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Identifying invertebrate species in Arctic muskox dung using DNA barcoding

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Abstract

The Arctic is undergoing strong environmental changes, affecting species and whole biological communities. To assess the impact on these communities, including their composition and functions, we need more information on their current distribution and biology. In the High-Arctic tundra, dung from animals, such as muskoxen (*Ovibos moschatus*), is a relatively understudied microhabitat that may be attractive for organisms like dung-feeding insects as well as gastro-intestinal parasites. Using a DNA barcoding approach, we examined muskox droppings from two Greenlandic regions for dung-dwelling invertebrates. In 15% of all samples, we found the DNA of insect species in the orders Diptera and Lepidoptera. The saprophagous Diptera colonized dung differently in west versus north-east Greenland and summer versus winter. In addition, we found muskox dung harbouring endoparasitic nematodes in samples from both regions. However, we could not find traces of saprophagous arthropods, such as collembolans and mites, from the soil sphere. Our pilot study sheds a first light on the invertebrates living in this neglected Arctic microhabitat.

Keywords

Arthropods; eDNA; Greenland; nematodes; tundra; *Ovibos moschatus*

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Abbreviations

COI: cytochrome c oxidase subunit I
eDNA: environmental DNA
PCR: polymerase chain reaction

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Introduction

The Arctic tundra is characterized by a shortage of readily available nutrients, which is why the urine and faeces of Arctic birds and mammals represent important sources of nitrogen, phosphorous and other elements necessary for plant growth (Mosbacher et al. 2016; Barthelemy et al. 2018; Thomas 2021). They allow plant roots and soil organisms to take a fast route of nutrient uptake, bypassing yearlong decomposition processes of animal and plant tissue that are predominantly controlled by weathering and microbial activity (van der Wal et al. 2004). The positive effects of droppings and urine on vegetation growth are particularly visible in the presence of colonial breeders, such as geese (Bazely & Jefferies 1985; Gauthier et al. 1995; Person et al. 2003). Along with animal carcasses, vertebrate dung and urine may therefore form attractive 'nutrient hot spots' for many species, which are directly or indirectly linked to decomposition processes, including fungi, bacteria and arthropods (Sutcliffe et al. 2000; Richardson 2001; Floate 2011; Sigsgaard et al. 2021). Similarly, fresh, moist faeces may be attractive for species in an otherwise dry tundra region. Hence, animal faeces may also have the potential to be a 'diversity hotspot' that can host high species diversity and, for several of these species, high abundances as well (Sigsgaard et al. 2021).

In the Arctic, research so far has focused on characterizing the microbial community (bacteria and fungi) on faeces of vertebrates, such as muskoxen (*Ovibos moschatus*; Andersen-Ranberg et al. 2018; Bird et al. 2019). However, we know little about the community of invertebrate species in this specific habitat. This is surprising, since the faeces of vertebrates in warmer regions of the world, such as cattle, are rapidly colonized and densely inhabited by a species-rich community of invertebrates (Sigsgaard et al. 2021). Whilst many specialist dung dwellers are absent from the Arctic (e.g., dung beetles), it is home to a large number and abundance of non-specialist invertebrates

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(Böcher et al. 2015) that are known to colonize faeces in other parts of the world, such as the larvae of calliphorid flies and, from the soil sphere, arthropods like collembolans and mites. Knowing which invertebrate species participate in nutrient cycling by breaking down dung may contribute to our understanding of Arctic ecosystem processes and how they will evolve with ongoing climate change (Post et al. 2009).

In the Greenlandic tundra, vertebrate faeces can be found for many months or even years after dropping, suggesting a long potential time for colonization. Although desiccation of the surface of faeces and their deposition in remote and inhospitable places, such as mountain ridges and sand banks, may slow down colonization by soil arthropods and flies, latrines (near fox dens) or large dung left by Arctic megaherbivores such as caribou/reindeer (*Rangifer tarandus*) and muskox may be a prime target for invertebrate colonization. For example, a typical population of muskox in north-east Greenland, which consists of about 10 individuals per km^2, produces 4.11 tonnes of dung during the main growing season (Mosbacher et al. 2016), suggesting ample opportunities for invertebrate colonization from the surrounding environment.

In addition, vertebrates are often carriers of a highly diverse community of invertebrate parasites in their guts, and dung allows for an effective spreading of infective parasite stages, potentially resulting in higher infection rates within a vertebrate host community. For example, reindeer populations in Svalbard avoid areas of defecation to minimize re-infection with endoparasitic nematodes (van der Wal et al. 2000). Knowing if and how much dung is inhabited by invertebrates will allow us to not only draw decomposition routes and quantify decomposition rates but also track transmission routes of parasites and pathogens, both being subject to significant changes in a warming Arctic (Post et al. 2009; Hueffer et al. 2011; Kutz et al. 2013).

Identifying invertebrates in dung samples is often complicated. Species here are mostly present in their immature stages (eggs and larvae), so that even with time and expert knowledge, a species-specific identification based on a physical inspection is not guaranteed. Novel molecular approaches, such as DNA barcoding of eDNA, are simple yet efficient ways to characterize whole invertebrate communities within a mixed sample (Beng & Corlett 2020). Even if target species lack important morphological characteristics needed for visual determination, barcoding allows for a thorough identification of both free-living arthropods and gastrointestinal parasites in dung samples (Ondrejicka et al. 2014). The method does not come without limitations, as summarized by Beng & Corlett (2020). For example, repeated freeze–thaw stress and exposure to ultraviolet radiation degrade eDNA in Arctic environmental samples, potentially hampering correct species assignment (Strickler et al. 2015; Matange et al. 2021). Moreover, the simple detection of DNA of target species may not be sufficient for estimating the abundances or biomasses necessary for quantitative analyses (Beng & Corlett 2020).

In this study, we used a DNA barcoding approach to identify invertebrate taxa of muskox dung from two Greenlandic regions. We were specifically interested in finding out which arthropod groups were associated with the dung samples collected in different habitats and of different ages. We also tested the dung samples for the presence of gastrointestinal nematode parasites of muskox, which have already been shown to infect free-living muskoxen in Greenland (Davidson et al. 2014).

Material and methods

Study area

The study was carried out in two regions of Greenland—the first near Kangerlussuaq (66°55′N, −49°59′W) on the west coast and the second on the southern edge of Traill Island (72°30′N, 24°00′W), situated inside the Northeast Greenland National Park (Fig. 1). Both regions are characterized by a mosaic of tundra habitats. On Traill Island, we find High-Arctic vegetation such as dwarf shrub heathland (*Cassiope tetragona*, *Salix arctica* and *Dryas octopetala* × *integrifolia*), wet vegetation dominated by mosses and relatively tall sedges (*Carex* spp., *Eriphorum scheuchzeri*) as well as sandy and rocky zones without vegetation besides lichens. The region around Kangerlussuaq is dominated by Low-Arctic dwarf shrub heaths (*C. tetragona, Betula nana*) and *Ledum palustre* and *Sphagnum* spp. on moist sites (Fredskild 1996).

The climate in Traill island is High-Arctic, with summer (June to September) temperatures above 0 °C and winter temperatures (December-March) ranging −15 to −25 °C. Snow cover is usually present from September to June or July (Gilg et al. 2009). In Kangerlussuaq, the climate is more continental, with summer temperatures between 10 and 12 °C and winter temperatures as low as −20 °C (climate-data.org).

The population of muskox in north-east Greenland comprises over 12 000 individuals and forms part of the endemic relict population surviving the glacial periods (Cuyler et al. 2020). As in all north-east

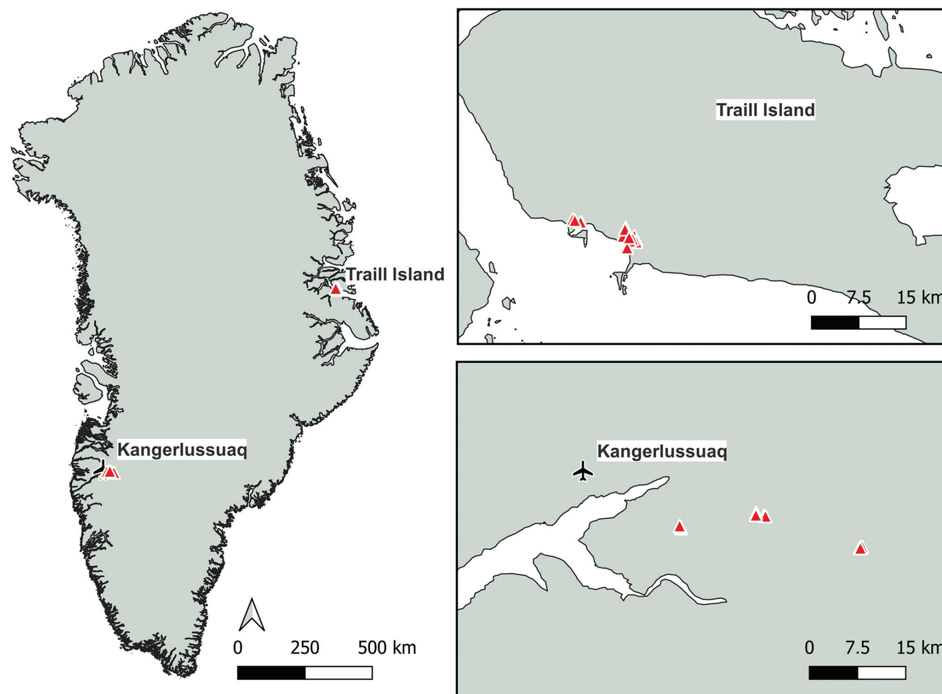

Fig. 1 Map of Greenland and enlarged sections marking the study areas of the south-west coast of Traill Island (above) and Kangerlussuaq (below). Red triangles indicate single collection sites of muskox dung.

Greenland, muskoxen on Traill Island are the only ruminant species. In Kangerlussuaq, however, muskoxen were introduced in the 1960s from the north-east metapopulation and developed to a population of over 20 000 individuals (Cuyler et al. 2020). Here, they share their habitat with caribou, another mammalian herbivore. The diet of muskoxen in summer primarily consists of leaves and stems of graminoids and willows, whilst senescent graminoids and willow twigs become more important during the winter (Larter & Nagy 2004; Mosbacher et al. 2016). In Traill Island, muskoxen have frequently been observed feeding on *Vaccinium* spp. in winter (own observation).

Sampling and DNA extraction

In the summers (June-September) of 2018 and 2019, a total of 32 muskox dung samples were collected in Kangerlussuaq (n = 15) and Traill Island (n = 17). Samples were picked using sterile nitrile gloves and dry-stored at −20 °C in paper bags until further processing. Dung was collected in different tundra environments (Fig. 2, Supplementary Table S1) and grouped into four age classes: fresh, previous winter, previous summer and over one year old. Dung deposited during wintertime consists of many small, round, dry pellets (Fig. 2a, b). It can therefore be distinguished from summer samples, in which the pellets are pressed into one big, moist lump (Fig. 2c, d). Whether the dung sample lies on the snow surface (recent winter dropping) or is buried under the snow layer (dropping from before winter) offers more clues to the timing of the deposit.

In the laboratory, all samples were weighed and checked for invertebrate remains and marks under a stereo microscope using heat-sterilized equipment. As samples had been exposed to freeze–thaw events, wind and solar radiation in the field, we only processed the inner part of the dung sample, promising higher chances of detecting intact DNA. Up to 50 mg of dung sample was used for DNA extraction. In cases comprising several dung pellets in one dung sample (as is common in winter dung samples), we pooled the inner parts of these pellets.

DNA was extracted using the NucleoSpin Soil Extraction kit (Macherey-Nagel), following the manufacturers protocol. A blank control was included to test for DNA carry-over contamination using universal invertebrate primers (Folmer et al. 1994). No contamination was detected when analysing PCR products of these blank controls on agarose gel.

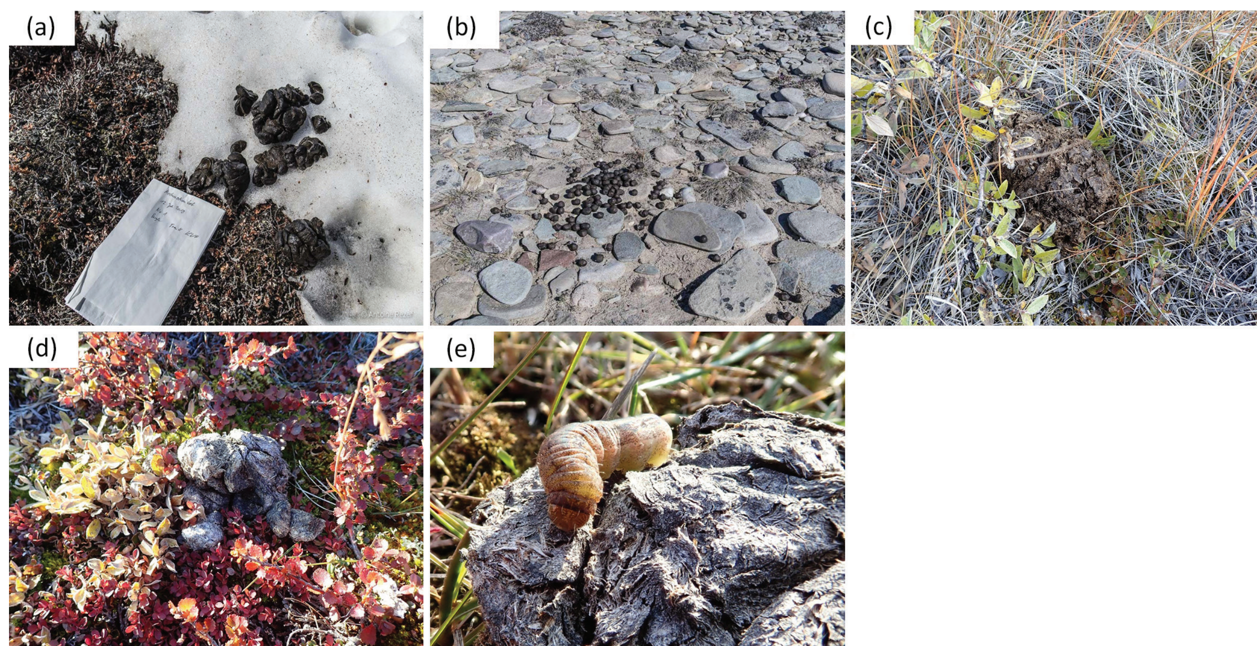

Fig. 2 Examples of muskox winter dung samples from Traill Island (photos Antoine Rezer), found on (a) snow and (b) dry sand/stone, and summer dung samples from the Kangerlussuaq region, found in (c) dry shrub heath and (d) moist shrub heath. (e) Noctuid caterpillar crawling on dried muskox dung in the Kangerlussuaq region.

Screening PCR

All dung samples were first analysed for the presence of invertebrate DNA using the universal primers LCO1490/HCO2198 amplifying a 710 bp long fragment of mitochondrial COI region (Folmer et al. 1994). Subsequently, we tested for the DNA of typical dung- and soil-inhabiting arthropod groups—collembolans, Diptera and oribatid and gamasid mites—using group-specific primers targeting the mitochondrial COI region and 18S ribosomal DNA, respectively (Kuusk & Agustí 2008; Eitzinger et al. 2013). We explicitly targeted these as they are present in abundant numbers per cell ('multiple copy gene'), promising a high recovery rate even if samples were exposed to unfavourable field conditions for long periods of time. Additionally, the amplicon length of maximal 272 bp allows a successful detection even when DNA is fragmented. Each 10 µL PCR contained 5 µL MyTaq Red Mix PCR mastermix (Bioline), 0.5 µL bovine serum albumin (3%; New England BioLabs), 1.5 µL sterile water, 0.5 µM of each primer and 2 µL of DNA extract. PCR cycling conditions for universal primers LCO1490/HCO2198 followed the protocol described by Folmer et al. (1994), for collembolan primers Col3F/Col5R, the protocol by Kuusk & Agustí (2008) and for primers DIPS16/DIPA17, ORIS14/ORIA16 and GAMS7/GAMA8, the protocol by Eitzinger et al. (2013). Each PCR was replicated once. PCR products were then separated in a 1.2% agarose gel stained with RotiLoad DNAstain (Carl Roth) and visualized under ultraviolet light.

Sequencing PCR

All DNA extracts were additionally subjected to PCRs with primers ZBJ-ArtF1c/ZBJ-ArtR2c (Zeale et al. 2011) and primer-cocktail C_NemF1_t1/C_NemR1_t1 (Prosser et al. 2013), targeting the COI barcoding region of arthropods and endoparasitic nematodes, respectively. Each 10 µL PCR contained 5 µL MyTaq Red Mix PCR mastermix (Bioline), 0.5 µL bovine serum albumin (3%; New England BioLabs), 1.5 µL sterile water, 0.5 µM of each primer and 2 µL of DNA extract. PCR cycling conditions followed the protocol by Zeale et al. (2011) and Prosser et al. (2013). Amplification success was tested by gel-electrophoresis (see conditions earlier).

PCR purification and Sanger sequencing were conducted at Microsynth (Balgach, Switzerland). Forward and reverse sequences were processed using software Geneious, and consensus sequences (contigs) were then identified by comparison with those in the BOLD database (https://v4.boldsystems.org/; Ratnasingham & Hebert 2007) and additionally compared with entries in the National Center for Biotechnology Information GenBank database (https://www.ncbi.nlm.nih.gov/), using the Basic Local Alignment Search Tool

(Altschul et al. 1990). Only samples with a minimum of 98% identity match were regarded as correct species identification.

Results

Only three dung samples from Kangerlussuaq (7W, 11W and 12W) exhibited visible remains of invertebrates under the stereo microscope. However, the bad condition of the specimens made visual identification impossible.

Screening for the DNA of collembolans, Diptera and oribatid and gamasid mites in all 32 samples using group-specific primers confirmed the presence of Diptera remains in only one sample (a sample from Kangerlussuaq). From all 32 DNA extracts, we obtained high-quality sequences of insect taxa in five samples (15.6% of all samples): three were identified as *Pegoplata tundrica* (Anthomyidae, Diptera), one as *Metriocnemus ursinus* (Chironomidae, Diptera) and one as *Scythris noricella* (Scythrididae, Lepidoptera; Supplementary Table S1). Sequences of another six DNA extracts derived from endoparasitic nematodes (Trichostrongylidae, Nematoda); however, the low quality of the sequence did not allow an exact species identification. The use of arthropod- or nematode-specific primers did not automatically result in specific detection of the respective groups: three sequences amplified by the nematode-specific primer cocktail were successfully identified as insect species. All samples identified as *P. tundrica* and *S. noricella* were collected in Kangerlussuaq in dung dropped during the summer, whilst the chironomid *M. ursinus* was found on Traill Island in winter faeces.

All three samples with *P. tundrica* were found in faeces collected in dry habitats, such as shrub heath and sandy locations, whilst *M. ursinus* and *S. noricella* were found in dung collected at moist sites. Dung with endoparasitic nematodes was predominantly fresh and was collected in moist and dry sites on Traill Island and in Kangerlussuaq.

Discussion

Dung-inhabiting arthropods

Diptera are a key group of dung-dwellers worldwide, so it came as no surprise that we also found DNA of two Diptera species in our muskox samples. In three of the Kangerlussuaq samples, we found DNA of *Pegoplata tundrica*, an anthomyiid fly with a circumpolar distribution in Subarctic and alpine regions (Böcher et al. 2015; Sorokina 2017). In Greenland, this species can be found in the south and south-west, Kangerlussuaq defining the northernmost range limit. This distribution may also explain the absence of the species in any of the dung samples from Traill Island, in north-east Greenland. The larvae of the genus *Pegoplata* are saprophagous, with a preference for bovine droppings (Böcher et al. 2015), which share many characteristics with muskox faeces. The DNA probably originates from maggots of *P. tundrica* developing within the dung pat, which is corroborated by the findings of two maggot-like structures in two of the three samples during microscopic inspection. Most coprophilous fly species are only attracted to ruminant dung that is less than two days old, after which a change in chemical cues and the formation of a hard crust make the dung pat less attractive and permeable to larvae (Sladecek et al. 2021). As all three samples are from summer, this suggests that colonization of the faeces must have happened when the droppings were fresh and moist.

In contrast to *P. tundrica*, DNA of the chironomid species was found only in winter dung samples from a site on Traill island, indicating that the dung pat must have been colonized after some time of exposure. Indeed, frozen (winter) dung samples may still attract insects after thawing (Bezanson et al. 2021), and chironomids are particularly attracted to dung volatiles that are emitted during late stages of dung succession (Sladecek et al. 2021). The free-living larvae of *Metriocnemus ursinus* are described as feeding on the substrate film in wet mosses in springs and terrestrial surroundings (Böcher et al. 2015), whilst our results suggest that they use faecal material or microbes living on it, as long as they provide moist conditions (Böcher et al. 2015). Since we removed the surface of the dung pats before our analysis, the finding of chironomid DNA suggests that the larvae used cracks in the surface to reach inner parts of the dung pat, potentially offering additional humidity and protection from predators.

The detection of DNA of the moth species *Scythris noricella* is interesting, as only adult butterflies and moths would spend short times puddling on fresh dung to ingest sodium and other nutrients (Molleman 2010), leaving only very few discernible DNA traces. Larvae of *S. noricella* feed until middle of July on the leaves and flowers of fireweed (*Chamaenerion latifolium*), whilst the adults fly from mid-July to September (Böcher et al. 2015). The detection of pupae-shaped material suggests that the DNA probably derives from individuals pupating in the cracks of the dried dung pat, which offers additional shelter from environment and predators.

To our surprise, none of the analysed dung samples exhibited traces of soil-dwelling arthropods, that is, collembolans and gamasid and oribatid mites. Collembolans and oribatid mites feed on dead organic matter and/or microbes and can reach high densities in Greenlandic soils: 129 000 individuals per m^2 and 27 500 individuals

per m^2, respectively (Sørensen et al. 2005). Globally, collembolans and oribatid mites have been found living on faecal matter and are abundant in areas with cattle dung (Suárez et al. 2009; Sigsgaard et al. 2021). In Greenland, many species of collembolans and oribatid mites are associated with dung (Böcher et al. 2015), where they feed on fungal spores, but potentially also take advantage of the humid microclimate (Hertzberg & Leinaas 1998). In contrast to flying insects, such as anthomyiid flies, dung colonization by soil-dwelling animals is probably slow and depends on the presence of a source population close to the site of the dung. Whilst this minimizes the possibility of colonization of dung collected on gravel, sand and snowfields, it does not explain the lack of colonization of faeces found in more suitable areas, like bogs and places with a cover of grasses or shrubs. Mites and collembolans may be predominantly active in fresh dung, where moist conditions allow feeding on the microbes living on it, whilst older and dry dung pats may be less attractive.

Gamasid mites do not feed on dung material, instead preying on the fly eggs, nematodes and enchytraeid worms found in this resource (Böcher et al. 2015). Many species are phoretic to specialized dung feeders, such as dung beetles (Hunter & Rosaria 1988), whilst in the Arctic, gamasid mite species are known to attach to flies as a means of transport and distribution (Petrova & Makarova 1992; Makarova 2013). Thus, gamasid mites colonize areas where flies forage, whilst also expanding into areas that would otherwise be difficult for gamasid mites to reach. As with collembolans and oribatid mites, gamasid mites may predominantly be found in fresh dung, which might explain the absence of these groups in most of our collected samples.

Gastrointestinal parasites

The majority of invertebrate DNA in the collected samples originated from Trichostrongylidae, a family of endoparasitic nematodes very common in sheep and goats worldwide (Hoberg et al. 2001). Whilst the percentage identity match is too low for an accurate species determination, the DNA most likely derives from the genus *Teladorsagia*, which is known to infect muskox in North America and Greenland (Hoberg et al. 1999; Kutz et al. 2012). In sheep, *Teladorsagia* is considered a significant pathogen, causing protein deficiency and reduced growth rate in lambs (Stear et al. 2003; Hoberg et al. 2001). In Greenland, this species colonizes the abomasum of ruminant mammals, such as caribou and muskox, which have a lower prevalence and intensity than observed in domestic sheep. However, infections of muskoxen may influence population cycling through impacts on host body conditions and reproduction (Kutz et al. 2012).

We found Trichostrongylidae in faeces of muskox in both Kangerlussuaq and Traill, which is probably a result of the introduction of 27 muskox individuals from northeast Greenland to the Kangerlussuaq-Sisimiut area in 1962 (Boertmann et al. 1992; Steele et al. 2013). This transfer did not only allow the spread of Trichostrongylidae in the newly established population of muskox in western Greenland but is also responsible for the spill-over to the local caribou population (Steele et al. 2013). Particularly during summer, muskoxen shed nematode eggs with their faeces; the eggs develop within days into the infective third larval instar before being ingested by new hosts where they complete their life cycle. The dominance of nematode infestation in fresh summer dung has also been noted in our study. Eggs of Trichostrongylidae (e.g., *Teladorsagia boreoarcticus*) can survive temperatures as low as −20 °C for one to two weeks (Kutz et al. 2012), suggesting that winter dung samples may be infective. Most of the dung containing gastrointestinal nematodes in our study was collected in shrub heath, suggesting high chances of nematode transmission, as graminoids and willows (*Salix* spp.) are preferred food for muskox and caribou (Larter & Nagy 2004; Mosbacher et al. 2016). In addition, moist conditions allow infective stages to migrate within vegetation, reaching high densities and consequently enhancing the chance of being ingested by new hosts (Korsholm & Olesen 1993; van Dijk & Morgan 2011).

The detection of endoparasites in 18.8% of all samples suggests a high prevalence within the muskox community, as has been noted by Korsholm & Olesen (1993). However, to validate this, we would need a larger sample size to rule out that faeces with nematode DNA came from only a few infected muskox individuals. In addition, our qualitative data cannot provide information on the parasite load within single muskox individuals, which would require counting of nematode eggs or quantitative real-time PCR of nematode DNA (qPCR).

Conclusion

The Arctic is experiencing strong climatic changes and has been warming four times faster than the global average (Rantanen et al. 2022). Predicting the future of Arctic species and communities is often difficult, as we lack information on their distribution and biology. Dung, in particular of herbivorous mammals, constitutes an understudied Arctic microhabitat with potentially important implications for the future. Rising temperatures and humidity in the Arctic will probably speed up

decomposition rate and nutrient cycling governed by biological (e.g., microbes) and physical processes (Thomas 2021). Our results from two different Arctic tundra areas indicate that currently only a small set of invertebrate species uses muskox dung as a resource. In addition, the absence of any soil-dwelling arthropods in our dung samples suggests that colonization processes are not driven by communities on a local (micro)scale but primarily involve species capable of covering longer distances. Future developments, such as the 'greening' of currently non-vegetated areas and 'shrubification' as shrubs expand (Myers-Smith et al. 2011; Berner et al. 2020), will potentially create better conditions for colonization of dung by Arctic invertebrates. Moreover, such an environment will preserve favourable microclimatic conditions needed for animal and microbial breakdown of dung material.

DNA-based analysis of environmental samples provides an exciting method to describe the community of a microhabitat such as dung and to assess the impact of environmental drivers (Zielińska et al. 2017; Sigsgaard et al. 2021). For this pilot study, we used a combination of diagnostic PCR with group-specific primers and Sanger sequencing, which allowed us to detect and identify invertebrate dung dwellers and nematode parasites, although invertebrate remains were not visible or could not be identified microscopically. The method allows DNA detection with high sensitivity and specificity even in material that was exposed to harsh Arctic conditions over more than one year. We are aware that our methods may have failed to detect some species in our samples, and that the invertebrate community within muskox dung may be richer than observed here. For example, direct Sanger sequencing of DNA from a mixed sample will only identify the most abundant species. A metabarcoding approach, using general invertebrate primers, would allow to simultaneously detect and identify insects, nematodes and allies within dung samples, illustrating the whole invertebrate community (Sigsgaard et al. 2021). Such an approach would provide important information of species distribution and would help us assess its impact on ecosystem processes, such as decomposition, as well as pathogen spread in a warming Arctic. We hope that our successful pilot study highlights the need to investigate Arctic faeces in more detail.

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Disclosure statement

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