PHYSIOLOGICAL AND BEHAVIORAL COSTS AND CONSEQUENCES OF FLY (DIPTERA) EXPOSURE FOR MOOSE (*ALCES ALCES*)

A Dissertation

by

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Submitted to the Graduate and Professional School of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

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May 2023

Major Subject: Wildlife and Fisheries Sciences

ABSTRACT

I studied the responses of wild ungulates to harassment by flies (Diptera) by reviewing the literature on North American ungulates and studying the behavior and physiology of female moose (*Alces alces*) in a boreal forest in Alaska. Hair and skin provide morphological resistance to flies at low exposures, but behavioral and physiological responses are used to reduce and tolerate flies as exposure increases. Caribou (*Rangifer tarandus*) avoid flies, which displaces foraging and reduces body mass gain in summer. Moose tolerate flies through the summer. I found that flies do not trigger a release of glucocorticoid hormones in either moose calves or adult moose as a stress response. The dense hair of the neonatal coat resists flies, but calves still suffer morbidity and mortality from parasitic diseases carried by flies. Adult moose molt and lose their barrier of protection in June and July when flies are the most abundant, and sores appear on their hind legs. Leg sores of adult moose were infected with a parasitic nematode within the genus *Onchocera*, which is likely transmitted by black flies (Diptera: Simuliidae). The number of sores on the hind legs of an individual was positively correlated with total body fat, which suggests that tolerance of flies increases gain of energy from foraging over the summer. Conversely, the number of sores was negatively correlated with serum albumin, which indicates the use of body protein to repair injuries from flies and parasites. Moose altered daily movements in response to flies; on cool days (8°C), movement rates increased with the abundance of flies. However, on warm days (20°C), movement rates were not affected by flies probably because cooling takes priority. Time spent in black spruce forests increased with both air temperature and with counts of flies. Warm summer temperatures create an ecological trap for moose when heat gained while foraging is dissipated at rest in shady, wet habitats preferred by flies that adversely affect the fitness of moose.

DEDICATION

I dedicate my dissertation to my family. I dedicate this to my dad who told me from a young age to stick with school and do well so that I could have a career that I love and am good at. I’m not sure he meant stay in school this long but here we are. I will never replace the original Dr. Benedict but I will sure try to make a mark like he did. Thank you to him and my mom for giving me such a strong appreciation for wildlife and the natural world around me. Thank you to Michelle, Leo, and Westley Watchman for bringing me so much joy and happiness.

ACKNOWLEDGEMENTS

I would like to thank my committee chair, Dr. Perry Barboza, for his guidance and support. It has been truly an honor to learn from you and be a part of your lab. Thank you to my committee members, Drs. Gabriel Hamer, Dominique Wiener, and Douglas Tolleson, for all of your help along the way. Thank you to Dr. Daniel Thompson and John Crouse for all of your time and support, and allowing me the privilege to work with the moose of the Kenai Moose Research Center and make the MRC my summer home. Thank you to my Texas lab mates Dr. Jeff Martin and Kaylee Hollingsworth, and lab manager Rachel Shively for showing me the ropes. Thank you to Drs. Matthew Hufford, Clare Aslan, and Robert Klinger for showing me that I could have a career in wildlife research, and that all of this was possible. Thank you to all of the graduate students that have been there for me over the years, from Carney's happy hours to helping me code and identify insects, graduate school would have been far less fun and successful without you all. Thank you all for being a part of my team and family.

CONTRIBUTORS AND FUNDING SOURCES

Contributors

This work was supervised by a dissertation committee consisting of Professors Perry Barboza and Douglas Tolleson of the Department of Rangeland, Wildlife, and Fisheries Management, Professor Gabriel Hamer of the Department of Entomology, and Professor Dominique Wiener of the Department of Veterinary Pathobiology.

Perry Barboza assisted in editing and theorizing Chapter 1. Jose Cruz drew the ungulate outlines used in the figures of this chapter. Chapter 1 was published in 2022 with Perry Barboza as co-author.

Daniel Thompson, John Crouse, Sarah Newberry, and Matt King of the Alaska Department of Fish and Game, with the assistance of Bronwyn Stephenson, Stacy Crouse, and myself raised the moose calves used in Chapter 2. Sarah Newberry and Matt King also assisted in collecting samples. Saliva samples were sent to Salimetrics LLC for processing. Phillip Shults of the Department of Entomology assisted in fly identification by teaching me how to identify flies and confirming identification via molecular analysis of representative flies. Daniel Thompson, John Crouse, Phillip Shults, Gabriel Hamer, and Perry Barboza are all co-authors on this chapter, assisting in study design and providing manuscript edits. Perry Barboza and Daniel Thompson additionally provided statistical advice. Chapter 2 was re-submitted and is in-review for publication in 2023.

Daniel Thompson, John Crouse, and I performed the live and dead moose skin biospies used in Chapter 3. Nicholas Fowler of the Alaska Department of Fish and Game retrieved the wild moose samples. Histology on the tissue samples was done by Katia Groch and Dominque

Wiener of the Department of Veterinary Pathobiology, and genomic analysis was done by Matthew Kulpa and Guilherme Verocai of the Department of Veterinary Pathobiology. Photographs taken in 2015 were provided by Daniel Thompson. Perry Barboza, John Crouse, Katia Groch, Matthew Kulpa, Daniel Thompson, Guilherme Verocai, and Dominque Wiener are all co-authors on Chapter 3, published in 2023, and were involved in writing and editing the manuscript. Additionally, Perry Barboza provided statistical advice, and was involved in conceptualization along with Dominique Wiener.

Habitat maps and 2015 photographs used in Chapter 4 were provided by Daniel Thompson. Moose were imobilized and rump fat was measured by Daniel Thompson and John Crouse. Blood was collected by Daniel Thompson, John Crouse, Sarah Newberry, and myself, and sent to Zoetis Reference Laboratories for processing. Salivary cortisol was sent to Salimetrics LLC for processing, and fecal samples were analyzed for glucorticoids by Scott Jaques of Applied BioSciences. Perry Barboza provided statistical advice and conceptualization. Daniel Thompson, John Crouse, Gabriel Hamer, and Perry Barboza are all co-authors on Chapter 4, involved in editing. Chapter 4 will be submitted in 2023 for publication.

Habitat maps and GPS collar data for Chapter 5 were provided by Daniel Thompson. Daniel Thompson assisted in HOBO temperature logger collection and vegetation sampling. Perry Barboza provided statistical advice and conceptualization. Daniel Thompson, John Crouse, Gabriel Hamer, and Perry Barboza are all co-authors on Chapter 5. Chapter 5 will be submitted in 2023 for publication.

All other work conducted for the dissertation was completed by the student independently.

Funding Sources

Graduate study was supported by a fellowship from the Boone & Crockett Club | Dr. James “Red” Duke Wildlife Science to Policy Program at Texas A&M University. It was also supported by multiple Charles & Frances Fleming Scholarships, multiple Will Roach Scholarships, the J H Benedict Sr Memorial Graduate Scholarship, multiple Graduate Students of Wildlife & Fisheries Scholarships, and multiple WB Davis Scholarships from Texas A&M University.

This project was funded under Alaska Department of Fish & Game (Pittman-Robertson funds from the Federal Aid in Wildlife Restoration program and ADF&G’s Fish and Game Fund) and the Boone & Crockett Dr. James H. “Red” Duke endowment for Wildlife Conservation and Policy at Texas A&M University. Its contents are solely the responsibility of the authors and do not necessarily represent the official views of the Alaska Department of Fish and Game or Texas A&M University.

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CHAPTER I

INTRODUCTION

ADVERSE EFFECTS OF DIPTERA FLIES ON NORTHERN UNGULATES: *RANGIFER*, *ALCES*, AND *BISON*[*]

Synopsis

Flies (Diptera) damage ungulates far beyond the injury of their bite wounds: they are vectors of diseases and cause ungulates to lose foraging opportunities due to avoidance behaviour. We can use the behavioural and physiological responses of bison *Bison* spp. (Artiodactyla: Bovidae), caribou/reindeer *Rangifer tarandus* (Artiodactyla: Cervidae), and moose/elk *Alces alces* (Artiodactyla: Cervidae) to assess the impacts of flies on these ungulates. Ungulates rely on morphological and physiological resistance to flies at low intensities of exposure. However, as fly exposure increases, ungulates begin to react with behavioural avoidance in addition to increasing their physiological response. *Rangifer tarandus* are highly sensitive to flies and respond quickly to their presence by avoidance behaviours that incur fitness costs through reduced body mass. *Alces alces* are less reactive to fly exposure, enduring the presence of flies and maintaining a low loss of fitness, sometimes dying from the cumulative effects of exposure. *Bison* spp. may use a facultative strategy that depends upon the prevalence of flies and associated diseases in their environment. Among these strategies, variables

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[*] Benedict BM, Barboza PS (2022) Adverse effects of Diptera flies on northern ungulates: *Rangifer*, *Alces*, and *Bison*. *Mammal Review*. DOI: 10.1111/mam.12287.

such as the type of fly bite, presence and degree of infection, and heritability of resistance affect individual host survival. Relationships between flies and ungulates can integrate multiple scales of organisation in the ecosystem to reflect system stability. Climate change is predicted to alter the species composition and seasonal phenology of flies and the associated effects of wounding and vector-borne disease on ungulate populations that are central to the functions of Arctic and temperate ecosystems in the Northern Hemisphere.

Introduction

Evolutionary radiations of artiodactyls and perissodactyls occurred in the middle Miocene (15 million years before present; Janis 2008). Climatic variation due to ice ages favoured large body size and mobility (Mann et al. 2019) that predisposed ungulates to becoming a mobile feast for large vertebrate predators and a wide variety of invertebrate parasites (Jolles & Ezenwa 2015). Gregarious behaviour such as seasonal aggregation for migration is an outcome of selective pressures that balance the benefits of finding food and mates with the risks of attack from predators and flies, and exposure to pathogens (Ezenwa & Worsley-Tonks 2018, Bowyer et al. 2020).

Some of the main arthropod parasites of ungulates are flies. Flies belong to the order Diptera that includes 150000 species with a common origin in the Mesozoic period (225 million years before present; Sarwar 2020). Over 100 independent lineages have developed structures, behaviours, and developmental patterns for parasitism in 13 families of Diptera, resulting in the widest array of hosts among the parasitic groups of insects (Feener & Brown 1997). Flies cause distress and injury to animals that become an immediate source of nutrients for the fly, a host for developmental life stages of the fly, and/or a host for organisms that flies convey (Burger & Anderson 1970, Krinsky 1976, Kutz et al. 2012). Diptera mouthparts can be categorised as piercing or sponging types (Appendices A.1–3). Piercing mouthparts, which create wounds, range from slender (mosquito, Culicidae) to broad and knifelike (horse fly, Tabanidae; Johnson & Triplehorn 2005). Sponging mouthparts (face flies) absorb fluids from mucus membranes and wounds, with the potential for a secondary effect of transferring pathogens. Adult bot and warble flies (Oestridae) have vestigial mouthparts, but the eggs they lay on ungulates hatch into larvae that penetrate the skin with proteases to migrate through tissues as endoparasites (Wolfe 1959, Johnson & Triplehorn 2005, Barbet 2014).

The effects of flies on ungulate populations depend upon their impacts on fitness. Fitness is the ability of individuals to survive and reproduce by gaining energy and nutrients, as fat and lean body mass, to meet environmental demands and the costs of mating, pregnancy, and lactation. The response to flies is morphological, physiological, and behavioural.

An ungulate's first line of defence against flies is the morphological barrier of skin and hair. At low exposure to flies, the costs of skin repair and hair growth are likely to be small and part of the greater cost of seasonal moult, but those costs would increase in a linear fashion with increasing fly exposure and with the extent of injuries to the body surface (Fig. 1.1). The energy, protein, and time required to repair bites increase with severity of the bite from small piercing wounds (e.g. mosquito bites) to open

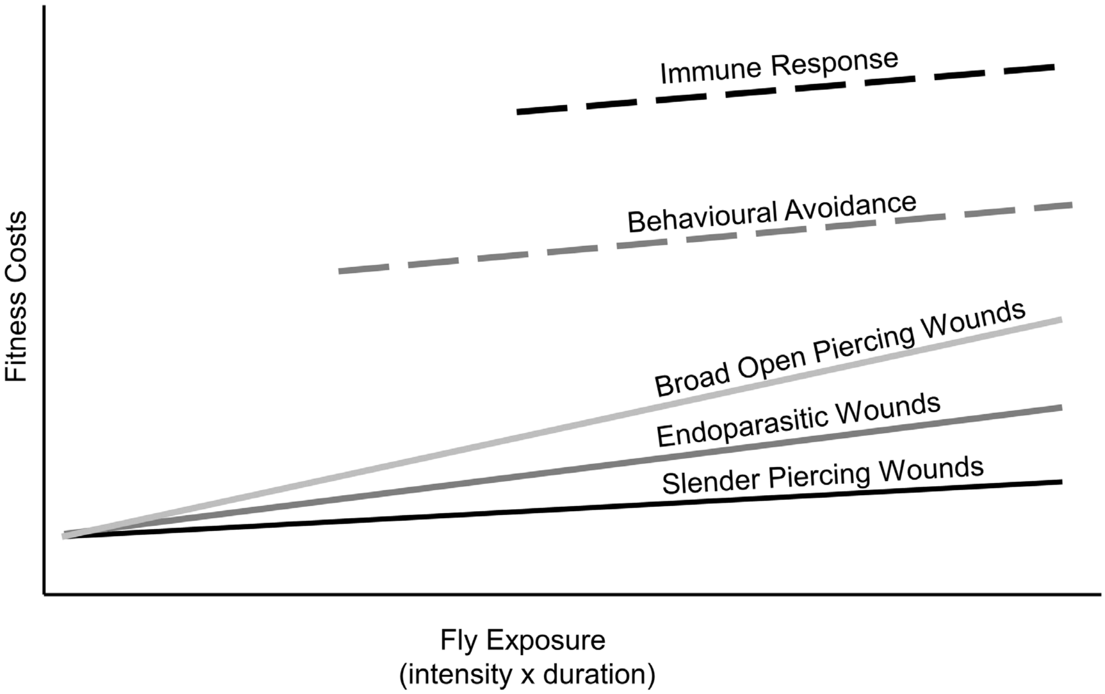

Figure 1.1 Hypothetical relationships between exposure to flies and the component costs of fitness for ungulates. Fitness costs can be measured as the change in body condition (e.g. gain of body mass, body fat, or body protein) or by indices of production (e.g. milk production, calf growth, and timing of seasonal moult) during the period of plant growth when animals are expected to gain mass and condition for survival and reproduction. Fly exposure is the product of fly activity (i.e. numbers of bites or encounters) and the duration of that activity (i.e. days) summed across fly groups. Solid lines indicate the physiological costs of physical injuries to the skin and underlying tissues by bites and secretions of flies and secondary infections from fly larvae or parasites. Dashed lines indicate higher and more complex costs of mounting an immune response or avoiding flies and their associated parasites.

wounds (e.g. horse fly bites; Fig. 1.1). Blood flow increases to supply cells and substrates to repair the wound, but this physiological response may be prolonged as the immune system reacts to secretions from the fly and subsequent infections of larvae or parasites (Nelson & Weintraub 1972, López et al. 2005, Dacal et al. 2009, Mauldin & Peters-Kennedy 2016). The activated immune system of a previously infested host often has strong cellular reactions to invasion, which can be effective in killing some of the penetrating parasites (Dacal et al. 2011). However, the costs of immune function rise with secondary infections and transmission of pathogens from the bite of adult or larval flies, or from contact of flies with an open wound. High costs of infection coincided with declines in *Alces alces* populations from 2004 to 2015 in north-east Minnesota, USA (DelGiudice 2020), where flies transferring nematode parasites cause neurological impairment, peritonitis, and death (Grunenwald et al. 2016, 2018). The added immunological costs of fly bites therefore depend greatly on what the fly leaves behind and the time course of the physiological response. Larvae and parasites that migrate through tissues leave internal wounds that provoke progressive physiological reactions of repair and defence through the developmental period of the parasite over a season (Fig. 1.1). The timing of infections may therefore impact seasonal growth and reproduction by diverting energy and protein from productive processes. For example, high metabolic demands in early lactation are associated with increased susceptibility to intestinal parasites in some breeds of sheep *Ovis aries*; if the increased susceptibility leads to infection, energy and protein are diverted away from lactation to repair the gut lining and to create proteins for the immune response (Doyle et al. 2014). Inflammation affects energy allocation, alters food selection, and reduces food intake (Kyriazakis 2010, Lacourt et al. 2018), to exacerbate the loss of body stores needed for survival and reproduction (Fig. 1.1).

Immunological resistance to intestinal parasites can improve the survival of host individuals, especially when food availability is low and environmental demands are high in winter (Graham et al. 2010, Nussey et al. 2014). However, immune responses incur trade-offs in resources (use of energy and nutrients) and function (e.g. risks of autoimmune reactions) that contribute to the low heritability of traits for resistance to parasites (Bisset & Morris 1996). Increasing host population density not only increases exposure to infectious parasites but also reduces food supply per individual; thus, the costs of resistance are not easily afforded when the exposure is greatest. In sheep, subsequent declines in density and exposure do not favour selection for these costly attributes, because individuals have a good likelihood of surviving and reproducing without the costly trait (Hayward et al. 2014). The specific ability to switch a response on and off may be a better indicator of resistance to parasites than the extent to which the immune system can mount a general response (Seguel et al. 2019). The major histocompatibility complex (MHC) is a large library of genes that code for antigens. High diversity of the MHC is associated with reduced survivorship in adult *Rangifer tarandus* (Gagnon et al. 2020), which suggests that sustaining physiological resistance to pathogens and parasites is costly. Sociality also affects transmission of pathogens; low genetic diversity of MHC in *Alces alces* is consistent with a solitary lifestyle and a low risk of transmission within the species (Swialocka et al. 2020).

Behavioural avoidance of flies can reduce exposure to pathogenic vectors, but foraging returns may decline because the warm wet conditions that favour flies also favour the growth of plants. Behavioural avoidance of flies can reduce the time available for feeding in favourable locations with abundant, high-quality food. Frequency-dependent selection for behaviours such as fly avoidance, or for fly resistance, is likely to be the outcome of reinforcement by physiological costs of each strategy (Hart 1990, Wolf & McNamara 2012). Solitary species with low exposure to pathogens and low competition for food are likely to develop greater morphological barriers (resistance) to fly bites as fly exposure increases (Fig. 1.2). However, this strategy depends on a low

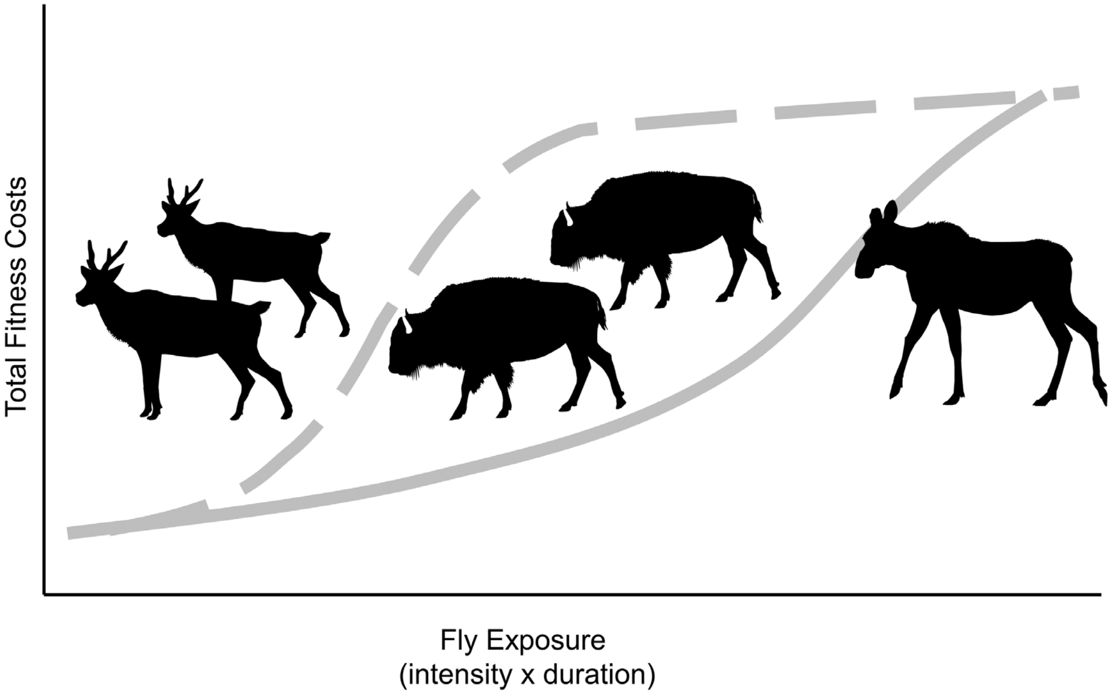

Figure 1.2 Hypothetical relationships between exposure to flies and total fitness costs of ungulates. Fitness costs are the sum of physical, behavioural, and immunological responses that can be quantified as changes in body condition (e.g. gain of body mass, body fat, or body protein) or by indices of production (e.g. milk production, calf growth, and timing of seasonal moult) during the period of plant growth when animals are expected to gain mass and condition for survival and reproduction. Fly exposure is the product of fly activity (i.e. numbers of bites or encounters) and the duration of that activity (i.e. days) summed across fly groups. The solid grey line indicates the progressive cost of increasing fly exposure as

wound repair transitions to immunological responses in a solitary ungulate such as *Alces alces* (right silhouette). The dashed grey line indicates the progressive cost of increasing fly exposure as wound repair transitions to behavioural avoidance with attendant foraging costs (e.g. declines in quality and quantity of food) in a social ungulate such as *Rangifer tarandus* (left silhouettes). Ungulates such as *Bison* spp. (middle silhouettes) may achieve the same fitness cost in either strategy by using avoidance or physical resistance to fly bites, depending upon the prevalence of pathogens associated with flies in the environment.

probability of incurring high physiological costs of infection, which are likely to rise rapidly with fly exposure. Hosts are likely to start avoiding flies as the probability of a costly immune reaction increases, especially when hosts aggregate and are likely to be infected (Fig. 1.2). High costs of foraging would increase fitness costs above a threshold exposure at which hosts start avoiding flies, but increasing exposure to flies would slowly increase physiological costs as pathogens are incurred (Fig. 1.2).

The resilience of ungulates to flies could be indicative of the stability of the population and its interactions with the ecosystem (Ostfeld & Keesing 2017, Scheffer et al. 2018, Magnusson et al. 2020). Relationships between flies and ungulates encompass multiple scales of organisation in the ecosystem, including interactions between biophysical elements (e.g. aquatic and aerial life stages of flies) and disparate functional groups in hosts (e.g. predators and herbivores). Complex communities and interactions with variability, multidimensionality, and shifting conditions can lead to ecological surprises (Doak et al. 2008) such as population declines in keystone species that accompany a state transition in the ecosystem (Dakos et al. 2014, Van de Leemput et al. 2017, Scheffer et al. 2018).

Ungulate populations drive many terrestrial ecosystems because they are large herbivores that often aggregate in large numbers and move long distances, resulting in strong feedback on the biomass and diversity of plant communities (Sinclair et al. 2007, Speed et al. 2019). For example, the introduction of the viral disease rinderpest to Africa impacted domestic and wild herbivores, as well as the human and ecological communities and underlying ecosystem processes for several decades (Normile 2008). Vector-borne diseases are the continued focus of monitoring programmes that sustain productivity of domestic herbivores (Galvani et al. 2016). However, much less is known about the relationships between flies and wild herbivores, which ultimately reflect ecosystem health (Galvani et al. 2016). Relationships between flies and wild ungulates may provide indicators of critical slowing down in the responses of multiple elements in a system to environmental change — an early warning sign that key elements such as herbivore populations are approaching a transition (Dakos et al. 2014, Van de Leemput et al. 2017, Scheffer et al. 2018).

In this review, we focus on bison *Bison* spp. (Artiodactyla: Bovidae), caribou/reindeer *Rangifer tarandus* (Artiodactyla: Cervidae), and moose/elk *Alces alces* (Artiodactyla: Cervidae) because they represent the two largest families of ungulates (Cervidae and Bovidae). *Bison* spp. and *Rangifer tarandus* are gregarious and predominately travel in herds, while *Alces alces* are mostly solitary (Mörschel & Klein 1997). Previous studies have been focused on one host species, one level of defence (morphological, physiological, or behavioural), and one parasitic species. The purpose of this review is to link these elements together in ungulates that differ greatly in their responses to flies, insects in the order Diptera. We structure this review as discussions of morphological barriers, physiological change, behavioural changes, and body condition for the three focal species. We emphasise the importance of understanding effects of Diptera, due to the order's diverse evolution and understudied nature. We then couple this new approach with the additive effects of environmental change with respect to possible tipping points and future research needs, ending the review with a summary of how climate change may influence interactions.

Methods

We used the web browsers Google Scholar and Web of Science to search for peer-reviewed publications. We used combinations of the keywords: 'ungulates', 'insect', 'Diptera', 'moose', '*Alces alces*', 'bison', '*Bison*', 'caribou', 'reindeer', '*Rangifer tarandus',* 'biting', 'climate', 'Northern Hemisphere', 'harassment', 'population', 'distribution', 'temperature', 'coat', 'disease', 'flies', 'immune', 'skin', 'behavior', 'stress', 'cortisol', 'morphology', 'physiology', 'mouthpart', and 'tipping point'. All query results were reviewed for relevance and used to find additional articles for inclusion. One hundred and thirty peer-reviewed publications were used in this review.

Results and discussion

Morphological barriers

The physical characteristics of the skin and coat are important factors in the vulnerability of ungulates to flies (Caro et al. 2014, Kynkaanniemi et al. 2014, Scasta & Smith 2019). *Bison* spp. have short coats in the summer, making them particularly vulnerable to flies and breaks in their skin barrier (Meagher 1973). For example, warble flies *Hypoderma* spp. lay eggs on the hairs of *Bison* spp. and *Rangifer tarandus*, they hatch, and the larvae migrate through the connective tissue and throat, to pupate in the subcutaneous tissues along the back (Kearney et al. 1991, Haigh et al. 2002). However, the short, dense winter hair of *Rangifer tarandus* may provide some protection against deer keds *Lipoptena cervi* (Kynkaanniemi et al. 2014). *Alces alces* infested with deer keds are often found with severe alopecia (hair loss), which is also associated with poor body condition (Madslien et al. 2011). As for other flies, they tend to feed on the areas of *Alces alces* where the hair is thin and short, such as the legs and around the anus (Lankester & Samuel 2007).

Physiological change

When a fly breaks through the skin barrier, either by direct penetration with biting mouthparts or by depositing larva into damaged skin, direct and indirect toxicity results (Mauldin & Peters-Kennedy 2016). Tissue damage from both bites and the immune response can be progressive and may eventually result in permanent loss of function (morbidity) and death (Samuel et al. 2001, Hosni et al. 2019). Severe conjunctivitis and blindness have been associated with flies on *Bison bison* at the National Bison Range Nature Reserve, Montana, USA (Burger & Anderson 1970, Meagher 1973). Flies are also vectors for secondary parasites and pathogens in *Bison* spp. (Haigh et al. 2002).

In *Rangifer tarandus*, oestrid flies are associated with an increase in secondary loads of parasites, allergic responses, localised secondary infections, restricted breathing, disease outbreaks, pneumonia, and deaths (Hughes et al. 2009, Laaksonen et al. 2009, Kutz et al. 2012). Mosquitoes and horn flies *Haematobia irritans* are intermediate hosts for adult nematodes *Setaria tundra* that live in the peritoneal cavity and create microfilariae that are then distributed via the bloodstream, leading to peritonitis and even death (Laaksonen et al. 2009). *Rangifer tarandus* are initially able to resist experimental infection of deer keds by maintaining blood parameters, including cell count, electrolytes, amino acids, enzyme activities, and fatty acid profiles (Paakkonen et al. 2011). However, other studies of flies have indicated high physiological costs to *Rangifer tarandus* (Kutz et al. 2012). For example, warble flies are negatively associated with the likelihood of pregnancy in the Dolphin-Union caribou *Rangifer tarandus* of Victoria Island, Canada (Hughes et al. 2009). In western Greenland, an infestation of warble flies reduced survival body weight of *Rangifer tarandus* calves and reduced fat depots in pregnant females (Cuyler et al. 2012). Parasite loads that cause decreased body condition and reproduction (Mallory & Boyce 2018) may lead to cycles of repeated infection and deteriorating body condition (Thomas & Kiliaan 1990, Beldomenico & Begon 2010). Additionally, the reindeer warble fly *Hypoderma tarandi* and reindeer throat botfly *Cephenemyia trompe* are obligate parasites causing myiasis, an infestation of the skin by developing fly larvae, among *Rangifer tarandus* in northern Norway (Kearney et al. 1991). Calves are particularly at risk because growth and development are impaired by interruptions to foraging, injury from bites, and responses to parasites that are new to their immune system (Åsbakk et al. 2005, Witter et al. 2012b). The effects of these exposures can continue into the first winter or even to the onset of reproduction after the second or third summer (Witter et al. 2012b).

Many flies are attracted to *Alces alces* for a bloodmeal and/or to complete their reproductive development, transmitting pathogenic bacteria and parasitic worms (Valimaki et al. 2011, Egan & Moon 2013). In particular, the moose fly *Haematobosca alcis* is an obligate parasite that uses the faeces of *Alces alces* to lay eggs and develop pupae (Burger & Anderson 1974). It is common for the hindquarters to be occupied by over 500 moose flies (Lankester & Samuel 2007). Moose flies are associated with open sores on the hind legs of *Alces alces* that appear in mid-June and decline in early September along with the number of flies (Murie 1934). Up to 12 round wounds of 1.5 cm diameter are typically observed on each back leg in the peak of the fly season (Lankester & Samuel 2007). Moose flies appear to have the mouthparts capable of creating the wounds and have been observed feeding on the periphery of the sores (Lankester & Samuel 2007), although it has been suggested that horse flies *Hybomitra* spp. are responsible for creating the sores (Lankester & Samuel 2007).

Moose flies and horse flies are not alone in bringing physiological impacts to *Alces alces*. There is a positive linear relationship between the number of adult legworms *Onchocerca cervipedis*, a filarioid nematode found beneath the leg skin, and the age of *Alces alces* when black flies *Simulium* spp. are present (Pledger et al. 1980). Additionally, wingless deer keds *Lipoptena cervi* that use *Alces alces* as a host are vectors for the spread of infections of *Bartonella* spp., an emerging zoonotic disease in humans (Duodu et al. 2013). However, a comparison of blood and plasma values of infested versus ked-free *Alces alces* in Finland showed only minor changes in values as a result of heavy parasitism (Paakkonen et al. 2012). Flies have transferred nematode parasites (e.g. *Rumenfilaria* spp. and *Setaria yehi*) to many individual *Alces alces*, causing severe disease, neurological impairment, peritonitis, and death (Grunenwald et al. 2018).

Behavioural changes

Flies may have a greater effect on the movement and distribution of *Bison* spp. herds than breeding activity and foraging (Meagher 1973). *Bison* spp. spend less time foraging and more time lying down, standing, grooming, and using wallows during periods of high fly activity (Melton et al. 1989, Mooring & Samuel 1998b, McMillan et al. 2000). *Bison* spp. herds are rarely observed in areas with abundant flies (Meagher 1973). The combination of radiant heat and flies drives *Bison* spp. to seek shade in forested areas rather than to continue foraging in open areas, and to choose resting sites with fewer flies and a denser canopy (Van Den Brink 1980, Belovsky & Slade 1986, Melton et al. 1989, Schneider et al. 2013).

Rangifer tarandus are even more sensitive to flies than *Bison* spp. Tabanids in particular influence *Rangifer tarandus* movements; Raponi et al. (2018) found that when there are more tabanids, female *Rangifer tarandus* are noticeably less active. *Rangifer tarandus* are only slightly less active in response to mosquitoes and black flies, but in response to oestrid flies, they strongly increase time spent standing and decrease time spent feeding (Mörschel & Klein 1997, Witter et al. 2012a, Raponi et al. 2018). Toupin et al. (1996) found that *Rangifer tarandus* at George River, northern Quebec, Canada, reduced their time spent feeding from 54% to 30% and increased standing time from 1% to 39% during periods of high fly activity. Witter et al. (2012a) found that interruptions to feeding activity were greatest when oestrid flies, black flies, and mosquitoes were all present in combination. Mörschel and Klein (1997) also found that there was an increase in long-range movement and other activities in the presence of mosquitoes and oestrid flies, and that resting cycles were disturbed when fly activity was high. The increase in time spent standing with increases in long-range movement and energy expenditure is due to *Rangifer tarandus* moving more on barren ground and using shorelines, exposed ridges, higher elevations, cool winds, snow patches, and ice patches to escape flies (Mörschel & Klein 1997, Hagemoen & Reimers 2002). The increase in long-range movement is also due to a dramatic response to oestrid flies – *Rangifer tarandus* run, jump, and travel erratically to seek refuge (Hagemoen & Reimers 2002). Other restless behaviours include shaking, scratching, and grooming (Kynkaanniemi et al. 2014). *Rangifer tarandus* in mountainous areas exploit lichen and low-quality forage during the day when they are retreating from flies, and only return to lower elevation areas of higher quality forage when temperatures cool in the evening (Galloway et al. 2012, Witter et al. 2012a). The response is, however, contextual to food availability and other disturbances; *Rangifer tarandus* do not compensate for interruptions from harassment by flies, or increase grazing time in the evening or in other periods when fly activity is low (Colman et al. 2003, Witter et al. 2012a, Mallory & Boyce 2018). In some *Rangifer tarandus* herds, flies influence distribution even more than people (Vistnes et al. 2008).

Rangifer tarandus may use aggregation to dilute or distribute the risk of being attacked by flies among members of a group (Helle et al. 1992, Fauchald et al. 2007, Kynkaanniemi et al. 2014); warble flies cause females and calves to form groups (Fauchald et al. 2007). A similar phenomenon has been seen with the Porcupine Caribou Herd *Rangifer tarandus granti* of Alaska and Yukon, USA and Canada, where groups of *Rangifer tarandus* aggregate for spring calving and increase in density on the foraging grounds when fly activity is at its highest (Russell et al. 1993). At low wind speeds, it is advantageous to be in the middle of the group to avoid flies, but it is the opposite in high wind speed situations, when there are fewer flies and more opportunities to forage at the periphery of the group (Russell et al. 1993).

Although avoidance of flies can be effective for *Rangifer tarandus*, avoidance behaviour reduces the time that can be spent feeding and fattening on high-quality forage, which, in turn, affects body condition (Kutz et al. 2012, Witter et al. 2012a, Raponi et al. 2018). *Rangifer tarandus* in poor condition are less likely to survive and reproduce; recruitment to the population therefore diminishes with loss of body mass and body stores (Colman et al. 2003, Kutz et al. 2012, Witter et al. 2012a, Raponi et al. 2018). Harassment by flies affects foraging of female *Rangifer tarandus* and thus the annual productivity of herds in Alaska (Johnson et al. 2018, 2021). The timing is particularly detrimental, as the end of fly season, and the resulting time of reduced body condition for *Rangifer tarandus*, is close to the start of winter (Mallory & Boyce 2018). Summer is an important time for *Rangifer tarandus* to gain mass to start the next reproductive cycle and to survive winter. In early spring and late autumn, there are brief periods of time when fly intensity is low and *Rangifer tarandus* are able to find and consume forage with less interruption (Mallory & Boyce 2018). Additionally, in some areas where deer keds are present the fly period is prolonged, as deer keds have the potential to remain on the host for the entire year (Härkönen et al. 2013, Kynkaanniemi et al. 2014).

Although flies can cause mortality in both *Rangifer tarandus* and *Alces alces*, the hosts' responses to exposure vary. *Alces alces* appear to be less bothered by flies than *Rangifer tarandus*, which is particularly remarkable because they are heavily infested, and moose fly are an obligate parasite of *Alces alces* (Lankester & Sein 1986, Samuel et al. 2001, Lankester & Samuel 2007). In a captive study, *Alces alces* calves reacted less to flies on their lower legs than *Rangifer tarandus* calves, which continually flinched and stomped when stable flies *Stomxys calitrans* were present (Lankester & Sein 1986, Lankester & Samuel 2007). *Alces alces* may increase their grooming or scratching in response to flies, similar to how grooming increases in response to winter ticks *Dermacentor albipictus*, which can lead to hair loss (Mooring & Samuel 1998a, 1999). In Yellowstone National Park, USA, *Alces alces* surrounded by 500 or more flies do not appear to be annoyed (Burger & Anderson 1974). Wallowing in mud and wading in water may provide relief from flies or from radiant heat loads (Van Wormer 1972, Wolfe 1974). However, *Alces alces* move to wetland communities and cool water to forage on cattails *Typha* spp. and aquatic plants on hot days, even though fly numbers are high in these warm wet areas (Renecker & Hudson 1992). It was believed that *Alces alces* move to roadside areas to avoid flies, but they are more likely to be looking for salt and forage there (Fraser & Thomas 1982, Lankester & Samuel 2007). However, it has been suggested that flies may cause *Alces alces* to change their foraging habitats on a small scale, resulting in changes in food quality and body weight (Renecker & Hudson 1992). In addition to lost foraging opportunities, *Alces alces* may lose weight when they expend energy on avoiding flies (Lankester & Samuel 2007).

Body condition

Low body condition of *Rangifer tarandus* has been directly linked with high fly intensity (Weladji et al. 2003). Energy focused on avoiding flies reduces the time spent feeding, and in turn fattening, which then decreases lactation and fecundity for the current year and future years (Reimers 1997, Weladji et al. 2003, Pachkowski et al. 2013). The time of year of highest fly intensity coincides with female lactation, when the bodies of female *Rangifer tarandus* have few reserves but experience high demand for energy and protein (Reimers 1997, Weladji et al. 2003). Flies cause blood loss, energy loss, and a loss of grazing time, which leads to reduced body weight and milk production (Steelman 1976, Pickens & Miller 1980, Byford et al. 1992). The depth of rump fat in *Rangifer tarandus* cows is negatively correlated with the number of warble fly larvae on the back (Cuyler et al. 2012). Harassment by flies occurs when females are producing milk for growing calves; flies can therefore impact the population by affecting the viability of calves and the likelihood of their mothers gaining sufficient body mass for conception in the autumn (Cameron et al. 1993, Reimers 1997, Weladji et al. 2003, Hughes et al. 2009). For example, in the George River *Rangifer tarandus* herd of northern Quebec and Labrador, Canada, high infestations of warble flies reduce the probability of pregnancy by reducing body mass and body fat (Pachkowski et al. 2013). Flies negatively affect the autumn weight of calves, which can later affect their reproduction, creating cohorts with small calves born in years when flies were plentiful (Weladji et al. 2003, Gurarie et al. 2019).

Flies can even drive natural selection. Warble fly larvae are more abundant on lighter coloured *Rangifer tarandus* calves, leading to lower body mass in lighter calves than in dark calves in the same herd (Rodven et al. 2009). Hence, warble fly infestation indirectly selects for less variable and darker colours of *Rangifer tarandus*, because body mass is positively related to survival of *Rangifer tarandus* calves over winter (Rodven et al. 2009).

Climate change impacts

The numbers of flies experienced by ungulates are heavily dependent on climatic variables such as wind, temperature, and precipitation (Downes et al. 1986, Anderson & Nilssen 1998, Colman et al. 2003, Culler et al. 2018). Warming causes faster development rates in some flies, which translates into fewer days of exposure to predation at the larval stages and an increase in parasitism for the host (Culler et al. 2015). Spatial variation in temperature causes a 10-fold range in the per capita growth of populations of Arctic mosquitoes *Aedes nigripes* in western Greenland (Culler et al. 2018).

Climate warming is changing the distribution and abundance of hosts and parasites, introducing invasive species and diseases to new latitudes and higher elevations (Danks 1992, Kutz et al. 2009, Vander Wal et al. 2014, Jolles et al. 2015, Mallory & Boyce 2018). Regions with low biodiversity, such as the Arctic, may be vulnerable to range expansion and invasions, as winters become milder, allowing new species to survive into the following spring (Kutz et al. 2009). The Arctic is already seeing the effects of warming on snowmelt and expansion of shrubs into the tundra, bringing more flies (Rich et al. 2013). It is unlikely that all flies will respond in the same way to changes in climatic variables (Witter et al. 2012a). A shift in habitats might not always mean an increase in a species; in north-east Greenland, the abundance of muscid flies decreased with warming summer temperatures (Loboda et al. 2018). Populations within a species may also not be uniform across the Arctic; a human community in northern Quebec, Canada, has seen an increase in mosquito abundance, size, and emergence, while other communities have seen lower abundance (Koltz & Culler 2021). For deer keds, rising winter and summer temperatures could extend their growing season, allowing them to expand their range northwards (Härkönen et al. 2010). The introduction of new species creates novel interactions and assemblages for a host's immune system (Jolles et al. 2015). Consequently, variation in fly populations across the large geographic range of *Rangifer tarandus* drives seasonal patterns of movement, body size, and body condition of populations (Witter et al. 2012a).

The seasonal pattern of fly activity is also predicted to change with summer warming; fly activity is predicted to start earlier and last longer as summers warm (Brotton & Wall 1997, Witter et al. 2012b, Mallory & Boyce 2018). This leaves *Rangifer tarandus* and *Bison* spp. with fewer fly-free foraging days, which means less time for fattening and, in turn, poorer body condition (Melton et al. 1989, Klein 1991, Brotton & Wall 1997, Mörschel & Klein 1997, Weladji et al. 2003, Mallory & Boyce 2018). *Rangifer tarandus* are already losing some of their relief areas; melting of ice fields in southwest Yukon, Canada, is leaving *Rangifer tarandus* with fewer fly relief areas (Farnell et al. 2004). However, climate change is heterogeneous, and some areas may experience increased summer precipitation and clouds, causing reduced fly intensity (Heggberget et al. 2002, Mallory & Boyce 2018).

Alces alces in the Arctic are better at surviving in cold environments than most fly vectors; however, vector-borne pathogens have been found in Eurasian moose *Alces alces alces,* indicating their host potential (Malmsten et al. 2019). *Alces alces* and *Rangifer tarandus* at the southern extent of their ranges are vulnerable to the combined effects of warm temperatures on forage supply and diseases, especially those borne by flies (Murray et al. 2006, Monteith et al. 2015).

Conclusions

The literature provides abundant information on the behaviour, physiology, and morphology of *Rangifer tarandus* in relation to flies, but much less is known about *Bison* spp. and *Alces alces*. The avoidance strategies undertaken by *Rangifer tarandus* are well documented, probably because changes in the movement of large groups of these large animals have marked effects on their availability to and use by human communities. Future research should focus on ungulate species for which the cost of flies is less apparent.

Rangifer tarandus forego foraging and fattening opportunities to avoid flies during the valuable short summer window of plant growth at high latitudes. Behavioural avoidance of flies is less apparent for *Alces alces*, which presumably rely upon physical barriers to limit wounding by flies. *Alces alces* may be able to endure the costs of repairing those wounds with a lower risk of infection, due to their solitary behaviour and their ability to attain high intakes of energy and nutrients in Boreal habitats with high forage growth (Shively et al. 2019). *Bison* spp. may use different strategies to contend with flies throughout their range – low foraging windows and cooler temperatures may allow northern *Bison* spp. to avoid flies like *Rangifer tarandus*, but southern *Bison* spp. may be unable to avoid exposure to flies and parasites, especially in hot, wet regions. The costs of flies are compounded over the summer. Individual ungulates may therefore reach tipping points between physiological compensation and morbidity that could precipitate sudden population declines and ecological surprises (Doak et al. 2008, Stoneberg Holt 2018).

The degree of fly intensity experienced by ungulates is largely controlled by climatic variables. Climate change will continue to alter the range of fly species, change the degree of intensity of fly exposure, and extend the period of exposure of ungulates to flies, depending on seasonal patterns of temperature and precipitation. The effects of harassment by flies and exposure to parasites are additive and contribute to the cumulative effect of warming on ungulates' food supply (i.e. forage quality and abundance) and exposure to extreme weather (i.e. hot spells, wildfires). These effects of climate change are likely to contribute to a tipping point for large mammals, especially for populations that are not only valued by human communities but also constrained in their distribution by human land use and activity, such as many ungulate species (Albon et al. 2002, National Research Council 2003, Johnson et al. 2005, 2015).

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CHAPTER II

PHYSIOLOGICAL RESISTANCE OF MOOSE CALVES TO FLIES: CORTISOL AND ENVIRONMENTAL STRESSORS[*]

Synopsis

Young animals are particularly vulnerable to environmental stressors that can impair growth and compromise survival. Moose (*Alces alces*) face threats from predators and infectious diseases from birth. We used salivary cortisol, a glucocorticosteroid hormone, to characterize the stress response of moose calves in relation to age, time of day, ambient air temperature, and the abundance and type of biting and non-biting flies. We measured salivary cortisol in 5 calves up to 4 times a day on 25 days through the summer with corresponding samples of on-host fly collections by sweep nets. Of the 2,618 flies sampled on the animals, 68% were moose flies (*Haematobosca alcis*), 13% were coprophagous flies, 9% were mosquitoes (Culicidae), 5% were horse and deer flies (Tabanidae), and 2% were black flies (Simuliidae). Salivary cortisol levels were low ($<0.2\ \mu g \cdot dL^{-1}$) from 25 to 89 d of age at ambient temperatures of 13 to 34 ºC from June through August. Cortisol did not increase with ambient air temperature or with counts of flies. A low-stress response to flies is consistent with low avoidance of flies by female adult moose and the calves that must follow their mothers. Moose calves apparently rely on their coat and their immune system to resist fly bites and pathogens associated with

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[*] Benedict BM, Thompson DP, Crouse JA, Shults PT, Hamer GL, Barboza PS (2023) Physiological resistance of moose calves to flies: cortisol and environmental stressors *Alces*: In Review.

wounds or bites. Recruitment and recovery of moose populations may be sensitive to increases in vector borne pathogens associated with a warming climate because calves appear to rely on physiological resistance to flies.

Introduction

Moose (*Alces alces*) do not appear to avoid biting and nuisance flies (Diptera), despite persistent attacks from species such as the moose fly (*Haematobosca alcis*) (Benedict & Barboza 2022). Even when surrounded by >500 flies, moose do not appear to be annoyed, exhibiting minimal flinching or stomping (Burger & Anderson 1974; Lankester & Sein 1986; Lankester & Samuel 2007). Moose may be able to avoid flies by using wetlands and lakes but this behavior is also associated with thermoregulation and foraging (Fraser et al. 1984; Renecker & Hudson 1992). Moose may also avoid flies by using roadside areas but it is more likely that moose are attracted to growing plants and salt (Lankester & Samuel 2007). Flies impose a physiological cost to moose because the wounds they create require energy and nutrients to heal. Furthermore, wounds increase the risk of infection by microbes and parasites that can affect maintenance and growth of the body (i.e., morbidity rate) and also increase the likelihood of death (i.e., mortality rate) (Benedict & Barboza 2022). The moose fly completes its life cycle in association with moose and is often implicated as the cause of open sores on adult moose (Murie 1934; Burger & Anderson 1974). As fly numbers increase in mid-June, round wounds (about 1.5 cm in diameter) develop on the hind legs of adult moose (Lankester & Samuel 2007). At the peak of fly season in summer, adult moose may have up to twelve open round wounds on their hind legs, blanketed with moose flies (Lankester & Samuel 2007).

The glucocorticosteroid hormones cortisol and corticosterone, have been used to assess the effects of environmental stressors on a wide variety of mammals (Cook & Schaefer 2002; Sheriff et al. 2011). When an animal experiences an environmental perturbation, the hypothalamic-pituitary-adrenal axis is activated and glucocorticoids are secreted above basal levels (Sapolsky et al. 2000; Reeder & Kramer 2005; Sheriff et al. 2011). While this stress response may help the animal initially, long-term or chronic stimulation can have negative effects on immunity, inflammatory responses, reproduction, and growth (Wingfield et al. 1998; Sheriff et al. 2011). Additionally, the stress response consumes energy, which can deplete body stores of fat and glycogen (Busch & Hayward 2009; Sheriff et al. 2011).

The principal goal of this project was to characterize the stress response of moose calves in relation to age, time of day, ambient air temperature, and the abundance and type of biting and non-biting flies. We studied calves because they should be most vulnerable to the stresses of flies and environmental variables because high requirements for growth and development coincide with a naive immune system (Campbell et al. 1977; Åsbakk et al. 2005; Witter et al. 2012). We used salivary cortisol as the primary measurement of stress because it reflects circulating cortisol in the blood, peaking 20-30 minutes after the onset of a stressor (Sheriff et al. 2011). Recent studies of adult female moose measured salivary cortisol levels in conjunction with fecal corticosterone (Thompson et al. 2020a). Rapid increases in ambient air temperature elevated salivary cortisol levels during the day while increasing daily heat loads from solar radiation increased fecal corticosterone levels among days (Thompson et al. 2020a). However, Thompson et al. (2020) did not measure fly abundance and diversity, which could be responsible for the increase in salivary cortisol with ambient temperature, as a positive relationship is often seen between fly numbers and ambient air temperature (Burger & Anderson 1974). We measured salivary cortisol of female calves repeatedly throughout the summer to assess the effects of ambient air temperature, time of day, and age as the calves, and the abundance and diversity of flies around the moose calves. We predicted a positive relationship between salivary cortisol and fly abundance, and ambient air temperature, which would support our hypothesis that calves were physiologically stressed by flies and warm temperatures.

Methods

Capture and captive facility

All procedures for care, handling, and experimentation of animals were approved by the Animal Care and Use Committee, Alaska Department of Fish and Game, Division of Wildlife Conservation (IACUC protocol no.0086-2019-38) and by the Agricultural Animal Care and Use Committee, Texas A&M AgriLife Research (AUP 2019-009A). From May through August of 2019, we studied 5 captive moose calves held at the Kenai Moose Research Center (MRC), operated by the Alaska Department of Fish and Game (ADFG) on the Kenai National Wildlife Refuge (60° N, 150° W).

The MRC was established to study the interrelationships between moose and their environment (Hundertmark et al. 2000). The MRC hand raises calves by bottle-feeding animals a milk replacer and providing forage and a pelleted ration through weaning (Thompson unpublished data). Calves are trained to allow collection of various non-invasive samples (e.g., saliva, fecal), to enter handling areas, and stand on scales for routine measures of body mass. Calves were born in May (21-24); three calves were born at the MRC and two calves were orphaned by wild moose from Soldotna and Anchorage, Alaska. The three calves born at the MRC were removed from their mothers at 18 hours post-partum after ensuring that the calves had begun suckling and received colostrum. Based on behavior and hoof wear, we estimated the two orphan calves to be 8 and 9 days old (Bragulla 1991). All five calves were placed into a 700 m^2 nursery pen that was enclosed by a 2.4 m high woven wire fence. The nursery pen was further protected by an electric fence to exclude predators in the adjacent mid-seral boreal forest. We provided a 4 m^2 covered shelter within the nursery pen along with feed buckets and ad libitum water. We initially placed a colored string (blue, green, white, yellow, and pink) around the neck of each calf for identification, which was then replaced with a colored, expandable VHF collar (Mod-415-3, Telonics, Mesa AZ, USA) at two weeks of age. Handlers minimized noise around the calves and spent time habituating the calves to human contact. Calves were monitored closely for alertness, milk intake, injury, and diarrhea. When calves were 21 days old, they began walking with handlers into a 0.23 km^2 enclosure which contained a large wetland, mixed age boreal forest, black spruce forest, and open meadow, allowing them to forage for 2-3 hours a day before returning to the smaller nursery pen for bottle feeding, sample collection, and nighttime security. At 10 weeks old, we walked the calves twice a day, and we progressively started leaving the calves in the large enclosure on their own for the entire day, returning for bottle feedings, sample collection, and nighttime security.

Sample collection

Calves were trained daily to allow for the collection of saliva and flies. All collections were done inside of the smaller nursery pen. Collection of saliva consisted of swabbing between the bottom teeth and gums using a synthetic swab (SalivaBio Children's Swab, Salimetrics LLC, Carlsbad, CA, USA), which was frozen for storage within an hour of collection. We collected flies aggregating on the calves with a 0.381 m diameter collapsible net (BioQuip, Rancho Dominquez, CA, USA) by swiping the net overhead and near the skin surface of all of the calves for two minutes (Lloyd & Dipeolu 1974). Flies were transferred to a kill jar with acetone. Apples, pellets, and bananas were offered as incentives to the calves during training and immediately after swabbing and collection of fly samples. Saliva was collected at 15:30 on June 18th and 23rd (Julian Day 169 and 174), and at 18:30 on June 21st (Julian Day 172). Saliva and flies were collected at 05:30, 12:30, 15:30, and 18:30 on June 22nd (Julian Day 173). We collected saliva from each calf on 3 consecutive days each week from July 6, 2019 to August 18, 2019 (Julian Day 187-230). On the first and third day, saliva was collected at 15:30. On the second day, saliva and flies were collected at 05:30, 12:30, 15:30, and 18:30. Daily collections were scheduled to collect saliva prior to a milk meal, which elicited a salivary response but precluded contamination of the sample with milk. Cortisol was analyzed in duplicate using a cortisol ELISA assay (µg/dL; Salivary Cortisol; Salimetrics LLC, Carlsbad, CA, USA) and reported as the mean concentration in each sample (Millspaugh et al. 2002; Thompson et al. 2020a; Thompson et al. 2020b). Weather variables (ambient air temperature (°C), wind speed ($m \cdot s^{-1}$), relative humidity (hPa)) were measured using a handheld weather meter (Kestrel 4400 Heat Stress tracker, Kestrel, Boothwyn, PA, USA).

Flies were identified and counted under a dissection microscope in the following groups: biting midges (Ceratopogonidae), mosquitoes (Culicidae), moose flies (*Haematobosca alcis*), coprophagous flies (various families), black flies (Simuliidae), horse and deer flies (Tabanidae), snipe flies (Rhagionidae), and other flies (Table 2.1; USDA Veterinary Permit 139420). These groups were chosen because they represent all fly families found on the calves, and the species most likely to be biting or harassing the calves. To confirm identification, total DNA was extracted from representative individual flies using a method modified from the Gentra Puregene Kit (Gentra Systems, Inc., D-5500A). PCR reactions targeting a 710 base pair barcoding region of the COI gene were performed using the primer set, LCO1490 and HC02198 (Folmer et al. 1994). Each reaction contained 2.0 µl of DNA, 0.75 µM of each primer, 12.5 µl Taq-Pro Complete (Thomas Scientific, C788T27), and 9.0 µl of deionized water. The amplification process consisted of the following thermal cycles: one cycle of 3 min at 95 °C, followed by 35 cycles of 1 min at 95 °C, 1 cycle of 1.5 min at 45 °C, one cycle of 2 min at 72 °C, and a final extension step for 5 min at 72 °C. PCR products were cleaned using the EXOSAP-IT protocol (ThermoFisher, 78201.1.ML). Each sample was prepared for sequencing using a BigDye Terminator v3.1 Cycle Sequencer Kit and protocol (Applied Biosystems, 4337454). Samples were sequenced in an Applied

Biosystems 3500 Genetic Analyzer. Chromatograms produced for each sequence were cleaned and aligned using the program Geneious v. 9.1 (Kearse et al. 2012). Sequences were assigned to species using BLAST search of the NCBI GenBank database, and the percent matches are reported in Table 2.1 (Ferrar 1974; Hanski & Stähls 1990; Kuchta & Savage 2008; Couri & Salas 2010). Fly counts are expressed on the basis of the number of calves in the group in a two-minute time window (flies•calf^{-1}).

Calculations and statistics

We used mixed-effects regression with individual moose and Julian day as random effects to account for repeated measures of dependent variables (STATA 15.1; StataCorp, College Station, Texas, USA). We used the robust “sandwich estimator” for standard errors to relax assumptions of normal distribution and homogeneity of variances (Rabe-Hesketh & Skrondal 2010). We used a reverse stepwise selection procedure for all mixed models, which removed coefficients that were not significantly different from zero. All statistical significance was set at $P \leq 0.05$.

We examined the effect of time with three metrics: age of the calf as a continuous variable (age_d from 1 to 132 d), age of the calf as a categorical variable (age_w; in nine weeks from 4 to 13) and the time of day as a categorical variable (hours at four collection times from 0530 to 1830). The model for salivary cortisol levels included ambient air temperature (T_a) with calf age (age_week) and time of day (collection) as categorical fixed effects: salivary cortisol = T_a + age_w + collection + ε.

We analyzed counts of flies as groups and as total counts with calf age (age_d) as a fixed effect: count = age_d + ε. We examined the effect of total combined fly groups on salivary cortisol levels with the model: salivary cortisol = flies + ε. We also used principal component analysis to derive two scores (PC1 and PC2) that indexed variation in the counts of flies across all 8 groups. Fixed effects for PC 1 and PC 2 were used to examine salivary cortisol levels: salivary cortisol = PC 1 + PC 2 + ε.

Results

Saliva was collected from each of the 5 calves up to 4 times a day for 25 days for a total of 49 samples per calf. We started collections when milk intakes were stable at 25-28 days of age and ceased collections at 86-89 days of age as calves foraged independently with only 2 or 3 milk meals each day. Saliva cortisol concentrations were normally distributed and analyzed without transformation. Time of day and age of the calf were significant effects on salivary cortisol. Salivary cortisol values at 05:30 were significantly different from values at 15:30 and 18:30 (Wald $X^2 = 18.19$, $P = 0.001$; Fig. 2.1A). Salivary cortisol was not significantly affected by age of the calf (Fig. 2.1B). Although morning temperatures were cooler than other times of the day, ambient air temperature at sampling was not significantly related to salivary cortisol level ($P = 0.182$).

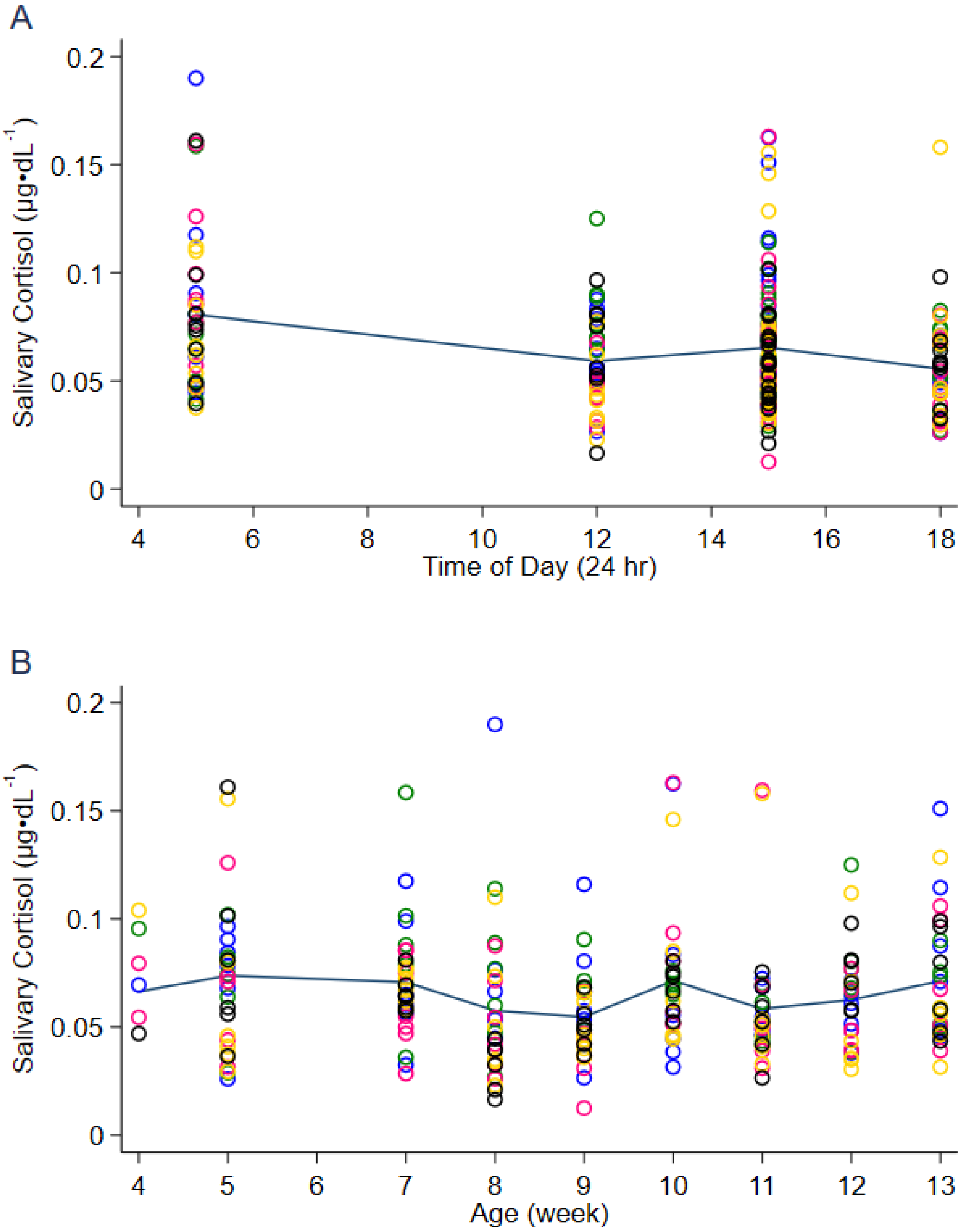

Figure 2.1 Marginal predictions and observations (circles), of time of day (A) and calf age (B) on salivary cortisol (µg•dL^{-1}) of moose calves (n=5 series of colors) based on a mixed model regression with individual and Julian day as random

effects to account for repeated measures within individual moose calves at the Kenai Moose Research Center, Alaska, USA from June to August 2019. Model parameters: 241 observations in 5 groups; χ^2 [4df] = 18.19; *P* = 0.001. Random effects within individuals were 1.00% of variance.

Flies were netted around the calves up to four times per day on 9 days over the summer for a total of 33 collections (Table 2.1). Flies were sorted into 8 groups through morphological identification, with representative specimens verified molecularly (Table 2.1). A total of 2,618 flies were processed and classified into the following groups: 68.4% moose flies, 13.5% coprophagous flies, 8.75% mosquitoes, 5.5% horse and deer flies, 2.0% black flies, and less than 1% other flies, biting midges, and snipe flies (Table 2.1).

Table 2.1 Flies collected during a two-minute sampling of all calves (n=33 collections) at the Kenai Moose Research Center, Alaska, USA from June to August 2019. Flies were grouped by morphological identifiers to calculate a rate (Count) per collection (mean ± SE) and a sum of all counts in the group (Total). Representative specimens were confirmed by genetic sequence using the NCBI GenBank database, and the BLAST search.

Common name	Species ID	Genbank Match	% Match	Count	Total
Moose fly	*Haematobosca alcis*	MF886185.1	99.8	54.30±8.13	1792
Coprophagous flies				10.70±3.83	353
-Black scavenger fly	Sepsidae sp.	Morphological ID			
-Dump fly	*Hydrotaea scambus*	MF891571.1	98.1		
-Dump fly	*Hydrotaea* sp.	KP049063.1	98.6		
-Dung fly	*Scathophaga suilla*	KR440263.1	100.0		
-Latrine fly	Fanniidae sp.	Morphological ID			
-n/a	*Mesembrina decipiens*	KR618635.1	100.0		
-n/a	*Morellia podagrica*	KU496783.1	100.0		
Mosquito	*Aedes* sp.	Morphological ID		6.94±2.26	229
Horse and deer flies				4.33±1.50	143
-Deer fly	*Chrysops exitans*	JF868977.1	99.8		
-Deer fly	*Chrysops frigidus*	KU874617.1	99.8		
-Horse fly	*Hybomitra affinis*	HM861001.1	99.8		
Black fly	*Simulium verecundum*	KR682101.1	99.8	1.58±0.38	52
Other Flies				0.79±0.20	26
-Dance fly	Hybotidae sp.	HQ551771.1	98.8		
-Long-legged fly	*Dolichopus* sp.	KM969513.1	100.0		
- n/a	*Pegomya* sp.	MG120915.1	99.9		
- n/a	*Thricops diaphanus*	HM412371.1	100.0		
-Tiger fly	*Coenosia conforma*	HM883164.1	99.5		
Biting midge	Ceratopogonidae sp.	JN291037.1	98.0	0.64±0.38	21
Snipe fly	*Symphoromyia* sp.	JF868466.1	100.0	0.06±0.04	2

Fly numbers were correlated with the age of the calves, with total flies increasing linearly with age and variation among the 8 fly groups (Fig. 2.2A). Of the 8 fly groups, moose flies exhibited the greatest increase in numbers with calf age, while coprophagous flies had the greatest decrease in numbers with age (Fig. 2.2A). Principal component 1 accounted for 79.9% of the variation in the fly groups, while principal component 2 accounted for only 12.9% percent of the variation (Fig. 2.2B). Moose flies, the largest group of flies, strongly influenced PC 1 whereas coprophagous flies strongly influenced PC 2 (Fig. 2.2B). Mosquitoes, horse flies and deer flies also influenced PC 2. Variation in the counts of coprophagous flies, horse flies, and deer flies were orthogonal to the counts of mosquitoes. Variation in the counts of black flies, biting midges, snipe flies, and other flies were not associated with either PC 1 or PC 2. The salivary cortisol levels of the calves were not significantly related to the total counts of flies (Wald $X^2 = 1.12$, $P > 0.05$). Scores for PC 1 and PC 2 were significantly related to salivary cortisol (Wald $X^2 = 59.44$, $P < 0.001$; Fig. 2.3). However, the absolute change in salivary cortisol levels was minimal (< 0.001 for PC 1 and -0.001 for PC 2) over the range of PC scores (Fig. 2.3).

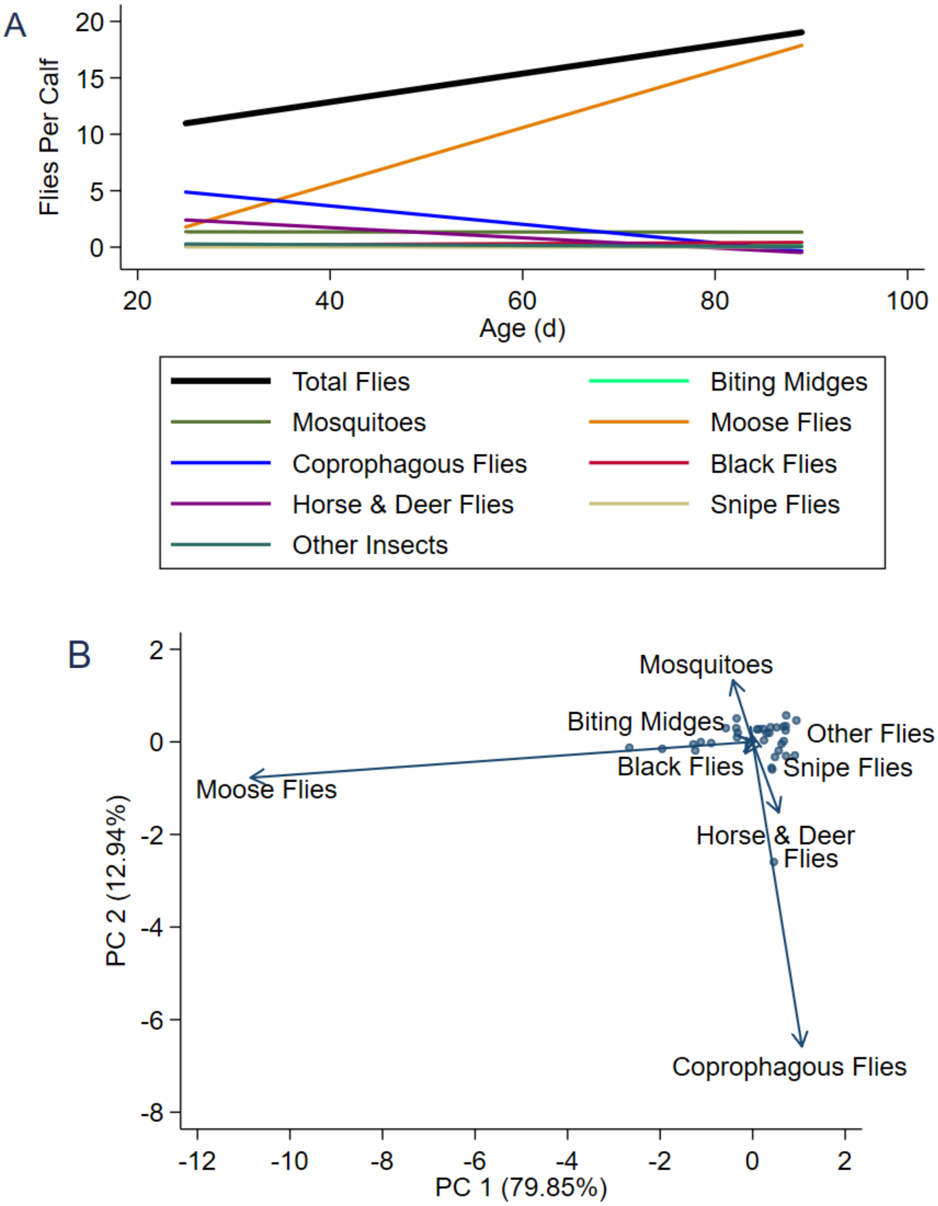

Figure 2.2 Plot of marginal predictions (A) of fly count per calf in each group of flies against age of moose calves at the Kenai Moose Research Center, Alaska, USA from June to August 2019. Effects of fly counts on age were all significantly different from zero in mixed model regressions with individual and time (Julian day) as random effects to account for repeated measures. Model parameters: 165 observations in 5 groups; total flies χ^2 [1df] = 9.38; *P* = 0.002, biting midges χ^2 [1df] = 0.97; *P* = 0.325, mosquitoes χ^2 [1df] = 0.000; *P* = 0.974, moose flies χ^2 [1df] =

53.12; $P < 0.001$, coprophagous flies χ^2 [1df] = 31.25; $P < 0.001$, black flies χ^2 [1df] = 4.40; $P = 0.036$, horse and deer flies χ^2 [1df] = 66.08; $P < 0.001$, snipe flies χ^2 [1df] = 0.72; $P = 0.397$, other flies χ^2 [1df] = 19.78; $P < 0.001$. Random effects within individuals were < 0.001% of variance for all models. Principal component analysis (B) of fly counts in 33 collections around moose calves during the summer. Arrows indicate vectors for each fly group in the first and second orthogonal components. Filled circles indicate observations.

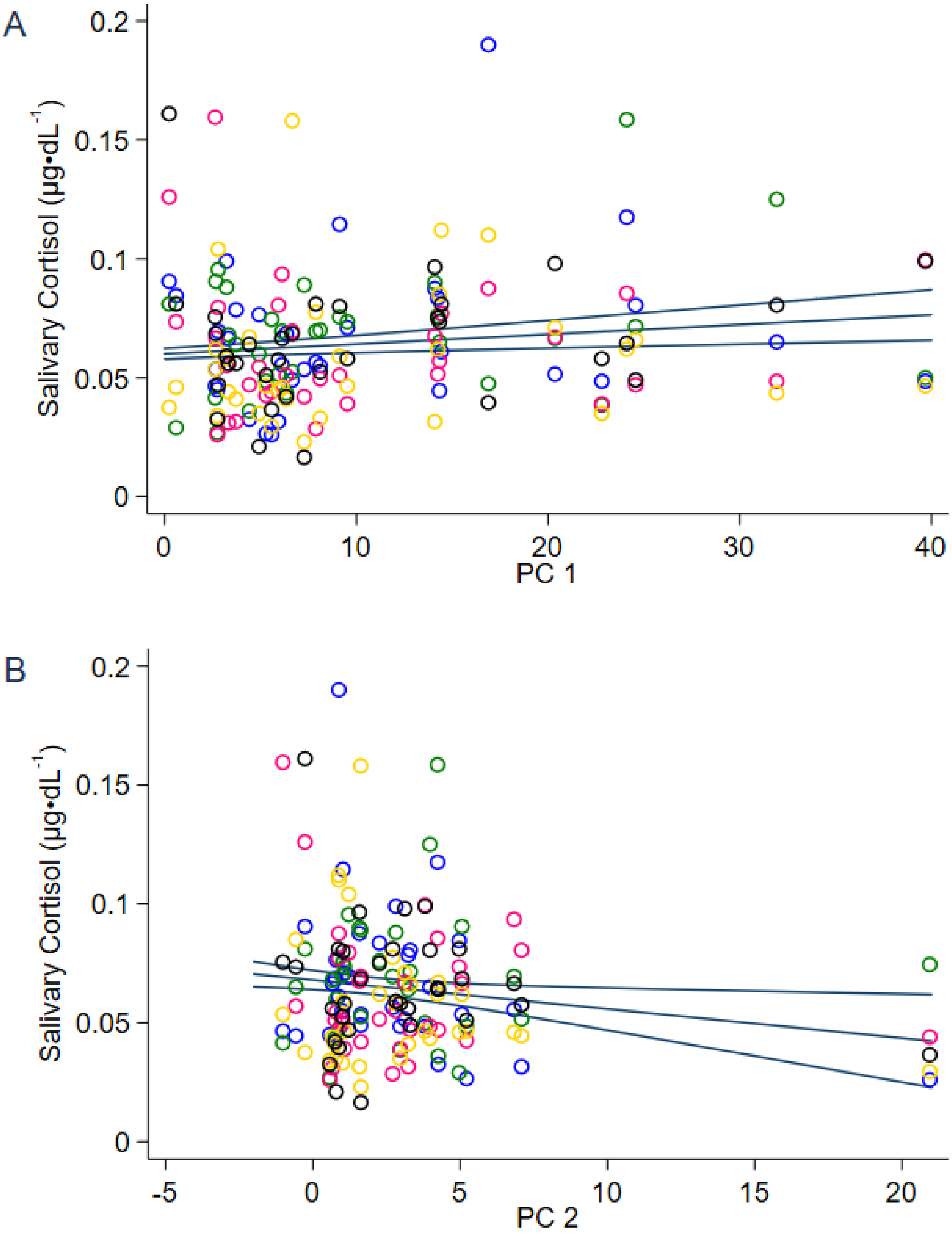

Figure 2.3 Relationship between salivary cortisol (µg•dL-1) and two indices of flies on moose calves: PC 1 (A) and PC 2 (B). Indices were derived from principal component analysis of counts of 33 collections of flies around calves (n=5 series of colors). Lines are predictions with 95% confidence intervals from mixed model regression with individual and Julian day as random effects to account for repeated measures within individual moose calves at the Kenai Moose Research Center,

Alaska, USA from June to August 2019. Model parameters: 165 observations in 5 groups; χ^2 [2df] = 33.68; $P < 0.001$. Random effects within individuals were < 0.001% of variance.

Discussion

Salivary cortisol levels of calves (0-0.2 $\mu g \cdot dL^{-1}$; Fig. 2.1) were at the lower end of the range observed for adult female moose (0-3.0 $\mu g \cdot dL^{-1}$) (Thompson et al. 2020a). The repeated measures design allowed us to account for any developmental changes in the stress response with age, diurnal effects of temperature and meal feeding as well as changes in temperature and fly activity among days. We found small transient changes in salivary cortisol with week and time of day over the season, even though fly abundance increased over the season (Fig. 2.1B, 2.2A). Although we found an increase in salivary cortisol with PC1 (Fig. 2.3A), the predicted rise in concentration (0.007 $\mu g \cdot dl^{-1}$) was less than the sensitivity of the assay (0.012 $\mu g \cdot dL^{-1}$) (Salimetrics LLC, Carlsbad, CA, USA). The same assay was able to show a correlation between change in air temperature and salivary cortisol for adult female moose, with the majority of the salivary cortisol values between 0 and 0.5 $\mu g \cdot dL^{-1}$ (Thompson et al. 2020a).

While there aren't any other moose calf studies for comparison, we expected a larger rise in salivary cortisol in relation to flies, based on the sensitivity of the assay for adult female moose. Consistently low levels of salivary cortisol in moose calves also suggest that hand-rearing was not a stressor. Similarly, a study of maternally raised musk deer (*Moschus berezovskii*) found stable cortisol values from birth until weaning (Li et al. 2021). Maternal stress responses in pregnancy may influence those of the offspring in utero and during lactation as observed in fallow deer fawns (*Dama dama*) (Amin et al. 2021). We do not know the levels of salivary cortisol in calves with their mothers, but we do know that the growth and development of these hand-reared calves are similar to those of maternally raised moose (J.A. Crouse, Alaska Department of Fish and Game, unpublished data). Habituated maternal moose have been used to measure food intake and milk production over summer, but the corresponding measures of maternally raised calves are limited because the calves do not share the habituation of their mothers and are easily stressed by handling (Reese & Robbins 1994; Shively et al. 2019).

Young moose appear to rely on morphological barriers to resist flies. Unlike adult moose, the calves did not have large sores on their hind legs (Lankester & Samuel 2007). Most flies collected from the moose calves were from their hind end, near their tail. Moose flies were particularly abundant in this region. Similar observations have been made on adult moose, making it difficult for moose of all ages to reach this area to disturb flies (Lankester & Samuel 2007). However, the coat of moose calves may offer a barrier of protection that is not present in the adults, particularly for species of flies that likely do not burrow (moose flies and mosquitoes). Calves were born with dense fuzzy coats that were replaced with a winter coats at the end of the sampling period, providing them with protection over the majority of their body (anus and around the eyes are left exposed) throughout the season (Samuel et al. 1986). Any injury to the calves' coat, resulting in a lack of hair, was bitten by flies.

Flies appear not to trigger the release of glucocorticoid hormones as an emergency response in moose calves, even though the flies may still be perceived as noxious (McEwen & Wingfield 2003; Busch & Hayward 2009). The moose calves exhibited some signs of annoyance; running, jumping, shaking their head, stomping, and twitching in response to some larger flies (horse and deer flies). Flies may be a tolerated stressor, in which moose calves are habituated. There also may be a tipping point in which moose can no longer resist exposure and a cortisol response is induced. The response of moose to deer ked (*Lipoptena cervi*) suggests a threshold response: hair cortisol concentrations do not increase at low intensities of exposure but prolonged exposure to deer ked triggers a release of glucocorticoids in moose (Madslien et al. 2020).

We found a correlation between salivary cortisol and time of day (Fig. 2.1A) and age (Fig. 2.1B). High cortisol values in the morning coincides with the beginning of milk-feeding for the day after calves were left alone overnight in the pen without a human handler (Carbonaro et al. 1992b, 1992a). The calves were not fed milk meals throughout the night, the greatest time gap between milk meals was between their last milk feeding at night and their first feeding the following morning, which may explain the higher levels of morning stress. The higher morning values could also be unrelated to stress and may be a circadian rhythm (Ingram et al. 1999). Regardless of the explanation, fly abundance and ambient air temperature does not explain this temporal pattern.

Although moose calves appear to resist fly harassment through a dense hair coat and a minimal cortisol response during the summer, moose calves on the Kenai Peninsula are still affected by biting flies. Both wild and captive moose calves on the Kenai Peninsula have shown elevated levels of the filarial nematode *Setaria yehi* during their first winter, with morbidity and mortality in calves from peritonitis associated with *S. yehi* migrating out of the blood vessels in the peritoneum (Thompson unpublished data). In Finland, warmer summers resulted in increased fly density, and subsequently increased peritonitis in reindeer from filarial nematodes (Laaksonen et al. 2007; Laaksonen et al. 2009b). Hematophagous insects are the intermediate hosts for filarial nematode larvae, ingesting microfilaria larvae, which then reside and develop in the insect under warm environmental conditions, before being transmitted back to the host mammalian species (Laaksonen et al. 2009a; Kutz et al. 2012; Grunenwald et al. 2016). Longer periods with warm temperatures may allow for multiple cycles of filarial nematodes, beyond the typical one cycle, to develop in fly vectors and be transmitted to hosts (Laaksonen et al. 2009b).

The resistance of moose to flies may be exceeded if flies and the associated parasites expand in range and seasonal activity with climate change (Kutz et al. 2012; Mallory & Boyce 2018). Moose populations may be even more vulnerable than caribou and reindeer to increases in flies because they rely on physiological tolerance. Wildlife managers may need to consider the abundance of flies and the prevalence of filarial nematode infections when making decisions on harvest rates and moose densities. High densities of moose could lead to high transmission and prevalence of parasites, leading to variation in recruitment and thus harvest (Brown 2011; Rempel 2011). Summer weather affects survival of moose calves through additive effects of predators (e.g., wolves and bears), arthropod pests (i.e. ticks) and forage supplies on both mother and calf that increase variation in population growth and harvest (Patterson et al. 2013; Jones et al. 2017).

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CHAPTER III

SORES OF BOREAL MOOSE REVEAL A PREVIOUSLY UNKNOWN GENETIC LINEAGE OF PARASITIC NEMATODE WITHIN THE GENUS *ONCHOCERCA*[*]

Synopsis

Long-standing reports of open sores on the hind legs of moose (*Alces alces*) have been recorded in Alaska (as well as Canada, Europe, and Michigan), eliciting concerns about causes and infection. We used histological and genomic methods to investigate the sores from 20 adult moose on the Kenai Peninsula, Alaska. We paired this with thermal imagery and molt scoring of adult moose to further describe sore formation and understand its timing. Severe, ulcerative and eosinophilic dermatitis was found in all moose with sores present, and microfilariae within intraepidermal pustules were additionally found in four samples. Genetic analysis of sores from moose revealed a previously unknown genetic lineage of *Onchocerca*. Adult moose molt and lose their barrier of protection against flies in June and July during peak fly activity, leaving them vulnerable and allowing the development of sores. In summary, our results indicate that the cause for the sores on the hindleg of moose is a previously unknown genetic lineage of *Onchocerca*, probably transmitted by black flies, in timing with the molt cycle of

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[*] Benedict BM, Barboza PS, Crouse JA, Groch KR, Kulpa MR, Thompson DP, Verocai GG, Wiener DJ (2023) Sores of boreal moose reveal a previously unknown genetic lineage of parasitic nematode within the genus *Onchocerca*. *PLoS ONE* 18(1): e0278886. https://doi.org/10.1371/journal.pone.0278886.

adult moose. These sores leave moose exposed to pathogens, making them vulnerable, and challenging their health and fitness.

Introduction

A mammal's coat and integument is an important barrier between the animal and its environment, providing protection against pollutants, extreme temperatures, pathogens, and irritants such as insects (Caro et al. 2014; Mauldin & Peters-Kennedy 2015; Benedict & Barboza 2022). Hair and melanin in the integument offer thermoregulation and photoprotection. Any break in the integument is a potential site of infection by a wide variety of pathogens that can cause morbidity and mortality (Mauldin & Peters-Kennedy 2015). The ability to repair these breaks can be an indication of the viability of an animal.

Ungulates such as horses (*Equus caballus*), donkeys (*Equus asinus*), mules (*Equus asinus* x *Equus caballus*), and a dromedary camel (*Camelus dromedarius*) often have been documented with round sores/breaks in the integument called "summer sores" (cutaneous habronemiasis) (Mauldin & Peters-Kennedy 2015). These summer sores are caused from a fly (Diptera) bite transferring spirurid nematodes (*Habronema majus*, *H. microstoma*, *H. muscae*, and *Draschia megastoma*), whose larvae erratically migrate through the tissue, initiating an infection, and causing a gross lesion with ulcerated granulated tissue to grow (Mauldin & Peters-Kennedy 2015). From the surface, the lesions range from 5-15 cm in diameter and there may be one or many, starting small and irregular and growing to circular sores (Mauldin & Peters-Kennedy 2015).

Another type of lesion known as "legworm" or "footworm" has been found on the distal legs of moose (*Alces alces*), caribou (*Rangifer tarandus*), white-tailed deer (*Odocoileus virginianus*), mule deer (*Odocoileus hemionus*), elk (*Cervus canadensis*), and pronghorn (*Antilocapra americana*), and has been associated with *Onchocerca cervipedis* (DeNio & West 1942; Verocai et al. 2012; McFrederick et al. 2013). *O. cervipedis* is a filarioid nematode that is transmitted through an arthropod vector and, as an adult worm, infects primarily the subcutaneous tissues of ungulate legs and hooves. These adult parasites will produce microfilariae, which will move to the skin tissues throughout the ungulate body, to be taken up by intermediate black fly vectors (Diptera: Simuliidae) (Pledger et al. 1980; Anderson 2000). Once ingested, microfilariae will develop to the infective L3 stage and migrate to black fly mouth parts to infect other mammalian hosts upon a subsequent blood meal. These parasite-induced lesions seem to have no seasonal pattern, are often found along the metatarsus or metacarpus, and occur in subcutaneous tissues with gross lesions only visible when the area is skinned (Rush 1935; Herman 1945).

Contrary to the subcutaneous lesions associated with *O. cervipedis*, open sores have been found proximal on the hind leg of moose, on the area above the tibio-tarsal joint, also known as the hock (Lankester & Sein 1986; Lankester & Samuel 2007). Up to 12 round sores of approximately 1.5 cm in diameter have been recorded in moose from Alaska, Michigan, Canada, and Europe (Murie 1934; Lankester & Samuel 2007). Despite a superficial resemblance suggesting a similar cause, these sores have not been reported as cutaneous habronemiasis. The timing of the appearance of these sores in mid-June and declining in early September coincides with the timing of fly activity in boreal areas of North America, suggesting biting flies as the potential cause and the main factor delaying the healing (Murie 1934; Lankester & Sein 1986; Lankester & Samuel 2007). It has been suggested that moose flies (*Haematobosca alcis*) are responsible for creating these sores based on their mouth parts and observations of them feeding on the periphery of the sores, aligning themselves in whorl patterns over the sores (Lankester & Sein 1986; Lankester & Samuel 2007). Horse flies (*Hybomitra* spp.) and leeches (Clitellata) have also been suggested, though leeches are the least probable cause as they have rarely been observed (Lankester & Sein 1986; Lankester & Samuel 2007).

Areas on a moose with a lower density of hairs and shorter hairs are heavily targeted by feeding flies (Meagher 1973; Caro et al. 2014; Scasta & Smith 2019); after molting, the caudal aspect of the legs, the perianal region, and the eyes are most vulnerable to flies (Lankester & Samuel 2007). A moose's coat consists of guard hairs covering their entire body, wool hairs or underfur on their torso, and vibrissae around their eyes and nose (Rubenik 2007). Adult moose experience one annual molt beginning in late spring and summer, whereas moose calves molt from neonatal coats to winter coats in the late summer (Samuel et al. 1986; Thompson et al. 2020).

Very little is known about the open hind leg sores on moose. The principal goal of this project was to describe and analyze these sores, with the main objective being to identify the cause for the lesions. We first describe the progression of molt and the formation of sores in adult moose, using visual scoring and thermal imagery. We then sampled the sores of moose and used histological and genomic techniques to describe the sores and identify the parasitic agent associated with these lesions.

Materials and methods

Captive facility

All procedures for care, handling, and experimentation of animals were approved by the Animal Care and Use Committee, Alaska Department of Fish and Game (ADFG), Division of Wildlife Conservation (IACUC protocol no. 0086-2019-38 and 0086-2020-40) and by the Agricultural Animal Care and Use Committee, Texas A&M AgriLife Research (AUP 2019-009A and 2021-009A).

We studied captive moose held at the Kenai Moose Research Center (MRC), operated by the ADFG on the Kenai National Wildlife Refuge, Alaska, USA (60° N, 150° W). All captive female moose (2-18 y old) used in this study (2015: n=11; 2016: n=12; 2021: n=12) were held in 2.6 km^2 outdoor enclosures. Moose had unlimited access to water and natural forage habitats; mixed seral state boreal forest, black spruce (*Picea mariana*) forest, bogs, open meadows, and lakes.

Molt and hock scoring

We observed all MRC moose at 5-20 day intervals from May 5 to July 13, 2015; May 3 to July 18, 2016; and May 19 to August 13, 2021. During these observations, or from photographs taken during these observations, each individual was assigned a molt score based on their progress in whole body molting (Table 3.1 and Fig. B.1). Individuals were also assigned a hock score to further track the progression from hair coverage to the appearance of sores on their hind legs, in the area above the hock (Table

3.1 and Fig. B.2). Our molt and hock scoring system was designed to best capture the molt sequence that we observed in previous years and during our observations. Adult molt is characterized by the loss of the long guard hairs and underfur and the replacement with shorter guard hairs, which continues to grow into the long guard hairs associated with a winter coat. During 2021 observations, we also recorded the number of sores per individual's leg.

Table 3.1 Molt and hock scores. Scores and description of each score used to describe the stages of the annual molt. Scores and description of each score used to describe the hair loss and progression towards sore being apparent on the hind leg area above the hocks of the moose.

Molt Score	Description
1	None – no signs of molt
2	Start of Ears – ears starting to molt
3	Ears Molted – ears molted and/or nose and eyes molting
4	Loose Body Hair – loose winter hair on body
5	Around Eyes Molted – molted around eyes and thin winter hair coverage on legs
6	Thin Shoulders & Legs – face molted from eyes forward, thin winter hair coverage on shoulders and legs
7	Mostly Molted – mostly short summer body hair, face mostly molted
8	Molted – full summer coat
Hock Score	**Description**
1	Hair – hair covering the area above the hock
2	Hair Loss – small amounts of hair loss above the hock
3	No Hair – clear hair loss above the hock
4	Sores – sores above the hock

Hind leg thermal imagery

We took thermal images of the moose from May 19 to August 13, 2021 to track warming of their hind legs across the season. We used a forward-looking infrared thermal camera (FLIR T1030sc; FLIR Systems) with a 12° x 9° lens (f/1.2), and pixel resolution of 1,024 x 768. We stood approximately 5 m away directly facing the rear end of the moose, and captured the area from the rump to the hocks while the moose were standing. These images were taken in the shade, under clouds or forest canopy. Multiple images were taken and the sharpest image with the best view was used for analysis. Ambient air temperature (°C) and relative humidity (%) were recorded digitally with a portable weather meter (Kestrel 4400 Heat Stress tracker or Kestrel DROP, Kestrel, Boothwyn, PA, USA) to correspond with each image. We analyzed the images using FLIR ResearchIR Max software (Version 4.40.1; 64 bit, FLIR Systems), and corrected them to an emissivity of 0.95, distance of 2 m, and the correlated ambient air temperature and relative humidity, using spatial calibration calculated for focal length of 83.2 mm with pixel pitch of 17 microns (Martin & Barboza 2019; Thompson et al. 2020). For each selected image we created three regions of interest (ROI) (Fig. 3.1). First, an ellipse ROI was drawn to encompass the perianal region and vulva of the moose to calculate a reference temperature for the exposed skin. Then, a polygon ROI was drawn on each hind leg encompassing the hind leg area from the center line of the hock to the base of the leg to calculate the maximum and minimum leg temperatures.

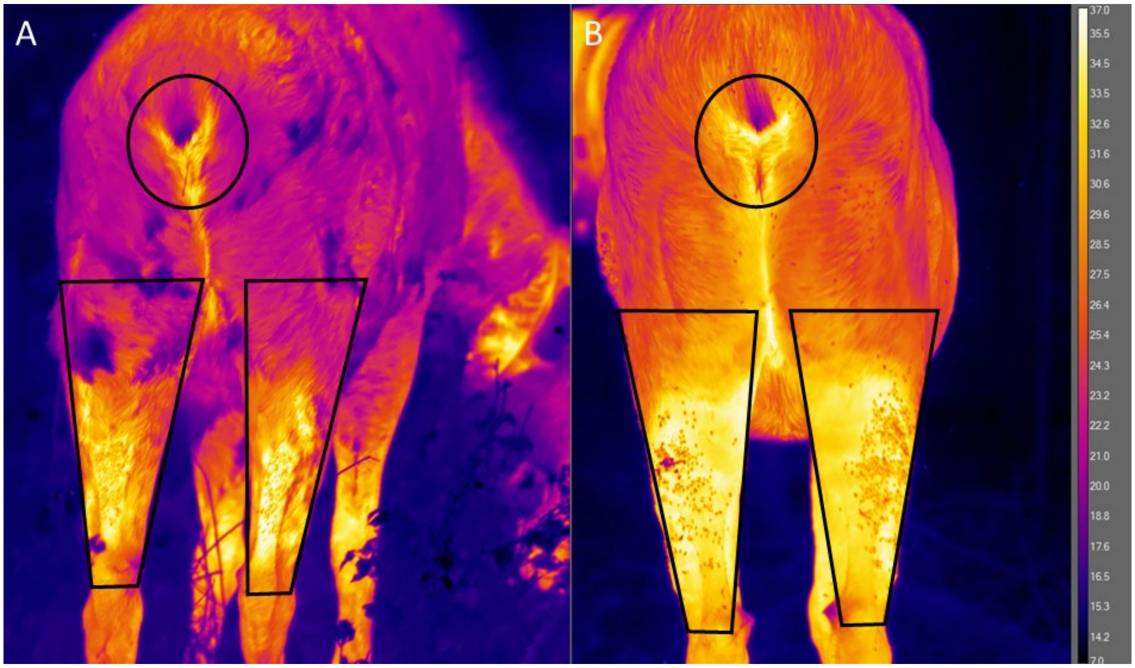

Figure 3.1 Thermal analysis. Surface temperature of the same moose on May 28, 2021 (A) and July 30, 2021 (B). Surface temperatures were measured inside of the ellipses (black circles) and polygons (black irregular quadrilaterals) drawn over the images, representing perianal and leg temperatures respectively.

We regressed leg and perianal temperature against date and climatic variables (STATA 15.1; StataCorp, College Station, Texas, USA). We used the robust "sandwich estimator" for standard errors to relax assumptions of normal distribution and homogeneity of variances (Rabe-Hesketh & Skrondal 2010). We used a reverse stepwise selection procedure for all regressions, which removed coefficients that were not significantly different from zero. All statistical significance was set at $P \leq 0.05$. The model for maximum leg temperature (max_temp) included Julian day (day) and ambient air temperature (T) as continuous fixed effects: max_temp = day + T. Similarly, the model for minimum leg temperature (min_temp) included Julian day (day) and ambient air temperature (T) as continuous fixed effects: min_temp = day + T. The model for maximum perianal temperature (perianal) included Julian day (day) and ambient air temperature (T) as continuous fixed effects: perianal = day + T.

Tissue collection

We immobilized the moose from May 12 to July 22, 2021 with Thiafentanil oxalate (0.001-0.004 mg/kg estimated body mass; 10mg/mL; ZooPharm Wildlife Pharmaceuticals Inc., Windsor, CO, USA) and Xylazine (0.03-0.05 mg/kg estimated body mass; 100mg/mL; Lloyd Laboratories, Shenandoah, IA, USA) hand-injected deep into shoulder muscle using a luer-lock syringe and 21Ga x 25mm hypodermic needle. Immobilization lasted less than 45 minutes in duration and was reversed with Atipamezole HCl (0.005 mg/kg estimated body mass; ¼ dose intravenous, ¾ dose intramuscular; 5 mg/mL; Zoetis, Parsippany, NJ, USA) and intramuscularly administered Naltrexone HCl (100 mg/mg Thiafentanil oxalate intramuscular; 50mg/mL; ZooPharm LLC, Laramie, WY, USA). We monitored the heart, respiration rate and blood perfusion to the mucous membranes of the eyes and gums throughout handling. When hind leg sores were present, we took a biopsy of the sore most proximal on the accessible leg (the other leg was beneath the immobilized moose). If a vessel was directly beneath the sore, the next closest sore was selected. If sores were not present, we took a biopsy in the same area that sores are known to occur later in the year. Scarring and changes in hair density and color were used as indicators for this location. We took the biopsy by gently rotating and pressing a sterile, disposable 6-mm punch (Miltex biopsy Sterile Disposable Dermal Punch, Integra, Princeton, NJ, USA) in the middle of the sore. The punched tissue sample was then removed with forceps and cut at the base with a scalpel blade. The tissue sample was then cut down the center line with a scalpel, half was placed in 2.5 ml RNAlater Stabilizing Solution (Invitrogen, Carlsbad, CA, USA) and half was placed in 2.5 ml 10% buffered formalin. We flushed the wound with isotonic saline (0.9% sodium chloride) and antiseptic cream (4% Chlorhexidine gluconate) was applied to the wound. After 24 hours, we removed the samples in formalin and rinsed them with 70% ethanol and placed in 2.5 ml ethanol. We later shipped the samples to Texas A&M University; prior to shipment we drained the ethanol-soaked samples of ethanol and placed an ethanol-soaked gauze pad for shipment.

Wild moose sampling

We also collected tissue samples from wild moose that were killed from collisions with vehicles (2020: n=2 adults; 2021: n=8 adults, n=1 calf) on the Kenai Peninsula, Alaska, USA from September 3 to 24, 2020 and from June 30 to July 23, 2021. Excised pieces of skin and muscle from the hock area were collected by ADFG personnel. We trimmed the hair on these samples and cut out the sores and some surrounding tissue using scissors and a scalpel, stopping at muscle (≤ 5mm). Skin without sores was also sampled for comparison. At least one sore from each individual was placed in RNAlater Stabilizing Solution and the remainder were placed in 10% buffered formalin. If only one sore was present, then the sample was cut in half and one side was placed in RNAlater Stabilizing Solution and the other half was placed in 10% buffer formalin. We prepared and shipped the samples as described above.

Histology

We trimmed the tissue sections and placed them in cassettes for processing. The tissue was embedded in paraffin, cut at 4 µm thick sections and stained with hematoxylin and eosin using the standard procedures. The sections were evaluated by a board-certified veterinary pathologist (DJW).

Genomic DNA

We performed Genomic DNA extraction from RNAlater preserved sections of moose leg tissue using DNeasy Blood & Tissue Kits (Qiagen, CA, USA) according to the manufacturer's recommendations. In total 26 samples were processed; 13 samples from dead wild moose and 13 samples from MRC moose. We amplified DNA extracts for the partial cytochrome *c* oxidase subunit 1 (COI) of the mitochondrial DNA (mtDNA) and performed polymerase chain reaction (PCR) reactions in 25 µL containing 0.25µM of each primer, 1x GoTaq® Green Master Mix (Promega Corporation, Madison, Wisconsin, United States) and 2.5 µL of DNA template. We amplify the COI using primers COINT (forward) 5'-TCAAAATATGCGTTCTACTGCTGTG-3'and COINT (reverse) 5'-CAAAGACCCAGCTAAAACAGGAAC-3' using a protocol modified from Hassan et al. (Hassan et al. 2015). Briefly, the cycling conditions consisted of an initial denaturation 95 °C for 2 min, followed by 35 cycles of 95 °C for 45 s, 50 °C for 45 s, and 72 °C for 30 s, and a final extension at 72 °C for 5 min. We used nuclease-free water as negative a control and DNA of *Dirofilaria immitis* as positive control.

We purified obtained PCR products using the E.Z.N.A.® Cycle Pure Kit (OMEGA Bio-Tek Inc., Norcross, GA, USA) according to the manufacturer's instructions. We aligned generated sequences and compared them to a variety of homologous *Onchocerca* sequences, including those reported in North America (Verocai et al. 2012; McFrederick et al. 2013; Lefoulon et al. 2017; Verocai et al. 2018; Kulpa et al. 2021), in the nucleotide sequence database at National Center of Biotechnology Information (NCBI) using MEGA X 10.1 (Kumar et al. 2016). We conducted phylogenetic analysis using a maximum likelihood method and a General Time Reversible best fit model with gamma distribution (2,000 bootstrap replicates) and *Dirofilaria immitis* served as the species outgroup. All newly sequenced samples were submitted and accessioned in GenBank (OP265723-39).

Results

Molt and sores

We compiled 322 observations of 15 individual moose (2015: n=11; 2016: n=12; 2021: n=12) during molt. The first sign of molt starting was on May 5 and all moose completed molt by August 1 (Fig. 3.2A). The first observation of a moose completing molt was on June 29 and the last was on July 25. Sore appearance was in coordination with molt; the first sore was observed June 9, 35 days after the start of molt (Fig. 3.2). All moose had sores by July 5, and continued to have sores through summer (Fig. 3.2B). In 2021, moose reached an average of 12 sores per hind leg per individual from July 22 to August 9, ranging from 3 to 25 sores (Fig. 3.3). The first evidence of sores starting to heal was on July 30, and all moose had some evidence of sores starting to heal by August 10. All moose had sores that remained open through August.

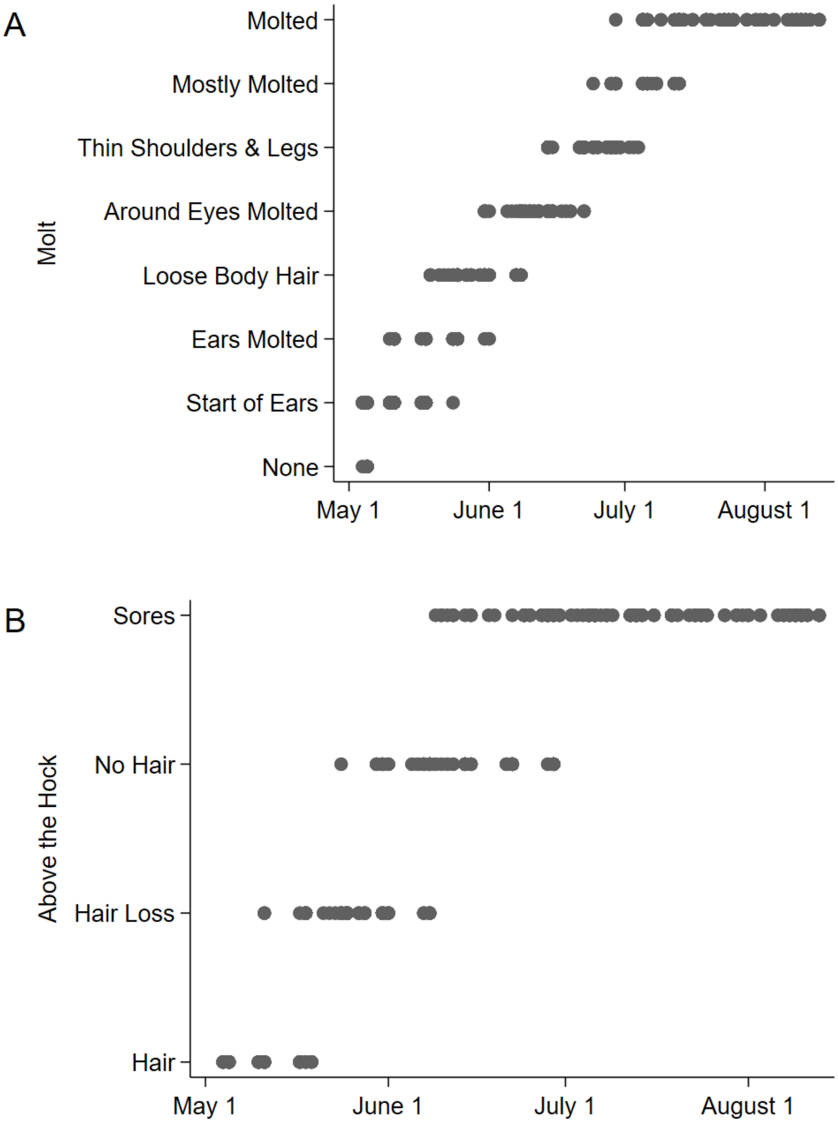

Figure 3.2 Molt and sores. A. The progression from winter coat to molted, summer coat in adult female moose (2015: n=11; 2016: n=12; 2021: n=12). B. Molt of the hind leg area above the hocks and the appearance of sores. Points represent individual observations.

Figure 3.3 Sores. Variation in sores on the hind leg of adult female moose. A. Two sores (July 21, 2021). B. 25 sores (July 22, 2021).

Thermal analysis

A total of 93 images from 12 moose were used for analysis. Maximum hind leg temperature was correlated with Julian day and ambient air temperature (R^2 [2df] = 0.2485, $P < 0.001$; Fig. 3.4). Similarly, minimum leg temperature was correlated with Julian day and ambient air temperature (R^2 [2df] = 0.6128, $P < 0.001$; Fig. 3.4). Maximum perianal temperature was correlated with Julian day and ambient air temperature as well (R^2 [2df] = 0.2489, $P < 0.001$; Fig. 3.4). Maximum perianal and maximum hind leg temperature were consistently correlated with similar values across Julian days, unlike minimum hind leg temperature which increased across the season (Fig. 3.4). Increasing temperature coincided with molt, the loss of hair, and the appearance of sores (Fig. 3.2, 3.4).

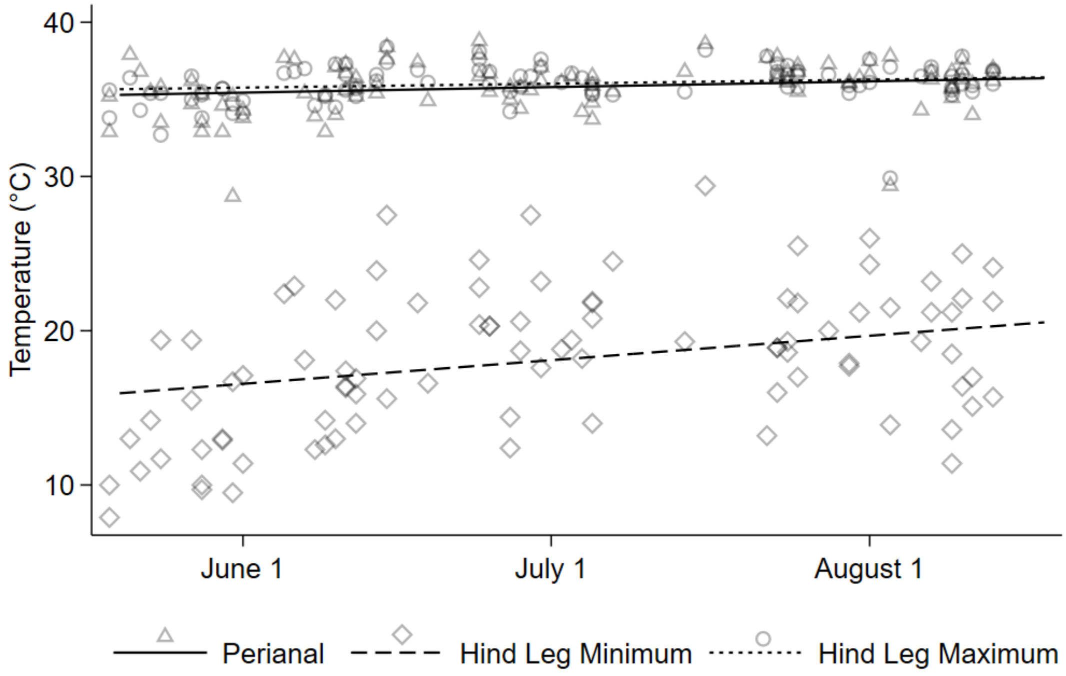

Figure 3.4 Observed skin temperature (open symbols) in regions of interest on the caudal surface of adult female moose (n=12) over summer. Lines are predicted relationships between skin temperature and day of year for the temperature of the perianal (solid line and open triangles), hind leg maximum (dotted line and open circles), and hind leg minimum (dashed line and open diamonds).

Tissue samples and histology

Biopsies were taken from eight MRC moose from May 12 to June 6, 2021 prior to the appearance of sores. In two of the animals, there was a mild eosinophilic and lymphocytic dermatitis (Fig. 3.5A). The skin biopsies from the other six animals were without significant pathologic findings. The sores of 10 MRC moose were biopsied from July 20 to July 22, 2021. In the skin biopsies from all 10 animals, there were similar histologic lesions. On the surface there were multifocally extensive serocellular crusts. The epidermis was frequently ulcerated. In the dermis there was a diffuse infiltration with many eosinophils (severe, multifocal, chronic, ulcerative and eosinophilic dermatitis; Fig. 3.5B). However, no microfilariae were visible in the skin samples from live moose at the MRC. Tissue samples were collected from eight wild dead adult moose and one wild calf from the Kenai Peninsula from June 30 to July 23, 2021. In all of the adult animals, there were similar lesions as observed in the MRC moose with severe, ulcerative and eosinophilic dermatitis. In addition, there were multifocally large intraepidermal pustules filled with eosinophils (Fig. 3.5C). In four of the dead animals, there were microfilariae within intraepidermal pustules surrounded by degenerated eosinophils as an eosinophilic sleeve (Fig. 3.5D).

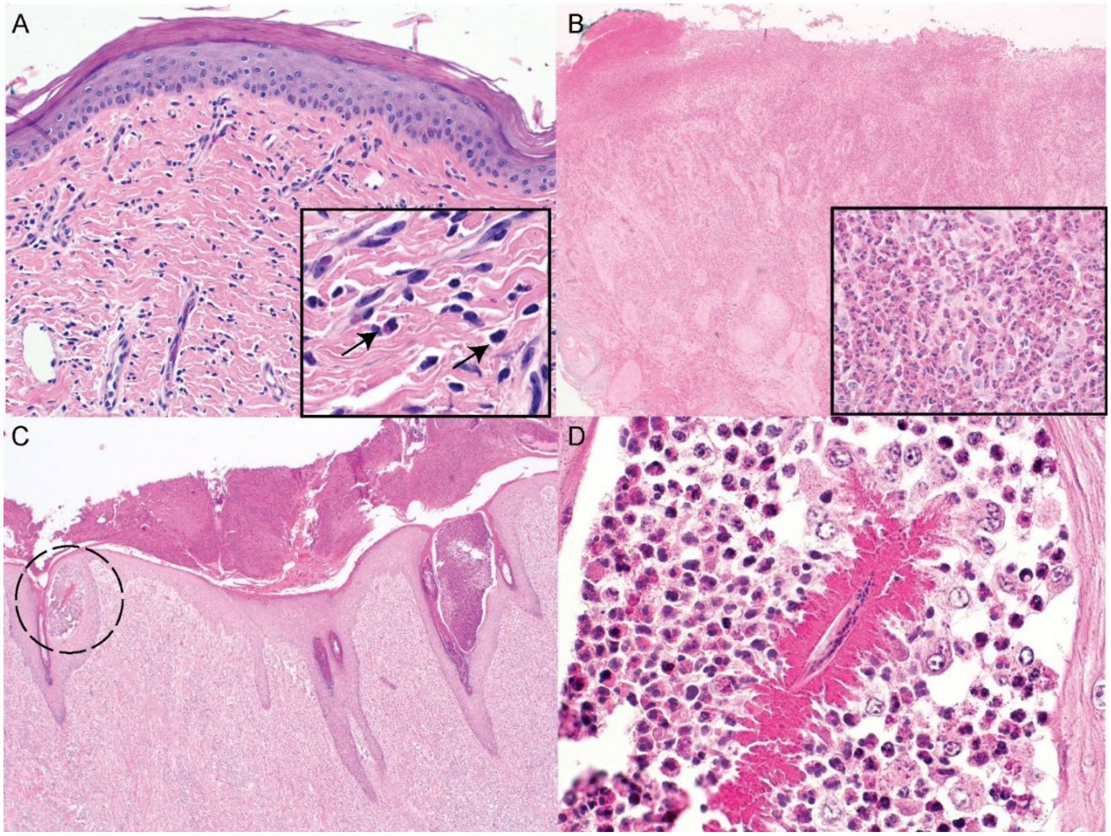

Figure 3.5 Representative histologic photomicrographs of moose skin collected between May and July 2021. A. Skin biopsy taken before appearance of sores in

May, 2021. There are small numbers of perivascular eosinophils and lymphocytes in the dermis. No pustules or microfilariae are observed. Hematoxylin and eosin, 200x magnification; insert: higher magnification of small numbers of eosinophils (black arrows) and lymphocytes. Hematoxylin and eosin, 600x magnification. B. Biopsy from a leg sore of a live adult female moose. There is a diffuse, severe infiltration with eosinophils throughout the dermis. Note the diffuse ulceration and a serocellular crust on the left. Hematoxylin and eosin, 40x magnification; insert: higher magnification of the eosinophilic infiltration. Hematoxylin and eosin, 600x magnification. C. Skin sample from a dead wild moose sampled in July, 2021. There is a diffuse, severe infiltration with eosinophils throughout the dermis. Note multiple intraepidermal pustules (dashed circle). D. Higher magnification of the pustule highlighted in C. The pustule is filled with eosinophils with a microfilaria in the center surrounded by degenerated eosinophils forming an eosinophilic sleeve. Hematoxylin and eosin, 600x magnification.

Genomic DNA

Out of the 26 samples processed, 17 produced a band from gel electrophoresis. Out of these samples 2/4 (50%) were from MRC moose taken before July, 4/9 (44.44%) were from MRC moose taken in July, and 7/8 (87.50%) were from wild moose taken between July and August, 2021. The remaining positives came from wild moose in 2020 (3/3; 100%) and samples from the same moose but different lesions (1/2; 50%). The sequences (396bp) were blasted using BLASTn tool to analyze their similarity with other published sequences available in online databases and it revealed very little similarity to other filarioid species. The closest parasites were *Onchocerca*, specifically *O. gutturosa* (94.59%), and decreased from there on. In addition, all 17 sequences had little intraspecies diversity (average pairwise identity of 99.85% with range of 100.00-98.96%) and likely belong to one *Onchocerca* species. Phylogenetic analysis revealed no specific group that clusters with the newly sequenced filarioid nematode DNA and it creates one distinct clade (Fig. 3.6)

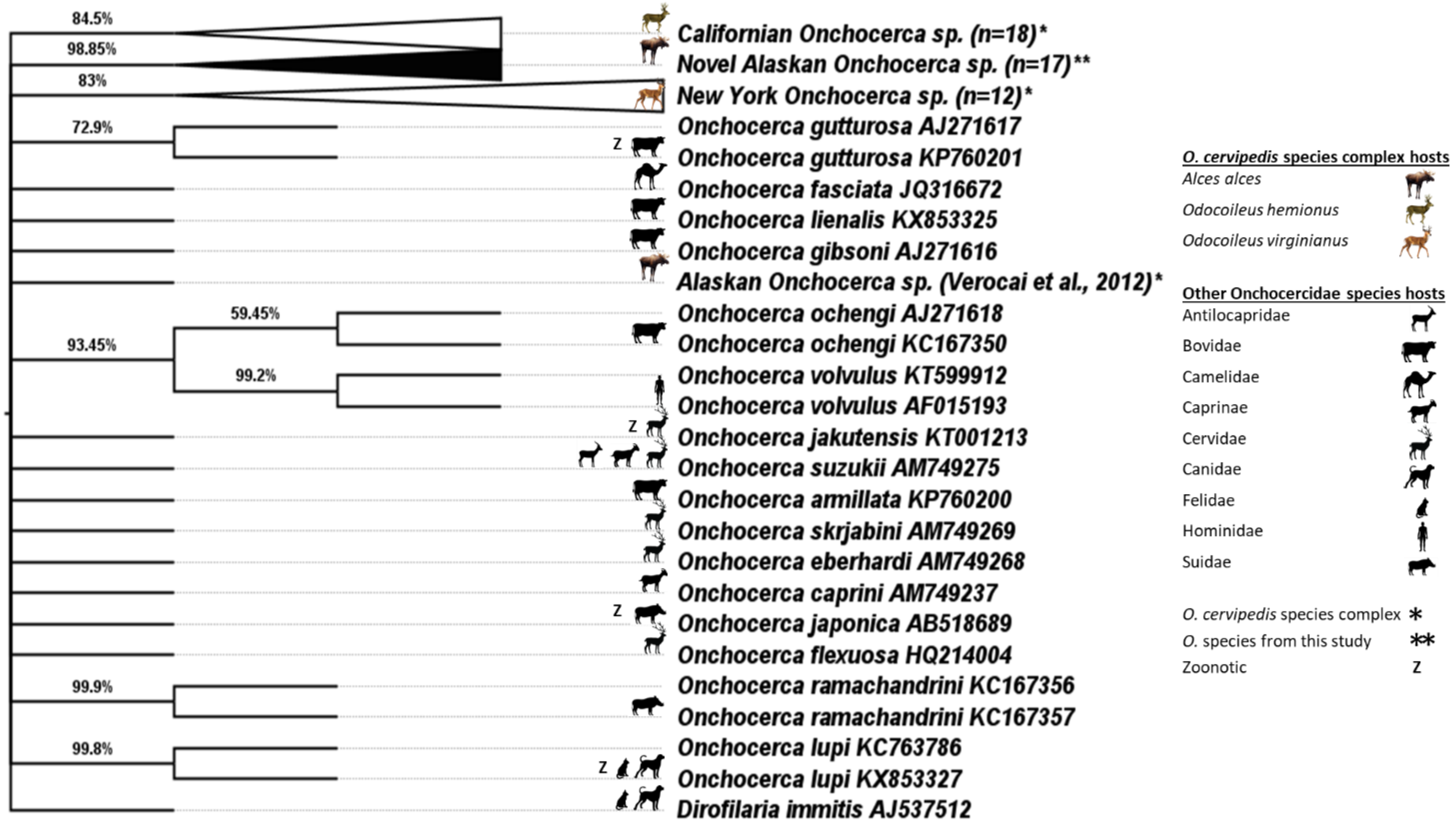

Figure 3.6 Phylogenetic tree. Phylogenetic tree created using a maximum likelihood method and a General Time Reversible best fit model with gamma distribution (2,000 bootstrap replicates) showing the relationship of all known *Onchocerca cervipedi*s species complex species in conjunction with other Onchocercidae species. At this time, all *Onchocerca* species comprised within the *O. cervipedi*s species complex (n=4, denoted with *) form distinct clades including the positive samples (n=-17) from this study (denoted in black with **). These samples were accessioned in GenBank (OP265723-39).

Discussion

Morphological barriers such as skin and hair are the first line of defense used by ungulates to resist flies (Benedict & Barboza 2022). Moose calves do not develop sores, likely because their skin is covered by hair throughout the summer (Benedict et al. 2023). Calves molt from a fuzzy natal coat to a winter coat that covered the majority of their body. Any area of the body exposed is heavily attacked by flies; the anus, around the eyes, and any injury resulting in a lack of hair. In adult moose, skin was exposed over the course of the molt from May to July (Fig. 3.2). Fly numbers increased at the onset of molt, with the greatest numbers of flies on moose in July and August (Benedict et al. 2023). As molt was achieved in June and July, sores began to appear on the moose (Fig. 3.2), which suggests that the flies are associated with the development of the sores. While there was a range in number of sores (3 to 25 sores per leg per individual), all adult moose had sores in July. Peak sore numbers (end of July and beginning of August) coincided with peak fly season (Benedict et al. 2023) (Fig. 3.2, 3.3).

Hair as a physical morphological barrier and timing of the damage to the hind legs of the adult moose was further shown in the form of heat emitted, as captured by the thermal images, with the minimum temperature approaching perianal temperature and continuing to increase throughout the season (Fig. 3.4). The perianal is a part of the moose that is hairless, an approach of minimum hind leg temperature towards perianal temperature shows the loss of a barrier (Fig. 3.4). The perianal temperature (mean 35.8°C) and maximum leg temperature (mean 36.0°C) were slightly lower than summer core body temperature of moose (38.2°C; Thompson et al. 2019). The initial rise in minimum hind leg temperature occurred with the loss of hair, and continued through the summer, coinciding with the breaks in the integument and abundance of sores, showing warming of the skin surrounding the sores and inflammation (Fig. 3.1, 3.4).

Eosinophilic dermatitis usually is a sign of allergic dermatitis/hypersensitivity, which is commonly associated with ectoparasites in wild animals. Twenty-five percent of the moose (2 of 8) had eosinophilic dermatitis before the sores developed, indicating that these animals may have been more susceptible and reacted earlier to the impact of flies than others. Additionally, *Onchocerca* DNA was found in 50% (2 of 4) of samples from moose without open sores. However, we cannot exclude hypersensitivity due to other environmental causes (Mauldin & Peters-Kennedy 2015). Interestingly, every single animal (10 of 10 moose) had developed severe eosinophilic and ulcerative dermatitis ten weeks later. It is unclear if the severe eosinophilic infiltration is due to skin-swelling nematodes/microfilariae, or if the eosinophils are associated with a more severe reaction to ectoparasites. Even though no microfilariae were found histologically in these live animals (Fig. 3.5), filarioid nematode DNA was found by genomic DNA extraction. *Onchocerca* DNA was found in 68% (14 of 21) of samples taken from open sores, 4 of which were from live MRC moose. Histology may therefore not be the most sensitive method to detect pathogens and is largely dependent on the sampling of the right location. For example, biopsies from horses with cutaneous habronemiasis often do not show nematodes in histological evaluations, despite their clear causation. The samples from the dead wild moose were much larger and more numerous per individual than the live moose biopsies, which increased the likelihood of finding microfilaria in histopathology.

Genetic analysis revealed a previously unknown genetic lineage within the genus *Onchocerca*, which is distinct from other genetic lineages with available COI sequence data, including those reported from North America (Fig. 3.6). We use the terminology 'previously unknown genetic lineage' due to a lack of clustering with any specific group in online databases, and clustering into one distinct clade. Further work would need to be done to determine if the species is newly evolved, or just newly sequenced. The need for genetic evaluation of *Onchocerca* beyond morphological evaluation makes identification sparse. The serendipitous finding of a previously unknown genetic lineage of *Onchocerca* provides further evidence that, prior to the last decade of research, diversity of *Onchocerca* associated with North American ungulates was largely underestimated. At this time, *O. cervipedis* is no longer viewed as a single species, but rather a species complex with at least three to four genetic isolates, infecting at least three different ungulate species¸ with two of these distinct lineages infecting moose from northwestern North America (Verocai et al. 2012; McFrederick et al. 2013; Verocai et al. 2018). It remains unclear which of these genetic lineages characterized to date correspond to the originally described *O. cervipedis*, which was based on specimens from white-tailed and mule deer (McFrederick et al. 2013; Verocai et al. 2018; Kulpa et al. 2021). Until recently, all reports of *Onchocerca* in various North American ungulates were assumed to involve *O. cervipedis*.

This previously uncharacterized genetic lineage of *Onchocerca* is the likely cause of the sores on the hind legs of moose, probably transmitted by black flies. This was a surprising finding as *O. cervipedis* predominately affects the subcutaneous tissue in the lower forelegs and below the hock in the hind legs of moose (Pledger et al. 1980; Verocai et al. 2012), whereas the previously uncharacterized *Onchocerca* species seem to induce lesions further proximal on the legs. The impact of *Onchocerca* in moose is largely unknown, with findings of *Onchocerca* species in healthy moose and reports of only localized inflammatory reactions (Verocai et al. 2012). Black flies have been identified as biological vectors for various *Onchocerca* species, and have been observed feeding on moose (Pledger et al. 1980; Verocai et al. 2012; Benedict et al. 2023). Other species of *Onchocerca* use biting midges (Culicoides: Ceratopogonidae) as biological vectors. We observed that the majority of flies congregated on the hind end of the moose, from their rump to their hocks, and fed predominately in the areas that sores were observed. Further research is needed to determine which dipterans serve as vector for this newly characterized *Onchocerca*.

Sores and the previously uncharacterized species of *Onchocerca* were found in both MRC moose and wild moose from the Kenai Peninsula, showing the prevalence throughout the area. The impact of *Onchocerca* infection on individual moose health or moose populations has never to our knowledge been studied in detail. However, the location of infections and lesions associated with parasitism have been suggested to make infected animal more prone to predation (Verocai et al. 2012). Moose are an important part of the diet of many Alaskans, particularly rural Alaska where 60% of households harvest wild game and 86% consume wild game (Titus et al. 2009). Therefore, concerns around food safety and security, including those associated with parasitic diseases deserve attention.

In conclusion, we found that the sores developing on the proximal aspect of the hind limbs of moose in the summer months are caused by a previously uncharacterized *Onchocerca* species. We correlated the emergence of sores with molt, and a lack of a barrier of protection in adult animals. These breaks in integument leave an ungulate exposed to pathogens, making them vulnerable, and challenging their health and fitness.

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CHAPTER IV

WOUNDED BUT UNSTRESSED: MOOSE TOLERATE INJURIOUS FLIES IN THE BOREAL FOREST

Synopsis

Moose (*Alces alces*) in boreal habitats feed and rest where they are exposed to Dipteran flies and the parasites they carry. We collected 31,905 flies during the summer from 12 habituated moose on the Kenai Peninsula, Alaska. Collected flies included mosquitoes (Culicidae), black flies (Simuliidae), and deer flies (Tabanidae), but moose flies (Muscidae: *Haematobosca alcis*), a species that completes its entire life cycle on or around moose, accounted for 91% of flies collected. Flies impose physiological costs on moose including hind leg sores likely caused by legworm (*Onchocerca* sp.), which is transmitted by flies. We found that the number of sores present on the hind legs of moose is positively correlated with body fat, which suggests a trade-off between foraging gains of energy and exposure to flies. We also found that the number of sores is negatively correlated with serum albumin, which is consistent with the use of body protein to repair injuries from flies and parasites. The number or type of flies present on a moose were not correlated with the concentration of corticosteroids in saliva or feces. Flies do not elicit a stress response in moose even though the costs of repairing wounds and resisting infections of those wounds likely reduce gains of protein from summer foraging. Moose can tolerate the injuries from biting flies with regular gains from

summer foraging but exposure to insect-borne parasites pose a risk to reproduction and survival.

Introduction

Wild ungulates face persistent attacks by arthropods and arthropod-borne parasites while needing to forage to obtain enough energy and protein reserves for growth and reproduction (Samuel et al. 2001). Ectoparasites such as ticks or deer keds can contribute to alopecia which increases risk of winter mortality (Kynkaanniemi et al. 2014; Bondo et al. 2019) while vector-borne internal parasites such as filarioid nematodes contribute to skin lesions or other pathology (Grunenwald et al. 2016; Benedict et al. 2023a). The threat posed by nuisance haematophagous Diptera and other arthropods, and associated vector-borne pathogens, has considerable spatial and temporal variation and the consequence of this burden on wild ungulate population health. Animal coping mechanisms to deal with these direct and indirect impacts of biting arthropods remains poorly understood.

Ungulates use a combination of resistance and tolerance responses to contend with arthropods and their parasites (Rauw 2012; Hayward et al. 2014; Benedict & Barboza 2022). Ungulates can resist attack from arthropods by investing in behaviors (e.g., avoiding exposure to insects, grooming to displace arthropods) and morphologies (e.g., thick coats and skins) that reduce the number of bites and thus avoid ensuing infections. Behaviors that avoid arthropod attack incur a cost of movement as well as an opportunity cost (e.g., lost foraging time) with increased geographical risks to mortality (e.g., predation or crossing water bodies, mountains, roads, and fences). Tolerance of repeated injury from arthropod attack may entail suppression of a stress response that is associated with costly behavioral and physiological reactions (e.g., corticosteroid response) (Boonstra 2004; Defolie et al. 2020; Benedict et al. 2023c). Tolerance of arthropod bites incurs physiological costs for repair to wounded tissues as well as a cascade of immune responses (e.g., reactions to antigens from arthropod saliva, parasites or microbes) (Benedict & Barboza 2022; Benedict et al. 2023a). Costs of immune reactions vary over time from first exposure (Froy et al. 2019). Initial costs of immune response are high at first exposure to a pathogen, but subsequent exposures are less expensive when antibodies are maintained and reactivated by repeated exposure to the pathogen (Derting & Compton 2003). Sustained tolerance of a wide variety of pathogens is costly and may increase the risk of an auto-immune reaction (i.e., anti-self) (Rauw 2012). The resulting suite of resistance and tolerance responses to insects depends upon the functional response of the host ungulate and the productivity of the habitat.

Moose (*Alces alces*) are attacked by an abundance of biting and non-biting flies (Diptera) throughout the spring and summer months (Lankester & Samuel 2007; Benedict & Barboza 2022). Stretching from Alaska to Norway to Minnesota, fourteen families of flies have been collected from moose, including the moose fly (*Haematobosca alcis*), which is thought to feed exclusively on moose and oviposit in fresh moose feces (Lankester & Samuel 2007; Rolandsen et al. 2021; Benedict & Barboza 2022; Benedict et al. 2023c). The most evident costs of flies for moose are the hind leg sores likely caused by legworm (*Onchocerca* sp.), which is thought to be carried by black flies (Diptera: Simuliidae) (Benedict et al. 2023a). During the summer, moose molt from a long winter coat to a short summer coat, losing a protective barrier of hair at a time when flies are abundant (Benedict et al. 2023a). During June and July, moose populations in Alaska molt, fly numbers increase, and *Onchocerca* parasites invade; likely creating the round wounds that have been observed on the hind legs of adult moose in the area above the hock (the tibio-tarsal joint) (Benedict et al. 2023a). Up to 25 sores and associated inflammation have been observed on a single leg at one time on moose in Alaska, Michigan, and Canada (Lankester & Samuel 2007; Benedict et al. 2023a). Even if impacts from these wounds remain local, tissue repair and immune response to local infection by parasites and secondary microbes may reduce summer mass gains of affected individuals (Samuel et al. 2001; Grunenwald et al. 2016; Benedict & Barboza 2022). Parasitic nematodes transferred by flies can cause life threatening internal infections and neurological impairment (Grunenwald et al. 2016; Grunenwald et al. 2018).

The wide variety of parasites associated with moose reflects their resilience as a host species and strong functional response to seasonal pulses of food to ensure sufficient nutrition for survival and reproduction (Allen et al. 2017; Benedict et al. 2023b). Growth of moose populations vary with climate as annual cycles of summer supply and winter demand change across regions (Grøtan et al. 2009). Female moose rely on high food intakes in a short summer of plant growth to grow their calves and to gain mass for winter survival and the next pregnancy (Renecker & Hudson 1986; Grøtan et al. 2009; Shively et al. 2019). Female moose produce one to three calves from 2 to 17 years old, having the greatest fecundity when primary plant production is high and winter severity is low (Boer 1992; Sand 1996; Schwartz 2007). Flies can reduce energy and nutrient intake of ungulates by reducing foraging time and displacing foraging from preferred areas (Hagemoen & Reimers 2002; Benedict & Barboza 2022; Benedict et al. 2023b). Nematode parasites can also reduce body mass gains of ungulates by reducing protein intakes (Ezenwa 2004). The burden of biting flies and risk of parasitic infection are likely dynamic and influenced by global climate change. Consequently, changes in exposure to flies and other biting arthropods and associated parasites create the potential for moose population regulation that could explain population declines observed in many regions of North America (Murray et al. 2006; Grunenwald et al. 2018).

We studied the tolerance of female moose to fly exposure during summers in Alaska. We used adult moose habituated to human contact to collect flies around the animals for our measure of fly exposure. We measured the abundance and diversity of flies collected from the moose in relation to environmental variables (vapor pressure, ambient air temperature, wind, and habitat type) and time (time of day and Julian day) to understand the composition and phenology of the fly community on moose. We measured changes in salivary and fecal corticosteroids to monitor the stress response to fly exposure through the summer as morphological resistance to flies declined through the annual molt. We predicted that increasing exposure to flies would not affect corticosteroid concentrations if moose were tolerant of flies. Furthermore, we predicted that number of sores on the hind legs of moose would be inversely related to body fat, showing the physiological costs of tolerance. We also predicted that serum protein concentrations would decline as leg sores increased and the costs of repairing wounds from fly parasites increased.

Methods

Study system

This study was conducted at the Kenai Moose Research Center (MRC) operated by the Alaska Department of Fish and Game (ADFG) on the Kenai National Wildlife Refuge (60° N, 150° W) on the Kenai Peninsula, Alaska, USA. All procedures for care, handling, and experimentation of animals were approved by the Animal Care and Use Committee, Alaska Department of Fish and Game, Division of Wildlife Conservation (IACUC protocol no. 0086) and by the Institutional Animal Care and Use Committee, Texas A&M AgriLife Research (AUP 2019-009A and 2021-009A). We studied free ranging tame adult (2-18 y old) female moose (2015: n=11; 2016: n=12; 2021: n=12) held in 2.6 km^2 outdoor enclosures with seral stages of boreal forest, wetlands, open meadows, and lakes.

Molt and sores

We observed the moose for signs of molt and the appearance of sores at intervals of 5-20 d during three summers: May 5 to July 13, 2015; May 3 to July 18, 2016; and May 19 to August 13, 2021. Signs of molt and completion of molt were recorded at the time of observation, or from photographs taken during these observations. Start of molt was characterized as loss of hair on the backs of the ears. Molt was considered complete when exposed skin was covered with short hair. Additionally, we paid careful attention to observing the loss of hair and the emergence of sores on the area above the hock on the hind legs of the moose. We also recorded the number of sores on each leg throughout the summer in 2021 (n = 12 moose).

Body condition

We chemically immobilized the moose as follows in 2021: four on May 12, two on May 20, ten between July 20 and July 22, and ten between December 7 and December 8. Moose were immobilized with Thiafentanil oxalate (1-4 $\mu g \cdot kg^{-1}$ estimated body mass; 10mg/mL; ZooPharm Wildlife Pharmaceuticals Inc., Windsor, CO, USA) and Xylazine (30-50 $\mu g \cdot kg^{-1}$ estimated body mass; $100 mg \cdot mL^{-1}$; Lloyd Laboratories, Shenandoah, IA, USA). Immobilizing drugs were hand-injected deep into the shoulder muscle using a luer-lock syringe and 21Ga x 25mm hypodermic needle. Heart, respiration rate and blood perfusion to the mucous membranes were monitored during immobilization. We collected up to 60mL of blood via jugular venipuncture with a 20Ga x 38mm needle. Blood was sent to Zoetis Reference Laboratories (Mukilteo, WA) for Complete Blood Count Equine and ClinChem25 panels. We also measured maximum rump fat thickness (MAXFAT) via ultrasonography (Ibex Pro, E.I. Medical Imaging, Loveland, Colorado) and converted it to ingesta-free body fat (IFBFAT) using the equation: IFBFAT = 5.61 + 2.05 x MAXFAT (Stephenson et al. 1998). We reversed the animals within 45 minutes by injection of Atipamezole HCl (5 $\mu g \cdot kg^{-1}$ estimated body mass; ¼ dose intravenous, ¾ dose intramuscular; 5 $mg \cdot mL^{-1}$; Zoetis, Parsippany, NJ, USA) and Naltrexone HCl (100 $mg \cdot mg^{-1}$ Thiafentanil oxalate intramuscular; $50 mg \cdot mL^{-1}$; ZooPharm LLC, Laramie, WY, USA).

Salivary and fecal cortisol

We first collected saliva from May 23 to August 19, 2019 from one moose ten times to develop our sampling technique. We collected saliva and fecal samples from twelve moose up to eight times each from May 19 to August 13, 2021. Moose were approached with a familiar food reward to ease salivary collection and elicit a salivary response. Prior to consuming the offered food, saliva was collected by swabbing between the teeth and gums using a synthetic swab (SalivaBio Children's Swab, Salimetrics LLC, Carlsbad, CA, USA). We then placed the swabs in a sterile tube (Swab Storage Tube; Salimetrics LLC) which we kept on ice, and later frozen at -20°C. Salivary cortisol was analyzed in duplicate using a cortisol ELISA assay ($\mu g \cdot dL^{-1}$; Salivary Cortisol; Salimetrics LLC, Carlsbad, CA, USA) and reported as the mean concentration in each sample (Thompson et al. 2020). We collected freshly deposited fecal samples using a plastic bag, placed them on ice and later froze them at -20°C. We dried the fecal samples to constant mass in a freeze-drier (Labconco Model 7,752,020, Kansas City, MO, USA) and then milled them through a 1.0 mm mesh (Shively et al. 2019). Fecal samples were analyzed for glucocorticoids by radio-immunoassay ($\mu g \cdot g^{-1}$; Applied BioSciences Endocrinology Laboratory, College Station, TX, USA). At the time of saliva and fecal collection, we measured ambient air temperature (°C) and relative humidity (hPa) using a portable weather meter (Kestrel 4400 Heat Stress tracker or Kestrel DROP, Kestrel, Boothwyn, PA, USA). Vapor pressure (vap_pres) was later calculated from ambient temperature and relative humidity by the equation: $\text{vap\_pres}=6.11\times 10^{\frac{7.5\,\text{Ta}}{237.3+\text{Ta}}}\times\frac{\text{relative humidity}}{100}$.

Flies

We collected flies from one moose on 7 occasions in 2019 from June 16 to August 19 to establish the collection method. In 2021, we collected flies from 12 moose on up to eight occasions from May 19 to August 13. Flies were collected by sweep netting near the skin surface with a 0.381 m diameter collapsible net (BioQuip, Rancho Dominquez, CA, USA) while a moose was laying down (Lloyd & Dipeolu 1974; Lankester & Samuel 2007; McGregor et al. 2019). Netting was focused on the hind end of the moose, where most flies congregated. Netting stopped at 60s or when the moose stood up. Duration of net sweep was recorded along with habitat type and weather variables at the time of collection. We determined the habitat type by overlaying netting location, pinned by GPS (Oregon 650t; Garmin, Olathe, Kansas, USA), with vegetation polygons (ArcMap 10.6.1; ESRI, Redland, CA, USA) of early-seral boreal forest (2–5 years post-disturbance, open canopy), mid-seral boreal forest (25 years post-disturbance), old growth boreal forest (65+ years post-disturbance), black spruce forest, wetland (kettle ponds and/or sphagnum peat bogs with areas of standing water) and open meadow (Thompson et al. 2021). We measured ambient air temperature (°C), wind speed ($m \cdot s^{-1}$), and relative humidity (hPa) with Kestrel 4400 Heat Stress tracker or Kestrel DROP and Kestrel 1000 Pocket Wind Meter (Kestrel, Boothwyn, PA, USA). Flies were killed by acetone exposure and stored frozen for analysis. Insect samples were transported under a USDA Veterinary Permit (139420 Research). We identified the flies morphologically and counted them under a dissection microscope into the following groups: mosquitoes (Culicidae), moose flies (Muscidae), coprophagous flies (various families), black flies (Simuliidae), horse and deer flies (Tabanidae), snipe flies (Rhagionidae), and other flies (Benedict et al. 2023c).

Calculations and statistics

We performed all statistical analysis in STATA version 16.0 (StataCorp, College Station, Texas, USA). We used a reverse stepwise selection procedure for all models, which removed coefficients that were not significantly different from zero. We used the robust "sandwich estimator" for standard errors to relax assumptions of normal distribution and homogeneity of variances (Rabe-Hesketh & Skrondal 2010). All statistical significance was set at $P \leq 0.05$.

We examined the effect of Julian day (julian) on the start of molt (molt_start) using logistic regression with intragroup correlation of individual moose to account for repeated measures, assigning a value of 1 to moose starting to molt and 0 for all other stages of molt: $\text{molt\_start} = \text{julian} + \varepsilon$ (error). The same methods were repeated for completing molt (molted), hair loss on the hind legs above the hock (hair_loss), and the emergence of sores (sores): $\text{molted} = \text{julian} + \varepsilon$, $\text{hair\_loss} = \text{julian} + \varepsilon$, $\text{sores} = \text{julian} + \varepsilon$. We used robust regression to examine the effects of IFBFAT, blood proteins (total protein (protein), albumin, globulins, fibrinogen) and blood cells (eosinophils and lymphocytes) on the average number of hind leg sores (max_sores) observed on a moose, in July: $\text{max\_sores} = \text{IFBFAT} + \text{protein} + \text{albumin} + \text{globulins} + \text{fibrinogen} + \text{eosinophils} + \text{lymphocytes} + \varepsilon$.

We used mixed-effects regression with individual moose as random effects to account for repeated measures of dependent variables to examine the effects of ambient air temperature (Ta), time of day (time), and Julian day on salivary cortisol levels (salivary cortisol): salivary cortisol = Ta + time + julian + ε. The same methods were repeated for fecal corticosteroids (fecal cortisol): fecal cortisol = Ta + time + julian + ε.

To examine the effects of environmental variables on number of flies netted per second (flies; not grouped by taxa) we regressed vapor pressure, ambient air temperature, Julian day, wind, time of day, habitat type (habitat), and individual moose (individual) against flies netted per second: flies = vap_pres + Ta + julian + wind + time + habitat + individual + ε. The same methods were repeated for each of the fly groups (mosquitoes, moose flies, coprophagous flies, black flies, horse and deer flies, snipe flies, other flies). The final significant model for flies netted per second (of all groups combined) was then used to predict fly numbers at every saliva and fecal collection by predicting the margins using each corresponding individual and Julian day. Prediction accuracy was checked by regressing observed against predicted flies netted per second. We then used mixed-effects regression with individual moose and Julian day as random effects to account for repeated measures of dependent variables to examine the effects of predicted flies (predict_flies) on salivary cortisol levels: salivary cortisol = predict_flies + ε. The same method was used for fecal corticosteroids: fecal cortisol = predict_flies + ε. The same method was repeated again for fecal corticosteroids but using predicted flies from the prior Julian day to account for a 24-hour lag in fecal glucocorticoids in response to a stressor (Crouse 2003; Lechner et al. 2010).

Results

A total of 325 observations of molt were made across fifteen female moose (2015 n=11; 2016 n=12; 2021 n=12). Julian day predicted the start of whole-body molt (X^2 [1df] = 94.35, $P = 0.000$; Fig. 4.1A and Table C.1) with a negative slope of -0.173 ± 0.02 and the completion of molt (X^2 [1df] = 65.35, $P = 0.000$; Fig. 4.1A and Table C.1) with a positive slope of 0.286 ± 0.04. Whole-body molt began May 5 - 18 and ended June 29 - July 25. Loss of hair from above the hock was from May 11 to June 29, which coincided with the appearance of sores on June 9 (X^2 [1df] = 213.20, $P = 0.000$; Fig. 4.1B and Table C.1). All moose had sores by July 5 and continued to have sores through August (X^2 [1df] = 93.10, $P = 0.000$; Fig. 4.1B and Table C.1).

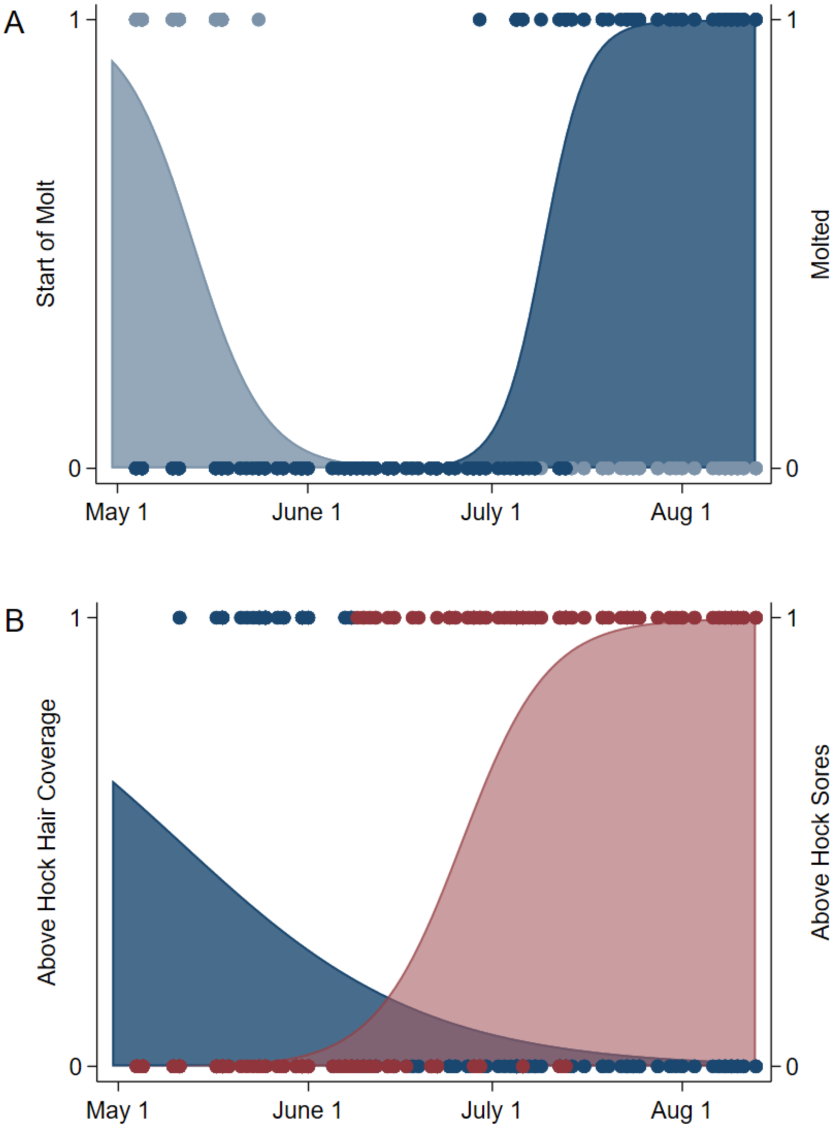

Figure 4.1 A. Marginal predictions of Julian day on the start of molt (light blue; X^2 [1df] = 94.35, $P = 0.000$) and marginal predictions of Julian day on completion of molt (dark blue; X^2 [1df] = 65.35, $P = 0.000$) from a winter coat to a short summer coat, based on logistic regression, with the completion of molt being reached by July 25th in all female adult moose (n=15) at the Kenai Moose Research Center, Kenai Peninsula, Alaska, USA. B. Marginal predictions of Julian day on the loss of hair in the area above the hock (dark blue; X^2 [1df] = 213.20, $P = 0.000$) and marginal predictions of Julian day on the emergence of sores in this area (red; X^2

[1df] = 93.10, *P* = 0.000), based on logistic regression, with all moose having sores by July 5th. Points represent individual observations.

Ingesta-free body fat increased from May to July but had declined by December (Fig. C.1). In July, ingesta-free body fat and serum albumin were correlated with the number of sores (3-25 sores) observed on the hind legs (n = 10; R^2 [1df] = 0.60, *P* = 0.014; Fig. 4.2 and Table C.2). The number of sores present on the hind legs of moose was positively correlated with the ingesta-free body fat of a moose (Fig 4.2A), while the number of sores was negatively correlated with serum albumin (Fig. 4.2B). The number of sores were not significantly related to serum protein, serum globulin, serum fibrinogen or to the counts of eosinophils, and lymphocytes in whole blood.

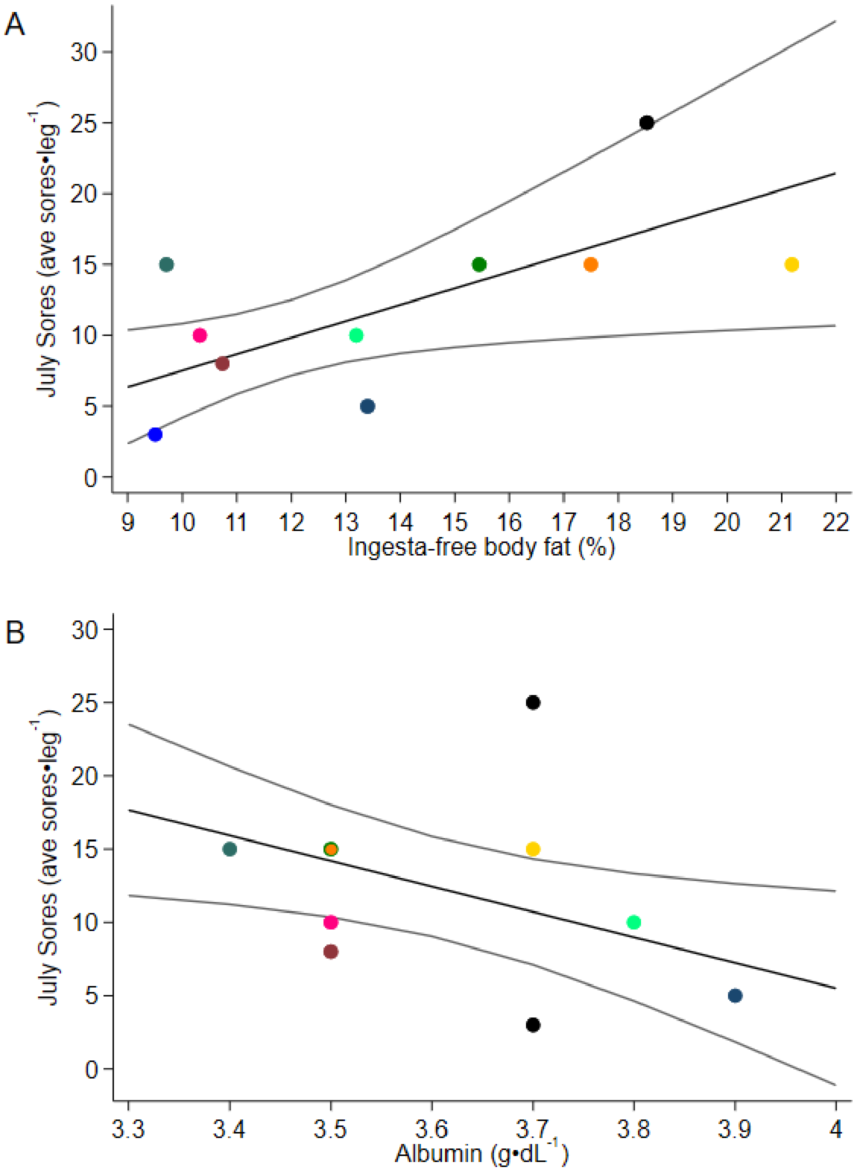

Figure 4.2 Marginal predictions of ingesta-free body fat (%; A) and serum albumin (g•dL^{-1}; B) on the average number of sores per hindleg of female adult moose (n=10 series of colors) in July at the Kenai Moose Research Center, Kenai Peninsula, Alaska, USA, based on robust linear regression ($R^2 = 0.599$, *P* = 0.014).

Saliva was collected from 11 moose for a total of 73 collections, with two collections removed from analysis for extreme values and one for contamination with food. Salivary cortisol ranged from 0.16 to 0.12 $\mu g \cdot dL^{-1}$ but was not significantly related to ambient air temperature, time of day, or Julian day. Fecal samples were collected from 12 moose for a total of 95 collections. Fecal corticosteroids declined significantly with the time of day from 3.17 $\mu g \cdot g^{-1}$ at 9:00am to 2.90 $\mu g \cdot g^{-1}$ at 6:00pm (X^2 [1df] = 5.12, P = 0.024; Table C.3), but effects of ambient air temperature and Julian day were not significantly related to fecal corticosteroids.

Flies were collected from 12 moose for a total of 98 collections. A total of 31,905 flies were collected; the majority of which were moose flies (28,968; 90.79%; Fig. 4.3), the remainder were coprophagous or necrophagous flies (1,440; 4.51%), mosquitoes (873; 2.74%), black flies (494; 1.55%), horse and deer flies (58; 0.18%), other flies (71; 0.22%), and snipe flies (1; <0.01%). Up to 450 flies were netting from a single moose in one second (Fig. 4.3). Julian day and individual moose were the only significant effects on the number of flies (R^2 = 0.216, P = 0.007; Fig. 4.3, C.2, and Table C.4) and moose flies (R^2 = 0.206, P = 0.007; Fig. C.2 and Table C.4) netted per second. Ambient air temperature was the only significant effect on the number of coprophagous (R^2 = 0.021, P = 0.046), black flies (R^2 = 0.036, P = 0.024), horse and deer (R^2 = 0.014, P = 0.049) netted per second (Table C.4). Julian day was the only significant effect on the number of other flies netted per second (R^2 = 0.041, P = 0.043; Fig. C.2 and Table C.4). The number of mosquitoes netted per second were significantly affected by Julian day, ambient air temperature, wind, time of day, and vapor pressure (R^2 = 0.203, P = 0.000; Fig. C.2 and Table C.4). The sample size was too small to model the environmental variables against snipe flies (n=1). Due to the parallel between the models for flies and moose flies (Fig. C.2 and Table C.4), which represent 90.79% of flies collected, the model for all flies combined was used in further analysis. The final significant model for flies netted per second (of all combined groups) was able to predict fly numbers (from 0 to 90 flies per second) at every saliva and fecal sampling location, with a slope close to one (1.139 ± 0.211) when predicted values were regressed on observed values ($R^2 = 0.233$, $P < 0.001$). Neither salivary cortisol nor fecal corticosteroids were significantly related to the predicted number of flies on the day of collection of saliva (X^2 [1df] = 0.01, $P = 0.92$; Fig. 4.4A) or feces (X^2 [1df] = 0.45, $P = 0.50$; Fig. 4.4B), or 24 hours prior to collection of feces (X^2 [1df] = 0.45, $P = 0.50$).

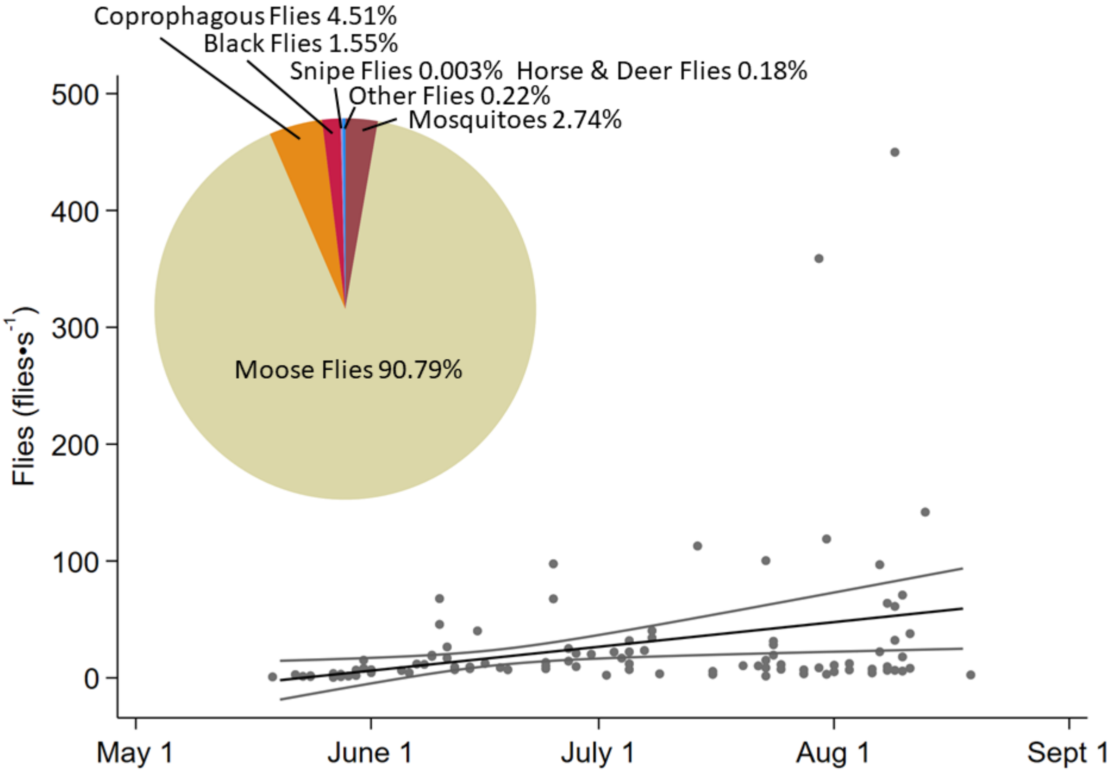

Figure 4.3 Marginal predictions and observations (dots), of Julian day on flies (flies•s^{-1}) netted from female adult moose (n=12) at the Kenai Moose Research

Center, Kenai Peninsula, Alaska, USA, based on linear regression ($R^2 = 0.216$, $P = 0.007$). Pie chart shows the percent of total flies collected in each group (colors).

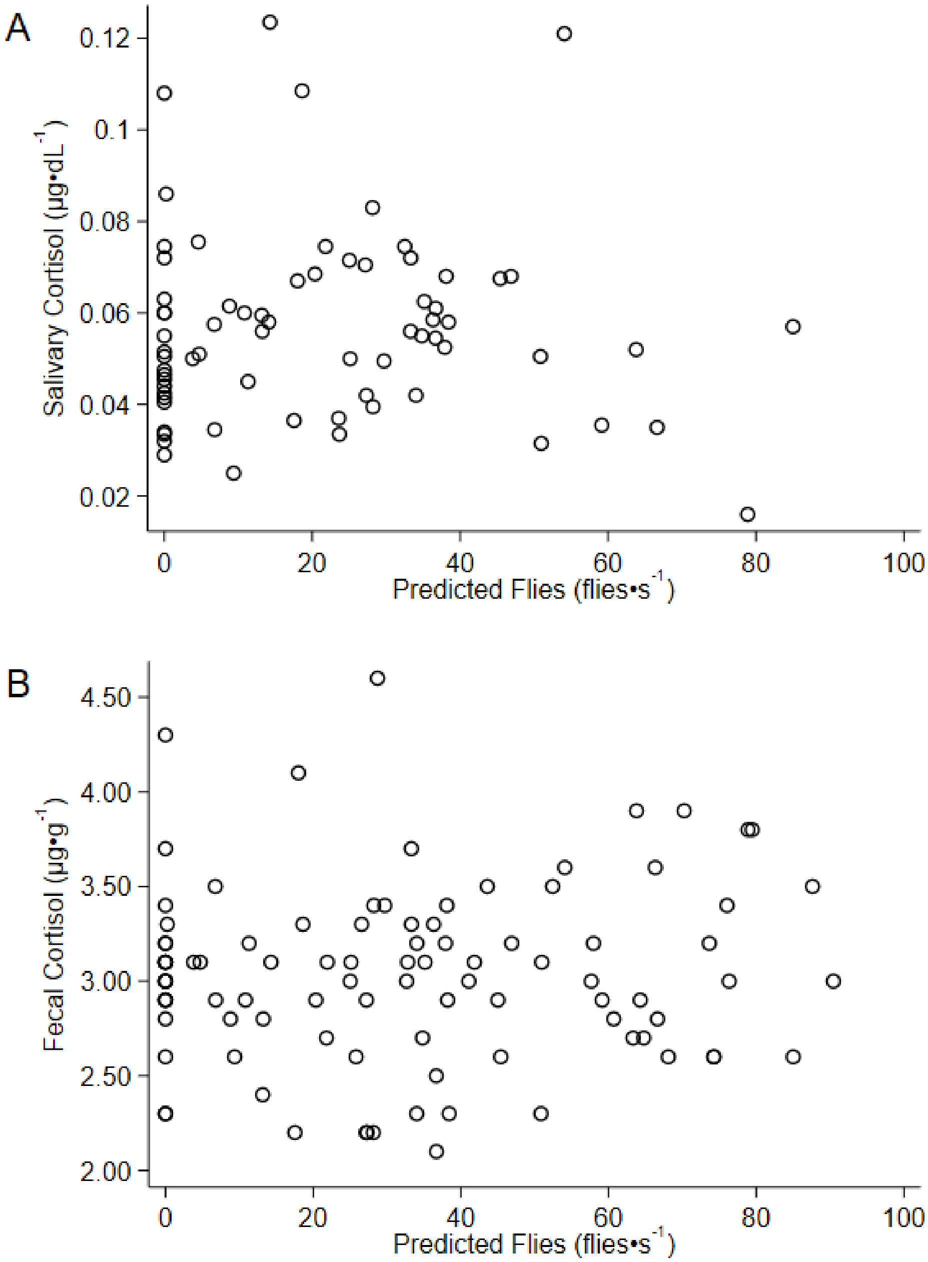

Figure 4.4 Flies (flies•s^{-1}) predicted to occur at time of collection and the stress response of female adult moose (n=12) at the Kenai Moose Research Center, Kenai Peninsula, Alaska, USA, measured as cortisol in saliva (A; µg•dL^{-1}) or corticosteroids in feces (B; µg•g^{-1}).

Discussion

Moose face a trade-off in summer between molting to reduce insulation as air temperatures increase and retaining a layer of hair as a barrier to fly attack. As moose completed molt in July (Fig. 4.1A), flies were abundant (Fig. 4.3) and likely contributed to the formation of sores in late-summer (Fig. 4.1B). These sores are likely created by legworm transmitted by black flies (Benedict et al. 2023a).

The moose also make a trade-off between fly exposure and body fat in an unexpected way. Summer body fat, measured as ingesta-free body fat, was positively related to the number of hind leg sores (Fig. 4.2A). Our study suggests that moose with higher levels of body fat may have a trade off in foraging in higher quality habitat that could also have higher fly abundance, in particular black flies. Moose are likely tolerating flies and foraging through fly exposure, incurring a cost of tissue damage, but taking in enough forage to more than offset any effect of flies on body fat. Alternatively, moose with more body fat might have more surface area on their legs for sores to form, again implying that foraging leads to more damage from flies.

The cost of moose foraging through fly exposure was seen in serum albumin concentrations. We saw a negative relationship between serum albumin concentration and the number of sores on a moose (Fig. 4.2B). A decrease in albumin is generally indicative of disease, malnutrition, and blood loss (Kaneko 1997; Crouse 2003; Reshetnyak et al. 2021), and in our study likely indicative of body protein being used to repair sores (Franzmann & Leresche 1978). Our albumin measurement (3.4-3.9 $g{\cdot}dL^{-1}$) were on the lower end of the range of past adult female MRC moose (3.8-5.2 $g{\cdot}dL^{-1}$), in the month of July (Franzmann & Bailey 1977). Past studies found a negative relationship between albumin and body condition (Franzmann 1977; Franzmann & Bailey 1977). In our study, serum albumin was 3.4-3.9 g·dL^{-1}, which is below the threshold of 4.5 g·dL^{-1} for moose in average or better condition (Franzmann & Leresche 1978) and therefore consistent with an elevated demand for protein to repair sores.

Moose are tolerant of flies throughout the summer; up to 1,515 flies were netted from a single moose in 60 seconds, with no measurable response in salivary cortisol (Fig. 4.4). Salivary cortisol reflects cortisol in the blood and peaks 20-30 minutes after the onset of stress, while fecal glucocorticoids reflect stressors within 15-22 hours (Crouse 2003; Merlot et al. 2011; Sheriff et al. 2011; Majchrzak et al. 2015). Previous studies of moose have looked at body condition and stress in relationship to environmental variables (Becker et al. 2010; Thompson et al. 2020), but the effect of flies on the stress response of adult moose has not been characterized. The only significant effect on salivary or fecal corticosteroids that we found was time of day, likely due to diel rhythms in the excretion of glucocorticoids in feces (Jachowski et al. 2015). Our measurements of salivary cortisol (0.016-0.124 μg·dL^{-1}; Fig. 4.4A) were on the lower end of the range observed for adult female moose (~0-3.0 μg·dL^{-1}) (Thompson et al. 2020) and moose calves (0-0.2 μg·dL^{-1}) (Benedict et al. 2023c), but still within the sensitivity of the assay (0.012 μg·dL^{-1}) (Salimetrics LLC, Carlsbad, CA, USA). Our measurement of fecal glucocorticoids (2.1-4.6 μg·g^{-1}; Fig. 4.4B) overlapped with ranges found in past studies of adult female MRC moose (~1.0-2.75 μg·g^{-1}) (Thompson et al. 2020) but were on the higher end, possibly due to within year weather differences.

Thompson et al. (2020) found a correlation between salivary cortisol and rapid increases in ambient air temperature, indicating that cortisol can be a measure of stress in moose. While moose may not be releasing glucocorticoid hormones as an emergency response to flies, we did observe moose displaying anti-fly behaviors by shaking their head, running, blowing their nose, trying to nudge flies off their hind legs (most cannot reach the back of their legs and none can reach their rump), and twitching in response to flies. This suggests that they still may be experiencing stress without a release of glucocorticoid hormones, showing their tolerance of flies. Moose calves that must follow their mothers and incur the same exposure to flies also do not increase salivary cortisol as the number of flies increase (Benedict et al. 2023c).

The majority of flies collected directly from adult moose (90.79%; Fig. 4.3) and calves (68.4%) (Benedict et al. 2023c) are moose flies, with abundances increasing through summer, but less than 0.03% of flies collected off host using CO_2-baited light traps and sticky traps were moose flies (Benedict et al. 2023b). The abundance of flies in a moose's habitat is significantly affected by environmental variables such as vapor pressure, ambient air temperature, and habitat type (Benedict et al. 2023b), while the abundance of flies on a moose is not. Instead, there were significant differences between the number of flies netted and the individual moose. These comparisons suggest that moose are their own moving habitats for flies, and in particular moose flies. The costs and benefits of the insect-host relationship between moose flies and moose awaits further studies of the transfer of nutrients from moose to fly and of parasites from fly to moose.

Moose are able to tolerate flies because they attain high intakes of energy and protein that can offset the added costs of tissue repair and injury (Shively et al. 2019). Tolerance of parasites carried by flies may vary widely because exposure to nematode parasites depends on transmission dynamics of vector and host populations (Hayward et al. 2014; Laaksonen et al. 2015; Buckingham & Ashby 2022). Moose populations ultimately attempt to tolerate outbreaks of parasitic infection by replacing themselves quickly (i.e., a strong functional response to food) when resistance is lowered by loss of morphological barriers (e.g., molt and hair loss) or constrained movement (e.g., geographic barriers).

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CHAPTER V

TRAPPED BETWEEN FOOD, HEAT, AND INSECTS: MOVEMENTS OF MOOSE (*ALCES ALCES*) AND EXPOSURE TO FLIES IN THE BOREAL FOREST OF ALASKA

Synopsis

Moose (*Alces alces*) in the boreal habitats of Alaska are unlike other northern ungulates because they tolerate high densities of flies (Diptera) even though flies cause wounds and infections during the warm summer months. Moose move to find food and to find relief from overheating (hyperthermia) but do they avoid flies? We used GPS collars to measure the rate of movement (m·h^{-1}) and the time spent (min·d^{-1}) by moose in four habitats: bogs, black spruce, early seral forest, and late seral forest. Average daily air temperatures increased until July when peak biomass of forage for moose was greatest in early seral forest habitats (424 vs. 46 – 2 kg·ha^{-1} in the other habitats). Average daily air temperatures were 1.5 – 2.8°C cooler in black spruce than other habitats, but exposure to flies was greatest in black spruce (688 vs. 110 – 181 flies·24h^{-1}). On cool days (8°C) moose increased their movement rate with counts of flies, but on warm days (20°C) movement declined with counts of flies. Moose spent 718 min·d^{-1} in early seral forest on cool days (8°C) but less than 63 min·d^{-1} in the same habitat on warm days (20°C). Time spent in black spruce increased with both air temperature and with counts of flies. Warm summer temperatures can create an ecological trap for moose

when heat gained while foraging is dissipated at rest in shady, wet habitats preferred by flies that adversely affect the fitness of moose.

Introduction

Moose (*Alces alces*) shape boreal ecosystems (Bowyer et al. 1997) by altering plant composition and succession, affecting nutrient cycling, fertilizing plants, and by serving as a valuable source of food for predators (Bowyer et al. 1997; Olmsted et al. 2021). Moose are also an important game species, particularly for native people of North American boreal forests. Approximately 7,300 moose are killed by 29,000 hunters each year in Alaska to yield 45 million pounds of wild food consumed by 86% of rural Alaskan households (Titus et al. 2009; ADFG 2019). Families and communities work together sharing, harvesting, and processing wild game, passing on skills and traditions to future generations, and a respect for wildlife and sustainable practices (ADFG 2019). Most wild food cannot be purchased in stores and alternative sources of animal protein are expensive and hard to come by in many rural areas (ADFG 2019). Moose are also an integral part of many native ceremonies, particularly a funeral potlatch; a burial ceremony that ends in a feast (Sumida & Alexander 1985).

The effects of people, wolves, and bears on moose populations are well studied (Boutin 1992), but much less is known about the effects of smaller consumers such as flies (Diptera) that can alter both behavior and physiology of ungulates. The skin and coat is the first line of defense against flies, creating a morphological barrier to their exposure (Benedict & Barboza 2022). Breaks in this barrier, either from injury or molt, leave the animal vulnerable to flies. Flies can directly penetrate the skin with biting mouthparts to ingest tissue or blood while transmitting secondary parasites. Fly bites can cause allergic responses, blood loss, secondary infection, restricted breathing, pneumonia, peritonitis and neurological impairments. All of which can decrease body condition to reduce birth rates and increase death rates in a population (Samuel et al. 2001; Ezenwa 2004).

As fly exposure increases, many ungulates react with behavioral avoidance (Benedict & Barboza 2022). Bison (*Bison bison*) herds are rarely observed in fly abundant areas. In the presence of flies, bison trade-off foraging for wallowing, grooming, and standing (Meagher 1973; Melton et al. 1989; McMillan et al. 2000). Caribou (*Rangifer tarandus*) escape flies by moving to exposed ridges and higher elevations with cold winds and ice, which increases energy expended on movement and reduces time for feeding on high quality forage (Mörschel & Klein 1997; Hagemoen & Reimers 2002; Weladji et al. 2003). Fly harassment of ungulates coincides with the highest demands for lactation when females must spend most of their time foraging (Shively et al. 2019; Cook et al. 2021).

Moose are less reactive and more tolerant to flies than caribou and bison (Benedict & Barboza 2022). Moose do not make large-scale movements to evade flies even though an individual moose may be surrounded by thousands of flies at any one time in the summer (Benedict & Barboza 2022; Benedict et al. 2023b). The majority of flies on a moose are moose flies (Muscidae: *Haematobosca alcis*); a biting species that completes its entire life cycle on or around moose (Lankester & Sein 1986; Benedict et al. 2023b). The amount of time moose spend in different habitats and microclimates is affected by environmental variables (e.g. temperature and humidity), predation, and habitat attributes such as canopy cover, understory composition, and water (Timmermann & McNicol 1988; Thompson et al. 2021; Verzuh et al. 2022). Movement of moose in summer is influenced by foraging and the effects of warm temperatures, radiant heat loads, and metabolic heat from movement and metabolism (Thompson et al. 2021). Movement rates are greatest in the morning when moose forage in early seral forest (Thompson et al. 2021). Some groups of flies may cause moose to move faster and further to seek habitat attributes that reduce exposure to flies (Renecker & Hudson 1990; Thompson et al. 2021). Radiant heat loads, wind, and fly activity all change the heart rate of bedded female moose, which indicates that resting energy expenditure is influenced by environmental conditions, especially radiant heat loads (Renecker & Hudson 1990). Moose spend the majority of daylight hours (68%) in the summer bedded (Verzuh et al. 2022) where both shade and wet soils allow cooling, and provide cover from predators (Jennewein et al. 2020; Verzuh et al. 2022). Moose also wallow in mud and wade in water to dissipate heat and gain relief from radiant heat loads, and possibly flies (Benedict & Barboza 2022). Moose calves and adults exhibit signs of annoyance with flies, especially large horse and deer flies, by shaking their head, blowing their nose, running, jumping, twitching, stomping, scratching, and trying to nudge flies off with their nose (Benedict et al. 2023b; Benedict et al. 2023c). However, neither calves nor adults release glucocorticoid hormones (an indicator of stress) as an emergency response to flies, showing that even though flies affect behavior, flies do not cause a physiological stress response in moose.

This strategy of tolerating and living with flies has consequences. During the summer, adult moose loose a barrier to flies when flies are most abundant because they molt to a short winter coat that flies can penetrate (Benedict et al. 2023a). As molt is achieved, up to 25 round sores with severe eosinophilic and ulcerative dermatitis progressively appear on the hind legs above the hock (tibio-tarsal joint) (Benedict et al. 2023a; Benedict et al. 2023b). The sores are likely caused by legworm (*Onchocerca* sp.), carried by black flies (Diptera: Simuliidae) (Benedict et al. 2023a). The sores leave the moose exposed to further pathogens and the cost of tissue repair and immune response. Moose with more sores have lower concentration of serum albumin probably because body protein is used for wound repair (Benedict et al. 2023b). Heavier moose have more sores, which suggests that tolerating flies may allow individual moose to attain high intakes of energy and protein to offset the costs of repairing wounds from flies (Shively et al. 2019; Benedict et al. 2023b).

Exposure to flies is affected by season, habitat, and weather conditions including ambient temperature, wind, relative humidity, precipitation, light, and cloud cover (Russell et al. 1993; Weladji et al. 2003; Rogy et al. 2019; Shety et al. 2022). Fly life cycles are often complex because development includes multiple stages that cross between aquatic and terrestrial habitats (Culler et al. 2018). Variables do not uniformly effect all species of flies to the same extent; Anderson and Nilssen (1998) found more tabanids in the morning and more mosquitoes in the evening and overnight, as temperature decreased, in a tundra-like biome of northern Norway. Shipp et al. (1987) found that the energy and water balance of a black fly (*Simulium arcticum*) was correlated to vapor pressure, air temperature, light intensity, and wind gust velocity in a prairie of central Alberta, Canada. Many flies have an upper and lower limit for activity; Russell et al. (1993) did not catch any mosquitoes below 7°C or at wind speeds above 6 $m \cdot s^{-1}$ in the northern Yukon, Canada.

Moose are resident to an area in the summer, but likely make daily movements in response to seasonal and diurnal changes in food supplies, heat loads, and flies. We used adult female moose habituated to people at the Moose Research Center to study daily movements in relation to the food, temperature, and number of flies in four habitats: bogs, black spruce (*Picea mariana*) forest, early seral forest, and late seral forest. We measured the amount of available forage in each habitat at peak biomass in July based on previous studies of moose forages and food intake at this study site by Shively et al. (2019). Environmental conditions of temperature and humidity were monitored in each habitat along with the counts of flies from two types of insect traps. We predicted that the moose would move faster when ambient air temperatures were cooler (Fig. 5.1A). We predicted that movement rates ($m \cdot h^{-1}$) would increase with counts of flies across habitats if moose were avoiding flies, and thus minimizing their risk to wounds and infections from flies (Fig. 5.1A). Alternatively, if moose were tolerating flies, movement rates would decrease with exposure to flies (Fig. 5.1A). We predicted that moose would spend most of their time in habitats with high forage abundance but that high ambient temperatures would increase the use of closed habitats with shade from the sun (i.e., less radiant heat; Fig. 5.1B) and wet habitats with more heat dissipation (i.e., conductive heat loss) as observed by Thompson et al. (2021). We predicted that moose would avoid flies by decreasing their time spent in habitats with high abundances of flies (Fig. 5.1B). Alternatively, if moose were tolerating flies, time spent in the habitat would increase with counts of flies (Fig. 5.1B).

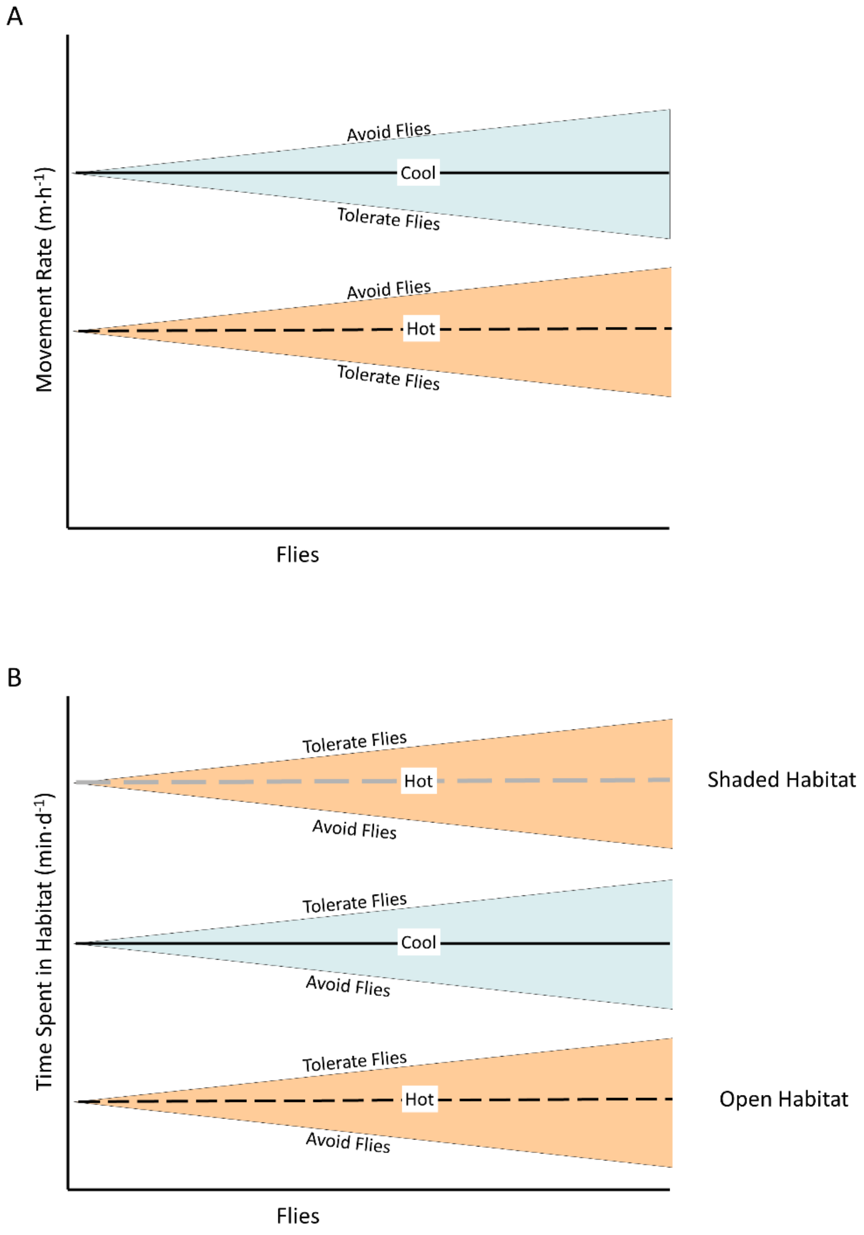

Figure 5.1 Hypothetical relationships between the movement of moose and exposure to flies during cool and hot days. A) Movement rate (m·h^{-1}) is predicted to

be slower on hot days than cold days. Increasing movement rates would indicate avoidance of flies, while decreasing movement rates would indicate tolerance of flies. B) In comparison with cool days, time spent in shaded habitats would increase on hot days while time spent in open habitats would decrease. Time spent in each habitat is predicted to increase when moose tolerate flies and decrease when moose avoid flies.

Materials and methods

Study system

This study was conducted at the Kenai Moose Research Center (MRC) operated by the Alaska Department of Fish and Game on the Kenai National Wildlife Refuge (60° N, 150° W), a boreal forest in south central Alaska, USA with minimal affects from human disturbance on insect populations. All procedures for care, handling, and experimentation of animals were approved by the Animal Care and Use Committee, Alaska Department of Fish and Game, Division of Wildlife Conservation (IACUC protocol no.0086) and by the Institutional Animal Care and Use Committee, Texas A&M AgriLife Research (AUP 2019-009A and 2021-009A). Free ranging tame adult (2-19 y old) female moose (2019: n=11; 2021: n=12), some of which gave birth (2019: n=5; 2021: n=5) and were lactating (2019: n=3) throughout the summer, occupied two 2.6 km^2 outdoor enclosures. Moose had unlimited access to water and natural forage habitats; mixed seral state boreal forest, black spruce forest, bogs, open meadows, and lakes.

Flies

In 2019, flies were collected 18 times from each of 12 sites between May 21 and August 17; three black spruce, three early seral forest (2–5 years post-disturbance, open canopy), three late seral forest (65+ years post-disturbance), and three bog (kettle ponds and/or sphagnum peat bogs with areas of standing water) habitats (Thompson et al. 2021) for a total of 216 collections. Flies were also collected from one open meadow habitat site, adjacent to a National Oceanic and Atmospheric Administration (NOAA) weather station on 50 days. From May 21 to August 14 of 2021, flies were collected 14 times from the same three black spruce sites and one open meadow site used in 2019, for a total of 56 collections. The locations of the sites were chosen to represent the physiographic range of each type of habitat in the study area. A trapping session at each of the 13 sites consisted of one CO_2-baited CDC miniature light trap with ultraviolet light (John W. Hock Company, Gainesville, FL, USA) and one sticky trap (Knight Stick Biting Fly Trap BugJammer, Inc., Pennington, NJ, USA) set with their base at approximately 1.3 m above the ground for 24 hours (one trap-night). Flies were killed by either acetone exposure or citrus adhesive remover (Goo Gone, Gurnee, IL, USA), and stored frozen for analysis. Flies were transported under a USDA Veterinary Permit (139420 Research). Flies were identified morphologically and counted under a dissection microscope into the following seven groups: biting muscid flies (Muscidae), coprophagous flies (various families), mosquitoes (Culicidae), black flies (Simuliidae), horse and deer flies (Tabanidae), biting midges (Ceratopogonidae), and snipe flies (Rhagionidae). Functional groups of flies were chosen due to previous findings of these species on moose (Benedict & Barboza 2022; Benedict et al. 2023b; Benedict et al. 2023c).

Weather

Weather was recorded in conjunction with fly sampling in 2019: one HOBO Pro V2 temperature and relative humidity data logger and one HOBO pendant temperature and light data logger (Onset, Bourne, MA, USA) were set at all 13 of the trap sites across 5 habitat types (i.e., bog, black spruce, early seral and late seral forest, open meadow at the weather station). Loggers were installed at approximately 1.2 m high on steel T-posts. In addition, at each habitat type one operative temperature logger was installed, consisting of a HOBO water temperature Pro V2 data logger (Onset, Bourne, MA, USA) installed in a black globe, hung 0.75m above ground and 15cm from the trunk on the northeast side of a tree (Olson et al. 2014). In 2021, we recorded ambient air temperature, light and humidity at the same 13 trap sites with the addition of two sites in mid-seral forest and two sites in open meadow habitats. We also added one temperature logger within a black globe at each of the 5 habitat types to record operative temperature that included the effect of radiant heat load. Loggers recorded ambient air temperature (°C), operative temperature (°C), relative humidity (%) every 5 to 15 min. The single open meadow fly trap site used both years was chosen due to its location adjacent to a NOAA U.S. Climate Reference Network weather station (AK Kenai 29 ENE; Diamond et al., 2013) which recorded ambient air temperature and relative humidity at 5 min intervals, and was used to validate our HOBO loggers.

Vegetation

Vegetation was sampled on July 28 and 29, 2022, in all six habitats (bog, black spruce, early seral forest, mid seral forest (25 years post-disturbance), late seral forest, open meadow) on the MRC by stripping leaves and cutting above ground parts to mimic direct observations of feeding in habituated moose (Thompson unpublished data). Moose forage on fireweed (*Chamerion angustifolium*), and the leaves of rose (*Rosa acicularis*), highbush cranberry (*Viburnum edule*), shrub birch (*Betula glandulosa*), Alaska birch (*Betula neoalaskana*), aspen (*Populus tremuloides*), Scouler's willow (*Salix scouleriana*), and Bebb willow (*Salix bebbiana*). Twigs of shrubs and trees are not being consumed at this time. This knowledge of vegetation species consumed and mimicking how moose bite was then used to estimate the number of "moose bites" available. Three 50 m transects were randomly conducted in five of the habitats, and six were conducted in the mid seral forest due to the variability of the habitat. At 0 m and 50 m of each transect, tree biomass surveys were conducted in a 5.64 m radius circle. The number of forage trees by species were counted; with forage trees being defined as any tree that is >1.37 m tall, and either <5 cm diameter at breast height (DBH, moose can break over even if the tree is taller than 3 m), or any available forage <3 m off the ground (DBH>5 cm, tree cannot be broken over but branches within the reach of a moose). "Moose bites" (sections where leaves would be stripped by a moose from the woody stems) on up to ten of the nearest trees to point center of each forage tree species were counted and DBH was recorded. At every 5 m from 0 m to 45 m, along each transect, 1 m^2 herbaceous plots were conducted. All available "moose bites" for each forage species of herbaceous, shrub, and tree seedlings (<1.37m tall) that originate within the plot were counted. Representative "moose bite" samples were collected on odd number transects in each habitat, with additional new species collected on even number transects. Number of "moose bites" collected ranged from 10-40 depending on the size of the species (ex. 10 bites for large paper birch leaves, 40 bites for small dwarf birch leaves). Vegetation samples were collected into plastic bags, frozen, and later dried to constant mass with a freeze dryer to determine moisture content and dry mass per bite.

GPS collar deployment

Moose were chemically immobilized in 2021 on three occasions: May 7 – 20, July 20 – 22, and November 23 – 24. Moose were immobilized with Thiafentanil oxalate (0.001-0.004 $mg \cdot kg^{-1}$ estimated body mass; $10mg \cdot mL^{-1}$; ZooPharm Wildlife Pharmaceuticals Inc., Windsor, CO, USA) and Xylazine (0.03-0.05 $mg \cdot kg^{-1}$ estimated body mass; $100mg \cdot mL^{-1}$; Lloyd Laboratories, Shenandoah, IA, USA) hand-injected deep into shoulder muscle using a luer-lock syringe and 21Ga x 25mm hypodermic needle. Moose were reversed within 45 min by intramuscular injection of Atipamezole HCl (0.005 $mg \cdot kg^{-1}$ estimated body mass; ¼ dose intravenous, ¾ dose intramuscular; 5 $mg \cdot mL^{-1}$; Zoetis, Parsippany, NJ, USA) and intramuscularly administered Naltrexone HCl ($100mg \cdot mg^{-1}$ Thiafentanil oxalate intramuscular; $50vmg \cdot mL^{-1}$; ZooPharm LLC, Laramie, WY, USA). Heart, respiration rate, and blood perfusion to the mucous membranes of the mouth were monitored during immobilization. Vertex plus-4 GPS collars (Vectronics, Berlin, Germany) were deployed on all 12 moose during the May immobilizations and removed during November immobilizations.

Calculations and statistics

All daily fly counts were corrected to represent flies collected for 24 hours by the equation: $\text{flies} = \frac{\text{flies collected}}{\text{length of time trap was set}} \times 24 \text{ hours}$ ($\text{flies} \cdot 24h^{-1}$). Vapor pressure (VP;

mb) was calculated by the equation: $\mathrm{VP} = 6.11 \times 10^{\frac{7.5\,\mathrm{Ta}}{237.3+\mathrm{Ta}}} \times \frac{\text{relative humidity}}{100}$. Radiant heat (°C) was calculated by subtracting ambient air temperature from operative temperature. The average daily vapor pressure, ambient air temperature, and radiant heat of each habitat was used in analysis. Total dry mass ($\mathrm{kg \cdot ha^{-1}}$) was calculated for each vegetation species by multiplying dry mass measurements by bite counts, and then summing to get total dry mass per habitat. The locations of the moose by habitat were determined by overlaying moose GPS collar locations with vegetation polygons (ArcMap 10.6.1; ESRI, Redland, CA, USA) (Thompson et al. 2021) to calculate the amount of time each individual moose spent in each habitat, each day (time spent; $\mathrm{min \cdot d^{-1}}$). Euclidean distance was calculated for each successive GPS location to calculate movement rate ($\mathrm{m \cdot h^{-1}}$).

Statistical comparisons were performed in STATA version 16.0 (StataCorp, College Station, Texas, USA). We used simple linear regression to analyze the relationship between HOBO temperature and vapor pressure as compared to NOAA temperature and vapor pressure at the weather station site (i.e., open meadow). We used mixed-effects regression to examine the effects of Julian day on ambient air temperature (Ta), with site and Julian day as random effects to account for repeated measures: Ta = Julian + ε. We used simple linear regression to analyze the relationship between fly groups collected in a sticky trap versus a CO_2 baited light trap, in each of the five habitats (i.e., bog, black spruce, early seral and late seral forest, open meadow at the weather station). We then log corrected total daily counts of flies and each group of flies for use in further analysis. We used mixed-effects regression to examine the effects of

NOAA vapor pressure (NOAA VP) and NOAA ambient air temperature (NOAA Ta) on flies (flies): flies = NOAA VP + NOAA Ta + ε. We used the following independent variables in mixed model regressions for flies: vapor pressure, ambient air temperature, Julian day, year, habitat, and the interaction between habitat and each of these variables (interactions). We used site and Julian day as random effects to account for repeated measures of total flies: flies = VP + VP^2 + VP^3 + Ta + Ta^2 +Ta^3 + $julian^1$ + $julian^2$ + $julian^3$ + year + habitat + interactions + ε. The same method was applied to model counts of mosquitoes and black flies, respectively. Sample sizes were not sufficient for log likelihood models to converge on a solution in the remaining groups: biting muscid fly, coprophagous fly, horse and deer fly, biting midges, and snipe. The top model for total counts of flies was used to predict total counts of flies (flies predict) at every GPS collar location for each moose.

We first used mixed-effects regression to describe the effects of age of the moose (age), season (Julian day), and time of day (time) on movement rate of moose: movement rate = age + julian + $julian^2$ + time + $time^2$ + $time^3$ + ε. To further analyze movement rate on a daily time step we calculated average daily movement rate (av movement rate) and the corresponding daily averages for flies experienced (av flies) vegetation experienced (av veg) and ambient air temperature experienced (av Ta). We then used mixed-effects regression to examine the effects of age of the moose (age), Julian day, and the interaction between average daily flies and temperature (interaction) on movement rate: av movement rate = age + julian + av flies + av veg + av Ta + interaction + ε. We used mixed-effects regression to describe time spent in each habitat

(habitat-min) in each day for bogs, black spruce, early seral and late seral forest. The model for habitat-min included age of the moose, Julian day, average daily experience of flies, ambient air temperature experienced, and the interaction between average daily flies and temperature: habitat-min = age + age^2 + julian + $julian^2$ + av flies + av $flies^2$ + av Ta + av Ta^2 + interaction + ε. We included individual moose and Julian day as random effects to account for repeated measures of dependent variables for all movement mixed-effects regression.

We used the robust “sandwich estimator” for standard errors to relax assumptions of normal distribution and homogeneity of variances (Rabe-Hesketh & Skrondal 2010). We used a reverse stepwise selection procedure for all mixed models, except for flies, which removed coefficients that were not significantly different from zero. All statistical significance was set at $P \leq 0.05$. Due to the difficulty in selecting the top model for flies and the effects of vapor pressure, ambient air temperature, Julian day, year, habitat, and the interaction between habitat and each of these variables, we performed model selection for this model using Akaike’s information criterion (AIC), selecting the simplest model with the lowest AICc within two AICc units of the top model (Burnham and Anderson, 2002).

Results

Habitat

Temperature and vapor pressure were consistently recorded by HOBO devices when compared with records of the NOAA weather station in the open meadow. HOBO temperature was closely correlated with NOAA temperature ($R^2 = 0.926$, $P = 0.000$; Fig.

D.1A) with a positive slope of 1.056 ± 0.017. Likewise, HOBO vapor pressure was closely correlated with NOAA vapor pressure ($R^2 = 0.928$, $P = 0.000$; Fig. D.1B) with a positive slope of 1.085 ± 0.017.

Early seral forest habitats had the greatest amount of forage, but the warmest temperatures. We recorded an average of 91.70 $kg \cdot ha^{-1}$ of dry mass across six habitats in July across the MRC; 424.46 $kg \cdot ha^{-1}$ early seral forest, 45.88 $kg \cdot ha^{-1}$ open meadow, 35.61 $kg \cdot ha^{-1}$ bog, 24.72 $kg \cdot ha^{-1}$ late seral forest, 17.32 $kg \cdot ha^{-1}$ mid seral forest, and 2.2 $kg \cdot ha^{-1}$ black spruce (Fig. 5.2A). Similarly, early seral forests had the highest average daily air temperature at 17.39°C compared with 16.64°C in bogs, 16.33°C in late seral forests, and 14.96°C at the open meadow of the NOAA weather station (Fig. 5.2B). Air temperatures were coolest at 14.61°C in black spruce (Fig. 5.2B). Air temperatures increased with Julian day (X^2 [1df] = 1190.87, $P = 0.000$). Average daily radiant heat was lower in late seral forests (0.03°C) and black spruce (0.54°C) than all other habitats; 3.36°C at the open meadow of the NOAA weather station, 2.21°C in bog habitats, and 1.98°C in early seral forest habitats (Fig. 5.2C).

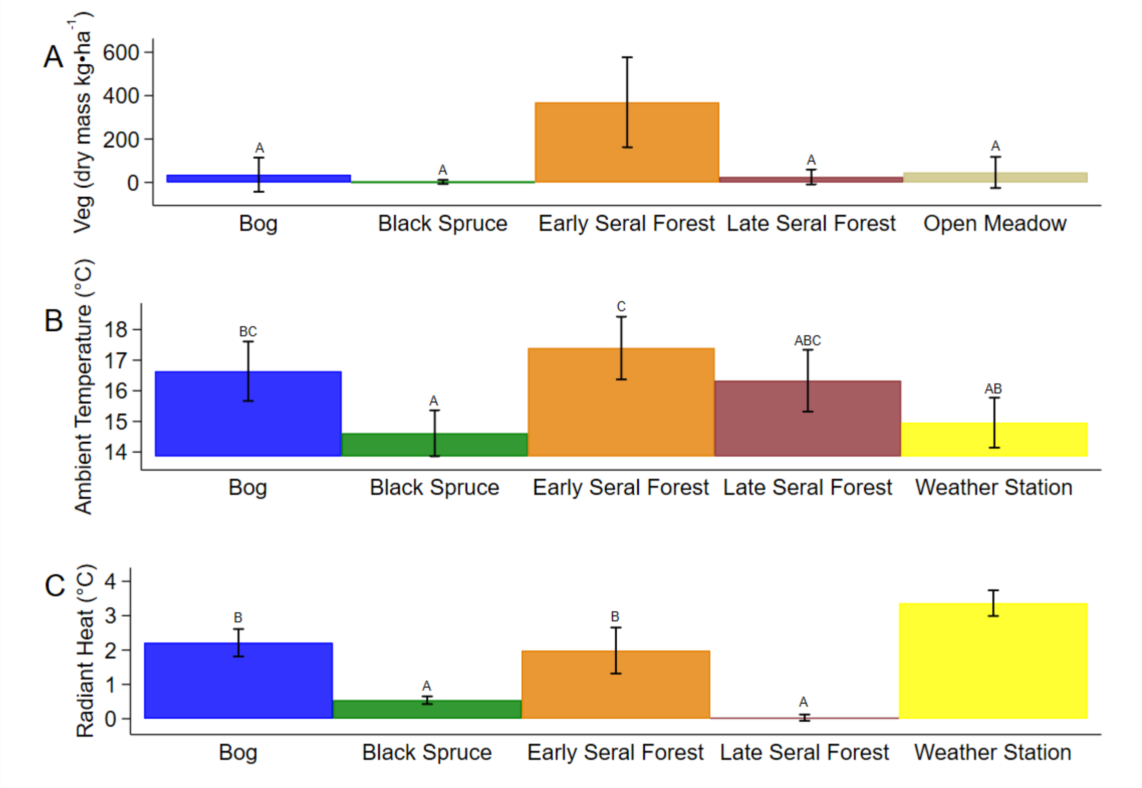

Figure 5.2 Average of vegetation (kg·ha^{-1}; A), average daily ambient air temperature (°C; B), and average daily radiant heat (operative temperature-ambient air temperature; °C; C) in bog (blue), black spruce (green), early seral forest (orange), late seral forest (maroon), and open meadow (yellow) habitats at the Kenai Moose Research Center, Kenai Peninsula, Alaska, USA. Vegetation was measured in open meadow at multiple sites, but temperature was measured only at the NOAA weather station for the open meadow habitat in 2019. Error bars indicate 95% confidence intervals around the estimate. Letters above each error bar indicate significant difference between means (*$P<0.05$*).

Flies

A total of 102,812 flies were trapped, identified, and assigned to seven functional groups. We collected 88.00% of all flies in CO_2 baited light trap, with the remainder in sticky traps (Fig. 5.3 and Table D.1). CO_2 baited light traps were most effective in capturing biting midges (99.88%), mosquitoes (94.51%), biting house flies (93.75%), and black flies (67.22%; Fig. 5.3A-C). Sticky traps were most effective in capturing snipe flies (61.57%), and horse and deer flies (72.04%; Fig. 5.3D and 5.3G). Trap success varied within taxa, by habitat. For coprophagous flies, CO_2 baited light traps were most effective in black spruce, but both traps were equally effective in late seral forest (Fig. 5.3E). For horse and deer flies, sticky traps were more effective in bog and early seral forest, but both traps were equally effective in late seral forest and black spruce (Fig. 5.3G).

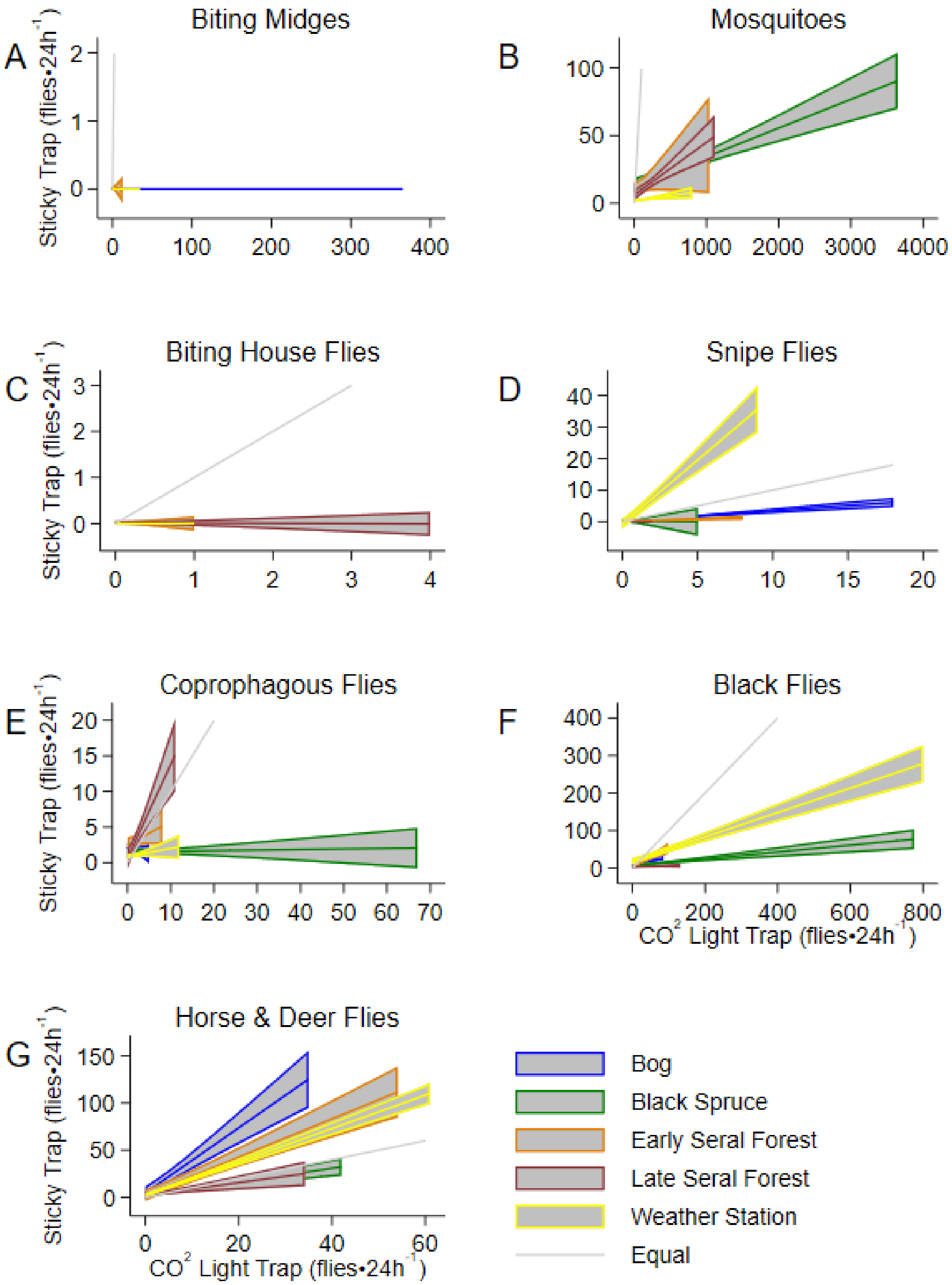

Figure 5.3 Relationship between capture rates of flies in two types of traps in each habitat. Linear regression between flies (flies·24h^{-1}) collected in a sticky trap versus a CO$_2$ baited light trap by fly group (A-G) at the Kenai Moose Research Center, Kenai Peninsula, Alaska, USA, with habitat collected shown in colors.

The majority of flies collected from both traps at each site were mosquitoes (80.82%) followed by black flies (13.54%), horse and deer flies (3.22%; Fig. 5.4). Less than 3% of remaining flies were coprophagous flies (1.34%), biting midges (0.79%), snipe flies (0.26%), and biting muscid flies (0.03%; Fig. 5.4). We collected an average of 320 flies per site in each trap period of 24 hours (Fig. 5.4). The greatest number of flies collected were from black spruce habitats (62.41%, 668 flies·$24h^{-1}$), followed by 11.40% (181 flies·$24h^{-1}$) from the open meadow at the NOAA weather station, 10.84% (206 flies·$24h^{-1}$) from late seral forest habitats, 9.56% (185 flies·$24h^{-1}$) from bog habitats, and 5.80% (110 flies·$24h^{-1}$) from early seral forest habitats (Fig. 5.4).

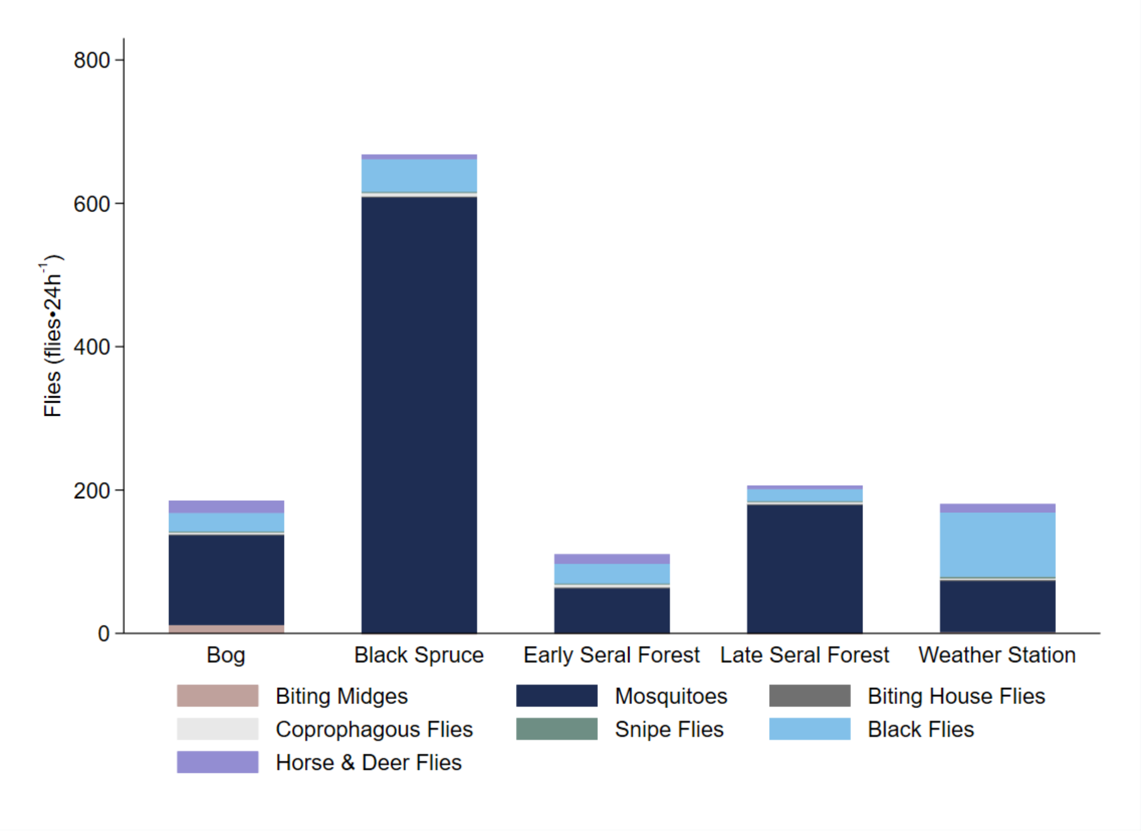

Figure 5.4 Average flies collected (flies·24h^{-1}) in bog, black spruce, early seral forest, late seral forest, and open meadow habitats, with fly groups stacked by color, at the Kenai Moose Research Center, Kenai Peninsula, Alaska, USA.

Fly abundance was related to Julian day, average daily air temperature, and average daily vapor pressure in the top model (X^2 [12df] = 4255.89, $P = 0.000$; Fig. 5.5A-C and Table D.2). Total fly abundance was greater in 2021 than 2019. Counts of all flies rose to a peak at Julian day 169 and then declined slowly through summer (Fig. 5.5A). Fly counts increased with average daily vapor pressure (Fig. 5.5C). Flies also increased with average air temperature to a peak at 12°C. Increasing daily air temperatures through the end of summer were associated with declines in fly abundance in all habitats, except black spruce (Fig. 5.5B). At the NOAA weather station, total fly counts decreased linearly with NOAA temperature and increased linearly with NOAA vapor pressure (X^2 [2df] = 11.63, $P = 0.003$; Fig. D.2). The top models for mosquito (X^2 [22df] = 783.89, $P = 0.000$; Fig. 5.5D-F and Table D.2) and black fly (X^2 [12df] = 6037.21, $P = 0.000$; Fig. 5.5G-I and Table D.2) abundances were also related to Julian day, average daily air temperature, and average daily vapor pressure. Counts of mosquitoes rose to peak at Julian day 176 (Fig. 5.5D), and at an average air temperature of 11°C (Fig. 5.5E). Mosquitoes increased with average daily vapor pressure (Fig. 5.5F). The abundance of black flies was highest at the beginning and end of the summer, with the lowest counts at Julian day 196 (Fig. 5.5G). Counts of black flies rose to a peak at an average air temperature of 13°C, and declined in all habitats except black spruce and late seral forests (Fig. 5.5H). Black fly counts increased with average vapor pressure in all habitats except for black spruce, where black fly counts decreased with average vapor pressure (Fig. 5.5I).

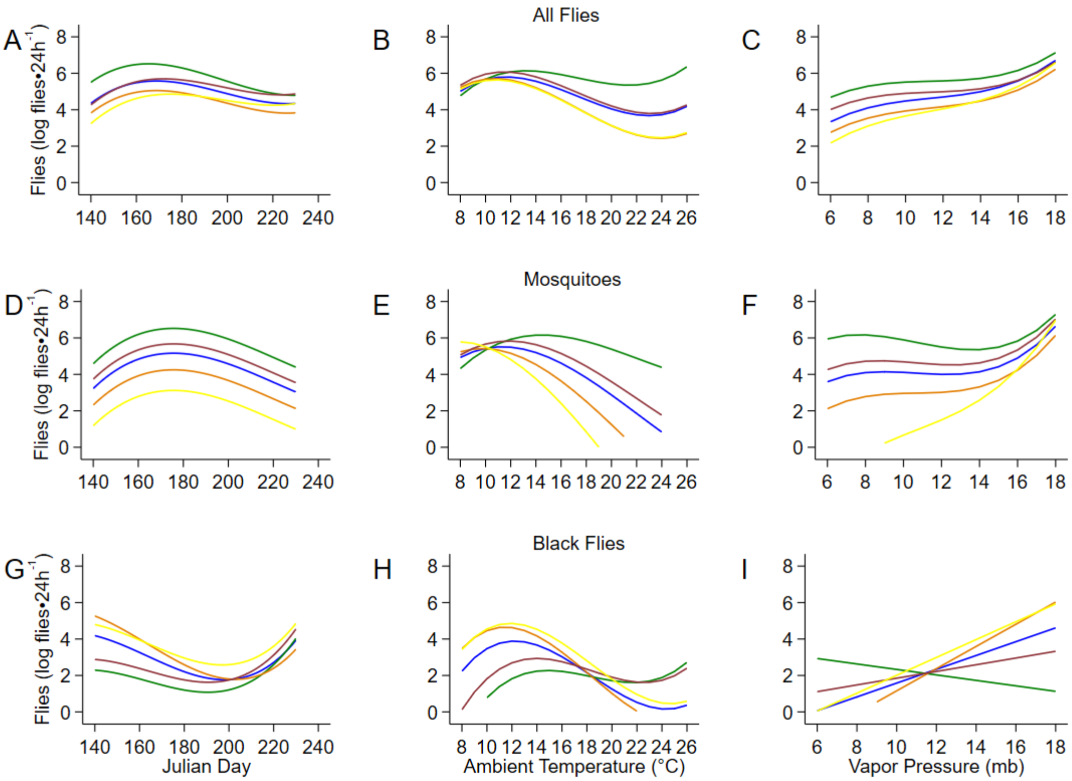

Figure 5.5 Relationships between total rate of capture of flies (log flies·24h^{-1}) and environmental conditions (Julian day, ambient air temperature (°C), vapor pressure (mb)). Lines are marginal predictions from mixed-effect regression models for all flies (log flies·24h^{-1}; A-C), mosquitoes (log flies·24h^{-1}; D-F), and black flies (log flies·24h^{-1}; D-I) in each habitat (bog = blue, black spruce = green, early seral forest = orange, late seral forest = maroon, weather station = yellow; see Fig. 5.2 key) at the Kenai Moose Research Center, Kenai Peninsula, Alaska, USA.

Behavior of moose

Movement rate (m·h^{-1}) was correlated with age, Julian day, and time of day (X^2 [6df] = 344.17, $P = 0.000$; Fig. 5.6 and Table D.3). On average, older moose moved less in each hour than younger moose over the summer (Fig. 5.6A). Average movement rates declined by 38.39 m·h^{-1} as summer progressed (Fig. 5.6B). Movement rates were greatest at 6:00am and slowest at 5:00pm (Fig. 5.6C).

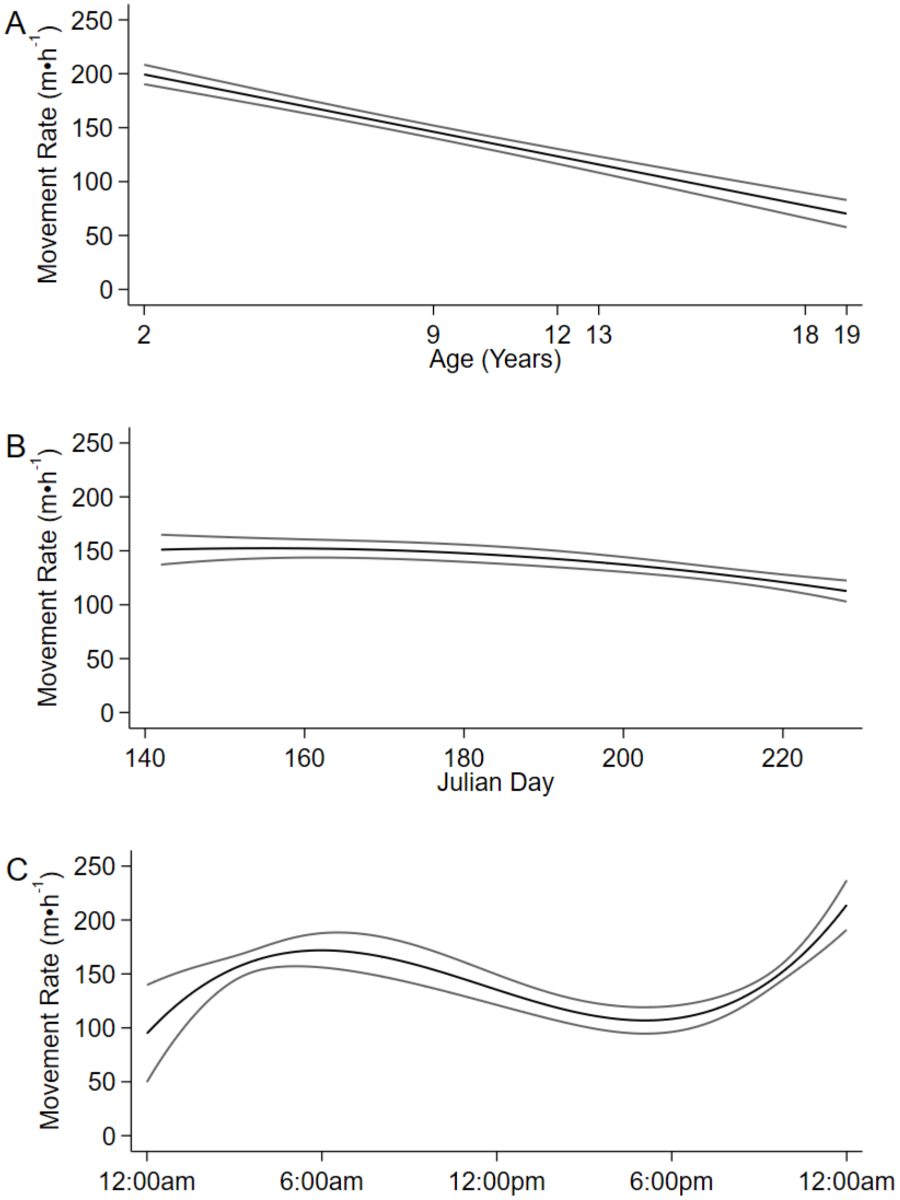

Figure 5.6 Relationships between movement rate (m·h^{-1}) and metrics of time for moose (n=12) at the Kenai Moose Research Center, Kenai Peninsula, Alaska, USA. Lines are marginal predictions with 95% confidence intervals from mixed-effect regression models with age of the moose (years; A), Julian day (B), and time of day (C) as independent variables.

On a daily average basis, movement rate was related to the average daily vegetation experienced, average daily flies experienced and its interaction with average daily ambient air temperature (X^2 [6df] = 220.99, *P* = 0.000; Fig. 5.7 and Table D.4). Moose moved slower as forage density increased (Fig. 5.7A). Moose moved faster (177.12 vs. 112.29 m·h^{-1}) as fly abundance increased (7.5 vs. 4.5 log flies·24h^{-1}) on cool days (8°C) (Fig. 5.7B). However, on warm days (20°C), movement rates were not significantly affected by flies (Fig. 5.7B).

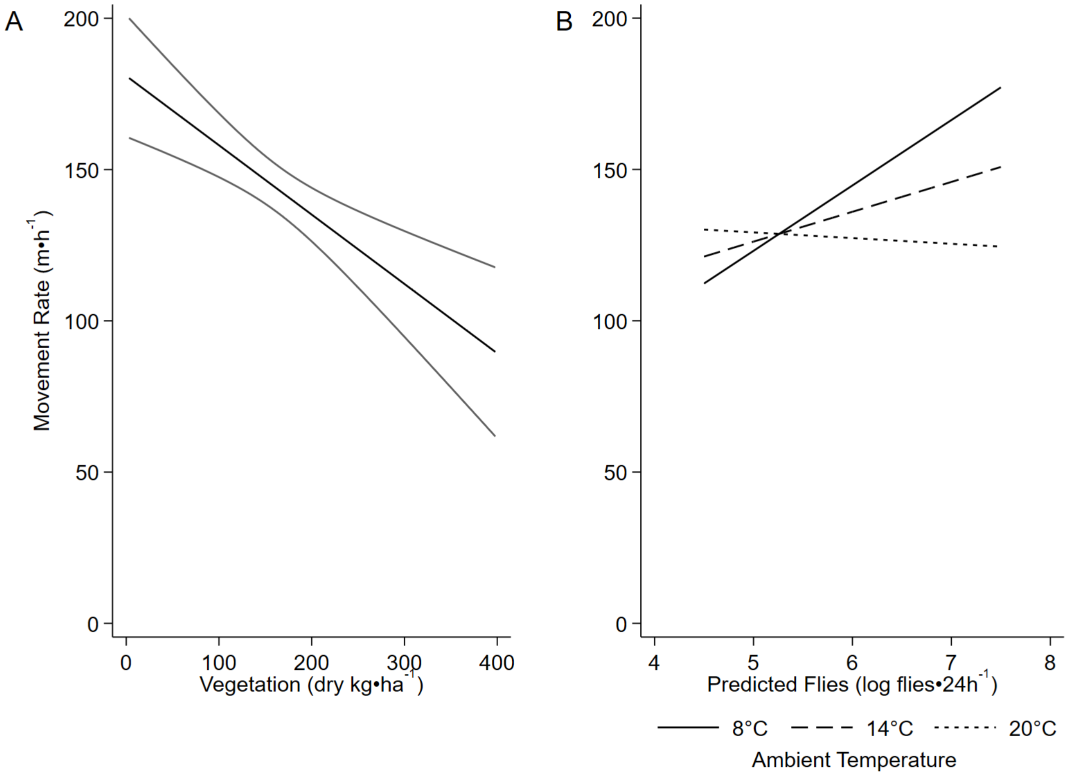

Figure 5.7 Relationships between average daily movement rate (m·h^{-1}) of moose (n=12) and exposure to vegetation and flies at the Kenai Moose Research Center, Kenai Peninsula, Alaska, USA, based on mixed-effects regression. A) Average daily dry mass of vegetation (kg·ha^{-1}); lines indicate the marginal prediction with 95% confidence interval. B) Average daily exposure to flies (log flies·24h^{-1}); lines indicate the marginal prediction at 8°C, 14°C, and 20°C.

The amount of time the moose spent in any one habitat was related to their age, Julian day, average daily exposure to flies, and average daily ambient air temperature (bog: X^2 [4df] = 19.58, $P = 0.001$; black spruce: X^2 [9df] = 1669.93, $P = 0.000$; early seral forest: X^2 [9df] = 511.33, $P = 0.000$; X^2 [5df] = 56.77, $P = 0.000$; Fig. 5.8, 5.9, and Table D.5). Across the summer, old moose (> 18 years) spent less time in late seral forest than young moose (2 years), whereas at intermediate age (10 years), moose spent more time in black spruce than either old or young moose (Fig. 5.8A). In the middle of summer (Julian day 181), moose spent most time in early seral forests, and the least time in black spruce and late seral forest (Fig. 5.8B). Time spent in bogs did not vary across the season. Although effects of temperature and fly exposure were significant in bogs, those effects were much smaller than predicted for other habitats (Fig. 5.9A). Overall, the amount of time the moose spent in black spruce and late seral forest increased with air temperature and counts of flies (Fig. 5.9B and 5.9D). Conversely, the amount of time moose spent in early seral forest decreased with increasing counts of flies and increasing air temperatures (Fig. 5.9C). Moose spent 718 $\text{min}\cdot\text{d}^{-1}$ in early seral forest on cool days (8°C) but less than 63 $\text{min}\cdot\text{d}^{-1}$ in the same habitat on warm days (20°C). Late seral forest was the only habitat in which there was no significant interaction between flies and air temperature on the time spent by moose (shown by parallel slopes in Fig. 5.9D).

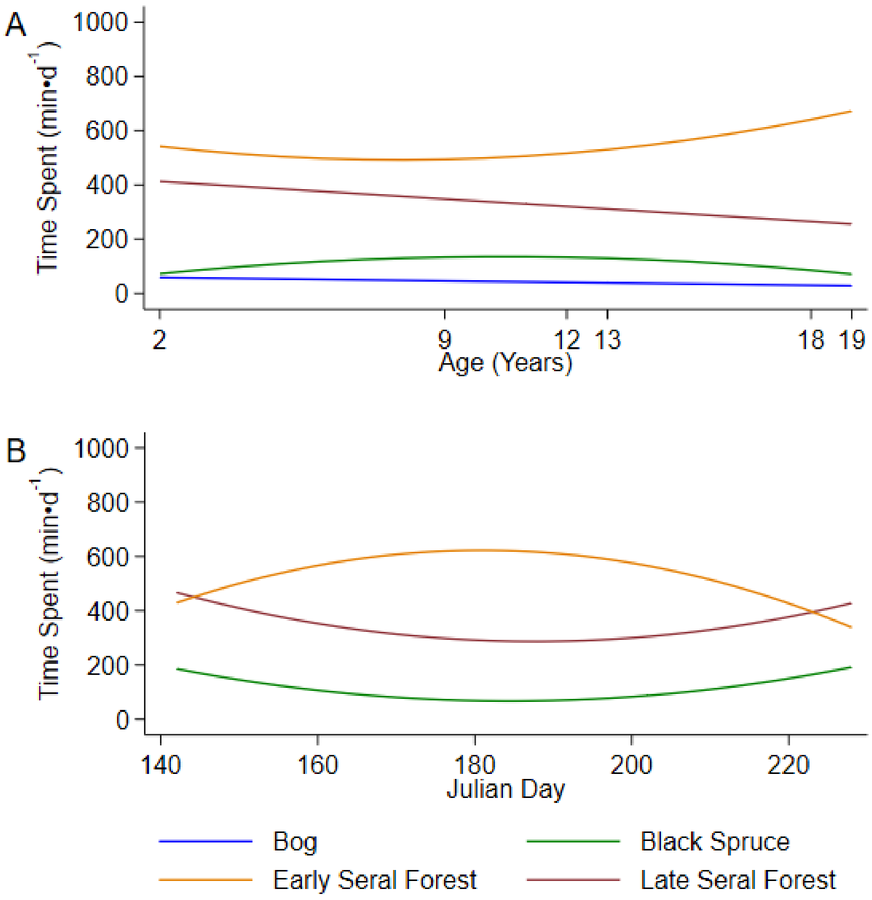

Figure 5.8 Relationships between daily time spent by moose (n=12, min·d^{-1}) in each habitat and the age of the indvidual (A) or the time of year (Julian Day). Lines are marginal predictions from mixed-effect regression models for bog (blue), black spruce (green), early seral forest (orange) and late seral forest (maroon) at the Kenai Moose Research Center, Kenai Peninsula, Alaska, USA.

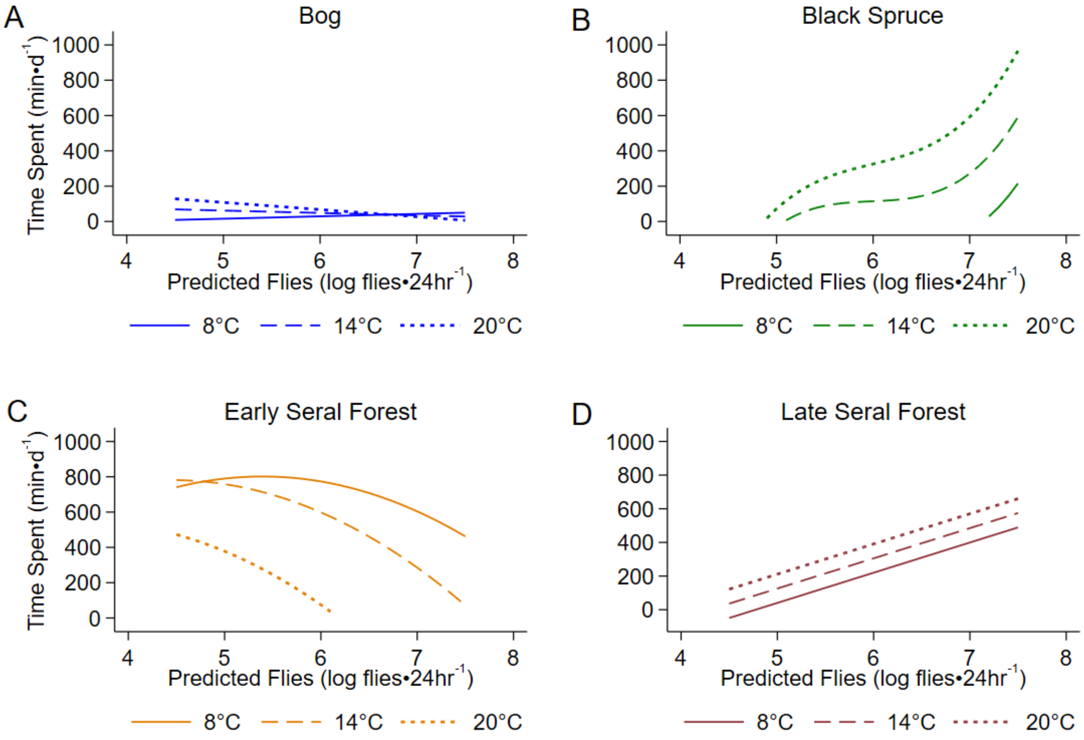

Figure 5.9 Relationships between time spent in a habitat (min·d^{-1}) and the exposure to flies (log flies·24h^{-1}) at three ambient air temperatures: 8°C, 14°C, and 20°C. Lines are marginal predictions from mixed-effect regression models for moose (n=12) in bog (A), black spruce forest (B), early seral forest (C), and late seral forest (D) habitats at the Kenai Moose Research Center, Kenai Peninsula, Alaska, USA.

Discussion

As predicted, moose spent the majority of their time in the forage abundant early seral forest (Fig. 5.8 and 5.9). On warm days, moose avoided flies by decreasing their time spent in early seral habitat, but tolerated flies by increasing time spent in black spruce and late seral forest (Fig. 5.9). Movement rate did increase with exposure to flies as predicted, but only on cool days (Fig 5.7B). Our data indicate that air temperature is the most important driver for moose — warm days increased time spent in shaded habitats (late seral forests and black spruce) that reduce radiant heat loads as well as shaded wet habitats that allow conductive heat loss (black spruce, not bog) (Fig. 5.8 and 5.9). However, the use of cooling habitats (black spruce and late seral forest) can increase exposure to flies especially when heat loads increase as moose move slowly to forage in open habitat (early seral forest) on warm days in mid-summer.

Foraging

Foraging accounts for most of the movement of moose as spring ambient air temperatures rise, and both growing plants and flies emerge (Renecker & Hudson 1986; Shively et al. 2019; Thompson et al. 2021). High movement rates in spring coincide with emergence of plants (Fig. 5.6B). High movement rates in the morning and low movement rates in the evening (Fig. 5.6C) probably reflect daily patterns of foraging and resting that coincide with rising and declining body temperatures in moose (Thompson et al. 2021). Time spent in early seral forest increased at the beginning of the season, and this quickly became where the moose spent most of their time (Fig. 5.8B), browsing the slowest (Fig. 5.7A) in this forage abundant habitat (Fig. 5.2A). Movements differed between life stages, likely with experience; older moose had lower rates of movement than the younger moose (Fig. 5.6A) and spent less time in late seral habitats (Fig. 5.8A), where forage is sparse (Fig. 5.2A). The benefits of foraging probably diminished as flies became abundant (Fig. 5.5A) and ambient air temperatures rose from spring to summer.

Behavioral Thermoregulation

As ambient temperatures increased moose spent significantly less time browsing in early seral forest and more time resting under canopy in late seral forest and black spruce habitats, with the greatest increase of time spent in black spruce (Fig. 5.9) (Thompson et al. 2021). In comparison to early seral forest, late seral forest provides less forage but more shade from radiant heat loads (Fig. 5.2). Black spruce also provided shade with moist bedding sites that allow conductive heat loss (Fig. 5.2B and C) (Van Cleve et al. 1983; Alston et al. 2020). Mosses insulate the soil, reducing soil temperatures, and contribute to the formation of permafrost beneath black spruce forests (Van Cleve et al. 1983; Oechel & Van Cleve 1986). Thermoregulatory models of moose indicate that canopy cover can mitigate heat stress up until 10°C ambient air temperature, but temperatures above 10°C require access to wet ground and water for conductive cooling (McCann et al. 2016; Alston et al. 2020; Verzuh et al. 2022). Increasing time spent in black spruce likely reflects cooling at rest (Fig. 5.9). Wet ground (bog habitats) and canopy cover (late seral forest habitats) alone do not meet the needs of both requirements, like black spruce habitats.

Flies and temperature

An interaction occurred between ambient temperature and flies; as ambient temperature and fly numbers increased, moose spent more time in late seral forest and black spruce habitats (Fig. 5.9). This is counterintuitive because black spruce was the most fly abundant habitat, where 62.41% of flies were collected (Fig. 5.4). At high temperatures moose trade-off browsing in fly sparse habitats for cooling and resting in fly abundant habitats (Fig. 5.9). They must tolerate flies to cool down. Warm summer temperatures can create an ecological trap for moose when heat gained while foraging is dissipated at rest in shady, wet habitats preferred by flies that adversely affect the fitness of moose (Hale & Swearer 2016). This trap of preferring the most fly abundant habitat, with little to no forage, for cooling will likely worsen as climate change progresses (Robertson & Hutto 2006; Hale & Swearer 2016; Thompson et al. 2021)

Consequences

Many ungulates follow waves of spring green-up (Merkle et al. 2016; Abrahms et al. 2021), however, heat loads and flies impact a moose's ability to track the changing abundance and quality of forage through summer. Feeding is probably curtailed by flies in spring when movement rates are increased on cool days. Moose also miss opportunities to forage in summer when they seek relief in cool shady habitats with less forage. Although movement costs are reduced by resting on hot days, thermoregulation expends energy and water to increase conductive heat loss by peripheral vasodilation and by evaporative heat loss through panting (Renecker & Hudson 1990; Alston et al. 2020). Moose, especially in the north temperate zone of the boreal forest, already have a narrow summer window for capturing a short period of growth of vegetation through high intakes of browse (Renecker & Hudson 1986; Shively et al. 2019). Summer temperatures, particularly late spring, create a foraging window in which moose must assimilate enough protein and energy before winter when forage abundance and quality are low. Moose are capital breeders, calving rates are dependent on a female's body condition at the time of rut, in the fall (Allen et al. 2017). What is gained in the spring and summer effects their health, fecundity, survival, and the survival of their calves throughout the year (Allen et al. 2017).

The costs of tolerating flies may extend into winter because flies carry parasites. We recently described the effect of filarial nematodes on moose in the Kenai Peninsula, Alaska. Legworm likely causes the open sores on the legs of adult moose and led to a decrease in serum protein (Benedict et al. 2023a; Benedict et al. 2023b), while *Setaria yehi* led to morbidity and mortality in calves (Benedict et al. 2023c). Nematode parasites were also found to cause neurological impairments, peritonitis, and death in a declining moose population in northern Minnesota (Murray et al. 2006; Grunenwald et al. 2016; Grunenwald et al. 2018).

Moose resident in warming regions at the southern end of their distribution may be stuck in an ecological trap that exposes animals to flies in thermal refugia. Moose populations are constrained by predators, heat stress, and parasites in the southern range, but the northern range has expanded historically (Murray et al. 2006; Monteith et al. 2015; Tape et al. 2016). Since 1850, climate warming has facilitated the growth of riparian shrubs and earlier snowmelt to provide foraging corridors for moose to expand from the boreal forest into the arctic slope (Tape et al. 2016). Flies are however abundant in the tundra and likely to limit foraging gains at the northern limits of the distribution.

Management

Boreal moose need abundant black spruce and early seral forest in summer to cope with the effects of flies and heat stress. They need black spruce with moist, wet understories for cooling and early seral forests for browsing. They must maintain high levels of browsing to offset periods of lost foraging opportunities and for tissue repair, and to maintain high fecundities that replace cohorts of calves lost to parasitic infections. Fire is the primary driver of succession in boreal forests, creating valuable early seral forest with increased forage quantity and quality (Davis & Franzmann 1979; Brown et al. 2018). Black spruce habitat is historically resilient to fire, having a high flammability, but depending on fire for regeneration and replacing itself quickly after being burned (Baltzer et al. 2021). Drier climatic conditions and more severe fires have limited the ability of black spruce to regenerate (Baltzer et al. 2021). An outbreaks of spruce bark beetle (*Dendroctonus rufipennis*) on the Kenai Peninsula in the 1990's also increased fire and fire severity by creating surface fuels and killing the less resilient white spruce (*Picea glauca*) (Hansen et al. 2016). A careful balance of fire severity is important for maintaining moose habitat and thus populations of boreal moose in Alaska.

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CHAPTER VI

CONCLUSIONS

Introduction

Ungulates are a mobile feast for many invertebrate parasites, their large body size and mobility expose them to a wide variety of parasites (Jolles & Ezenwa 2015). Flies are one of the main arthropod pests of ungulates, they have developed structures, behaviors, and developmental patterns in over 100 independent lineages and 13 families resulting in a wide array of specializations (Feener Jr & Brown 1997). Flies injure and distress ungulates with piercing mouthparts, spongy mouthparts, or endoparasitic invasion (Johnson & Triplehorn 2005). The effects of flies on ungulates depends on their impact on fitness. I found that an ungulates skin and hair are its first line of defense against flies, creating a morphological barrier. Ungulates can also physiologically resist flies at low intensities of exposure; making repairs to damaged skin and hair, activating their immune system to kill or resist invading parasites, fighting infection and transmission of pathogens. As fly exposure increases, many ungulates react with behavioral avoidance, incurring costs through reduced body mass from loss of foraging opportunities.

Moose appear much more tolerant of flies than other wild ungulates. I investigated how flies morphologically, physiologically, and behaviorally effect moose. I began by measuring salivary cortisol levels in moose calves, to characterize their stress response, in relation to the abundance and diversity of flies on their bodies. I then looked at the molt patterns of moose and the ability of moose to rely on hair as a morphological barrier to flies, and the damage done by flies to this barrier. I then looked at the physiological costs to this damage and the stress response of adult moose to flies. Lastly, I investigated how flies behaviorally influence moose.

Resistance of moose calves

To investigate the stress response of moose calves in relation to environmental variables and flies, I measured salivary cortisol in 5 calves up to 4 times a day on 25 days from June through August, 2019, with corresponding fly collections and weather measurement. I collected 2,618 flies from the calves, 68% of which were moose flies that predominately congregated on their hind end, near their tail. I found low salivary cortisol levels ($<0.2\ \mu g \cdot dL^{-1}$) in the calves, with no correlation to ambient air temperature or counts of flies. Flies did not trigger a release of glucocorticoid hormones in the moose calves as a stress response, even though flies may still be perceived as noxious. Moose calves appear to rely on their dense hair coat and their immune system to resist fly bites and pathogens associated with wounds or bites. However, resisting comes with risks of morbidity and mortality, especially when flies are vectors for parasites such – 2 of 5 calves died from infection with the parasitic worm *Setraria yehi* in their first winter.

Morphological barrier

I began by tracking and recording molt to investigate the ability of moose to rely on hair as a morphological barrier to flies, and the damage done by flies to this barrier. I created molt scores (322 observations) which described the phases of molt from May to

August 2015, 2016, and 2021, paired with thermal imagery (93 images) of the hind legs of moose from May to August, 2021. I found that sores began to appear on the hind legs of moose as the winter pelage molted; moose completed molt by July 25 and all moose had sores by July 5. Similarly, the temperature of the hind legs increased with the loss of hair, and the appearance of sores.

To further investigate the sores on the hind legs of the moose, I biopsied the tissue of 20 adult moose on the Kenai Peninsula, Alaska. Histological analysis showed severe, ulcerative and eosinophilic dermatitis in all of the sores, and microfilariae with intraepidermal pustules in four additional samples. Genomic analysis revealed a previously unknown genetic lineage of *Onchocerca*, likely transmitted by black flies.

Moose calves have a dense hair coat throughout the summer, protecting them from some effects of flies and sore formation. Adults do not have this barrier of protection; the number of sores found on the moose increased as fly numbers peaked at the end of July. These open sores leave a moose exposed to further pathogen invasion and the costs of tissue repair and fitness.

Physiological costs

To investigate the costs of tissue damage to adult moose from flies, I analyzed the relationship between the number of sores on moose and the condition of the animal. I used body fat measurements of 10 moose, recorded the number of sores present on the hind legs of each moose, and measured serum concentrations of protein and other metabolites. I found that the number of sores present on the hind legs of moose is positively correlated with body fat, which suggests a trade-off between foraging gains of energy and exposure to flies. I also found that the number of sores is negatively correlated with serum albumin, which is consistent with the use of body protein to repair injuries from flies and parasites.

I also looked at the stress response of moose to the abundance of flies on their bodies. I collected 31,905 flies from 12 moose in the summer of 2021 and one moose in the summer of 2019, had salivary and fecal samples analyzed for concentrations of corticosteroids, and measured associated climatic variables. I found that the number of flies present on a moose was not correlated with the concentration of corticosteroids in saliva or feces, nor with environmental variables (ambient air temperature, relative humidity, wind).

Flies do not elicit a stress response in moose even though the costs of repairing wounds and resisting infections of those wounds probably reduce gains of protein from foraging in summer. Moose can tolerate the injuries from biting flies with regular gains from summer foraging but exposure to insect borne endo-parasites risks subsequent reproduction and survival.

Behavioral costs

To assess the behavioral changes caused by flies to moose, I used 12 GPS collared moose to measure movement rate and time spent in 4 habitats (bog, black spruce, early seral forest, and late seral forests). Movement and duration in each habitat was compared to available forage, fly exposure, and ambient temperatures, across time and age. I measured the abundance and diversity of flies (102,812 flies) and associated climatic variables (ambient air temperature, relative humidity, and radiant heat) from 13 sites, across habitats in the summer of 2019 and 2021. I measured the abundance of available moose browse vegetation in these habitats in July of 2022. I found that on cool days (8°C) moose increased their movement rate with counts of flies, but on warm days (20°C) movement declined with counts of flies. On cool days moose spent 718 $\text{min}\cdot\text{d}^{-1}$ in early seral forest, the habitat with the greatest forage, but less than 63 $\text{min}\cdot\text{d}^{-1}$ in the same habitat on warm days. Time spent in black spruce, the coolest habitat, increased with both air temperature and with counts of flies. Warm summer temperatures can create an ecological trap for moose when heat gained while foraging is dissipated at rest in shady, wet habitats preferred by flies that adversely affect the fitness of moose.

Implications

I found that the abundance and diversity of flies in a moose's environment is heavily dependent on environmental variables such as temperature and vapor pressure. Mosquitoes make up the majority of flies in a moose's environment, in the boreal forests of Kenai, Alaska, with black flies being the second most abundant group of flies. Both of these groups of flies are known vectors of parasites and disease (Kutz et al. 2012). As the arctic warms, the northern expansion of parasites may follow (Kutz et al. 2009). Warmer temperatures will likely create longer fly seasons, and allow new species to survive until the following spring. Warming may also cause faster development rates in some flies, translating into less larval predation and growth of fly populations (Culler et al. 2015).

Increased populations of mosquitoes and black flies, and the diseases and parasites that they carry, translates into increased costs of living for moose and more uncertainty in population growth. Moose may have to contend with more tissue repair, losses of protein, and more foraging disturbance through increased fly abundance and warm days. While warming may also expand their summer foraging window, allowing moose to make gains in energy and protein to offset what is lost by flies, an increase in parasites and introduction of disease and new parasites may kill individuals or impair their growth and reproduction. Moose will need to maintain high levels to fecundity in order to replace cohorts of calves lost to parasitic infection.

However, the majority of flies that moose host are probably not vectors for parasites. Moose flies made up 90.79% of flies that I collected from adult moose and 68.4% of flies that I collected from calves. Less than 0.03% of the flies that I collected off host in the moose's environment using CO_2-baited light traps and sticky traps were moose flies. While the abundance of flies in a moose's habitat is significantly affected by environmental variables, the abundance of flies on a moose is not. Instead, there were significant differences in the number of flies netted among individual moose. Moose flies are thought to feed exclusively on moose and breed in fresh moose feces, overwintering as pupae in the feces (Lankester & Sein 1986). While they are blood feeders and come in contact with flies that are known vectors, mosquitoes and black flies, their reliance on moose as a habitat would suggest a co-evolution in which keeping moose healthy would be advantageous. Additionally, moose are largely solitary in the summer when flies are abundant, occasionally crossing paths with another moose, and staying with their calves for its first year of life (Geist 1963). It is in these few social interactions that moose flies would have the opportunity to move to a new host and transmit disease. Moose fly dispersal likely occurs when they initially emerge from moose feces, seeking the nearest moose host. I would suspect that the genetic diversity of the moose fly is much less mixed than that of moose, with a lower level of gene flow across Alaska (Schmidt et al. 2009), since moose are predominately social in the winter (Geist 1963) when flies are not present.

While there is much uncertainty in how climate change will influence host-insect dynamics, we can learn from declining moose populations at the southern end of their distribution. Warming regions at the southern end of the moose distribution have been constrained by predators, heat stress, and parasites (Murray et al. 2006; Monteith et al. 2015; Tape et al. 2016). I found that early seral forest is valuable forage habitat for moose, and black spruce habitat with cool, moist moss floors and a dense canopy are important for thermoregulation. Both habitats are more important than ever for boreal moose. Fire is the primary driver of succession in boreal forests, creating valuable early seral forest with increased forage quantity and quality (Davis & Franzmann 1979; Brown et al. 2018). Black spruce habitat is historically resilient to fire, having a high flammability, but depending on fire for regeneration and replacing itself quickly after being burned (Baltzer et al. 2021). Drier climatic conditions and more severe fires has limited the ability of black spruce to regenerate (Baltzer et al. 2021). An outbreak of spruce bark beetle (*Dendroctonus rufipennis*) on the Kenai Peninsula in the 1990's also increased fire and fire severity by creating surface fuels and killing the less resilient white spruce (*Picea glauca*) (Hansen et al. 2016). A careful balance of fire severity is important for maintaining moose habitat and thus populations of boreal moose in Alaska.

In valuing moose for hunting, viewing, native ceremonies, and the ecosystem services that they provide, we must value their habitat as well.

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APPENDIX A

CHAPTER 1 SUPPLEMENTAL DATA

Table A.1 Flies known to occur on *Bison* spp.

Family	Species	Bite Type
Ceratopogonidae		
	Ceratopogon sp.	piercing
Culicidae		
	Aedes sp.	females piercing
Hippoboscidae		
	Lipoptena cervi	piercing
	Melophagus ovinus	piercing
Muscidae		
	Haematobia irritans	piercing
	Musca autumnalis	lapping
	Stomoxys calcitrans	piercing
Rhagionidae		
	Symphoromyia sp.	piercing or lapping
Simuliidae		
	Simulium sp.	females piercing
	Simulium venustrum	females piercing
Tabanidae		
	Crysops sp.	females piercing
	Tabanus bromius	females piercing

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Table A.2 Flies known to occur on *Rangifer tarandus*.

Family	Species	Bite Type
Culicidae		
	Aedes sp.	females piercing
Hippoboscidae		
	Lipoptena cervi	piercing
Oestridae		
	Cephenemyia trompe	endoparasitic larvae
	Hypoderma tarandi	endoparasitic larvae
Simuliidae		
	Simulium venustum	females piercing
Tabanidae		
	Hybomitra affinis	females piercing

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Table A.3 Flies known to occur on *Alces alces*.

Family	Species	Bite Type
Ceratopogonidae		piercing
Culicidae		females piercing
Hippoboscidae		
	Lipoptena cervi	piercing
Muscidae		
	Haematobosca alcis	piercing
	Hydrotaea sp.	piercing or lapping
	Stomoxys calcitrans	piercing
Oestridae		
	Cephenemyia sp.	endoparasitic larvae
	Cephenemyia jellisoni	endoparasitic larvae
	Cephenemyia phobifera	endoparasitic larvae
Rhagionidae		piercing or lapping
Simuliidae		
	Prosimulium decemarticulatum	females piercing
	Prosimulium exigens	females piercing
	Prosimulium formosum	females piercing
	Simulium arcticum	females piercing
	Simulium aureus	females piercing
	Simulium croxtoni	females piercing
	Simulium decorum	females piercing
	Simulium euryadminiculum	females piercing
	Simulium furculatum	females piercing
	Simulium jenningsi	females piercing
	Simulium latipes	females piercing
	Simulium meridionale	females piercing
	Simulium pictipes	females piercing
	Simulium pugetense	females piercing
	Simulium venustum	females piercing
	Simulium vittatum	females piercing
Tabanidae		
	Chrysops celvus	females piercing
	Chrysops cincticornis	females piercing
	Chrysops cuclux	females piercing
	Chrysops excitans	females piercing
	Chrysops frigidus	females piercing

Table A.3 Continued

Family	Species	Bite Type
	Chrysops lateralis	females piercing
	Chrysops mitis	females piercing
	Chrysops montanus	females piercing
	Chrysops niger	females piercing
	Hybomitra affinis	females piercing
	Hybomitra arpadi	females piercing
	Hybomitra criddlei	females piercing
	Hybomitra epistates	females piercing
	Hybomitra illota	females piercing
	Hybomitra lasiophthalma	females piercing
	Hybomitra lurida	females piercing
	Hybomitra microcephala	females piercing
	Hybomitra nuda	females piercing
	Hybomitra trepida	females piercing
	Hybomitra trispila sodalis	females piercing
	Hybomitra typhus	females piercing
	Hybomitra zonalis	females piercing
	Tabanus marginalis	females piercing
	Tabanus nigripes	females piercing

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APPENDIX B

CHAPTER 3 SUPPLEMENTAL DATA

Figure B.1 Representative adult molt score photographs. Photographs showing the eight stages of molt scores. Numbers correlated to molt scores in Table 3.1.

Figure B.2 Representative adult hock score photographs. Photographs showing the four stages of hock scores. Numbers correlated to hock scores in Table 3.1.

APPENDIX C

CHAPTER 4 SUPPLEMENTAL DATA

Table C.1 Results for the logistic regression between Julian day and start of molt, completion of molt, loss of hair on the hind legs above the hock, and the emergence of sores, with intragroup correlation of individual moose, to account for repeated measures, at the Kenai Moose Research Center, Kenai Peninsula, Alaska, USA. Standardized beta coefficients only of significant fixed effects (*P*<0.05) are shown.

Parameters and main effects	Dependent Variable (Y)			
	Start of Molt	Completion of Molt	Loss of Hair Above the Hock	Emergence of Sores
Observations	325	325	325	325
X^2 [df]	94.35 [1]	65.35 [1]	213.20 [1]	93.10 [1]
Intercept	23.10	-54.49	6.59	-25.10
Julian day	-0.17	0.29	-0.05	0.14

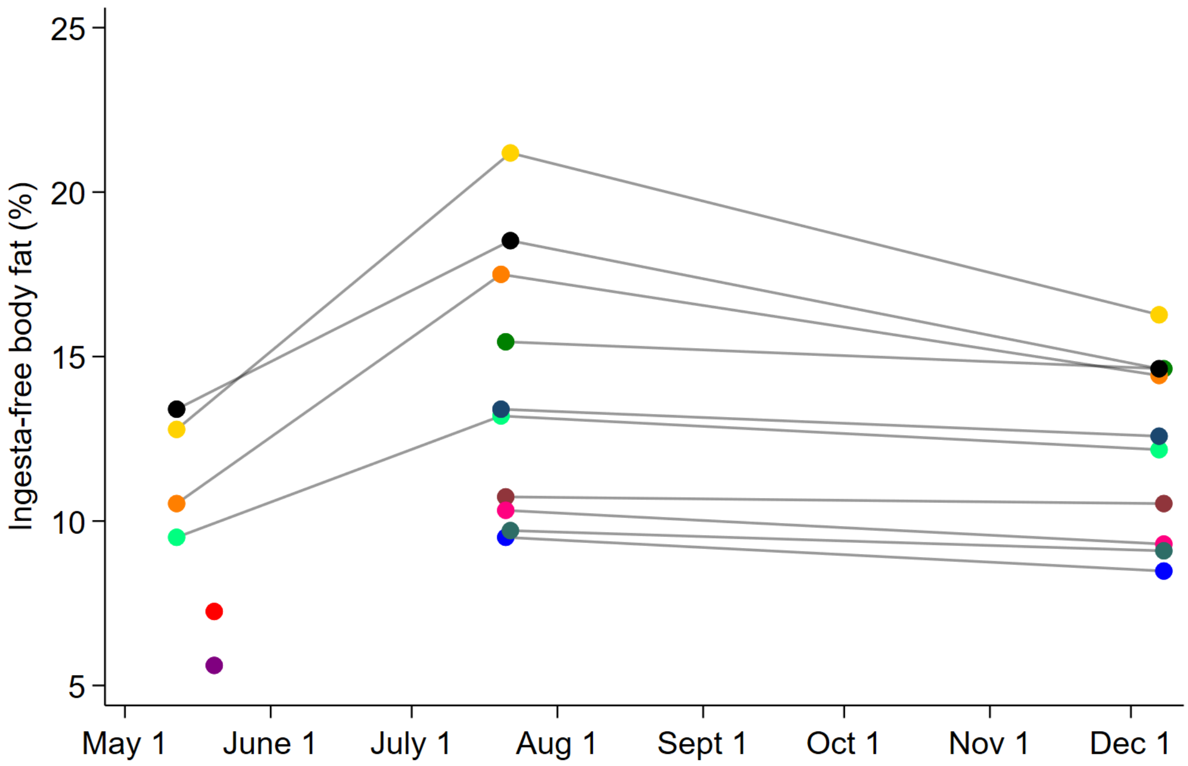

Figure C.1 Ingesta-free body fat (%) measurements across female adult moose (n=12 series of colors, individuals) at the Kenai Moose Research Center, Kenai Peninsula, Alaska, USA, across Julian days.

Table C.2 Results for the robust regression of ingesta-free body fat (IFBFAT), blood proteins (total protein, albumin, globulins, fibrinogen) and blood cells (eosinophils and lymphocytes) on the average number of hind leg sores observed on a moose, in July at the Kenai Moose Research Center, Kenai Peninsula, Alaska, USA. Standardized beta coefficients only of significant fixed effects (*P*<0.05) are shown.

	Dependent Variable (Y)
Parameters and main effects	Hind Leg Sores
Observations	10
R^2	0.60
Intercept	58.78
IFBFAT	1.16
Albumin	-17.36

Table C.3 Results for the regression of the effects of ambient air temperature (Ta), time of day (time), and Julian day on fecal corticosteroids (fecal cortisol) of moose at the Kenai Moose Research Center, Kenai Peninsula, Alaska, USA. Individual moose were included as random effects to account for repeated measures of dependent variables. Standardized beta coefficients only of significant fixed effects (*P*<0.05) are shown.

	Dependent Variable (Y)
Parameters and main effects	Fecal Cortisol
Observations	95
X^2 [df]	5.12 [1]
Intercept	3.51
Time	-0.91

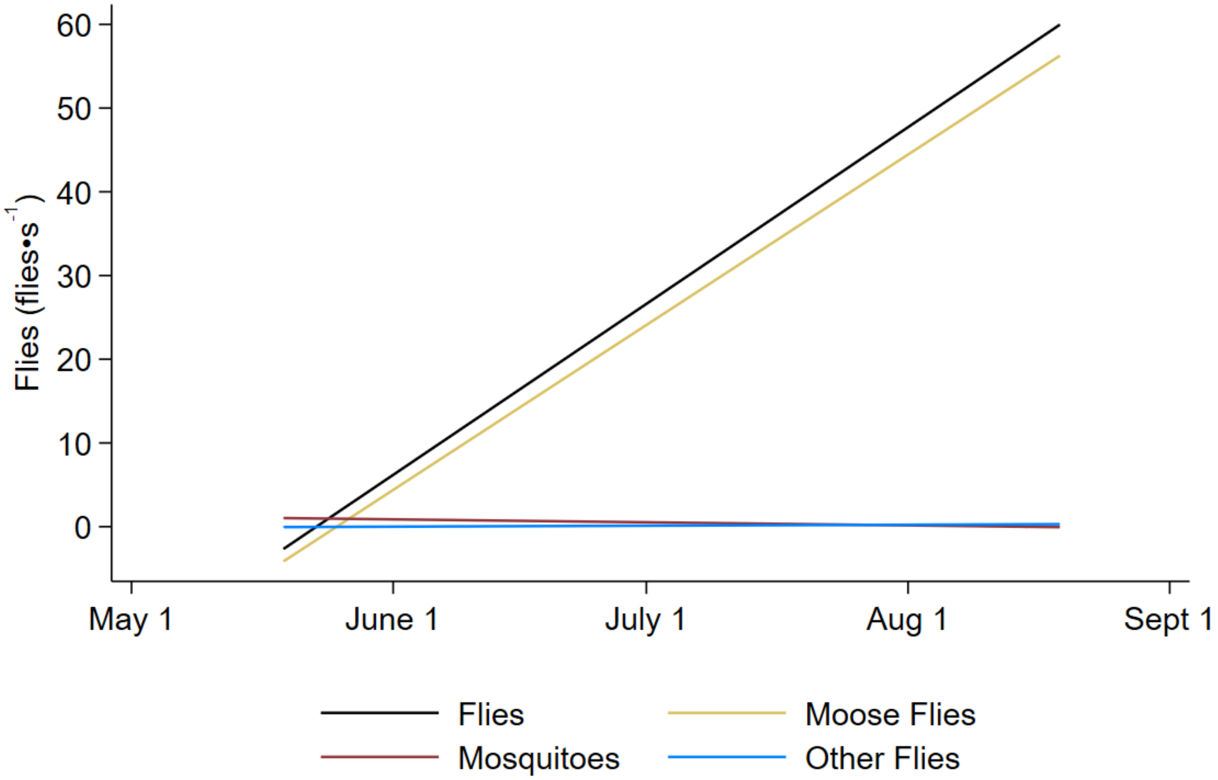

Figure C.2 Marginal predictions of Julian day on flies (R^2 = 0.216, *P* = 0.007), moose flies (R^2 = 0.206, *P* = 0.007), mosquitoes (R^2 = 0.203, *P* = 0.000), and other flies (R^2 = 0.041, *P* = 0.043) netted per second from female adult moose (n=12) at the Kenai Moose Research Center, Kenai Peninsula, Alaska, USA, based on linear regression.

Table C.4 Results for the regression of the effects of vapor pressure (vap_pres), ambient air temperature (Ta), Julian day (julian), wind, time of day (time), habitat type (habitat), and individual moose (individual) against flies netted per second (combined (flies) and by group) at the Kenai Moose Research Center, Kenai Peninsula, Alaska, USA. Standardized beta coefficients only of significant fixed effects (*P*<0.05) are shown.

	Dependent Variable (Y)						
Parameters and main effects	Flies	Moose Flies	Coprophagous Flies	Mosquitoes	Black Flies	Horse and Deer Flies	Other Flies
Observation	98	98	98	98	98	98	98
R^2	0.22	0.21	0.02	0.20	0.04	0.02	0.04
Intercept	-115.31	-111.70	-1.45	-0.64	-1.52	-0.07	-0.57
Julian	0.68	0.66	–	-0.01	–	–	0.00
Ta	–	–	0.17	–	–	0.01	–
Ta^2	–	–	–	-0.01	-0.01	–	–
$Wind^2$	–	–	–	-0.48	–	–	–
Time	–	–	–	-1.78	–	–	–
Vap_pres	–	–	–	0.09	–	–	–
Individual			–	–	–	–	–
	40.52	32.14					
	39.08	33.62					
	35.79	35.26					
	5.26	4.29					
	51.12	48.82					
	-9.44	-9.04					
	30.52	26.61					
	0.20	0.10					
	55.32	53.58					
	-1.15	-1.2					
	2.97	2.76					

APPENDIX D

CHAPTER 5 SUPPLEMENTAL DATA

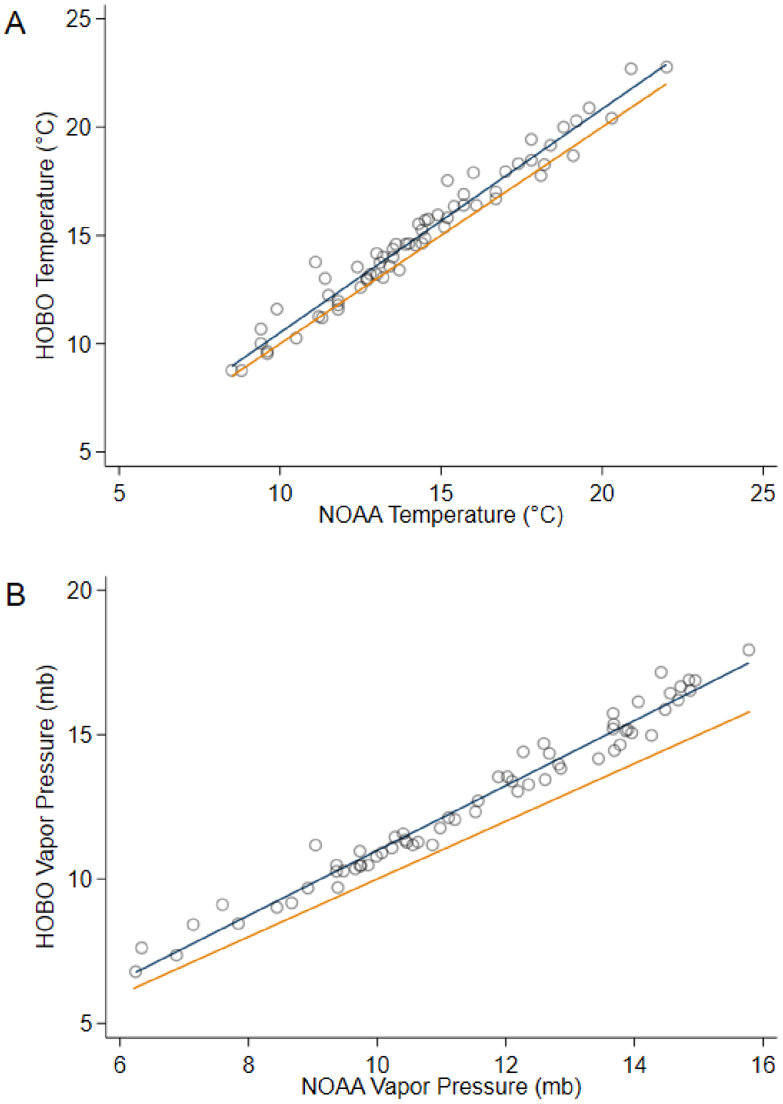

Figure D.1 Validation of the HOBO loggers at the NOAA weather station open meadow site at the Kenai Moose Research Center, Kenai Peninsula, Alaska, USA. A) Linear regression between HOBO measured ambient air temperature (°C) and NOAA weather station measured ambient air temperature (°C) plotted (circles). B) Linear regression between HOBO measured vapor pressure (mb) and NOAA weather station measured vapor pressure (mb) plotted (circles). Solid orange lines are 1:1 comparison between HOBO and NOAA measures.

Table D.1 Summary statistics of linear regression between flies (flies·24h-1) collected in a sticky trap versus a CO_2 baited light trap at the Kenai Moose Research Center, Kenai Peninsula, Alaska, USA, by fly group and habitat.

Fly Group	Habitat	R^2	*P*	Slope	Intercept
biting midges	bog	failed to run			
biting midges	black spruce	failed to run			
biting midges	early seral forest	0.002	0.764	-0.003 ± 0.009	0.020
biting midges	late seral forest	failed to run			
biting midges	weather station	failed to run			
mosquitoes	bog	0.087	0.032	0.029 ± 0.013	9.018
mosquitoes	black spruce	0.300	0.000	0.021 ± 0.003	13.076
mosquitoes	early seral forest	0.058	0.080	0.032 ± 0.018	10.024
mosquitoes	late seral forest	0.305	0.039	0.039 ± 0.008	6.120
mosquitoes	weather station	0.089	0.017	0.008 ± 0.003	1.756
biting house flies	bog	failed to run			
biting house flies	black spruce	failed to run			
biting house flies	early seral forest	0.001	0.811	-0.020 ± 0.082	0.020
biting house flies	late seral forest	0.001	0.847	-0.006 ± 0.033	0.019
biting house flies	weather station	failed to run			
coprophagous flies	bog	0.001	0.826	0.066 ± 0.299	2.022
coprophagous flies	black spruce	0.001	0.731	0.008 ± 0.023	1.527
coprophagous flies	early seral forest	0.051	0.100	0.342 ± 0.204	2.403
coprophagous flies	late seral forest	0.325	0.000	1.316 ± 0.263	0.638
coprophagous flies	weather station	0.030	0.167	0.110 ± 0.079	0.892
snipe flies	bog	0.548	0.000	0.336 ± 0.043	0.068
snipe flies	black spruce	0.000	0.891	-0.062 ± 0.454	0.277
snipe flies	early seral forest	0.170	0.002	0.145 ± 0.044	0.084
snipe flies	late seral forest	failed to run			
snipe flies	weather station	0.580	0.000	4.047 ± 0.437	-0.686
black flies	bog	0.223	0.000	0.339 ± 0.088	4.918
black flies	black spruce	0.213	0.000	0.091 ± 0.018	6.484
black flies	early seral forest	0.396	0.000	0.502 ± 0.086	3.302
black flies	late seral forest	0.032	0.194	0.028 ± 0.021	2.981

Table D.1 Continued

Fly Group	Habitat	R^2	*P*	Slope	Intercept
black flies	weather station	0.614	0.000	0.325 ± 0.033	17.898
horse & deer flies	bog	0.525	0.000	3.454 ± 0.460	4.611
horse & deer flies	black spruce	0.278	0.000	0.722 ± 0.120	1.958
horse & deer flies	early seral forest	0.536	0.000	2.027 ± 0.261	2.186
horse & deer flies	late seral forest	0.178	0.001	0.665 ± 0.198	2.256
horse & deer flies	weather station	0.851	0.000	1.771 ± 0.094	2.111

Table D.2 Summary statistics of model selection using AIC (lowest AIC units within 2 AIC units of the top model) to select the best* mixed effects regression of vapor pressure (VP), ambient air temperature (T), Julian day, year, habitat, and the interaction between habitat and each of these variables on all flies, mosquitoes, and black flies at the Kenai Moose Research Center, Kenai Peninsula, Alaska, USA. Site and Julian day were used as random effects to account for repeated measures of dependent variables. ΔAIC = difference between model AIC and lowest AIC in the model set. ω = Akaike model weight. k = number of estimable parameters. Deviance = measure of model fit.

no.	Model	ΔAIC	ω	*k*	Deviance
All Flies					
1*	VP^3 + T^3+ julian^3 + year + habitat + habitat w/ VP + habitat w/T + habitat w/julian	0.00	0.48	11	636.28
2	VP + T + julian + year + habitat + habitat w/ VP + habitat w/T + habitat w/julian			11	
3	VP + T + julian + year + habitat + habitat w/T + habitat w/julian	121.42	0.00	10	777.70
4	VP + T + julian + year + habitat + habitat w/julian			9	
5	VP + T + julian + year + habitat	131.88	0.00	8	804.15
6	T + julian + year + habitat			7	
7	julian + year + habitat			6	
8	year + habitat	182.64	0.00	5	860.92
9	habitat			4	
10	VP + T + julian + year	151.39	0.00	7	831.67
11	VP + T + julian + year + habitat	131.88	0.00	8	804.15
12	VP^3 + T^3 + julian^3 + year + habitat + habitat w/ VP + habitat w/T + habitat w/julian	0.12	0.45	11	642.40
13	VP^2 + T^3 + julian^3 + year + habitat + habitat w/ VP + habitat w/T + habitat w/julian	3.95	0.07	11	-321.11
14	VP + T^3 + julian^3 + year + habitat + habitat w/ VP + habitat w/T + habitat w/julian			11	
15	VP^3 + T^2 + julian^3 + year + habitat + habitat w/ VP + habitat w/T + habitat w/julian	16.57	0.00	11	654.85
16	VP^3 + T + julian^3 + year + habitat + habitat w/ VP + habitat w/T + habitat w/julian	20.10	0.00	11	660.38
17	VP^3 + T^3 + julian^2 + year + habitat + habitat w/ VP + habitat w/T + habitat w/julian	9.80	0.00	11	648.08
18	VP^3 + T^3 + julian + year + habitat + habitat w/ VP + habitat w/T + habitat w/julian	19.68	0.00	11	659.96
19	VP^3 + T + julian + year + habitat + habitat w/ VP + habitat w/T + habitat w/julian	67.05	0.00	11	711.32
20	Null	261.09	0.00	3	949.37

Table D.2 Continued

no.	Model	ΔAIC	ω	k	Deviance
Mosquitoes					
1*	VP^3+ T^3 + $julian^3$ + year + habitat + habitat w/ VP + habitat w/T	0.00	1.00	10	744.48
2	VP + T + julian + year + habitat + habitat w/ VP + habitat w/T	142.72	0.00	10	907.20
3	VP + T + julian + year + habitat + habitat w/ VP + habitat w/T + habitat w/julian	154.07	0.00	11	902.55
4	T + julian + year + habitat + habitat w/ VP + habitat w/T	142.72	0.00	9	907.20
5	julian + year + habitat + habitat w/ VP + habitat w/T	142.72	0.00	8	907.20
6	julian + habitat + habitat w/ VP + habitat w/T	212.73	0.00	7	971.21
7	year + habitat + habitat w/ VP + habitat w/T	183.52	0.00	7	942.00
8	julian + year + habitat w/ VP + habitat w/T	151.25	0.00	7	915.73
9	julian + year + habitat + habitat w/T	224.62	0.00	7	991.09
10	julian + year + habitat	247.10	0.00	6	1031.58
11	VP + T + habitat + habitat w/ VP + habitat w/T			8	
12	T + habitat + habitat w/ VP + habitat w/T			7	
13	$julian^2$ + year + habitat + habitat w/ VP + habitat w/T	60.90	0.00	8	815.37
14	$julian^3$ + year + habitat + habitat w/ VP + habitat w/T	43.51	0.00	8	795.99
15	$julian^3$ + $year^2$ + habitat + habitat w/ VP + habitat w/T	43.51	0.00	8	795.99
16	$julian^3$ + $year^3$ + habitat + habitat w/ VP + habitat w/T	43.51	0.00	8	795.99
17	Null	330.16	0.00	3	1118.64
Black Flies					
1*	VP + T^3 + $julian^3$ + year + habitat + habitat w/ VP + habitat w/T + habitat w/julian	0.00	0.69	11	840.84
2	VP + T + julian + year + habitat + habitat w/ VP + habitat w/T + habitat w/julian	67.58	0.00	11	916.43
3	T + julian + year + habitat + habitat w/ VP + habitat w/T + habitat w/julian	67.58	0.00	10	916.43
4	julian + year + habitat + habitat w/ VP + habitat w/T + habitat w/julian	67.58	0.00	9	916.43
5	julian + habitat + habitat w/ VP + habitat w/T + habitat w/julian	93.08	0.00	8	943.92

Table D.2 Continued

no.	Model	ΔAIC	ω	k	Deviance
6	year + habitat + habitat w/ VP + habitat w/T + habitat w/julian				
7	julian + year + habitat w/ VP + habitat w/T + habitat w/julian	83.47	0.00	8	940.31
8	julian + year + habitat + habitat w/T + habitat w/julian	72.26	0.00	8	931.11
9	julian + year + habitat + habitat w/VP + habitat w/julian	84.09	0.00	8	942.93
10	julian + year + habitat + habitat w/VP + habitat w/T	76.84	0.00	8	933.68
11	julian^2 + year + habitat + habitat w/ VP + habitat w/T + habitat w/julian	59.46	0.00	8	906.30
12	julian^3 + year + habitat + habitat w/ VP + habitat w/T + habitat w/julian	31.44	0.00	9	876.29
13	VP^3 + T^3 + julian^3 + year + habitat + habitat w/ VP + habitat w/T + habitat w/julian	1.61	0.31	9	838.45
14	Null	168.12	0.00	3	1056.97

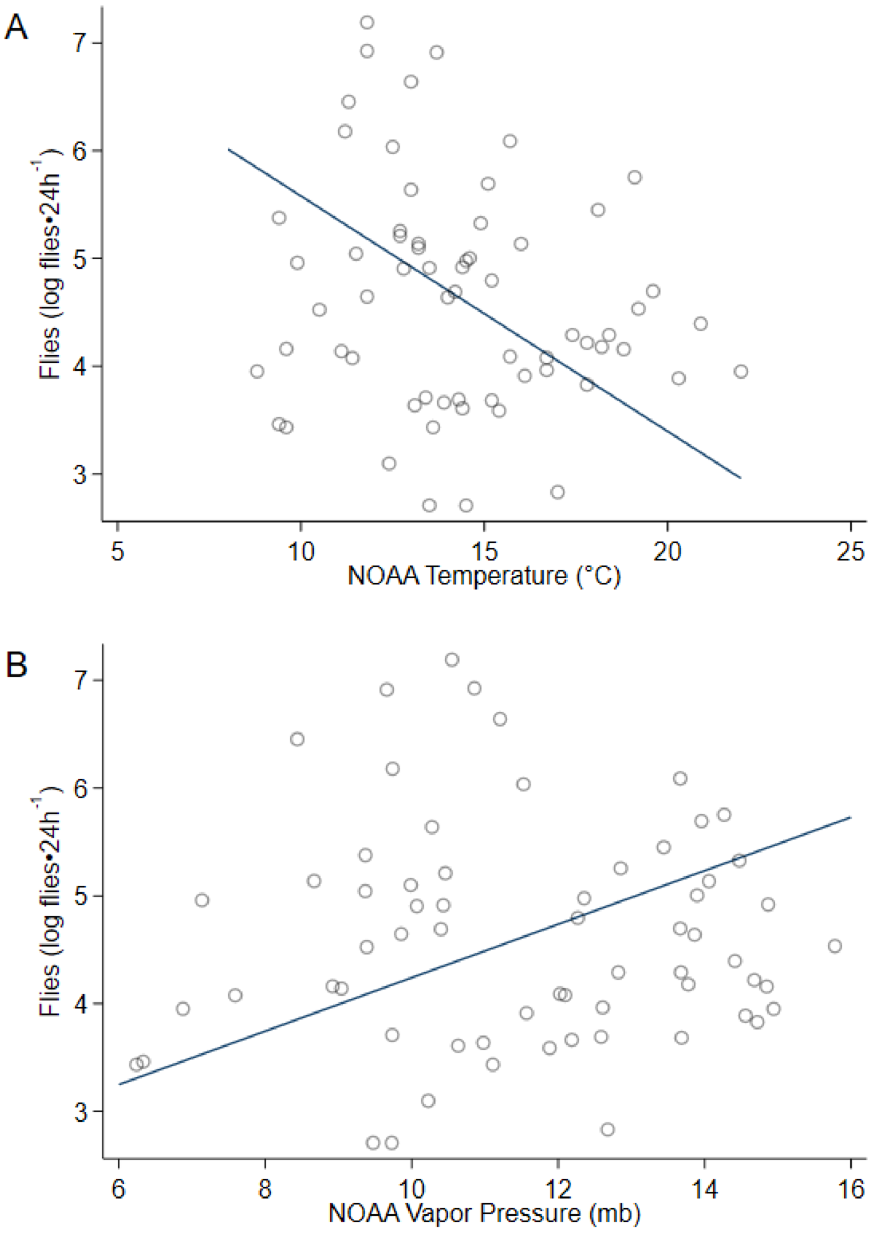

Figure D.2 Marginal predictions of ambient air temperature (°C; C) and vapor pressure (mb; D) on flies (log flies·24h^{-1}) at the NOAA weather station open meadow site at the Kenai Moose Research Center, Kenai Peninsula, Alaska, USA, based on mixed-effects regression.

Table D.3 Results for the top mixed model regression of movement rates (dependent variable) of moose at the Kenai Moose Research Center, Kenai Peninsula, Alaska, USA, with standardized beta coefficients of fixed effects. Individual moose and Julian day were included as random effects to account for repeated measures of dependent variables. Asterisks denote significant effects (P<0.05).

Parameters and main effects	
Observations	99677
X^2 [df]	344.17 [6]
Intercept	-0.40
Age of moose	-7.59*
Julian day	2.32
Julian day^2	-0.01*
Time of day	1.95*
Time of day^2	-0.02*
Time of day^3	0.00*

Table D.4 Results for the top mixed model regression of average daily movement rates (dependent variable) of moose at the Kenai Moose Research Center, Kenai Peninsula, Alaska, USA, with standardized beta coefficients of fixed effects. Individual moose and Julian day were included as random effects to account for repeated measures of dependent variables. Asterisks denote significant effects (P<0.05).

Parameters and main effects	
Observations	1044
X^2 [df]	220.99 [6]
Intercept	111.52
Age of moose	-7.08*
Julian day	-0.38*
Average flies experienced	37.27*
Average vegetation experienced	-0.23*
Average ambient air temperature	10.29
Interaction between average flies and ambient air temperature	-1.96*

Table D.5 Results for the top mixed model regressions for time spent by moose in bog, black spruce, early seral forest, and late seral forest habitats at the Kenai Moose Research Center, Kenai Peninsula, Alaska, USA, with standardized beta coefficients of fixed effects. Individual moose and Julian day were included as random effects to account for repeated measures of dependent variables. Asterisks denote significant effects (P<0.05).

Parameters and main effects	Dependent Variable (Y)			
	Bog	Black Spruce	Early Seral Forest	Late Seral Forest
Observations	1044	1044	1044	1044
X^2 [df]	19.58 [4]	1669.93 [9]	511.33 [9]	56.77 [5]
Intercept	-277.91	-23944.25	-6946.80	2121.61
Age of moose	-1.77*	18.37*	-22.88*	-9.25*
Age of moose^2	—	-0.88*	1.45*	—
Julian day	—	-24.41*	46.36*	-32.60*
Julian day^2	—	0.07*	-0.13*	0.09*
Average flies experienced	49.79*	13358.49*	1015.92*	179.48*
Average flies experienced^2	—	-2271.68*	-76.45*	—
Average ambient air temperature	30.28*	126.56*	220.91*	14.23*
Average ambient air temperature^2	—	-74.71*	-4.84*	—
Interaction between average flies and ambient air temperature	-4.52*	18.30*	-23.92*	—