Science of the Total Environment 601–602 (2017) 132–141

Contents lists available at ScienceDirect

Science of the Total Environment

journal homepage: www.elsevier.com/locate/scitotenv

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

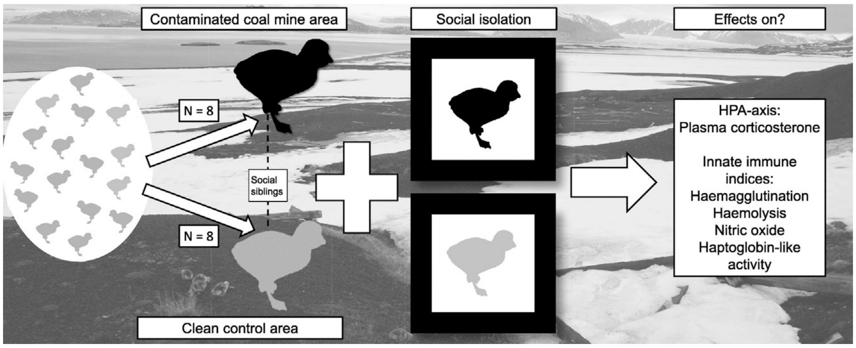
Margje E. de Jong [a],*, Isabella B.R. Scheiber [b], Nico W. van den Brink [c], Anna Braun [b], Kevin D. Matson [d], Jan Komdeur [b], Maarten J.J.E. Loonen [a]

[a] *Arctic Centre, University of Groningen, Aweg 30, 9718 CW Groningen, The Netherlands*
[b] *Behavioural and Physiological Ecology, The University of Groningen, Groningen Institute for Evolutionary Life Sciences (GELIFES), Nijenborgh 7, 9747 AG Groningen, The Netherlands*
[c] *Department of Toxicology, Wageningen University, Stippeneng 4, 6708 WE Wageningen, The Netherlands*
[d] *Resource Ecology Group, Wageningen University, Droevendaalsesteeg 3a, 6708 PB Wageningen, The Netherlands*

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



ARTICLE INFO

Article history:
Received 4 March 2017
Received in revised form 8 May 2017
Accepted 20 May 2017
Available online xxxx

Editor: F.M. Tack

Keywords:
Heavy metals
Natural antibodies
Nitric oxide
Acute phase proteins
Complement
Corticosterone

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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* Corresponding author.
E-mail addresses: m.e.de.jong@rug.nl (M.E. de Jong), nico.vandenbrink@wur.nl (N.W. van den Brink), kevin.matson@wur.nl (K.D. Matson), j.komdeur@rug.nl (J. Komdeur), m.j.j.e.loonen@rug.nl (M.J.J.E. Loonen).

http://dx.doi.org/10.1016/j.scitotenv.2017.05.183

in the other measures. Social isolation increased corticosterone and decreased haptoglobin-like activity in all goslings. Immunology and corticosterone levels of barnacle goslings thus seem unaffected, at least on the short term, by Arctic coal mining contamination.

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: Moroń 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 Lehikoinen, 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 (Hawkley 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 Hawkley 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 Hawkley 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, MJJEL 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, MJJEL). 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 Biomedicals/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 Hõrak (2009) to measure the concentration of nitric oxide (μmol/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 Hõrak, 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, age^2 or age^3 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 Pbkrtest 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 ± SD]) than in the control area (0.005 ± 0.0004 mg/kg dry weight [average ± 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: $F = 20.07$, $ddf = 13$, $p < 0.001$).

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 Dzubin 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			
	Age	19.602	1.567	155.52	334	<0.001
	Age^2	1.233	0.066	345.43	334	<0.001
Total tarsus	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 (β ± SE: −1.175 ± 1.938, F: 0.367, df = 1, 13, p = 0.555). χ^2 statistics are given for the analyes of haemagglutination and haemolysis (*), while F statistics are given for all other analyses.

Variable	N (mine/control)	Intercept (β ± SE)	Exposure				Sex			
			Effect: exposure to mine (β ± SE)	F/χ^{2*}	df	p	Effect: being male (β ± 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 μmol/l [95% CI: 0.37], mean control: 0.69 μmol/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 μmol/l [95% CI: 0.47], mean Δ control: −0.23 μmol/l [95% CI: 1.10]). Social isolation did not influence nitric oxide in any way (mean mine: 0.30 μmol/l [95% CI: 0.35], mean control: 0.36 μmol/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 (β ± SE: −0.263 ± 2.951, F: 0.008, df = 1, 13, p = 0.930). χ^2 statistics are given for the analyes of haemagglutination and haemolysis (*), while F statistics are given for all other analyses.

Variable	N (mine/control)	Intercept (β ± SE)	Exposure				Sex			
			Effect: exposure to mine (β ± SE)	F/χ^{2*}	df	p	Effect: being male (β ± 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

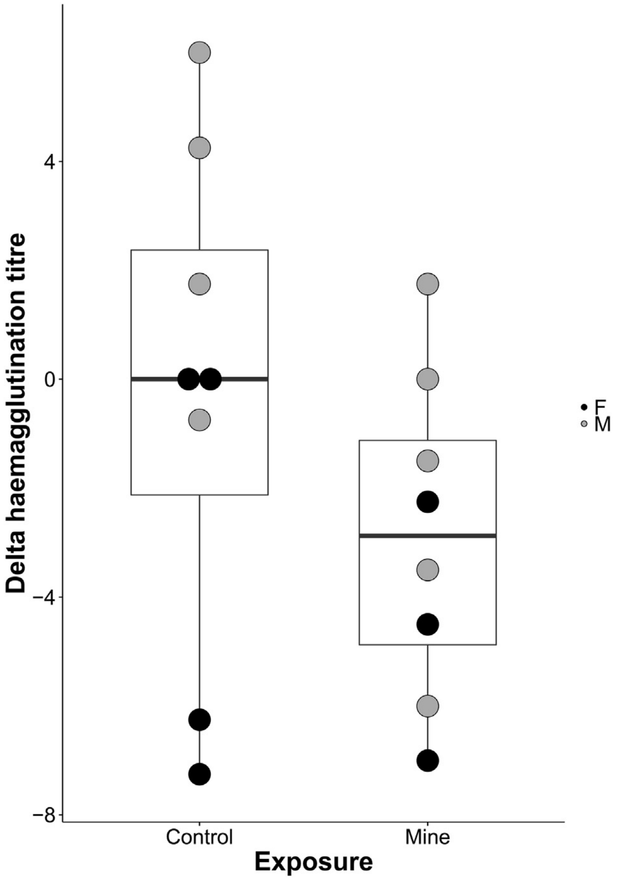

Fig. 1. Barnacle goslings that were exposed to the coal mining area tended to show a decrease in haemagglutination titres (Δ haemagglutination) after social isolation in comparison with control goslings, when controlling for sex effects (F = female, black circles; M = male, grey circles). Boxes show 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 Hawkley 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 greylag 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 (Hawkley 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.

Acknowledgements

This study was financed by the Netherlands Organisation for Scientific Research (NWO grant to JK: 866.12.406 and MJJEL: 866.12.407). It was performed under FOTS 6331 (Norwegian Animal Research Authority to MJJEL). We are grateful to Marco van der Velde (GELIFES, University of Groningen) for molecular sexing, Virginie Canoine (Dept. of Behavioural Biology, The University of Vienna) for corticosterone analyses and Maaike A. Versteegh (GELIFES, University of Groningen) for all her help with the laboratory analyses. We thank Kings Bay AS and the Governor of Svalbard for their cooperation. MEdJ and KDM thank all of their “Molecular Methods in Ecology and Evolution” teammates at the University of Groningen: Maravillas Ruiz Miñano, Marco van der Velde, B. Irene Tieleman, Joana F. Salles, and Bregje Wertheim. KDM also thanks his current colleagues in the Resource Ecology Group for their support. We would like to thank Rienk Fokkema, Frigga Kruse and three anonymous reviewers for valuable comments on the manuscript.

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