owen & Black, 1989). Recently, with increasing spring temperatures, Kongsfjorden barnacle geese have advanced their timing of reproduction (Lameris et al., 2019).

2.2 | General goose monitoring; sex and age

Nest monitoring took place each year approximately every other day during the incubation and hatching period from June to the beginning of July. Geese were identified by individually recognizable engraved plastic leg rings (see below) when the researcher approached the nest and sex was attributed to them on the basis of these observations. Sometimes, however, individuals were attributed different sexes in subsequent years. Of 1,134 females and males in the database, 479 individuals were sighted $\geq$ three times and had the same sex attributed to them every year or in $\geq$ 75% of the sightings. For these individuals we considered the sex that was attributed to them during nest monitoring as plausible. For individuals that were sighted less often or had a different sex attributed to them during different sightings, we used data from moult catches as follows. Mass captures of moulting goose flocks happened at the end of July/beginning of August (Loonen et al., 1997). During these catches, geese were ringed with individually recognizable engraved plastic leg rings on one leg and metal rings on the other and were sexed by cloacal inspection. There was a very strong correlation between the sex of the individuals as assigned to them at the nest and the sex that was attributed to them during catch ($N = 445$, Kendall's rank correlation tau: 0.95, $z = 20.12$, $p < 0.001$). Therefore, we assigned the sex observed during catch to individuals that were sighted less than three times or of which less than 75% of the sightings attributed to them were of the same sex.

Age was assigned to an individual during moult catch. Geese that were caught as goslings could be assigned an exact age (age is 0 in year of first catch), while we assumed that other geese that were caught for the first time as adults were 2 years old, as this is the minimum age barnacle geese are physically capable of reproduction

(Prop et al., 1980). The median age of female geese in this dataset was 7 years ($N = 448$).

2.3 | Observations on flight initiation distance

During incubation, multiple observations of nest defence of individually identifiable females against a threatening stimulus (human observer) were conducted every season by assessing FID. FID is defined as the number of paces between the observer and the female at the moment the female goose flees of her nest, during a straight and slow walking approach of the observer towards the nest (see for similar methodology; e.g. Miller et al., 2013; Osiejuk & Kuczyński, 2007; Quillfeldt et al., 2005; Sjöberg, 1994). Most females walked or ran off their nest, only in some cases (usually when they bred on the edge of an island) they flew off and landed in the water. FID measurements were not randomized in space and time as they were collected during standard nest checks, but the route past the nests changed between visits to the colony. We assume that a small FID equals high nest defence and high risk-taking, and the opposite for a high FID (Blumstein et al., 2016). Observations of FID within a year were not taken into account when these observations were made (a) after a female goose was caught on the nest or (b) when eggs were collected for other research purposes, as these activities might have affected further measurements. Females that fled their nests before the observer could make his/her approach were not used in the analyses. Over all study years, 4353 successful FID observations of 465 females were done, with on average 290 observations per year, by nine different observers.

2.4 | Statistical analyses

We analysed the data using R version 3.5.1 (R Core Team, 2018). For all analyses of the FID data, we applied a square root transformation to normalize the data.

2.4.1 | Repeatability

We analysed the repeatability of female FID at different temporal scales to assess the consistency of this behaviour, using linear mixed-effects models (LMM; R package `lme4`: Bates et al., 2015) with a random effects structure as proposed by Araya-Ajoy et al. (2015). We fitted a model with two random effects: individual identity (ID) and the combination of ID and year ('ID-year'). To control for the possible confounding effect of observer, we fitted observer as a fixed effect, which enabled the calculation of 'adjusted repeatability' (Nakagawa & Schielzeth, 2010). Using the variance values from this model, we assessed the repeatability of female FID on the long term (i.e. between-individual) versus short-term (i.e. within-individual-between-year). Long-term repeatability was calculated by dividing the variance of ID (i.e. variance between-individuals) by the sum of

variance components ID, ID-year (i.e. variance within-individuals/between-years) and residual variance (i.e. variance within-individuals/within-years). Short-term repeatability was calculated by dividing the sum of ID and ID-year by the sum of variance component ID, ID-year and residual variance (Araya-Ajoy et al., 2015; Clermont et al., 2019).

2.4.2 | Testing state dependence of FID

To test whether nest defence behaviour increased with reduced asset, that is, with date within year or age across years, we conducted two sets of analyses quantifying how FID varied in relation to either date or age (model #1).

First, we analysed variation in FID as a function of date by fitting centred June day (from now on only 'June day') on which FID was measured as a fixed effect. June day was mean-centred for each year to correct for annual variation when the FID measurements were done: we subtracted the mean June day of each year from each June day on which a FID measurement was taken of an individual during this particular year. We used June day to model the seasonal gradient as opposed to nest stage (which is used in comparable studies e.g. Clermont et al., 2019; Thys et al., 2019), because this allowed us to take the whole dataset into account as we only have hatch dates for successful nests and lay dates were not determined. In general, there is limited variation in timing of hatch (average hatch day over all years ($\pm SD$) = 32 ± 4 June days) and, therefore, we did not expect a marked difference in including season as June day or days until hatch for the subset of individuals for which hatch dates were known. This expectation was confirmed when we compared the outcome of two sets of analyses for the subset of females with a known hatching date ($N = 1,082$ FID observations, $N = 212$ females), including, respectively, days until hatch in the first models (see Table S1 for results) or June day in the second models (see Table S2 for results). We found no difference in the outcome of the first and second models and the parameter estimates were virtually similar. We therefore decided that modelling the seasonal component by using June day was justified and report on this in the results.

Second, we analysed if FID varied as a function of age by fitting age and age² as fixed effects. The relationship between age and FID could be simply linear if females invest increasingly in reproduction over their lifetime (i.e. terminal investment: Clutton-Brock, 1984). However, we also included age² in the models because in long-lived species the relationship between age and FID is expected to follow a U-pattern (Møller & Nielsen, 2014; Ortega et al., 2017). Hence, we expected that risk-taking would first increase with age and then gradually decline to values associated with lower fitness.

Behavioural habituation through repeated nest visits represents a general issue with repeated behavioural testing (see e.g. Araya-Ajoy & Dingemanse, 2017; Class & Brommer, 2016; Knight & Temple, 1986). In this study, we dealt with habituation by fitting the number of visits to a female's nest across all study years as a fixed

effect in all our analyses with 0 being the first visit to an individuals' nest in the entire dataset, 1 the second visit etc. This method is deemed efficient for individuals that are visited several times per year (sensu Class & Brommer, 2016). However, because habituation and seasonal or age effects are inherently correlated (Figures S1 and S2), we performed additional analyses to better judge how estimates of plasticity in nest defence with season and age could be affected by habituation. To that end, we analysed variation in FID using models fitted with either June day or age as explanatory variables and compared them with models fitted with (a) repeated nest visits within years and June day (Table S3) and (b) repeated nest visits across years and age (Table S4) respectively.

ID, ID-year and observer were fitted in all models as random effects. All variables were z-transformed to a mean of zero and a standard deviation of one to improve interpretability of model estimates (Schielzeth & Forstmeier, 2009).

2.4.3 | Unravelling the underlying mechanisms of state dependence

To determine whether the covariation between FID and state (date or age) was caused by plasticity or selection (Figure 1), we used a within-individual centring technique to separate within-individual effects (involving phenotypic plasticity), from between-individual effects (involving selection) of state on behaviour (de Pol & Wright, 2009; van Dingemanse et al., 2010). Within-individual centring is necessary when not all individuals experience identical conditions for any within-individual fixed effect; this happens for instance when individuals are not sampled over exactly the same range of the environmental gradient (here date and age; Araya-Ajoy et al., 2015; Dingemanse & Dochtermann, 2013). We therefore calculated, for all females, the between-individual variation component which was the individuals' mean June day/age ('Mean'), and the within-individual variation component by subtracting the individuals' mean June day/age from each observation value ('Diff'; van de Pol & Wright, 2009). Models #2 thus included Mean June day/Mean Age, Diff June day/Diff Age and the number of nest visits as fixed effects and random intercepts for ID, ID-year and observer. We found within-individual effects (see Section 3) and therefore the logical next step was to examine whether there was significant between-individual variation in the slopes of the within-individual effect. Therefore, in the third models (#3) we further quantified between-individual variation in slopes over the environmental gradient and compared these with models #2 using likelihood ratio tests (LRT). Next, we tested if intercepts and slopes were correlated. Significance of such a correlation was assessed using likelihood ratio tests between models that estimated or did not estimate covariance between intercepts and slopes (van de Pol, 2012).

For all models, we give model estimates of fixed (β) and random effects (variance) with their 95% credible intervals. For this, we used the 'sim' function of the package ARM to simulate posterior distributions of the model parameters based on a 1,000 simulations

(Gelman et al., 2018). 95% credible intervals (CIs) around the estimate were then extracted by calculating the highest posterior density intervals (Hadfield, 2010). We assessed the statistical significance of fixed effects on the basis of the 95% CIs. We regard a fixed effect to be significant in the frequentist's sense when the 95% CI does not overlap with 0 (see Nicolaus et al., 2016).

3 | RESULTS

3.1 | Long- and short-term repeatability of female FID

Variance estimates were 1.17 for ID, 0.43 for ID-year and 0.76 for residuals. Thus, females were consistent in their FID both on the short- (i.e. within-individual-between-year: $r = 0.71$, CI = 0.69, 0.73) and long term (i.e. between-individual: $r = 0.55$, CI = 0.53, 0.58).

3.2 | FID and season

As predicted, FID decreased over the season, which indicated that female barnacle geese stayed on average longer on their nest upon approach later in the season (model #1, Table 1). Decomposing the seasonal effect into within- and between-individual effects revealed that the population decline in FID over the season was mostly driven by individual plasticity in FID (significant effect of 'Diff June day'), and not by selection ('mean June day' was not significant; model #2, Table 1). Further analyses revealed that females varied in their plastic response to date ($I \times E$; LRT between model #2 and #3: $\chi^2 = 22.41$, $df = 2$, $p < 0.001$). The model that allowed for a positive intercept-slope covariance (model #3, Table 1) was preferred over the model without covariance (correlation_{intercept-slope} = 0.66, LRT: $\chi^2 = 768.86$, $df = 2$, $p < 0.001$), indicating that 'bolder' females (with a lower mean FID) exhibited stronger degree of behavioural plasticity compared to 'shyer' females ('fanning out' pattern; Figure 2).

3.3 | FID and age

Supporting our expectation, FID also declined significantly with age: younger females were on average shyer than older females (model #1, Table 2). We did not find a quadratic effect of age on population mean FID. The population decline in FID with age was due to quadratic individual plastic adjustment of FID with age (within-individual effects; 'Diff Age') rather than selective (dis)appearance of shy females (non-significant between-individual effect 'mean Age': model #2, Table 2). Females differed significantly in their plastic adjustment of FID to age (significant $I \times E$, LRT between model #2 and #3: $\chi^2 = 45.75$, $df = 2$, $p < 0.001$) and plastic response was stronger for bolder females (correlation_{intercept-slope} = 0.20, LRT: $\chi^2 = 803.43$, $df = 2$, $p < 0.001$; 'fanning out' pattern; Figure 3, model #3 in Table 1).

TABLE 1 Model summary of three linear mixed-effects models investigating variation in flight initiation distance as a function of time of the season (June day). Model #1 was used to investigate the overall population trend, model #2 was used to separate within-individual effects ('diff June day') from between-individual effects ('mean June day') and model #3 was used to investigate whether there was between-individual variation in the slopes of the within-individual effect ($I \times E$). The included random effect ID represents individual identity and ID-year the breeding attempt identity. (A) For each model, the predictions of the random regression variance components are given with the 95% credible intervals (CIs) in parentheses and (B) the estimates of the fixed effects are given with the 95% CIs in parentheses. Significant fixed effects are highlighted in bold

Season (A)					
Model	Random regression variance				
	ID	ID-year	Observer	Residuals	$I \times E$
#1	0.66 (0.59, 0.73)	0.14 (0.13, 0.15)	0.07 (0.05, 0.09)	0.32 (0.30, 0.33)	
#2	0.66 (0.60, 0.72)	0.14 (0.13, 0.15)	0.06 (0.05, 0.09)	0.32 (0.30, 0.33)	
#3		0.15 (0.14, 0.16)	0.08 (0.05, 0.11)	0.31 (0.30, 0.32)	0.02 (0.003, 0.70)
Season (B)					
Model	Fixed effects				
	Intercept	# Visits	June day	Mean June day	Diff June day
#1	-0.01 (-0.13, 0.18)	-0.29 (-0.32, -0.24)	-0.07 (-0.09, -0.05)		
#2	-0.03 (-0.14, 0.18)	-0.28 (-0.32, -0.24)		0.04 (-0.02, 0.09)	-0.06 (-0.09, -0.04)
#3	0.06 (-0.12, 0.19)	-0.29 (-0.32, -0.24)		0.04 (-0.01, 0.10)	-0.06 (-0.08, -0.03)

FIGURE 2 Predicted individual mean values of flight initiation distance (FID) as a function of individual mean-centred June day. The grey lines represent a subset of 143 individuals with more than 10 measurements of FID (within-individual response, model #3). The black dotted line represents population level seasonal response (model #1)

3.4 | Habituation effect

The total number of visits to nests of female geese was negatively correlated with FID in all models, suggesting that over time females

became less sensitive to human disturbance (they stayed longer on their nest; Tables 1 and 2). The additional analyses revealed that plasticity of FID over the season occurred independently of habituation (Table S3) while plasticity of FID over age was in fact confounded with habituation ('Diff Age' became non-significant in model #2, Table S4). In this latter case, controlling for habituation further revealed that individuals with longer FIDs lived longer ('mean Age' became significant in model #2, Table S4).

4 | DISCUSSION

This study tested whether nest defence behaviour (flight initiation distance or FID; a measure of risk-taking) was tuned to variation in individual 'state' (future fitness expectations) in free-living female barnacle geese. We predicted that FID would decline as a function of time of the season and individual age, two state variables that can vary within and between females and reduce future fitness expectations. We detected that female FID was strongly repeatable at both the long term (i.e. between-individual: 0.55) and short term (i.e. within-individual-between-year: 0.71). As predicted, FID decreased over the season and over age for all females, showing that on average, females were bolder later in the season and at older age. Decomposing these population patterns into within- and between-individual effects revealed that the declines in population mean FID over the season and over age were driven by individual plasticity in FID and not by selection. Females exhibited significant variation in plastic response ($I \times E$) with bolder females being more responsive than shyer individuals (positive correlation between intercepts and slopes of the reaction norms).

TABLE 2 Model summary of three linear mixed-effects models investigating variation in flight initiation distance as a function of individual age. Model #1 was used to investigate the overall population trend, model #2 was used to separate within-individual effects ('diff Age/diff Age²') from between-individual effects ('mean Age/mean Age²') and model #3 was used to investigate whether there was between-individual variation in the slopes of the within-individual effect (I × E). The included random effect 'ID' represents individual identity and 'ID-year' the breeding attempt identity. (A) For each model, the predictions of the random regression variance components are given with the 95% credible intervals (CIs) in parentheses and (B) the estimates of the fixed effects are given with the 95% CIs in parentheses. Significant fixed effects are highlighted in bold

Age (A)								
Model	Random regression variance							
	ID	ID-year	Observer	Residuals	I × E			
#1	0.72 (0.62, 0.77)	0.13 (0.12, 0.14)	0.08 (0.06, 0.10)	0.32 (0.30, 0.33)				
#2	0.73 (0.63, 0.78)	0.12 (0.11, 0.13)	0.08 (0.06, 0.10)	0.32 (0.30, 0.33)				
#3		0.09 (0.08, 0.10)	0.08 (0.05, 0.09)	0.32 (0.30, 0.33)	0.03 (0.02, 0.81)			

Age (B)								
Model	Fixed effects							
	Intercept	# Visits	Age	Age ²	Mean Age	Mean Age ²	Diff Age	Diff Age ²
#1	-0.04 (-0.17, 0.15)	-0.21 (-0.30, -0.15)	-0.18 (-0.33, -0.04)	0.09 (-0.05, 0.21)				
#2	0.06 (-0.13, 0.21)	-0.20 (-0.28, -0.09)			0.17 (-0.07, 0.59)	-0.26 (-0.64, 0.04)	-0.09 (-0.16, -0.02)	0.06 (0.04, 0.10)
#3	0.01 (-0.14, 0.17)	-0.30 (-0.39, -0.18)			0.23 (-0.05, 0.57)	-0.24 (-0.61, 0.05)	-0.01 (-0.10, 0.07)	0.10 (0.06, 0.13)

FIGURE 3 Predicted individual mean values of flight initiation distance (FID) as a function of individual mean-centred age. The grey lines represent a subset of 134 individuals with a known age and with more than 10 measurements of FID in total (within-individual effect, model #3). The black dotted line represents population level response (model #1)

Below we discuss the ecological and evolutionary implications of our findings.

4.1 | Nest defence behaviour as a personality trait

Our finding of high long- and short-term repeatability of female FID is very similar to those found in a recent study on nest defence behaviour against a human intruder in a related goose species, the Canada goose *Branta canadensis* (Clermont et al., 2019: long-term repeatability: 0.50, short-term repeatability: 0.72). As found in previous studies (e.g. Kontiainen et al., 2009; Patrick et al., 2013), repeatability of nest defence appears to be relatively high for a behavioural trait (on average 0.37; Bell et al., 2009). Our study thereby adds to the evidence that individuals consistently differ in their tendency to take risks in protecting their offspring (e.g. Betini & Norris, 2012; Fresneau et al., 2014; Kontiainen et al., 2009; Patrick et al., 2013; Thys et al., 2019) despite exhibiting plasticity in that trait: that is, the relative rank of individuals is preserved along the environmental gradients. The substantial variation in the elevations of the FID behavioural reaction norms can originate from both from additive genetic variance and other, non-heritable, stable differences between individuals, that is, 'permanent environment effects' (e.g. Dingemanse et al., 2010). Future studies should identify sources of variation in FID and establish if variation in barnacle goose nest defence is linked to fitness differences. This knowledge would be valuable, because if nest defence is linked to fitness, and is heritable, then it would

have the potential to evolve under selection (Dingemanse & Reale, 2005).

4.2 | Plastic adjustment of nest defence behaviour to changes in asset

Female barnacle geese plastically increased their nest defence over the season, as reflected by a decrease in their FID. Parental investment theory and the reneesting potential hypothesis are two non-exclusive explanations for this pattern (see Introduction; Shew et al., 2016). Arctic-breeding birds such as barnacle geese experience a very short breeding season, as the snow-free season is limited (mean snow-free season length in Kongsfjorden is $81 \text{ days} \pm 12$; Lameris et al., 2019) which seems to prohibit reneesting (from laying till fledge takes approx. 71 days; see Methods). Reneesting has rarely been observed in the Arctic and only seems to happen when clutches are depredated by polar bears *Ursus maritimus* early in the season when geese are still laying or have just started to incubate (J. Prop, pers. comm.; Mitchell et al., 1988). Hence, we consider it likely that females breeding in the Arctic adjust their nest defence to a decrease in asset over the season.

Supporting the asset protection principle (Clark, 1994), we also found that females increased their nest defence with age, that is, with decreasing asset (or decreasing future fitness expectations). We further detected a negative quadratic relationship between FID and age, meaning that nest defence first increased with age, reaching a high point with middle age and then declined with older age. Such age-specific patterns that are bell- or inverted-U-shaped have been detected more often for reproductive rates, survival probabilities (e.g. Berman et al., 2009; Forslund & Pärt, 1995; Jones et al., 2008; Patrick & Weimerskirch, 2014; Rockwell et al., 1993) and, more recently, for offspring defence (Møller & Nielsen, 2014; Ortega et al., 2017). A possible explanation for our results is that, with increasing age, defence first increased with decreasing asset and improvements of competence (e.g. breeding experience, access to resources; Forslund & Pärt, 1995) and then gradually declined as a result of reduced reproductive performance (Rockwell et al., 1993).

The additional analyses on habituation revealed that the effect of season was independent of habituation (Table S3), but that the plastic effect of age on FID needs to be interpreted with more care. The within-individual effect of age on FID appeared indeed to be confounded with habituation (Table S4; Figure S2), but controlling for habituation revealed a significant and positive between-individual effect in this model which indicates selective disappearance of bold individuals (Table S4). These results imply that disregarding habituation can mask selective disappearance and cause an overestimated individual plasticity coefficient. Hence, future studies should include habituation effects.

4.3 | Female differences in plasticity

Interestingly, our study revealed the existence of individual differences in plasticity with bolder females being generally more plastic

than shyer individuals. Such differences in plasticity or reactivity between behavioural types have been extensively reported in the 'coping style' literature (Benus et al., 1987; Coppens et al., 2010; Koolhaas et al., 2010). However, in contrast to our findings, bolder individuals were often found to be less responsive to environmental change and assumed to rely mainly on internal routines ('pro-active' coping style), while shyer individuals were more responsive to environmental change ('re-active' coping style; e.g. Cornwell et al., 2019; Jolles et al., 2019; Kareklas et al., 2016; Koolhaas et al., 2010). The discrepancy found with the coping-style literature supports a recent review showing that the relationships between personality and plasticity are often equivocal and lack consistency (Stamps, 2016).

Relatively few other studies have used a behavioural reaction norm approach to test for $I \times E$ in parental behaviours in general (Royle et al., 2014; Westneat et al., 2011) or, in nest defence behaviours in particular (Betini & Norris, 2012; Kontiainen et al., 2009; Thys et al., 2019). In line with our results, both in tree swallows *Tachycineta bicolor* (Betini & Norris, 2012) and Ural owls *Strix uralensis* (Kontiainen et al., 2009), bolder birds were found to be more plastic. In female great tits *Parus major*, however, no evidence was found for individual differences in plasticity (Thys et al., 2019).

Adaptive hypotheses for individual differences in plasticity are still under development (Stamps & Biro, 2016). Presently, we can only speculate about the causes and consequences of the observed individual differences in plasticity in FID of female barnacle geese ($I \times E$). If the individual differences in plasticity are not caused by permanent environmental effects (e.g. maternal and natal effects) and are mirrored on the genetic level by genotype by environment ($G \times E$) or by genotype by age ($G \times A$) interactions, then this would imply that plasticity could evolve under selection (Brommer, 2013; Brommer & Class, 2015; Nussey et al., 2007). Furthermore, even though there may be a heritable basis to individual differences in plasticity, evolution can still be constrained because mean trait level and plasticity can be genetically correlated (Lande, 1979; Lande & Arnold, 1983). In our study, bolder females were more plastic. If this correlation translates at the genetic level, this means plasticity and boldness cannot evolve independently which may affect the trajectories and rates of evolutionary changes available to populations (Brommer, 2013).

Our study solely focused on females and it is currently unknown how nest defence behaviour varies in males, who also defend the nest. To unravel the exact adaptive mechanisms underlying the maintenance of variation in personality and plasticity, future studies on geese should investigate the realized fecundity and longevity of different behavioural types in both males and females.

5 | CONCLUSIONS

In line with adaptive personality theory, we found that nest defence of barnacle goose females differs consistently among individuals and is adjusted to individual state (season and age). Additionally, this study revealed that behavioural types differ in their level of

plasticity, with bolder females being generally more plastic than shy females. We thus show that variation in state can explain the emergence of variation in behaviour. The exact mechanism needs further scrutinizing and future studies should focus on the persistence/enhancement of such variation which requires studying feedback loops between state and behaviour. Furthermore, it would be worthwhile quantifying the direct fitness consequences of individual variation in nest defence to formally establish the adaptive nature of variation in personality, plasticity and their correlation.

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AUTHORS' CONTRIBUTIONS

M.E.d.J. and M.J.J.E.L. conceived the ideas and designed methodology; M.E.d.J., M.J.J.E.L. and R.W.F. collected data; M.E.d.J. analysed the data with the help of M.N.; M.E.d.J. led the writing of the manuscript; M.E.d.J., M.N. and R.W.F. contributed critically to the drafts and all authors gave final approval for publication.

DATA AVAILABILITY STATEMENT

Data available from the Dryad Digital Repository <https://doi.org/10.5061/dryad.sf7m0Cg50> (de Jong et al., 2020).

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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Chapter 8.

Synthesis

The overall aim of my PhD, as stated in **Chapter 1**, was to gain understanding on how barnacle geese are affected by and cope with several changes in their Arctic breeding area. The thesis had four objectives: 1) To quantify the effects of nest fleas (*Ceratophyllus vagabundus vagabundus*), a parasite whose role might become more prominent under climate warming in Arctic regions, on the behaviour and reproductive success of Svalbard barnacle geese (**Chapter 2**), 2) To investigate the rate of advancement in the timing of reproduction in three Arctic barnacle goose populations (Russia and Svalbard) with earlier springs and what the possible effects are of climate warming on some aspects of their reproductive success (**Chapter 3**), 3) To examine the effects of exposure to contaminants from a historic coal mine area on physiology and behaviour in Svalbard barnacle goslings (**Chapters 4, 5 & 6**), and 4) To study the potential for behavioural change in Svalbard barnacle geese by investigating individual variation in nest defence behaviour over season and age (**Chapter 7**). Below, I shortly discuss the results per chapter.

In **Chapter 2**, my co-authors and I investigated the influence of nest fleas on barnacle goose reproductive success. Nest fleas (*C. vagabundus vagabundus*) have been documented as early as 1921 in goose nests near Longyearbyen, Svalbard (Summerhayes & Elton, 1923), but in other parts of the Arctic they may be a new parasite and their presence may increase through climate warming (Harriman, Alisauskas, & Wobeser, 2008). We were able to show that nest flea abundance was negatively associated with Svalbard barnacle goose reproductive success, but that these effects did not seem mediated through effects of fleas on female incubation behaviour. However, further research will be needed to understand the exact relationship between nest fleas and goose reproductive success. More knowledge

on interactions between nest fleas and their hosts are important, as fleas can be a vector of diseases that are new to the Arctic. For example, in the Canadian Arctic, migratory geese appear to play a role in the spread of flea-borne pathogenic *Bartonella* spp., the bacterium causing cat scratch fever, which was assumed to be absent from the Arctic. Arctic foxes are infected with this bacterium, probably through nest flea bites or dirt while depredating geese, eggs or goslings (Buhler et al., 2020).

From the results in **Chapter 3**, we learn that both in the high- and low-Arctic, barnacle geese have changed their reproductive timing in relation to advanced dates of snowmelt. In all colonies, geese advanced the date of egg laying equally, but this did not go at the same pace as the advancement of date of snowmelt. Geese in the high Arctic laid their eggs before the date of snowmelt, while geese in the low Arctic laid their eggs close to the date of snowmelt. To our surprise, geese in the high-Arctic laid their eggs much earlier than in the low-Arctic, even though in the northern colonies the snow melted on average 16 days later. It seems that in the high Arctic barnacle geese are able to have sufficient body stores to start earlier reproduction. Nevertheless, in contrast to low-Arctic geese, high-Arctic geese seemed to be constrained in advancing their reproduction sufficiently as most geese laid their eggs after the expected peak in reproductive output. If high-Arctic geese suffer fitness reductions due to this phenological mismatch, they may be more sensitive to the increasing advancement of spring under climate warming than low-Arctic geese. This warrants further investigation.

It may challenge general assumptions that certain Arctic areas, which several terrestrial animal species make use of, have been contaminated through earlier mining activities. In **Chapters 4, 5 and 6**, we discuss effects of such contamination on human-raised

goslings which grazed in a contaminated mine area near the former mining village of Ny-Ålesund while their social siblings foraged in a clean control area. My co-authors and I investigated mercury levels in soil, vegetation and gosling livers and if barnacle goslings were affected both in their physiology and behaviour when raised in such contaminated areas. In **Chapter 4**, we found that the mercury levels in soil and vegetation from the mine area were respectively 3- and 2-times higher than in the control area. These levels fell within ranges found at other Arctic sites, but were very low compared to e.g. soils in industrialized areas in The Netherlands. Mine goslings had higher hepatic mercury levels than control goslings. Mercury is known to be neurotoxic and in this context we studied the levels of two neuroreceptors, the NMDA-receptor and the D2-receptor. For both it has been shown earlier that dysregulation can affect behaviour. We did not find a difference in NMDA- and D2-receptor levels between mine and control goslings. D2-receptor levels were positively correlated hepatic mercury concentrations, which could potentially indicate that there are subtle changes in D2-receptors due to mercury exposure. It would be useful to investigate other contaminants in future studies to close knowledge gaps; e.g. selenium and their possible influence on mercury toxicokinetics and dynamics and how this may influence neuroreceptors and eventually gosling behaviour in a larger setup.

In **Chapter 5**, we showed that exposure to a contaminated coal mining area had no effects on baseline immune indices and plasma corticosterone levels of barnacle goslings. After experimental social isolation, all goslings had higher blood corticosterone concentrations irrespective of mine-exposure. This indicated that this type of isolation is a strong stressor in barnacle goslings, but that contamination did not dysregulate adrenocortical function. Interestingly, mine goslings tended to show

a decrease in haemagglutination titres after social isolation, which indicates decreased natural antibody activity, but we did not find other differences between the groups in immune parameters after social isolation. We detected a negative effect of social isolation on haptoglobin-like activity similarly for both gosling groups. This may expose the goslings to increased levels of oxidative stress, especially when infected with extracellular or intracellular pathogens. In conclusion, in this study we found no strong evidence that exposure to contaminants from a former mine area affected gosling corticosterone levels and immune parameters, also not when they experienced social stress.

In **Chapter 6**, we detected differences in behaviour and corticosterone metabolites between the mine and control goslings after three stress tests (group isolation, individual isolation, on-back restraint). In comparison with the study above, corticosterone metabolites measured in droppings represent an integrated measure over time rather than a single point in time when plasma corticosterone is measured. During the group isolation mine goslings had a higher vigilance than control goslings (i.e. the number of look-ups). Also, all goslings moved around less as the isolation progressed, but this decrease in movements was far less obvious in mine than in control goslings. Furthermore, control goslings moved closer together over time (i.e. group density), while this was significantly less for mine goslings. In addition, group cohesion differed between mine and control goslings over time, with mine goslings starting out with fewer subgroups (i.e. more cohesive) but fell apart into more subgroups over time, while the opposite pattern was the case for the control goslings. During the individual isolation, mine goslings tried to jump out of the cage more often, looked up more often and moved around more than control goslings. Goslings did not behave differently during the back test (i.e. goslings were placed in an 'on-

back' position and we measured the time until goslings righted themselves). This indicates that overall, control goslings behaved calmer than mine goslings after the stress tests. In addition, mine goslings excreted higher levels of corticosterone metabolites after the group isolation and individual isolation but not after the back test. Thus, grazing in a historic coal mine changed stress behaviour and stress physiology in barnacle goslings.

In **Chapter 7**, we studied the potential for behavioural change of Svalbard barnacle geese in response to the environment. Specifically, we investigated plasticity and consistency in nest defence of female barnacle geese over two environmental gradients; season and, as an internal environmental gradient, age. Season and age are two state variables that can vary within and between females and can determine asset (future fitness expectations). We used repeated measures of nest defence towards a human intruder (flight initiation distance or FID) of females of known age that were collected during 15 breeding seasons. We found that females showed strong and consistent differences in their FID within- and between-years. Females decreased their FID with season and age, as predicted by theory. By decomposing these population patterns into within- and between-individual effects we show that the declines in population mean FID over the season and over age were driven by individual plasticity in FID and not by selection (i.e. (dis) appearance of individuals with certain FIDs). In addition, females had significant variation in their plastic response ($I \times E$) with bolder females being more responsive than shy individuals. Consequently, we show that variation in state can explain the emergence of variation in behaviour, but that the exact mechanism needs further scrutinizing. Future studies should focus on the persistence/enhancement of such variation by studying feed-back loops between state and behaviour and quantify the direct fitness consequences

of individual variation in nest defence. One very intriguing aspect of our results in a population context would also be to establish whether individuals that are more in plastic in their nest defence are more plastic in general (Cornwell, McCarthy, Snyder, & Biro, 2019; Stamps, 2016). Individuals that are behaviourally plastic in one context, may also be better at adjusting to environmental cues in a different context. These individuals, experienced in interpreting and responding to environmental cues, may then face lower costs of being plastic than inexperienced individuals (Wolf, van Doorn, & Weissing, 2008). In such a long-distance migrant as the barnacle goose it would for example be very interesting to examine if individuals that are more plastic in their nest defence behaviour are also the ones that can more easily learn how to adjust their migration behaviour in response to climate warming (Lameris et al., 2019; Oudman et al., 2020; Tombre, Oudman, Shimmings, Griffin, & Prop, 2019).

In conclusion, my PhD thesis demonstrates the diverse ways in which individual Svalbard barnacle geese are affected and can cope with diverse changes in their Arctic breeding area. The main insights of my PhD are that:

- Barnacle goose reproductive success was negatively correlated with nest flea abundance, but this association was not mediated through a negative effect of fleas on female incubation behaviour.
- Barnacle geese in the high and low Arctic showed a similar advancement in the timing of egg laying with earlier springs, but they did not fully compensate for the advancement in snowmelt. High-Arctic geese might be especially vulnerable to the negative effects of climate warming, as their advancement in reproduction seems inadequate to reach the optimal level of reproductive success associated with earlier laying dates.
- Exposure to contaminants from a historic

coal mine area increased mercury levels in gosling livers, but there were no strong neurotoxic effects, nor strong negative effects on immune parameters or plasma corticosterone levels. Grazing in a historic coal mine changed stress-related behaviours and corticosterone metabolite levels in barnacle goslings.

- Female nest defence behaviour differed consistently between individuals and was adjusted to individual state in a direction predicted by adaptive personality theory.

Overall, on a population level, a warming climate may have the largest influence of all the changes in the Arctic breeding area that have been under investigation in this thesis. Earlier work by Layton-Matthews et al. (2019) in the same population demonstrated that egg production and hatching success was increased due to earlier springs and warmer summers. Nevertheless, the effects of factors such as parasites and contamination can become more pronounced under climate warming and should not be overlooked in future studies. Behaviourally, geese are known for their plasticity, but our study on the tendency of females to take risks in protecting their offspring, adds to the evidence that there are limits. Geese consistently differ from each other, which indicated that the range of plasticity an individual can display is smaller than the behavioural diversity that exists in the entire population. For now, the Svalbard barnacle goose population seems to be on the top of their game, a conservation success story and thriving in the current environment. However, with the growing array of changes in the Arctic and along their flyway, which are often also increasingly fast, behavioural plasticity may at a certain point no longer suffice. Time will tell whether the adaptive capacity of Svalbard barnacle geese proves sufficiently strong to cope with these new conditions.

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Samenvatting

Broeden in een veranderende arctis: fysiologie en gedrag van Brandganzen

Dit proefschrift combineert vier jaar onderzoek en lange-termijn data aan brandganzen (*Branta leucopsis*) die in het Arctisch gebied broeden. Het doel van dit onderzoek was om inzicht te krijgen in de wijze waarop Arctisch broedende brandganzen omgaan met veranderingen in hun omgeving. Het proefschrift heeft vier specifieke doelstellingen:

1. Het kwantificeren van de effecten van nestvlooiën (*Ceratophyllus vagabundus vagabundus*) op het gedrag en broedsucces van Spitsbergen brandganzen (Hoofdstuk 2).
2. Het vergelijken van veranderingen in de timing van reproductie en het broedsucces in drie Arctische brandganzen populaties als gevolg van klimaatverandering (Hoofdstuk 3).
3. Het bestuderen van de effecten van blootstelling aan vervuiling, veroorzaakt door een historische steenkoolmijn, op fysiologie en gedrag van brandganzen-kuikens (Hoofdstuk 4, 5 & 6).
4. Het inschatten van flexibiliteit in gedrag van brandganzen door individuele variatie in nestverdediging te bestuderen over het seizoen en met het ouder worden van de ganzen (Hoofdstuk 7).

Wat betreft het effect van nestvlooiën (hoofdstuk 2) vonden we een negatief verband tussen de aantallen nestvlooiën en het broedsucces van Spitsbergen brandganzen, maar deze vondst konden we niet verklaren door effecten van vlooiën op het gedrag van de vrouw gedurende het broeden. Verder onderzoek is nodig naar het oorzakelijk verband tussen nestvlooiën en broedsucces. Dergelijk onderzoek is belangrijk, want vlooiën zijn een potentiële vector van nieuwe

ziektes in het Arctisch gebied.

In hoofdstuk 3 lieten we zien dat, zowel in de hoge als lage Arctische gebieden, de timing van broeden van brandganzen is veranderd tussen de jaren 2000-2016 vanwege de steeds eerdere datum van het smelten van de sneeuw. In alle kolonies vervroegden de ganzen het leggen van hun eerste ei, maar dit ging niet gelijk op met het vervroegen van de sneeuws melt in het voorjaar. Ganzen in de hoge arctis legden hun eieren voor de datum van sneeuws melt, terwijl ganzen in de lage arctis hun eieren dichtbij de sneeuws melt datum legden. Tot onze verrassing legden ganzen in de hoge arctis hun eieren veel eerder dan ganzen in de lage arctis, hoewel in de noordelijke kolonies de sneeuw gemiddeld gezien pas 16 dagen later smolt. Het lijkt er dus op dat brandganzen in de hoge arctis genoeg vetvoorraden hadden opgebouwd om eerder met reproductie te beginnen. Maar op basis van data aan nestsucces en aantallen kuikens uit de hoge en lage arctis, lijken ganzen in de hoge arctis hun reproductie niet vroeg genoeg te beginnen, want de vroegst leggende ganzen hebben het grootste nestsucces. Als ganzen die in de hoge arctis broeden hierdoor een vermindering in fitness ondervinden, zouden ze mogelijk gevoeliger kunnen zijn voor de steeds vroegere voorjaren dan ganzen in de lage arctis. Dit is een belangrijke invalshoek voor toekomstig onderzoek.

Uit de hoofdstukken 4, 5 en 6 komt naar voren dat Spitsbergen niet de onaangetaste wildernis is zoals vaak gedacht wordt. Al vier eeuwen vindt er commerciële exploitatie van natuurlijke rijkdommen zoals steenkool plaats met vervuiling tot gevolg. We onderzochten experimenteel de effecten van vervuiling bij een historische steenkoolmijn op brandganzenkuikens. We deden dit door brandganzenkuikens na uitkomst te laten inprenten en opgroeien bij de onderzoekers. Deze kuikens werden verdeeld in twee groepen, waarvan één groep graasde in het

vervuilde mijngebied en de andere groep in schone gebieden *rondom het voormalige mijn*dorp Ny-Ålesund. In hoofdstuk 4 tonen we aan dat de kwikgehalten in grond en vegetatie in het mijngebied respectievelijk 3- en 2-maal hoger waren dan in het controle gebied. Deze gehalten waren vergelijkbaar met niveaus die in andere Arctische gebieden zijn gevonden, maar ze waren erg laag in vergelijking met bijvoorbeeld grond in industriële gebieden in Nederland. Kuikens die graasden in het mijngebied hadden hogere kwikwaardes in hun lever in vergelijking met controle kuikens die graasden in het schone gebied. Kwik kan neurotoxisch zijn en daarom bestudeerden we de gehalten van twee neuroreceptoren; de NMDA receptor en de D2-receptor. Voor beide receptoren heeft men eerder laten zien dat ontregeling hiervan gedrag kan beïnvloeden. We vonden geen verschillende gehalten van NMDA- en D2-receptoren tussen beide groepen kuikens, maar D2-receptor gehalten waren positief gecorreleerd met het kwikgehalte in de levers. Het zou nuttig zijn om het effect van andere vervuulende stoffen in dit mijngebied verder te onderzoeken.

In hoofdstuk 5 lieten we zien dat experimentele blootstelling aan een vervuuld koolmijngebied geen effecten had op immuun parameters en corticosteron gehalten in bloedplasma van brandganzenkuikens. Het immuunsysteem moet de ganzen beschermen tegen ziektes en corticosteron is een hormoon dat gedrag en fysiologie reguleert in stress situaties. Na sociale isolatie, hadden alle kuikens hogere corticosteron concentraties in hun bloed ongeacht of ze in het mijngebied hadden gegraasd of niet. Dit betekende dat, hoewel isolatie een sterke stressor was voor brandganzenkuikens, de vervuiling het functioneren van de bijnierschors, waar dit hormoon geproduceerd wordt, niet verstoorde. Interessant genoeg leken de kuikens uit het mijngebied een afname te hebben in hemagglutinatietiters na sociale isolatie in vergelijking met controle kuikens,

wat kan duiden op een verlaagde natuurlijke antilichaamactiviteit en dus een minder functionerend immuunsysteem. We vonden geen andere verschillen tussen de groepen in immuun parameters na sociale isolatie. We detecteerden wel een negatief effect van sociale isolatie op 'haptoglobin-like activity' die vergelijkbaar was in beide groepen. Dit kan de kuikens blootstellen aan verhoogde niveaus van oxidatieve stress, wanneer ze geïnfecteerd raken met pathogenen. In deze studie vonden we dus geen sterk bewijs dat blootstelling aan vervuiling in een voormalige koolmijn corticosteron gehalten en immuun parameters beïnvloedde, ook niet wanneer de kuikens sociale stress ondervonden.

In hoofdstuk 6 ontdekten we verschillen in corticosteron metabolieten gemeten in feces en in gedrag tussen beide groepen kuikens na drie stress testen (groepsisolatie, individuele isolatie en een 'rug-test' waarbij de kuikens op de rug worden gelegd en gekeken wordt hoe snel ze weer rechtop staan). In vergelijking met de studie in hoofdstuk 5, geven corticosteron metabolieten gemeten in feces een geïntegreerde maat over tijd in plaats van een enkel punt in tijd wanneer corticosteron in bloedplasma wordt gemeten. Gedurende de groepsisolatie, waren de kuikens die in het mijngebied gegraasd hadden, waakzamer dan controle kuikens (i.e. ze keken vaker op). Verder bewogen alle kuikens zich minder gedurende de isolatie, maar deze afname in bewegingen was minder duidelijk bij de kuikens uit het vervuilde gebied dan bij de kuikens uit het schone gebied. Bovendien kwamen de kuikens die in het schone gebied hadden gegraasd steeds dichter bij elkaar (i.e. grotere groepsdichtheid), terwijl dit beduidend minder was voor de kuikens die in het mijngebied hadden gegraasd. Ook verschilde de groepscohesie tussen de beide groepen kuikens, waarbij de kuikens uit het vervuilde gebied begonnen met weinig subgroepen (i.e. grotere groepscohesie)

die uiteen vielen over de tijd, terwijl voor de kuikens uit het schone gebied het tegenovergestelde het geval was. Gedurende de individuele isolatie, probeerden kuikens, die in het vervuilde gebied hadden gegraasd, vaker uit de kooi te springen, keken ze vaker op en bewogen ze meer rond dan kuikens uit het schone gebied. De kuikens verschilden niet in de snelheid van bewegen in de 'rug-test'. Over het algemeen gedroegen de kuikens uit het schone gebied zich dus kalmer dan de kuikens uit het vervuilde mijngebied tijdens de stress testen. Bovendien scheidden de kuikens uit het vervuilde gebied hogere gehalten aan corticosteron metabolieten uit na isolatie, maar niet na de 'rug-test'. Het lijkt er dus op dat opgroeien in een vervuild koolmijngebied gedrag en fysiologie kan veranderen.

In Hoofdstuk 7 onderzochten we de gedragsmatige flexibiliteit van Spitsbergen brandganzen door te kijken of vrouwelijke brandganzen hun gedrag veranderen binnen het seizoen en met het ouder worden. We gebruikten meerdere metingen van nestverdediging van vrouwelijke ganzen met een bekende leeftijd tegen een menselijke bezoeker van het nest (vluchtinitiatieafstand). Deze metingen werden verzameld gedurende 15 broedseizoenen. We ontdekten dat vrouwen consistente verschillen vertoonden in hun vluchtinitiatieafstand binnen en tussen jaren. Verder verkleinden ze hun vluchtinitiatieafstand gedurende het seizoen en hoe ouder ze werden. De vermindering in de vluchtinitiatieafstand over het seizoen en over de leeftijd werden gedreven door veranderingen in individueel gedrag en niet door veranderingen in de populatiesamenstelling. Verder verkleinden ganzen die aan het begin een grote vluchtinitiatieafstand hadden deze minder over de tijd in vergelijking met ganzen die aan het begin al een kleine vluchtinitiatieafstand hadden.

Concluderend laat mijn proefschrift zien op welke manieren individuele Spitsbergen brandganzen worden beïnvloed door veranderingen in hun broedgebied. De voornaamste inzichten zijn:

- Nestvlooiën zijn opvallend aanwezig in een broedkolonie van brandganzen op Spitsbergen en we vonden een negatief verband tussen aantallen nestvlooiën en het broedsucces van brandganzen. Deze vondst viel echter niet te verklaren door negatieve effecten van vlooiën op het gedrag van de vrouw gedurende het bebroeden van de eieren.
- Brandganzen in de hoge en lage arctis vervroegden het leggen van hun eerste ei over de jaren 2000-2016, maar dit ging minder snel als het vervroegen van het smelten van de sneeuw in het voorjaar. Ganzen in de hoge arctis lijken niet vroeg genoeg te beginnen, want de vroegst leggende ganzen hebben het hoogste uitkomstsucces. Hierdoor zijn ze mogelijk kwetsbaarder voor de negatieve effecten van klimaatverandering.
- Blootstelling aan verontreinigingen uit een historische steenkoolmijn verhoogde het kwikgehalte in de levers van brandganzenkuikens, maar er waren geen sterke neurotoxische effecten, noch sterke negatieve effecten op immuunsysteem parameters of plasma-corticosterongehalten. Grazen in deze vervuilde omgeving veranderde stressgerelateerd gedrag en niveaus van corticosteron metabolieten in feces van brandganzenkuikens.
- Het nestverdedigingsgedrag van vrouwelijke brandganzen verschilde consistent tussen de individuen, maar nam toe gedurende het broedseizoen en met de leeftijd van de ganzen.

Author contributions

Author contributions

Chapter 2: MEJ, RW and MJJEL conceived the ideas and designed methodology. MEJ, RW and MJJEL collected data. MEJ analysed the data. MEJ led the writing of the manuscript. All authors gave final approval for publication.

Chapter 3: TKL, MEJ, MJJEL and JP conceptualized the study. TKL, MEJ, MJJEL, HPJ, KEL, BAN and JP conducted the fieldwork. MPB provided data. TKL analysed the data. TKL wrote the initial manuscript and all authors contributed to the final manuscript.

Chapter 4: The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Chapter 5: MEJ, IBRS, JK and MJJEL devised the study. MEJ, IBRS, NWvdB, AB and MJJEL participated in data collection. MEJ performed laboratory analyses under supervision of KM. MEJ analysed the data. All authors contributed to the writing of the manuscript and gave final approval for publication.

Chapter 6: IBRS, BMW and JK devised the study. IBRS, MEJ, AB, NWvdB and MJJEL participated in data collection. JK, MJJEL and IBRS received funding. BMW performed statistical analyses and EM supervised laboratory analyses. All authors contributed to the writing of the manuscript and gave final approval for publication.

Chapter 7: MEJ and MJJEL conceived the ideas and designed methodology. MEJ, MJJEL and RWF collected data. MEJ analysed the data with the help of MN. MEJ led the writing of the manuscript. MEJ, MN and RWF contributed critically to the drafts and all authors gave final approval for publication.

List of publications

List of publications

Part of my PhD research

de Jong, M. E., Scheiber, I. B. R., van den Brink, N. W., Braun, A., Matson, K. D., Komdeur, J. and Loonen, M. J. J. E. 2017. Indices of stress and immune function in Arctic barnacle goslings (*Branta leucopsis*) were impacted by social isolation but not a contaminated grazing environment. - Sci. Total Environ. 601–602: 132–141.

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Short Curriculum Vitae

Short Curriculum Vitae

2019 – present: Academic staff, Department of Behavioural Biology, University Vienna, Austria. FWF Project: P 32216 (I. Scheiber), project title: *“Diurnal and seasonal rhythmicity of hormones and behaviour in Arctic-breeding barnacle geese (Branta leucopsis)”*

2014 – 2021: PhD, Arctic Centre, University of Groningen, The Netherlands. Supervisors: Prof. P.D. Jordan & Dr. M.J.J.E. Loonen, NWO grant Dossier Nr. 866.12.407 (M.J.J.E. Loonen), thesis title: *“Breeding in a changing Arctic: Physiology and behaviour of barnacle geese”*

2012 – 2014: Master Ecology & Evolution, Faculty of Mathematics and Natural Sciences, University of Groningen, The Netherlands

2008 – 2012: Bachelor Biology, Major Ecology & Evolution and Major Biomedical Sciences, Faculty of Mathematics and Natural Sciences, University of Groningen, The Netherlands

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I would like to thank all the station leaders, logistics engineers and observatory engineers of AWIPEV I got to know over the years who made sure logistically we had all we needed and that we were safe out in the field. I really enjoyed chatting with you over dinner, during the bar nights and playing games.

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