

A retrospective analysis of the Vancouver Island marmot (*Marmota vancouverensis*)
conservation breeding and release program: Conservation physiology and hibernation

by

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Abstract

The Vancouver Island marmot (*Marmota vancouverensis*) is an endangered mammal whose population is being recovered with a conservation breeding and release program. This involves management both *in* and *ex situ*, so it is vital to understand how the different environments are affecting the populations to maximize program outcomes. The purpose of this thesis was to retrospectively analyze data collected from this program to address questions relevant to population recovery. The first chapter compared the physiology of the managed populations using the neutrophil: lymphocyte ratio and found significant differences in this metric among targeted comparisons. The second chapter assessed factors that may be contributing to hibernation-related mortality of marmots during their first winter post-release. I found a combination of environmental and intrinsic factors influencing this mortality. Overall, this thesis contributes to a growing body of literature surrounding the efficacy of conservation breeding and release programs to recover critically endangered species.

Keywords

Vancouver Island marmot, *Marmota vancouverensis*, conservation physiology, neutrophil: lymphocyte ratio, hibernation, conservation breeding

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General thesis introduction

With an increasing number of species facing the prospect of extinction, reintroduction biology has gained traction as a viable option to combat species declines (Kleiman, 1989; Seddon *et al.*, 2007; Lloyd *et al.*, 2019). Zoos and aquariums are recognized for their role in reintroduction or release programs for their capacity as conservation breeding and housing facilities (Tribe and Booth, 2003; Bowkett, 2009). There have been notable conservation successes involving the release of captive bred animals; for example, golden lion tamarins (*Leontopithecus rosalia* Kierulff *et al.*, 2012), black-footed ferrets (Jachowski and Lockhart, 2009), and California condors (*Gymnogyps californianus* Woods and Heinrich, 2007). However, releases for conservation remain highly contested because of high costs and logistical problems associated with the approach, limited establishment of released individuals, and inadequate monitoring resulting in poor definitions of success (Griffith *et al.*, 1989; Seddon, 1999; Synder *et al.*, 2008; Taylor *et al.*, 2017) Further research within the field of reintroduction biology is needed to ensure the continued success of these programs.

Stress in released animals is a key area of research pertinent to reintroduction biology. After the preliminary “fast acting” response of the sympathetic nervous system which involves the release of catecholamines, there is a slower acting secondary response that equips the animal to physiologically and behaviourally cope with a stressor (Lattin and Kelly, 2020). This process, known commonly as the stress response, is directed by the hypothalamus-pituitary-adrenal (HPA) axis whose activation results in the release of adrenocorticotrophic hormone (ACTH). ACTH then promotes the secretion of

glucocorticoids from the adrenal cortex (Tsigos and Chrousos, 2002; Denver, 2009). The two primary glucocorticoids are corticosterone, predominantly found in birds, amphibians, reptiles, and some rodents, and cortisol, dominant in fish and mammals (Palme, 2019). For the purposes of this thesis, “stress” will refer to the level of HPA-activation, “stress response” refers to an increase in HPA-axis activation measured by an appropriate stress metric, and “stressor” refers to a stimulus that induces a response from the individual (McEwen and Wingfield, 2003; Romero, 2004; Johnstone *et al.*, 2012).

Once the glucocorticoids (GCs) are released from the adrenal cortex, they facilitate a myriad of physiological effects to help the individual manage the stressor such as increasing blood pressure and altering blood parameters, accelerating metabolism, and suppressing immune function (Sapolsky *et al.*, 2000; Von Der Ohe and Servheen, 2002; Bjelaković *et al.*, 2010). Although there are diverse effects, the primary consequence of secreted GCs is an increase in energy readily available to the individual through processes such as gluconeogenesis and protein and fat metabolism (Von Der Ohe and Servheen, 2002; Reeder and Kramer, 2005). Measuring the concentration of GCs is the most common metric to quantify HPA axis activity or a stress response, however as GCs affect other systems in the body, metrics such as total lymphocyte counts, the neutrophil: lymphocyte ratio, stress proteins, immune function, and regenerative anemia may also be used (Johnstone *et al.*, 2012).

HPA axis activity and the mobilization of energy stores is part of normal function and is essential for processes such as the development of organ systems, regulating the

microbiome, and influencing behaviours such as food seeking and maternal care (Reeder and Kramer, 2005; Denver, 2009; Almanza-Sepulveda *et al.*, 2020; Williams *et al.*, 2020). However, prolonged activation of the HPA axis – referred to as long term or chronic stress (Johnstone *et al.*, 2012), can lead to a range of deleterious effects to an individual (Carlstead and Shepherdson, 2009).

The sources and consequences of chronic stress have been well studied in captive animals (Morgan and Tromborg, 2007). Stressors for captive animals can range from anthropogenic noise, high visitor traffic, and artificial housing conditions (Morgan and Tromborg, 2007; Webster *et al.*, 2017; Fischer and Romero, 2019; Marino *et al.*, 2019). Chronic exposure to these stressors can promote stereotypic behaviour, increase aggression, reduce reproductive rates, and influence mortality (Carlstead and Brown, 2005; Malmkvist *et al.*, 2011; Lind *et al.*, 2019; Marino *et al.*, 2019; Bastian *et al.*, 2020). While these effects can have negative consequences on individuals, most institutions that house captive animals are also on the forefront of combating these effects. This includes implementing techniques such as environmental enrichment, feeding systems that encourage foraging behavior, and positive reinforcement training regiments (Carlstead and Shepherdson, 2009; Shepherdson *et al.*, 2013; Joyce-Zuniga *et al.*, 2016).

Although stress in captive animals has been thoroughly investigated, there is less literature available on understanding and quantifying stressors that are unique to animals in reintroduction or release programs. Animals released from conservation breeding programs experience stressors such as trapping and handling, transportation, release

protocols, and introductions to novel environments (Teixeira *et al.*, 2007; Dickens *et al.*, 2010). Undue stress of animals in release programs can influence management decisions that ultimately have economic and logistical consequences (Teixeira *et al.*, 2007; De Milliano *et al.*, 2016; Batson *et al.*, 2017). For example, research using GCs has encouraged the use of “soft-release” protocols in some programs designed to mitigate a stress response prior to release (Teixeira *et al.*, 2007; Lèche *et al.*, 2016). It is therefore crucial that more research is done in the area of stress in released animals.

A stress response in conservation breeding and release programs has been attributed to altered behaviour post-release (Zidon *et al.*, 2009), as well as delayed acclimatization to the novel environment (Franceschini *et al.*, 2008; Jachowski *et al.*, 2013). While these metrics can certainly contribute to the success of a program, the most common measure of success is post-release survival. To that effect, studies that have experimentally implemented soft-release protocols have found that they helped regulate stress of animals post-release, and may have contributed to increased survival rates (Letty *et al.*, 2000; Lèche *et al.*, 2016). Furthermore, a synthesis of 41 studies that used pre-conditioning techniques designed to mitigate a stress response such as anti-predator training, soft-releases, and environmental enrichment determined that animals that underwent pre-conditioning were 1.55 times more likely to survive than their unconditioned counterparts (Tetzlaff *et al.*, 2019). While these studies suggest a relationship between stress and survival, it is important to note that few have directly quantified the physiological stress of animals using a stress metric and compared it to survivorship post-release, which

indicates a crucial next step in conservation breeding and release research (Letty *et al.*, 2000; Teixeira *et al.*, 2007; Lèche *et al.*, 2016; Batson *et al.*, 2017).

The recovery of the Vancouver Island marmot (*Marmota vancouverensis*) is an example of a conservation breeding program that involves release of individuals from captivity to supplement wild populations. The Vancouver Island marmot (VIM) is a colonial burrowing rodent endemic to subalpine habitats on Vancouver Island (Nagorsen *et al.*, 1996). VIMs were first listed as endangered by COSEWIC in 1978 (COSEWIC, 2019), however the VIM population declined significantly throughout the 1990's with as few as 105 counted in the wild in 1996 (McAdie, 2004). The recovery plan was therefore updated and concluded that the target population of 400-600 individuals would not be accomplished without supplementation from a conservation breeding program (McAdie, 2004). Consequently, VIMs were transported from the wild to establish a conservation breeding program at the Toronto Zoo in 1997, followed by the Calgary Zoo in 1998 (McAdie, 2004; Vancouver Island Marmot Recovery Team, 2017). Additionally the Mountain View Conservation and Breeding Society, and the Tony Barrett Mount Washington Marmot Recovery Centre have bred VIMs since the program's inception (McAdie, 2004). Over 500 VIMs have been released since 2003, and the current population is estimated to be over 200 individuals (Marmot Recovery Foundation, 2019). While this growth is certainly encouraging, there is still a need for further research to ensure wild meta-populations can be maintained without assistance from an *ex situ* program (Bryant and Page, 2005; Jackson *et al.*, 2016; Vancouver Island Marmot Recovery Team, 2017).

To date, there are few published studies on the stress physiology of VIMs. Hair cortisol concentrations were found to vary based on body region in a study of individuals at the Toronto Zoo (Acker *et al.*, 2018). Further analysis of hair cortisol levels from this thesis suggested differences in the stress response of newly released and established VIMs, such that newly released VIMs had significantly higher hair cortisol levels (Acker, 2017). A study evaluating a “stepping-stone” release approach, where individuals are introduced to Mt. Washington for a year to acclimatize before being translocated to more remote sites, implied that the success of this strategy could have been due in part to acclimatization from a stress response from introduction to a novel environment (Lloyd *et al.*, 2019). However, this study did not use a physiological measurement of stress. In addition, there has yet to be a study that compares the stress physiology of VIMs in different environments in the conservation breeding program. This is important, as the program involves the movement of individuals among facilities, wild sites, and between *ex situ* and *in situ* environments. It would be relevant to know if the physiology is conserved among these environments, or if there are changes that occur after translocations.

Hibernation of VIMs likewise remains a key area of interest for research in this program (Bryant and McAdie, 2003; Jackson *et al.*, 2016; Aymen *et al.*, 2021). In particular, it was identified that newly released VIMs have higher than expected hibernation-related mortalities in their first year post-release. A 2016 study found that overwinter survival in this group can be as low as 40% in some years (Jackson *et al.*,

2016). Interestingly, overwinter survival of newly released individuals in subsequent winters is comparable to wild VIMs (Jackson *et al.*, 2016), suggesting acclimation after one year. Given that there were significant differences in hair cortisol between newly-released and established VIMs, it is possible a stress response is contributing to these hibernation-related mortalities in the first year (Jackson *et al.*, 2016; Acker, 2017). This inference is supported by evidence from the closely related yellow-bellied marmot (*Marmota flaviventris*), where higher fecal glucocorticoid metabolites were associated with lower overwinter survival (Wey *et al.*, 2015). In addition to a physiological mechanism, there has yet to be a study investigating other factors that may be contributing to hibernation-related mortality, including climate variables and site-specific characteristics. More research is needed to address the gap in knowledge in what is contributing to these mortalities to maximize the survival of released individuals.

Quantifying stress

While there is a greater need for studies that physiologically quantify stress for released animals, it is also important that these studies are using appropriate metrics to have a comprehensive picture of stress in the animals studied. The extensive use of GCs as a stress metric has contentiously earned them a reputation of being “stress hormones”, whereby it is often accepted that the stress of an animal equates to GC concentrations (Johnstone *et al.*, 2012; MacDougall-Shackleton *et al.*, 2019). This conclusion has been contested by researchers who propose that because GC concentrations are only one aspect of the complex response, they are not necessarily a complete measure of physiological stress (Breuner *et al.*, 2013), and furthermore complexities in the timing of the release of

GCs lead to challenges in interpreting concentration measurements (Von Der Ohe and Servheen, 2002; Johnstone *et al.*, 2012; Davis and Maney, 2018). Additionally, animal care managers may be interested in the clinical significance of physiological reactions to a stress response, as that has the greatest implication for animal health. Therefore, the use of other metrics is recommended to have a better understanding of stress in animals.

One metric that is being increasingly used in stress research is the comparison of the proportion of neutrophils (or heterophils in birds and reptiles) and lymphocytes in blood plasma, otherwise known as the neutrophil: lymphocyte ratio (or heterophil: lymphocyte ratio; henceforth H/N:L ratio; Widowski *et al.*, 1989; Davis *et al.*, 2008). Neutrophils and heterophils are the primary phagocytic white-blood cell, and while found in different species they are functionally the same in terms of their role and physiological response (Davis *et al.*, 2008). Lymphocytes have a variety of roles, particularly in the creation of immunoglobulin (Davis *et al.*, 2008). While these white blood cells have a role in immunity, research has also validated their applicability to stress physiology. Exposure to a stressor has been shown to reduce total numbers of lymphocytes circulating in the blood through sequestration in tissues (Keller *et al.*, 1981; Dhabhar *et al.*, 1996) while increasing neutrophils (Jain and Stevenson, 1991; Dhabhar *et al.*, 1994). Thus, an increase in the N:L ratio is indicative of a stress response in the individual. The N:L ratio is an example of a physiological consequence to stress, as it is triggered by the release of glucocorticoids in vertebrates (Davis and Maney, 2018). An increase in N:L ratio in response to stressors is documented in domestic animals such as horses (Snow *et al.*, 1983; Stull and Rodiek, 2000; Cywińska *et al.*, 2010), pigs (Cooper *et al.*, 2009) and

cattle (Anderson *et al.*, 1999; Lomborg *et al.*, 2008; Lynch *et al.*, 2010) as well as common species used in laboratory research such as rodents (mice and rats; Dhabhar *et al.*, 1994; Swan and Hickman, 2014; Hickman, 2017), and non-human primates (Kim *et al.*, 2005; Schapiro *et al.*, 2012; Lee *et al.*, 2013). The N:L ratio has also been used to document stress in wild populations (Vera *et al.*, 2013; Książek *et al.*, 2017; Carbillet *et al.*, 2019).

There are several advantages to using the N:L ratio as a stress metric. First, compared to GCs which can increase within minutes of encountering a stressor (Romero and Reed, 2005), the N:L ratio is considered a “downstream” response, and can be detected hours and even days after exposure (Davis *et al.*, 2008; Martin, 2009; Breuner *et al.*, 2013). A delayed increase in the N:L (or H:L) ratio post-stressor has been documented in birds (Gross, 1990; Davis, 2005; Buehler *et al.*, 2008), reptiles and amphibians (Aguirre *et al.*, 1995; DuRant *et al.*, 2015), and in mammals (Burguez *et al.*, 1983; Bonacic *et al.*, 2003; Montané *et al.*, 2003; López-Olvera *et al.*, 2007; Vera *et al.*, 2011). Although this delay is species specific, typically the H/N:L ratio does not show increases for at least an hour post-stressor. Therefore samples taken some time after capture or handling could still be indicative of baseline HPA axis activity, unlike GCs, which may be confounded during these processes (Romero, 2004; Davis and Maney, 2018).

Second, the N:L ratio can be used as a metric of chronic stress as the response is more enduring than that of GCs (Davis *et al.*, 2008; Goessling *et al.*, 2015; Hickman, 2017). In a study by Swan and Hickman (2014) exposure to several stressors over 7 days

(simulating chronic stress) did not elevate serum corticosterone concentrations in rats, however the N:L ratio was significantly higher. The attenuation of GCs with elevated N:L ratios have been found in a variety of taxa using experimental methods (McFarlane and Curtis, 1989; Love *et al.*, 2003; Bilandžić *et al.*, 2006; Vera *et al.*, 2011), however few studies have used field based approaches to validate this phenomenon in wild populations, with the noteworthy exception of Muller *et al.* (2011) with their study of Eurasian kestrels (*Falco tinnuculus*).

Finally, there is increasing evidence that the N:L ratio has predictive value for survival. Often used in biomedical research to predict survival in humans (Shimada *et al.*, 2010; Chua *et al.*, 2011; Proctor *et al.*, 2012; Diao *et al.*, 2019), applications to wildlife biology have found the H:L ratio to be a reliable predictor of survival in various species of bird (Lobato *et al.*, 2005; Hylton *et al.*, 2006; Kilgas *et al.*, 2006). In captive chimpanzees (*Pan troglodytes*) the N:L ratio was a predictor of longevity and is being used as a diagnostic tool for overall health (Webb *et al.*, 2020). While still a burgeoning area of research, it is evident there is utility for this metric to predict survival in other species.

While not to discredit studies using GC concentrations, an important trend elucidated from the review of multiple studies using the two metrics by Davis and Maney (2018) is that the N:L ratio is not always correlated with GC levels. In fact, no correlation between the metrics was found in 65% of the 23 studies evaluated (Davis and Maney, 2018). This may be because they are representative of two different aspects of the vertebrate stress

response. As GC's are fast acting and short lived, they can be considered the 'messenger' of the stress response, while the slower responding N:L ratio is the 'response' (Davis and Maney, 2018). Therefore, while both metrics have value, they are not interchangeable. More studies using the N:L ratio can give insights into the physiological consequences of a stress response, in a way that studies using GCs cannot.

Thesis outline

The purpose of this research is to provide an analysis of the N:L ratio as a proxy of HPA axis activity in VIMs in different environments in the conservation breeding and release program and elucidate what factors are contributing to hibernation-related mortality in the first year post-release. This will be done in two separate approaches. Chapter 1 will compare the N:L ratio of VIMs in different target environments including among captive, released and wild populations, as well as among care facilities and wild meta-populations. Factors contributing to variations in the N:L ratio for each of the target populations will also be compared. The blood samples used for this analysis have been routinely collected since 1992 and therefore provide a robust sampling of individuals from a historical perspective.

In Chapter 2, factors that may be influencing first year hibernation-related mortality will be investigated, including an analysis of the N:L ratio at release. Other external factors such as release region, colony size at release and winter weather will also be assessed to determine their influence on first year hibernation-related mortality. Hibernation-related mortality is a limitation in the success of the VIM conservation

breeding program. By understanding factors that may be contributing to first year hibernation-related mortality in VIM, we can begin to address this limitation, and start working towards management solutions to increase survival rates of newly released marmots, and therefore contribute to the ongoing success of the program.

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Chapter One: Investigating the neutrophil to lymphocyte ratio in the Vancouver Island marmot (*Marmota vancouverensis*): A retrospective analysis

Abstract

Conservation breeding programs typically involve the management of individuals both *in* and *ex situ*, so it is vital to understand how the physiology of managed species changes in these environments to maximize program outcomes. The Vancouver Island marmot (VIM; *Marmota vancouverensis*) is one species that has been managed in a conservation breeding program to recover the critically low wild population. Although releases of captive-bred individuals have increased the population size within their natural habitat, research in the program found that captive-bred individuals have lower chances of survival than wild-born conspecifics. This study considered this difference in survival from the perspective of stress physiology. Using over 1000 blood samples collected since the program's inception, the neutrophil to lymphocyte (N:L) ratio was used as a proxy of HPA axis activity and compared between captive, pre-release, post-release and wild populations. *In situ* VIM populations were found to have a significantly higher N:L ratio than *ex situ* populations, suggesting that the wild is a more physiologically challenging environment than managed care. Moreover, the effect of age, sex and the month of sampling on the N:L ratio were found to be different for each population. Age had the greatest magnitude of effect in the wild population, and sex was only significant in the *ex situ* populations. Although this study provided previously unknown insights into the physiology of VIMs, increased post-release monitoring will be useful in the future to fully understand how physiology may be contributing to lower survival of captive-bred individuals.

Introduction

It is vital that conservation initiatives use scientific evidence not only to develop management strategies, but to monitor the success of the programs. Physiological studies are useful in conservation because they can provide cause and effect evidence of situations that are relevant to the program (Wikelski and Cooke, 2006; Cooke *et al.*, 2013). Conservation physiology as a discipline was introduced in 2006 and has been applied in a variety of contexts including evaluating anthropogenic disturbances and ecosystem management, however there is the opportunity to expand research within the field (Cooke *et al.*, 2013, 2020). A key area where conservation physiology can be applied is in conservation breeding programs, especially those that involve the release of bred animals (Cooke *et al.*, 2020). As these programs involve the management of species both *in* and *ex situ*, understanding changes in physiology in managed animals can ultimately increase the effectiveness of these programs.

The conservation breeding program for the Vancouver Island marmot (*Marmota vancouverensis*) could benefit from a conservation physiology approach. The Vancouver Island marmot (VIM) is a social, burrowing rodent endemic to Vancouver Island, British Columbia (COSEWIC, 2019). Although the VIM population grew throughout the 80's due to increased reproduction in newly logged habitats, over-predation in these habitats led to dramatic declines throughout the 90's and early 2000's (COSEWIC, 2019). In response to detrimentally low population numbers, the recovery plan for the VIM was updated to include supplementation from a conservation breeding program (McAdie, 2004). Consequently, four facilities began breeding VIMs with the intent of restoring populations in the wild. 540 VIMs have been released from the conservation breeding

program to Vancouver Island since 2003, and the current population is estimated to be just over 200 individuals at 20 colonies (Jackson *et al.*, 2020). While there has been population growth, the wild population is not yet considered stable and is still highly dependent on supplementation from the conservation breeding program (COSEWIC, 2019).

Research involving VIMs has focused on reproductive physiology (Bryant, 2005; Casimir *et al.*, 2007; Keeley *et al.*, 2012), the evolutionary genetics of the species (Armitage, 2000; Cardini *et al.*, 2007; Kruckenhauser *et al.*, 2009; Kerhoulas *et al.*, 2015) and behavioural studies (Blumstein *et al.*, 2001; Werner, 2017). Recently, there has been an emphasis on studies that monitor the survival of released VIMs to evaluate the success of the program (Bryant and Page, 2005; Aaltonen *et al.*, 2009; Jackson *et al.*, 2016; Lloyd *et al.*, 2019). There has been less published research on VIM physiology, and specifically studies that compare physiological processes among captive, released, and wild VIMs.

Stress physiology is an area that may be of particular interest to the management and release of VIMs. For vertebrates, a stress response is coordinated by the hypothalamus – pituitary – adrenal (HPA) axis, where stimulation of this axis ultimately results in the secretion of glucocorticoids (GCs) whose diverse effects on the body allow the individual to cope with the stressor (Denver, 2009). While the secretion of GCs is part of normal functioning, prolonged secretion (known as chronic stress) can have negative consequences on the individual (Johnstone *et al.*, 2012; Lattin and Kelly, 2020). In an effort to increase transparency and circumvent possible miscommunications of results, for

the purposes of this research we define “stress” as the level of HPA activity, a “stress response” as a measurable increase in HPA activation, quantified by an appropriate physiological metric, and a “stressor” as a stimulus that increases HPA axis activity (Johnstone *et al.*, 2012).

It is important to consider the activity of the HPA axis of animals in conservation breeding programs because they are exposed to unique stressors such as transportation and relocation to a novel environment (Teixeira *et al.*, 2007; Gelling *et al.*, 2010; Taylor *et al.*, 2017; Fischer and Romero, 2019). Moreover, because animals are maintained in *in* and *ex situ* environments and can be moved between these environments, they are exposed to different stressors which may influence how the HPA axis is functioning. Chronic stress in released animals can negatively impact their behaviour and survival post-release (Kelly *et al.*, 2008; Batson *et al.*, 2017; Lopes *et al.*, 2017). This can ultimately influence the management of conservation breeding and release programs, such as integrating soft-release protocols (Letty *et al.*, 2000; Reading *et al.*, 2013; Tetzlaff *et al.*, 2019).

Thus far, the only published study to assess HPA axis activity in VIMs determined that there was a significant effect of season and body region on hair cortisol measurements (Acker *et al.*, 2018). Further research found significantly elevated hair cortisol in newly released VIMs compared to wild or captive conspecifics (Acker, 2017) and higher hair cortisol in wild individuals compared to captive (Acker, 2017). While GCs are the most predominantly used metric to measure stress or a stress response, there are other metrics

that can be used (Johnstone *et al.*, 2012). In fact, many researchers are now supporting the use of metrics other than GCs to better understand stress (Dallman, 2003; Davis *et al.*, 2008; Müller *et al.*, 2011; Gelling *et al.*, 2012; Breuner *et al.*, 2013; MacDougall-Shackleton *et al.*, 2019). Specifically, there is concern over the quick response of GCs to a stressor, which may affect measurements because of handling and sampling procedures (Romero and Reed, 2005). In addition, the release of GCs is just one physiological reaction to a stressor and thus may not be indicative of the entire stress response, nor the physiological consequences of the response (MacDougall-Shackleton *et al.*, 2019). One metric that is becoming more prevalent in stress research is the relative proportion of neutrophils to lymphocytes in the blood, known as the N:L ratio (Davis *et al.*, 2008).

In response to a release of GCs, the number of circulating neutrophils in plasma increases while lymphocytes decrease due to sequestration in tissues, thus an increase in the N:L ratio is indicative of a greater stress response for an individual (Keller *et al.*, 1981; Dhabhar *et al.*, 1995; Davis *et al.*, 2008). The applications of the N:L ratio to stress physiology have been reviewed elsewhere (Davis *et al.*, 2008; Davis and Maney, 2018) and an increase in the N:L ratio in response to a stressor has been documented in a variety of taxa in both laboratory and field-based experiments (Vera *et al.*, 2013; Swan and Hickman, 2014; Książek *et al.*, 2017; Carbillet *et al.*, 2019). A key advantage to using the N:L ratio as a metric of stress, particularly for VIMs, is that an increase in the N:L ratio occurs hours to days after exposure to a stressor (Davis *et al.*, 2008; Martin, 2009; Breuner *et al.*, 2013). This circumvents issues of confounding stress measurements from trapping, handling, and sampling procedures which is common when using GCs (Davis

and Maney, 2018). This is particularly relevant for VIMs, as blood samples are routinely taken during other medical procedures such as surgeries to implant a small tracker pre-release, or annual medical check-ups (G. Mastromonaco, pers.comm. 2019). While there has yet to be a study investigating the response time of the N:L ratio in VIMs, typically this metric does not elevate for at least an hour post-stressor (Davis and Maney, 2018).

The purpose of this study was to investigate HPA axis activity of VIMs in the conservation breeding program using the N:L ratio. This study capitalized on amassed blood samples that have been routinely collected from individuals in the program since 1992. This provided robust sampling beyond those currently in the program. The objectives of the study were two-fold: first, to compare the N:L ratio of individuals in different environments in the program including captive, pre-release, post-release and wild populations. Moreover, comparisons were made within these populations to know if there was a difference among managed care facilities and wild meta-populations. The second objective was to determine what factors are contributing to variation in the N:L ratio in the four aforementioned populations. It was hypothesized that 1) the N:L ratio would vary between target populations such that captive individuals would have the highest N:L ratio as a result of artificial housing conditions, handling, and irregular social structures 2) there would not be a pronounced difference in the N:L ratio among captive facilities because animal care protocols are standardized and 3) factors contributing to variation in the N:L ratio for each population would vary due to the environment (e.g. captive vs. wild).

Methods

Sampling populations and locations

VIMs are considered a part of the captive population if they are born in captivity, or any wild VIM taken into captivity for breeding purposes that have remained in captivity for longer than one hibernation period (McAdie, 2018). The captive population of VIM are currently housed at three sites, The Toronto Zoo (TZ), Calgary Zoo (CZ), and the Tony Barrett Mt. Washington Marmot Recovery Centre (TBMWMRC; Carnio 2019). VIMs were bred at the Mountain View Conservation and Breeding Society (MVF) from 2000 until 2014. Blood samples are routinely collected from captive VIMs as part of annual health screens, pre-surgical or pre-shipment health screens.

VIMs are considered a part of the release population if they were born in captivity and released, or if they were born in the wild but spent at least one hibernation period in captivity and were then released back to the wild. This population is further subdivided into the pre-release and post-release populations, where the pre-release were VIMs temporarily housed at the TBMWMRC before release. Pre-release individuals could have housed at any of the other care facilities prior to release and may have only been housed at the TBMWMRC for a brief quarantine period. This population was treated as independent from the captive population as samples were taken from these individuals for specific purposes other than annual exams, the pre-release surgery and pre-release exam. The post-release population were released VIMs that were recaptured after release and sampled opportunistically during recapture or to replace the transmitter.

VIMs in the wild population were individuals born in the wild and had not spent extended time in captivity (McAdie, 2018). VIMs are being released to a number of sites within two primary areas - referred to as meta-populations - on Vancouver Island; Strathcona Provincial Park and Nanaimo Lakes (Aaltonen *et al.*, 2009; Vancouver Island Marmot Recovery Team, 2017). This analysis focused on the meta-population scale. Blood samples were opportunistically obtained from wild VIMs during recapture events or when transmitters were implanted or replaced. These blood draws typically happened in the field, and the individual was not taken into captivity.

Blood sample collection, storage and analysis

All samples used in this analysis were from clinically healthy marmots, which were defined as “marmots that do not display cardiovascular, respiratory, musculoskeletal, nervous, integumentary or urogenital abnormalities following an examination by a veterinarian” (McAdie, 2018). As outlined by McAdie (2018), whole blood samples were collected from the cephalic, saphenous, femoral or tarsal veins after immobilization using an intramuscular injection of ketamine hydrochloride and midazolam hydrochloride (10mg/kg and 0.25 mg/kg respectively). A portion of the blood collected was used to make two blood smears which were stained and dried at room temperature (or ambient temperature in field conditions), and stored in cool, dry, dark conditions (McAdie, 2018).

These blood smears were previously used to establish 16 reference values for the species including neutrophils, lymphocytes, monocytes, eosinophils, and basophils (See: McAdie, 2018). The N:L ratio was calculated using the total neutrophil count (segmented and banded), divided by the lymphocyte count (Hickman, 2017). For 50 samples

analyzed in 2020, the N:L ratio was calculated using the percentage neutrophil count divided by the percentage lymphocyte count.

Statistical analysis

All statistical analysis was conducted in R (R Core Team 2020). To compare the N:L ratio among target management groups, linear mixed models were created using the *lme4* package (Version 1.1.23; Bates *et al.*, 2015). The first model (referred to as the population model) compared the N:L ratio among the four populations: captive, pre-release, post-release, and wild. Three other models were created to compare individuals within these populations as follows: a model using only captive individuals was used to compare the four care facilities, a model using only wild individuals was used to compare the two meta-populations, and a model using individuals with multiple samples (referred to as the sampling model) was used to compare the purpose for the sample being taken. Because samples used in this study were taken opportunistically for a variety of reasons, the sampling model was created to determine whether variation in the N:L ratio was affected by the procedure in question (e.g., annual exam vs. transmitter surgery). For this model, samples were used from any individual who had at least two samples for different purposes in the program.

For each of the aforementioned models, the N:L ratio was log transformed to achieve normality and used as the response variable. Sex and age were evaluated as fixed effects. Additionally, the month the sample was collected was evaluated as a fixed effect to account for seasonal variation in haematological parameters. All blood samples were analyzed at one of four laboratories throughout the sampling period (IDEXX, Central

Veterinary Laboratory (CVL), Toronto Zoo lab, or Calgary Zoo lab). To control for differences in sampling technique, experience, and procedures between labs that may confound cell counts, the laboratory the sample was analyzed in was evaluated as a fixed effect. Finally, the year the sample was collected was evaluated as a random effect to account for unequal sampling effort between years, and a random effect of individual was evaluated to account for multiple samples from the same individuals. These effects were selected because of *a priori* hypotheses about their effects on the N:L ratio, however including all factors in some models sometimes led to problems with overfitting or multicollinearity. A full list of effects used in each model are listed in Table 1.1. See Appendix for a full list of excluded predictors and rationale.

Model visualization was done using the *visreg* package (version 2.6.1; Breheny and Burchett, 2017). To determine significant differences between the target categories displayed on the figures, an analysis of variance (ANOVA) test was performed on the model. The *emmeans* package (version 1.5.5; Lenth *et al.*, 2021) was used to calculate the estimated marginal means for the target effect (e.g, the population, the care facility etc.) and contrasted using a Tukey-honest significant difference test.

To compare the sources of variation in the N:L ratio among populations, a linear mixed model was created for each of the four populations. Each model used the log transformed N:L ratio as the response variable. The fixed effects in the model included sex, age and the month the sample was taken, and a random effect of year and a random effect of individual. Numeric predictors were scaled for comparison. For the model of the wild

population and the post-release population, including individual as a random effect led to overfitting in the model due to limited repeat samples from the same individuals.

However, a comparison between these models, and models that experimentally removed the random effect of individual by randomly selecting one sample per individual yielded no significant differences in outcome in terms of magnitude of effect or significance (see Appendix; Figure A1.1). Therefore, the random effect of individual was kept in the wild and post-release population models to compare to the other two populations. Models were visualized and compared using the *jtools* package (version 2.1.0; Long, 2020).

Results

A total of 1237 blood samples were analyzed from 509 marmots, collected between the years of 1992 and 2020. The number of samples per individual ranged from 1 to 15, with a mean of 2.43 ± 2.02 SD samples per individual. Of these 1237 samples, 739 were from captive individuals, 348 were from pre-release individuals, 24 were from post-release individuals and 126 were from wild individuals.

Comparing populations

There were significant differences in the N:L ratio among the populations [$F(3, 1097.94) = 54.81, p < 0.001$]. A Tukey-significant difference test of the estimated marginal means from the population model yielded significant differences in the N:L ratio between *ex situ* populations (captive and pre-release) and *in situ* populations (post-release and wild). The N:L ratio for captive individuals was significantly lower than post-release and wild ($p < 0.001$, respectively, Figure 1.1). Similarly, the N:L ratio was significantly lower for pre-release individuals compared to post-release and wild ($p <$

0.001, respectively). There was no significant difference between the captive and pre-release populations, nor between the post-release and wild populations.

Comparing the purpose for sample collection

To compare the purpose for sampling, 580 samples were analyzed from 184 individuals. These were all individuals that have at least two samples for different purposes. Of these 580 samples, 225 were from annual exams, 176 were from implant surgeries, 135 were from pre-release exams, 34 were from replacing implants, 2 were from routine field processing of wild individuals and 8 were samples taken when trapping individuals for captivity. No significant differences were found in the N:L ratio between the samples for routine field processing, replacing implants, or trapping for captivity so these samples were grouped as “wild processing” samples ($n = 44$) to increase power. There were significant differences N:L ratio based on the purpose for sampling [$F(3, 454.92) = 28.21, p < 0.001$]. A Tukey-significant difference test of the estimated marginal means from this model revealed the N:L ratio during annual exams was higher than during implant surgeries ($p = 0.01$), but no significant difference between the annual exam and the pre-release exam, or between the implant surgery and the pre-release exam (Figure 1.2). However, the N:L ratio was significantly elevated for all wild processing samples compared to the annual exam, implant surgery, and pre-release exam samples ($p < 0.001$ for all).

Comparing care facilities

A total of 736 samples from 300 captive individuals were analyzed. This consisted of 228 samples from CZ, 140 samples from MVF, 227 samples from the TBMWMRC and 141 samples from TZ. There were significant differences in the N:L ratio among the

facilities [$F(3,486.21) = 19.06, p < 0.001$]. Post-hoc tests suggest the N:L ratio was significantly higher at MVF and CZ compared to TBMWMRC and TZ ($p < 0.01$; Figure 1.3). There was no significant difference in N:L ratio between individuals at TZ and TBMWMRC or between MVF and CZ

Comparing wild populations

A total of 105 samples from wild individuals were analyzed. This consisted of 58 samples from Nanaimo Lakes and 47 samples from Strathcona. There were no significant differences in the N:L ratio between the two meta-populations (Figure 1.4).

Sources of variation in the N:L ratio for the populations

The effect of age, sex, and the month the sample was taken on the N:L ratio was compared among each of the four populations. The relative impact of each predictor was assessed through a comparison of the effect sizes (β). Sex was a significant predictor in captive and pre-release populations (captive: $p = 0.04, \beta = 0.16$ and pre-release: $p < 0.001, \beta = 0.39$; Figure 1.5), however not in the wild or post-release models.

Visualization of the predictor showed that males in the captive and pre-release populations had higher N:L ratios than females (Figure 1.6). Age was a significant predictor for all populations except for post-release and had the strongest effect in the wild population ($p < 0.001, \beta = 0.46$; Figure 1.5). Visualization of this predictor revealed that there is a strong positive correlation between age and the N:L ratio (Figure 1.7).

Finally, the month the sample was taken was only significant in the pre-release population, such that samples taken later in the year have a higher N:L ratio ($p < 0.001, \beta = 0.13$).

Discussion

This study used the N:L ratio to compare the stress physiology of VIMs among and within different managed groups in the conservation breeding and release program and assessed factors contributing to variation in this metric. The results show there are differences in the N:L ratio for captive, pre-release, post-release and wild population VIMs. VIMs *in situ*, either wild-born or post-release individuals have a significantly higher N:L ratio than those *ex situ*. Moreover, the N:L ratio differed based on which care facility the VIMs were housed in within the captive population. Finally, an analysis of the relative effect of three predictors known to affect stress indicated that there is a difference in what influences the N:L ratio in VIMs based on which population they are in. These results support the use of conservation physiology in this breeding program as the results suggests the physiology of this species is not conserved between *in* and *ex situ* environments, nor between care facilities.

N:L ratio among target comparisons

It was unexpected that the N:L ratio of VIMs *ex situ* was lower than *in situ*. Other studies that have compared stress of animals in captivity to their wild conspecifics have found captive animals exhibiting increased HPA axis activity. This was the case for African wild dogs (*Lycaon pictus*; Van der Weyde *et al.*, 2016), Canada lynx (*Lynx canadensis*; Fanson *et al.*, 2012)) and cheetahs (*Acinonyx jubatus*; Terio *et al.*, 2004). This activity has been attributed to visitor traffic, artificial housing conditions, and interactions through zoo husbandry practices such as feeding and cleaning (Terio *et al.*, 2004; Morgan and Tromborg, 2007; Fanson *et al.*, 2012; Van der Weyde *et al.*, 2016).

While VIMs in managed care are in artificial conditions compared to the wild environment and have feeding and cleaning schedules, they are housed separately from the public viewing collection at the Zoos. No public viewing and limited interaction with humans may be contributing to the lower-than-expected N:L ratio in this study.

There are two hypotheses for the difference in N:L ratio between *in* and *ex situ* VIM populations. One, that *in situ* VIMs are experiencing increased N:L ratios as a result of a more physiologically challenging environment, or two, that VIMs *ex situ* are experiencing acclimation of the HPA axis because of chronic stress. The first hypothesis is strengthened by studies that demonstrate that HPA axis activity can increase with concurrent stressors, although this effect is variable in some individuals (Blanchard *et al.*, 1998; Kim *et al.*, 2013; Spencer and Deak, 2017). It is possible that exposure to multiple, unpredictable stressors for VIMs in the wild such as contact with predators, climate variability, and social competition is resulting in elevated HPA axis activity, and thus an increased N:L ratio (Boonstra, 2013). As there are now two studies using two different stress metrics (hair cortisol and the N:L ratio) that show elevation in wild individuals, it supports the theory that the wild is a more challenging environment than managed care for this species (Acker, 2017).

An important consideration for this hypothesis, and for the interpretation of the N:L ratio as a representation of HPA axis activity, is the role that white blood cells play in an immune response. As many types of white blood cells increase in response to infections and parasites (Abramson and Melton, 2000), it is possible that the N:L ratio for *in situ*

individuals was due in part to an immune response from exposure to these agents in the wild as opposed to managed care when they are treated for parasites. This can be partially mediated by looking at other leukogram parameters that are also involved in an immune response, particularly monocytes and eosinophils (Davis *et al.*, 2008). If wild VIMs were experiencing an increased immune response, monocytes would likewise be elevated as they have a phagocytic role (Davis *et al.*, 2008). This was not seen in McAdie (2018), which found the proportion of monocytes to be lower in wild individuals compared to captive. Moreover, there was no significant difference in eosinophils among any groups, which have been documented to decrease as a result of glucocorticoid release (Vickovic, 1979, McAdie, 2018). This suggests that the elevated N:L ratio for *in situ* individuals was more likely a reflection of HPA axis activity rather than an immune response, however future studies with targeted sampling that considers infections in sampled individuals would be needed to confirm if this was the case.

The second hypothesis that may explain the difference in N:L ratio between *in* and *ex situ* populations involves the acclimation of HPA axis activity as a result of repeated stressors. Acclimation occurs when repeated stressors suppress HPA axis function, and can result in lower glucocorticoid release (Romero, 2004; Rich and Romero, 2005; Spencer and Deak, 2017). Despite being housed apart from public zoo visitors, the managed care of VIMs still involves a regimented schedule (e.g. daily feedings, cleanings, annual exams etc.) Therefore, it is possible that VIMs in managed care are exhibiting lower HPA axis activity, and thus a lower N:L ratio, than their wild conspecifics as a result of chronic stress (Romero, 2004). While it is impossible to say if this is happening using the results

of the present study, this seems unlikely given that VIMs in managed care are typically in good body condition, do not suffer from a compromised immune system (McAdie, 2018), and were shown to have similar breeding rates to wild individuals (Bryant, 2005) which can all be indirect indicators of chronic stress. However, as this cannot be ruled out entirely, it is encouraging that our results showed no significant difference in the N:L ratio between post-release and wild individuals, indicating they are in a similar physiological state with respect to the N:L ratio. This implies that if there is dysregulation of the HPA axis as a result of managed care, these effects are not lasting. However, future studies should challenge the HPA axis and focus on the ability of VIMs to cope with a stressor to confirm if there are fitness consequences as a result of the observed differences.

Although the N:L ratio has a slower response time than that of glucocorticoids (Davis and Maney, 2018), it is still important to consider the conditions in which these samples were obtained to ensure the sampling method did not confound the measurement of stress. There was no significant difference in the N:L ratio between the implant surgery and the pre-release exam. This result suggests that within the pre-release population, the acclimation period after surgery and before release is sufficient and these individuals are not experiencing a lasting stress response as a result of surgery. Interestingly, it appears as though samples taken during the implant surgery have significantly lower N:L ratios compared to annual exams. This may be because VIMs are afforded a convalescent period after their translocation to the TBMWMRC to recover from the transportation. As there was no significant difference between *in situ* samples taken for the implant

replacement surgery and other samples taken without a surgery, it is unlikely the surgery itself or associated handling accounted for this difference.

The N:L ratio did vary based on the care facility. Overall, VIMs housed at the TBMWMRC had the lowest N:L ratio, which was significantly lower than VIMs at CZ and MVF, but not TZ. A study using Asian elephants (*Elephas maximus*), found captive elephants with access to outdoor enclosures in their natural habitats had a significantly lower stress (Kumar *et al.*, 2019). While this might suggest that access to outdoor space within the natural habitat at the TBMWMRC resulted in a lower N:L ratio, it is important to note that this was not the case for individuals housed at the MVF, which was located on mainland B.C., close to Vancouver Island. The N:L ratio of VIMs housed at MVF was significantly higher than that at the TBMWMRC, despite these facilities being within British Columbia. There are notable differences in elevation between the TBMWMRC (1244m a.s.l.) and MVF (25m a.s.l), as well as differences in the housing infrastructure and the care that VIMs received at these facilities that may be contributing to these differences (M.McAdie pers.comm 2021). The N:L ratio was likewise significantly higher at CZ compared to TBMWMRC and TZ. These results together suggest that a combination of environmental factors, including access to outdoor space in the natural habitat, as well aspects of management within the facility such as daily housing and welfare management are contributing the stress of VIMs in managed care.

Habitat and geographic range may also influence stress, as demonstrated by a study of free-ranging roe deer (*Capreolus capreolus*) which found significant variation in the N:L

in three different habitats (Carbillet *et al.*, 2019). However, the N:L ratio was not significantly different for VIMs in the two meta-populations in this analysis. Due to a lack of samples from each colony within the meta-populations, this analysis was based over a broad geographic area which may not account for colony-level effects if analyzed at a finer scale. The similarity in N:L ratio between the two meta-populations may be due to the types of stressors and environmental characteristics experienced in the wild. HPA axis activity in wild animals can be influenced by climate, and weather, especially varying among seasons (Romero *et al.*, 2008; Edwards *et al.*, 2016; Ozella *et al.*, 2017; de Bruijn and Romero, 2018). The two meta-populations experience similar climates due to their proximities (< 100 km; Aaltonen *et al.*, 2009) Moreover, although the meta-populations differ slightly in elevation, all VIM colonies are found in sub-alpine meadows in elevations between 700 and 1500m (Bryant and Page, 2005; Aaltonen *et al.*, 2009; Vancouver Island Marmot Recovery Team, 2017). To fully understand how or if the stress of wild VIMs is different based on the colony location, more samples would need to be taken from each site.

Sources of variation in N:L ratio

The relative contribution of age, sex, and the month of sampling to variation in the N:L ratio was different for the four populations of interest. Age was a significant predictor of the N:L ratio for all populations except for post-release, most likely due to a smaller sample size ($n = 24$) as one sample drove the relationship in the opposite direction. While the N:L ratio increased with age in almost all populations, this effect was the largest in the model for wild VIMs. Other studies have found HPA axis activity increases with age

when measured using glucocorticoids (Reeder and Kramer, 2005) and the N:L ratio (Watson *et al.*, 2016; Carbillet *et al.*, 2019; Uchida *et al.*, 2021). Animals in managed care are tended to regardless of their age and may have specialized treatments to mediate age-related physiological deteriorations and diseases. For example, a survey of 63 zoos housing African and Asian elephants found that “managed time” (i.e. time spent engaged with staff for training, feeding, exercise etc.) increased by 20.2% with each year of age, primarily to combat health risks associated with older individuals (Greco *et al.*, 2016). The specialized care of older captive VIMs may mediate the effect of age on the N:L ratio. Moreover, older VIMs in the wild are subjected to increased social and territorial pressure from younger individuals, which could likewise contribute to a larger effect of age in that population (M. McAdie pers. comm. 2021; Uchida *et al.*, 2021). While it is unclear if there is a relationship among stress, age, and fitness, a study investigating age-specific survival rates of released VIMs found apparent survival of older age classes (> 3 years) was lower than in closely related species (Aaltonen *et al.*, 2009). Further investigation into the mechanism of age-related stress responses in VIMs are needed to determine if this survival rate was impacted by stress.

There were notable differences in the effect of sex on the N:L ratio in different populations. Sex was a significant predictor of the N:L ratio in captive and pre-release populations of VIMs, but not in post-release or wild. Specifically, males *ex situ* exhibited a higher N:L ratio than females, although overall this was still lower than for *in situ* males. In wild mammals, there is a general consensus that HPA axis activity (measured with glucocorticoids) is higher for females than males (Reeder and Kramer, 2005). This is

attributed to high energetic costs associated with reproduction. There is less of a consensus about the N:L ratio between sexes particularly in the context of stress. Studies using closely-related ground squirrel species have found no differences in the N:L ratio between sexes (Wenberg *et al.*, 1973; Barker and Boonstra, 2005; Desantis *et al.*, 2016), and similarly no difference in rats (Hickman, 2017). However, a recent study using the N:L ratio in roe deer did find the N:L ratio to be higher in males (Carbillet *et al.*, 2019).

Higher N:L ratios for males in managed care could be due to increased HPA axis activity for males, or it could be the case that aspects of managed care are suppressing HPA axis activity for females. The N:L ratio was significantly elevated in years captive females had pups compared to those that did not (see Appendix Figure A1.2), which was expected given that reproduction typically leads to increased HPA axis activity for females. This suggests that there is variability in HPA axis activity for female VIMs in response to a known stressor and thus supports the hypothesis that there may be some aspect of managed care that is contributing to elevated N:L ratios in males, rather than suppressing HPA axis activity in females. However, these data were collected for routine purposes and not targeted towards breeding cycles so these samples would have been collected after the pups were weaned and therefore not reflect physiological conditions during pregnancy or weaning. Further research into the stress profiles of pregnant females including wild individuals would be needed to fully understand how the HPA axis responds at this time.

The structure of social animals, particularly rank, can affect HPA axis activity (Creel *et al.*, 2013), and may explain some of the variation in N:L ratio for VIMs in managed care. In the wild, VIMs live socially in colonies, often with more than one family (Aaltonen *et al.*, 2009). In managed care, VIMs are most commonly housed in breeding pairs, or in same sex groups with siblings. While HPA axis functioning and social rank has been investigated, there is not uniformity in how the HPA axis responds. A recent study on the alpine marmot (*Marmota marmota*) found that the N:L ratio was higher in dominant males than in subordinate but sexually mature males (Cohas *et al.*, 2018). However, a study in yellow-bellied marmots found the opposite where the N:L ratio decreased with rank (Uchida *et al.*, 2021). While these two studies clearly show that the N:L ratio is affected by rank or social dominance in marmots, there is no consensus as to which direction. If we can infer dominance by access to sexually mature females and the ability to participate in breeding behaviors, then the results of the present study might suggest dominant male VIMs exhibit an increased N:L ratio, similar to the alpine marmot. It is possible that housing multiple “dominant” males in close proximity in managed care is eliciting an increased N:L ratio overall. However, a study examining the N:L ratio in breeding vs. non-breeding males, compared to wild conspecifics would be needed to confirm if this was the case.

Finally, hematological parameters including the relative portion of neutrophils and lymphocytes, as well as basal glucocorticoids have been shown to change seasonally in a variety of species (Boonstra *et al.*, 2001; Vera *et al.*, 2011). This is particularly relevant to hibernation in VIMs, as neutrophils have been shown to decrease during hibernation in

two similar species, woodchucks (*Marmota monax*; Wenberg *et al.*, 1973) and Arctic ground squirrels (*Spermophilus parryii*; Barker and Boonstra, 2005). However, in this study the month the sample was taken was only significant in the model of pre-release VIMs and the overall effect in the model was quite small. The lack of significance of this predictor may have been due to the restricted range of the sampling schedule. In each population, samples are taken around the same time and all within the active season. For example, 81% of annual exams happen in September or October and 96% of implant surgeries and pre-release exams happen between June and August. There are no samples taken during hibernation due to possible complications with arousal, concerns with diminished platelets, and hemostasis (McAdie, 2018). Therefore, it is possible that there were not enough samples collected in the months leading into hibernation (November) and emergence (April – May) to capture this seasonal variation.

While this study found clear differences in VIM physiology among managed groups, a key limitation of the presented research is that the data were not collected with an *a priori* research question or hypothesis in mind. This quandary was outlined as a key area of focus for reintroduction biology research in general by Seddon and Armstrong (2008) and again by Taylor *et al.* (2017). Although using historical blood samples provided a robust sample size, none of the samples collected for this study were done so with the object of analyzing stress. Therefore, we were unable to use these data to represent a stress response, nor account for all possible factors that might influence this metric. This was partly mediated by controlling for as many confounding variables as possible, including sex, age and month and assessing the purpose for sampling separately. An additional

limitation was that *ex situ* and *in situ* individuals were sampled unevenly, where wild samples made up 10% of the total data, and post-release only 2%. This highlights the importance of post-release monitoring in conservation breeding and release programs.

Conclusion

This study has shown not only that there are significant differences the N:L ratio of VIMs in different managed groups in the program, but there are also significant differences in what is contributing to variation in this metric. As the apparent survival of captive bred VIMs is lower than wild conspecifics (Lloyd *et al.*, 2019), it is important to consider physiological differences between *in situ* and *ex situ* individuals to maximize our understanding of fitness of all individuals in the population. As it is difficult to know whether the difference in the N:L ratio in *ex situ* individuals was a result of HPA axis attenuation from chronic stress, a study challenging the HPA axis, compared to *in situ* individuals, would confirm if these groups differ in their ability to cope with stress. While there has been encouraging population growth of VIMs because of this program, understanding if physiology is affecting fitness, especially in released VIMs, may be a way to increase efficacy of this program

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Table 1.1 Summary of the models used for comparisons of the N:L ratio for this analysis. For each model, the N:L ratio was log transformed and used as the response variable.

Model Name	Target comparison	Predictors
Population	Populations	Sex + Month + Laboratory + (1 Individual) + (1 Year)
Captive	Care facilities	Sex + Age + Month + (1 Individual) + (1 Year)
Wild	Wild meta-populations	Sex + Age + Month + Laboratory + (1 Year)
Sampling	Purpose for sampling	Sex + Age + Laboratory + (1 Individual) + (1 Year)

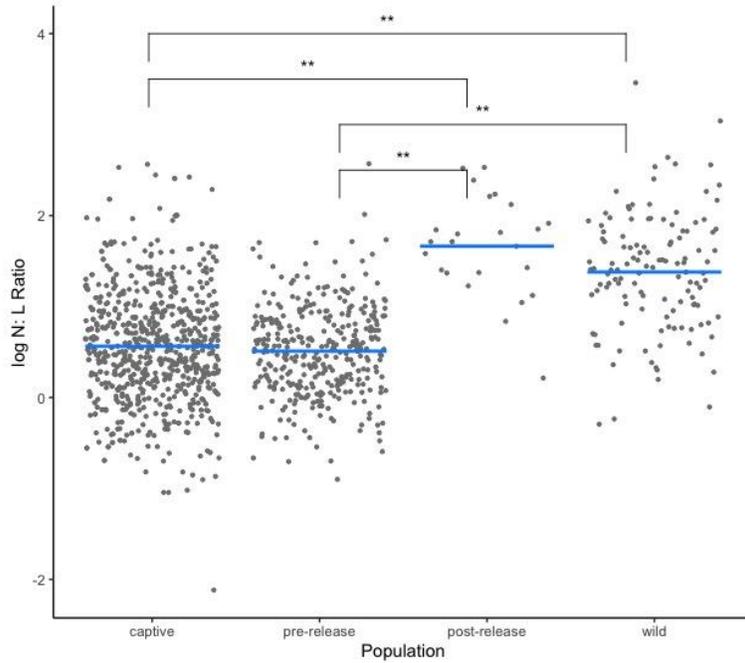


Figure 1.1 Partial residuals of log N:L ratio among each population. Captive samples ($n = 739$) are collected for annual exams, pre-release samples ($n = 348$) are collected for implant surgeries or pre-release exams, post-release samples ($n = 24$) are collected for replacement of transmitters and wild samples ($n = 126$) are collected opportunistically for replacement of transmitters, routine field processing and trapping for captivity.

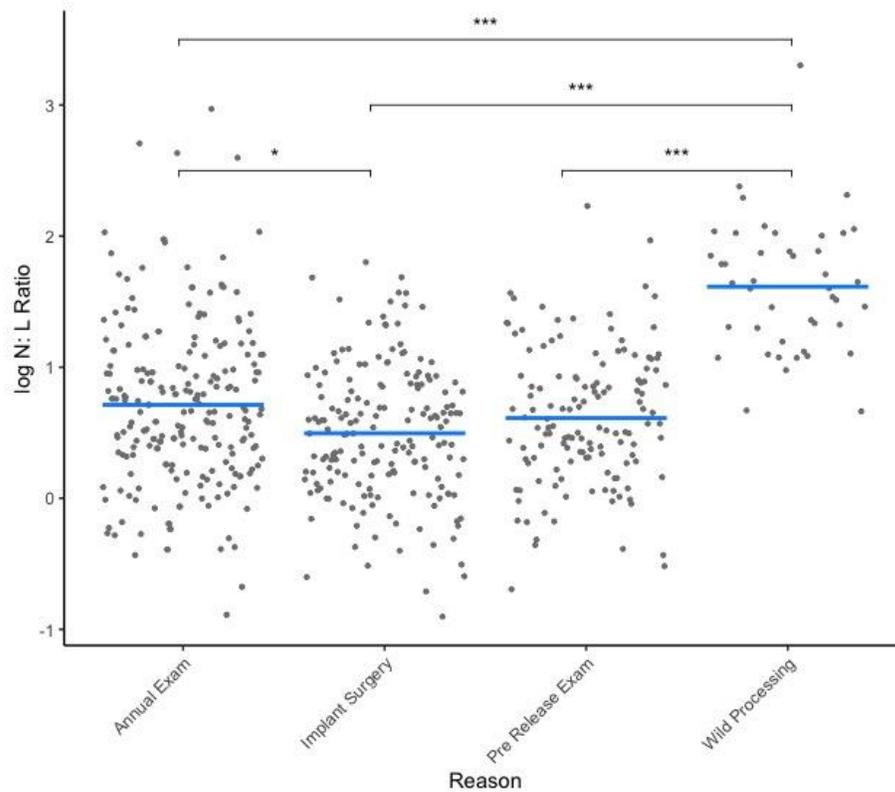


Figure 1.2 Comparison of partial residuals of log N:L ratio for the purpose of sampling. Sampling reasons include annual exams ($n = 225$), implant surgeries ($n = 176$), pre-release exams ($n = 135$) and “Wild Processing” which consists of replacing transmitters, routine field processing, and trapping for captivity ($n = 44$).

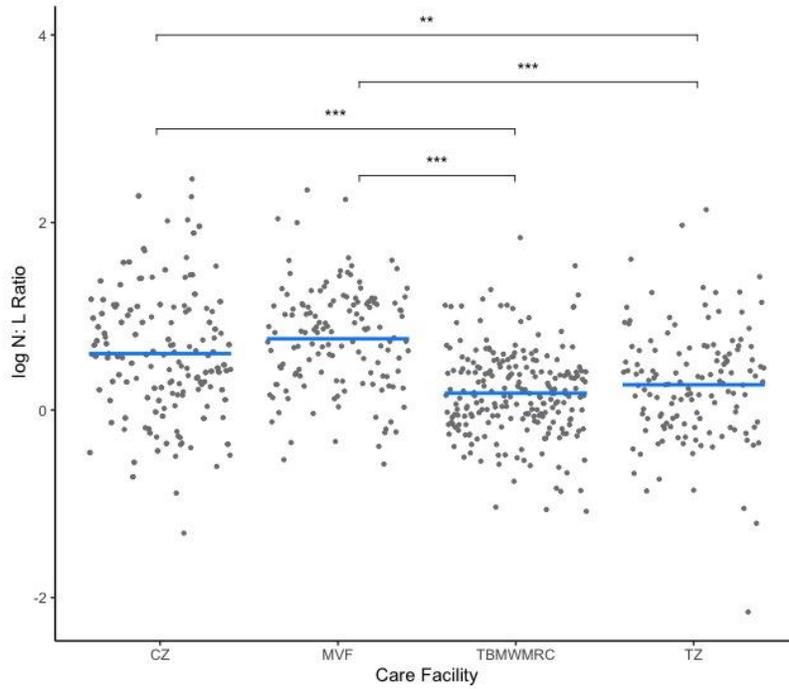


Figure 1.3 Comparison of partial residuals of log N:L ratio among care facilities. This consisted of Calgary Zoo (CZ; $n = 228$), Mountain View Facility (MVF; $n = 140$), the Tony Barrett Mt. Washington Marmot Recovery Centre (TBMWMRC; $n = 227$) and Toronto Zoo (TZ; $n = 141$).

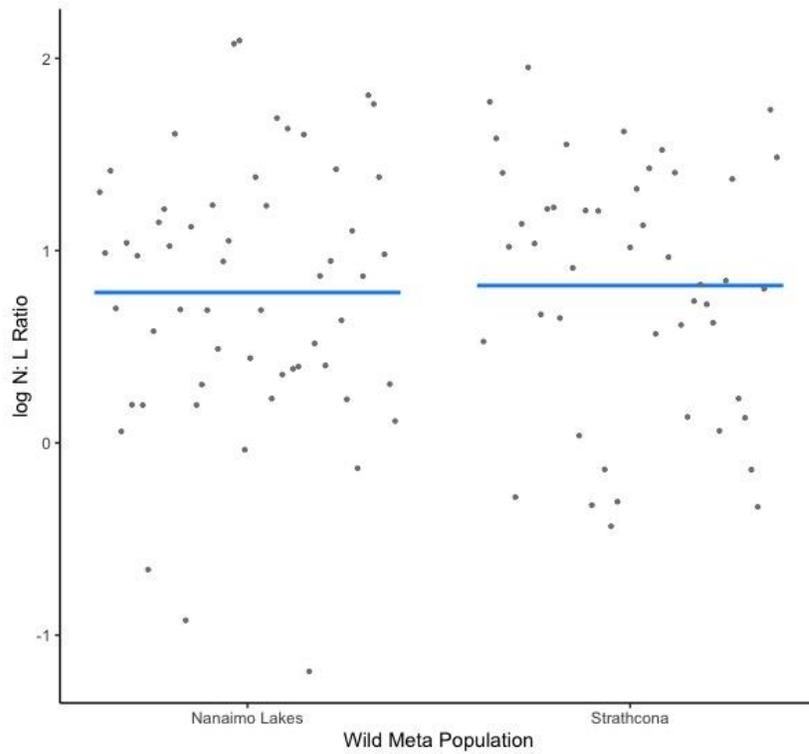


Figure 1.4 Comparison of partial residuals for log N:L ratio between Meta-populations.

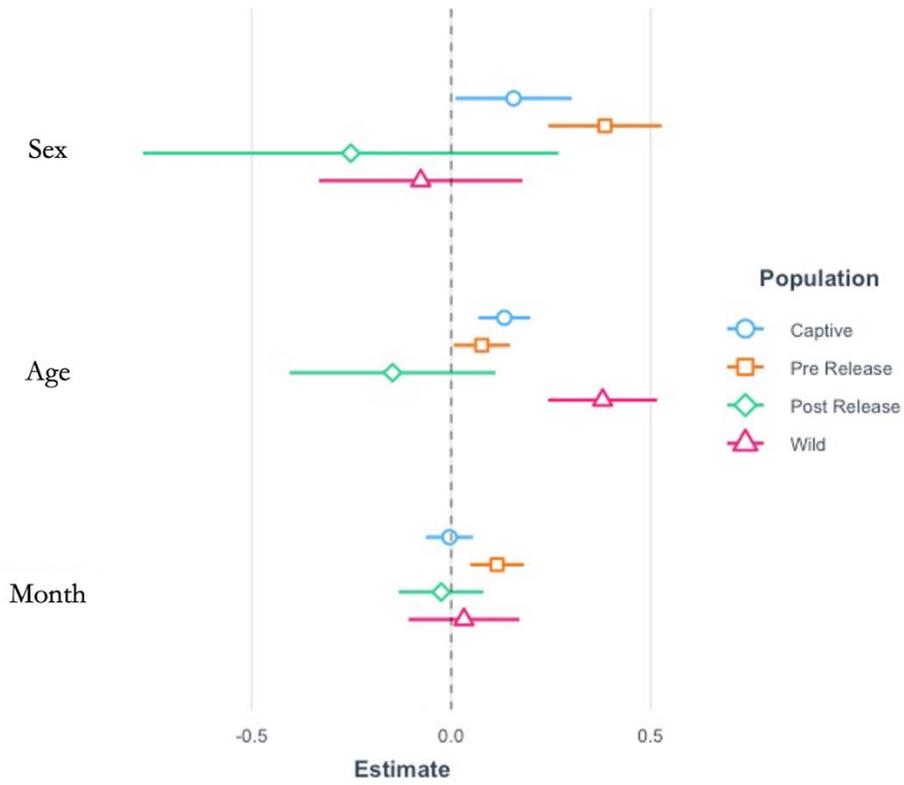


Figure 1.5 Comparison of estimates for three predictors for population models.

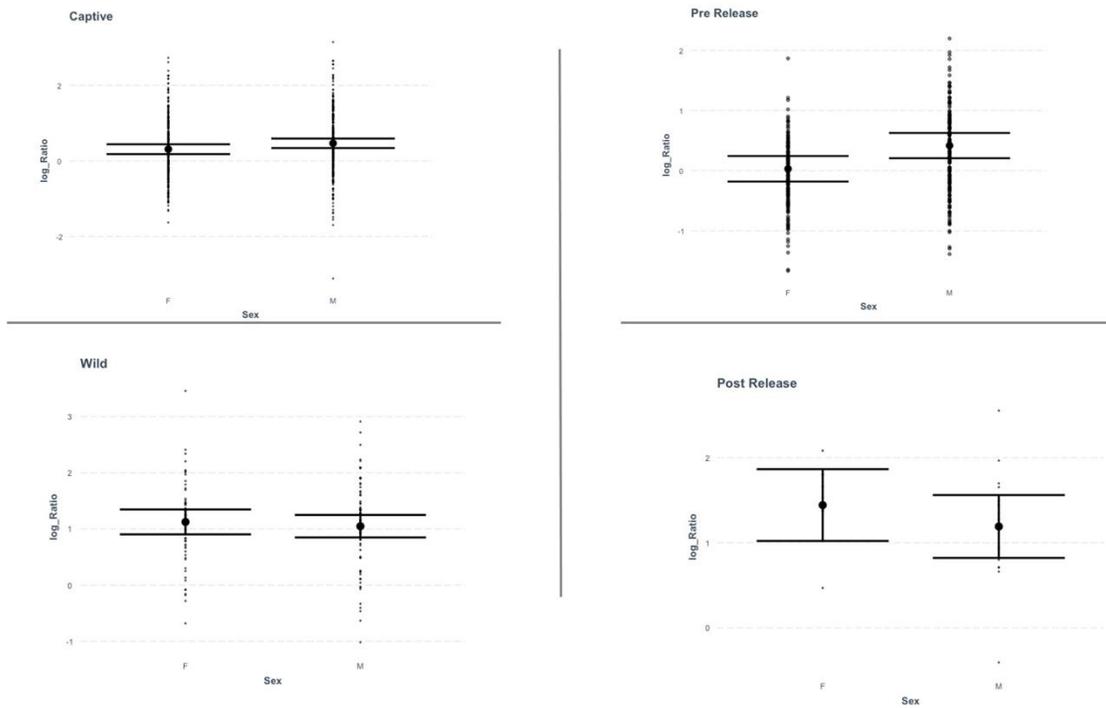


Figure 1.6 Comparison of log N:L ratio between sexes among each population included for analysis

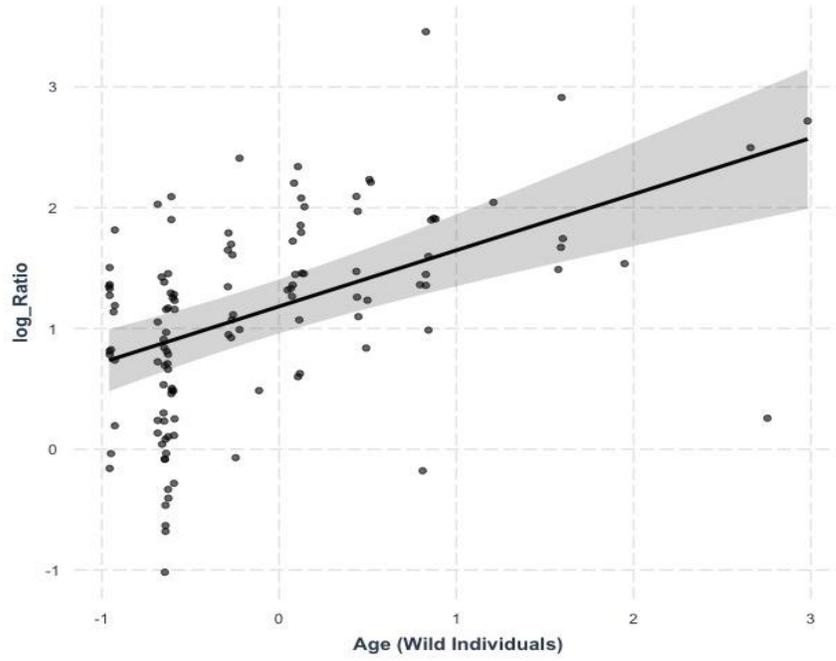


Figure 1.7 Visualization of age predictor from the model of wild population VIMs.

Appendix

Effects removed from linear mixed models

In the model that compared the N:L ratio among the captive, pre-release, post-release and wild populations including age led to overfitting in the model. This was because the variation in ages was not equal for all populations. For example, the majority of pre-release VIMs were less than a year old as this is the age they are typically released from managed care (Lloyd *et al.*, 2019). Similarly, post-release VIMs were largely between 2-3 years, as they are typically caught to replace the transmitter 2-3 years post-release. Therefore, the effect of age was removed from this model.

In the model that compared the purpose for sample collection, including month led to collinearity in the model. This is likely because sampling collection takes place at specific times in the year to correspond to management practices. For example, annual exams typically occur in September or October, whereas pre-release exams occur in June or July to facilitate releases during the active season. This was highly correlated to the purpose the samples were taken and was thus removed from this model.

In the wild model, using individual as a random effect led to overfitting due to limited repeat samples from the same individual. Therefore, in this model one sample from each wild individual was randomly selected and the individual effect was removed.

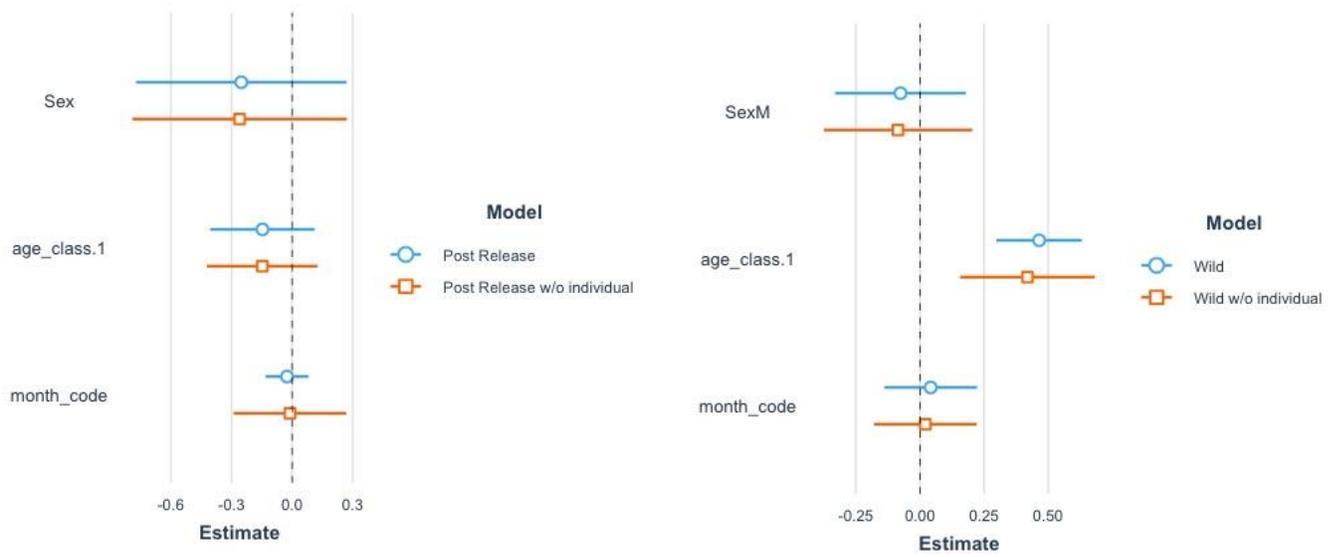


Figure A1.1 Comparison of estimates for the Post-release model (A) and Wild model (B) with and without the random effect of individual.

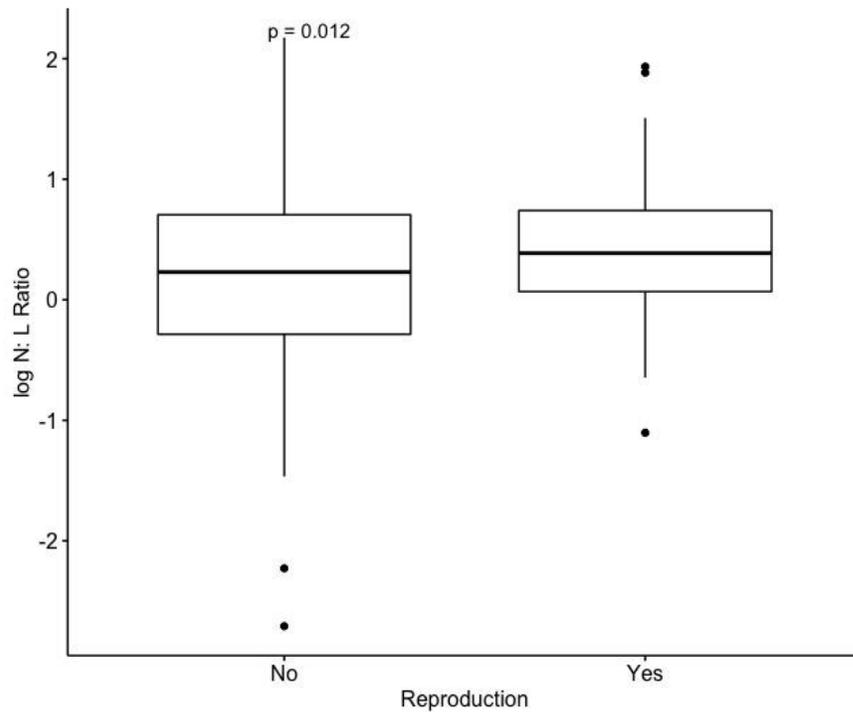


Figure A1.2 Comparison of N:L ratio of females in captivity in years they reproduced (Yes) and years they did not (No).

Chapter Two: Assessing factors that influence hibernation-related mortality in the endangered Vancouver Island Marmot (*Marmota vancouverensis*).

Abstract

Hibernation is a strategy used by many animals to limit energy expenditure during times of resource limitation in cold climates. Although often successful in maximizing survival, hibernation requires complex physiological trade-offs that can sometimes result in mortality to the individual. Additionally, environmental characteristics such as climate and snowpack can influence the overall success of hibernation. Recent research suggests that Vancouver Island marmots (VIMs; *Marmota vancouverensis*) bred in a conservation breeding program experience higher than expected hibernation-related mortalities compared to their wild conspecifics during their first winter post-release. This is unusual as VIMs are typically highly successful hibernators and moreover, experience similar hibernation survival rates during subsequent winters in the wild. This study used historical records of releases and hibernation-related mortalities to evaluate factors that may be contributing to low survival rates during the first year post-release. Three key areas related to hibernation were investigated using logistic regression models: release factors (e.g., region of release, colony size at release, year of release etc.), environmental factors (e.g., average snowpack, minimum and maximum temperatures etc.) and intrinsic factors relating to the individual VIM (e.g., age, sex, facility of origin etc.). Our analysis revealed that the region of release, colony size at release, average and standard deviation in snowpack, as well as the facility of origin were significant predictors of hibernation-related mortality. This supports the assertion that both intrinsic and extrinsic factors should be considered for this species to fully understand hibernation-related mortality and improve overwinter survival in the future.

Introduction

Hibernation is a strategy used by heterothermic animals to lower overall metabolic expenditure to conserve energy (Storey, 2010; Geiser, 2013). Although hibernation was previously attributed to long-term limits in available energy due to cold environments, recent research suggests that hibernation may also play a role in short-term processes such as protection from predation and water conservation (Boyles *et al.*, 2020).

Regardless of the purpose, hibernating animals have to make a trade-off between the benefits of conserving energy and the potentially fatal consequences of limiting energy such as immunosuppression, starvation, and oxidative stress (Humphries *et al.*, 2003). As limited energy could result in mortality to the individual, it is crucial to understand the mechanisms and effects that energy trade-offs can have for hibernating animals (Armitage, 2017).

Hibernation in mammals requires complex physiological adjustments that often occur months in advance (Storey, 2010). For example, animals prepare for hibernation in late-summer by increasing body fat, regulating enzymatic activity and altering gene expression to lower metabolic activity (Geiser, 2004; Storey, 2010). Once hibernation commences, animals undergo further physiological modifications to maintain the torpid state such as changes in hormones (Willis and Wilcox, 2014; Wey *et al.*, 2015) and suppression of immune function (Wenberg *et al.*, 1973; Havenstein *et al.*, 2016). Stress (defined as the level of hypothalamus-pituitary-adrenal (HPA) axis activity) in relation to hibernation has been more closely investigated recently, however there is no clear consensus of changes to HPA axis activity in hibernating mammals. Golden mantle

ground squirrels (*Callospermophilus lateralis*) showed a decrease in glucocorticoids (suggesting lower HPA axis activity) prior to emergence, while yellow pine chipmunks (*Neotamias amoenus*) showed an increase during the same time (Romero *et al.*, 2008). Likewise, yellow-bellied marmots (*Marmota flaviventris*) showed a decrease in glucocorticoids throughout the active season in preparation for hibernation (Smith *et al.*, 2012). The inconsistency in these results suggest that physiological modifications for hibernation can be highly species specific and warrant further study.

While these changes are all physiological adaptations to survive hibernation, survival through hibernation is also dependent on extrinsic factors. For example, overwinter survival has been associated with habitat characteristics such as canopy cover, soil composition, and ground temperature (James *et al.*, 2004; Williams *et al.*, 2012; Wheeler and Hik, 2014; Goldberg *et al.*, 2020). Additionally, the ambient climate has been shown to have significant influence on the success of hibernating mammals (Thogmartin and McKann, 2014; Turbill and Prior, 2015; Johnson *et al.*, 2018). This is particularly relevant in the context of climate change, as warming temperatures are typically attributed to poor survival in hibernating animals (Turbill and Prior, 2016; Johnson *et al.*, 2018). Understanding the factors that can impact hibernation success in a species requires the consideration of both intrinsic and extrinsic influences.

The Vancouver Island marmot (*Marmota vancouverensis*; henceforth VIM) is a critically endangered rodent endemic to Vancouver Island, British Columbia (B.C.; COSEWIC, 2019). VIMs are obligate hibernators and typically hibernate for ~8 months a

year immersing in late September – early October and emerging late April – May (Bryant and McAdie, 2003). In response to critical population declines throughout the late 90’s, a conservation breeding and release program was established to supplement the wild population (McAdie, 2004). Currently, VIMs are being managed at the Toronto Zoo (TZ), Calgary Zoo (CZ), and the Tony Barrett Mount Washington Marmot Recovery Centre (TBMWMRC) – a facility on Vancouver Island (COSEWIC, 2019). VIMs were also previously held at the Mountain View Conservation and Breeding Society (MVF) on mainland B.C. until 2014.

Research in the program found that newly released captive-born VIMs (< 1 year in the wild) have low survival rates during their first hibernation, which can be as low as 40% in some years (Aaltonen *et al.*, 2009; Jackson *et al.*, 2016). This is unusual, as VIMs are efficient hibernators both in managed care and in the wild, and do not typically experience hibernation-related mortality (Bryant and McAdie, 2003; Bryant and Page, 2005). There is some evidence to suggest stress may be contributing to this mortality. VIMs released earlier in the active season (late June – early July) had higher overwinter survival compared to those released later (September; Jackson *et al.*, 2016). A proposed mechanism for this observation is that longer time in the field before hibernation provided an acclimatization period for newly-released VIMs dealing with “release stress” (Jackson *et al.*, 2016). Furthermore, there is no significant difference in survival between wild and released VIMs beyond their first winter, which suggests acclimatization after their first hibernation (Jackson *et al.*, 2016). This hypothesis is supported by physiological evidence from two studies that examined hair cortisol concentration and

blood serum parameters and found significant differences among captive, wild, and captive-released VIMs (Acker, 2017; McAdie, 2018). Specifically, hair cortisol was found to be higher in newly-released VIMs compared to established VIMs that had spent a year in the wild (Acker, 2017). Although this suggests that there may be some aspect of HPA axis activity that may be influencing hibernation survival, there has yet to be a study that directly compares the two for VIMs (Bryant and Page, 2005; Jackson *et al.*, 2016; McAdie, 2018). However, in the closely related yellow-bellied marmot, high fecal glucocorticoid concentrations were associated with lower overwinter survival (Wey *et al.*, 2015), suggesting there is likely a relationship between physiological stress and hibernation survival.

Although HPA axis activity is most commonly measured using glucocorticoids, there are other metrics that can be used. The relative proportion of neutrophils to lymphocytes circulating in blood plasma, otherwise known as the N:L ratio (Davis *et al.*, 2008) is being increasingly used. An increase in the N:L ratio is indicative of increased HPA axis activity (Dhabhar *et al.*, 1996; Davis *et al.*, 2008). Recent research has highlighted the utility of the N:L ratio to predict survival. The N:L ratio has been used in biomedical research to predict survival in humans (Shimada *et al.*, 2010; Chua *et al.*, 2011; Proctor *et al.*, 2012; Diao *et al.*, 2019). Moreover, two recent studies have noted the capability of the N:L ratio to predict overwinter survival in obligate hibernators, the root vole (*Microtus oeconomus*; Książek *et al.*, 2014) and the edible dormouse (*Glis glis*; Havenstein *et al.*, 2016). An elevated N:L ratio was associated with greater late-winter survival in root voles (Książek *et al.*, 2014), but lower survival in the edible dormouse

(Havenstein *et al.*, 2016). These results suggest that while valuable, further studies are needed to elucidate a clearer relationship between the N:L ratio and predicting survival – especially in hibernating animals such as VIMs.

In addition to HPA axis activity, there are other factors that can be considered to understand the hibernation success of VIMs. The facility at which VIMs are housed has been shown to significantly affect hibernation duration in managed care. Individuals at facilities in British Columbia (MVF and the TBMWMRC) have hibernation cycles that are closest to wild populations (Bryant and McAdie, 2003). Moreover wild-born VIMs in managed care have longer hibernation cycles, typically immersing and emerging closer to their wild conspecifics (Aymen *et al.*, 2021). It is yet unclear if these differences in hibernation cycles from managed care affect the ability of captive-bred individuals to survive their first winter once released. Furthermore, there has yet to be a study investigating climate or release-site characteristics on the hibernation success of this species.

The purpose of this research was to investigate the factors that may be contributing to hibernation-related mortality in the first year post-release for VIMs from managed care. To have a comprehensive picture of hibernation-related mortality both extrinsic environmental factors including release regions, temperature, snowpack, and colony size and intrinsic factors including sex, age and an analysis of the N:L ratio of VIMs before release, were considered. This is the first study with VIMs to evaluate post-release hibernation survival using a physiological metric. Having a better understanding of the

influences on survival for released individuals will increase the efficacy of the program, and ultimately increase the population size of this critically endangered animal.

Methods

Description of facilities

For the period of time included in this study, VIMs were held in facilities at TZ (Toronto ON., 145m a.s.l.), CZ (Calgary AB., elevation 1027m a.s.l.) and the TBMWMRC (Comox-Strathcona, BC., elevation 1244m a.s.l.). VIMs were also held at MVF until 2014 (Fort Langley, BC., elevation 25m a.s.l.). In managed care, VIMs were housed in enclosures with straw and wood shaving substrates and had access to nest boxes as a replacement for natural burrows (McAdie, 2004). VIMs were fed a diet of alfalfa-based rabbit pellets, and supplemented with raw vegetables, primate biscuits, and hay (McAdie, 2004). At the TBMWMRC, VIMs were also provided natural vegetation from alpine meadows within the natural habitat. In preparation for hibernation at these facilities, food is either offered until it is refused, or gradually reduced. Hibernating VIMs were maintained at an ambient temperature of 5-7°C either in the VIM housing area, or the nest boxes are transferred to an environmentally controlled chamber (McAdie, 2004).

Data compiled

Data on the mortality of VIMs in the program have previously been collected by the Marmot Recovery Foundation (MRF) and was used in this analysis. Prior to release, captive VIMs were surgically implanted with a radio-transmitter to monitor their progress (McAdie, 2018). The mortality of implanted VIMs can be evaluated using transmitter pulse rates (Bryant and Page, 2005). As it can be difficult to pinpoint exactly when a mortality occurred, overwinter mortalities were classified by the MRF as hibernation-

related mortality. This included mortality during hibernation, as well as mortalities arising from complications of hibernation such as failure to emerge from the burrow and post-emergence emaciation. This study focused on mortality occurring within the first year post-release, as this is when hibernation-related mortalities typically occur (Jackson *et al.*, 2016). Hibernation mortality data was available from 1996 – 2015. Mortalities classified as hibernation-related by the MRF ($n = 123$) were included in this analysis as high-confidence mortalities. In addition to these, there were 40 unknown mortalities that were likely hibernation-related as indicated by comments in field reports. To increase the power of this analysis, these 40 mortalities were included, but considered low-confidence hibernation-related mortalities. A separate analysis was run using both high and low confidence mortalities and was compared to only the high confidence mortalities. There was no meaningful difference in the results in terms of the effect size of the predictors (See Appendix, Figure A2.1 – A2.3), so the results reported here are for all hibernation-related mortality, including low-confidence mortalities ($n = 163$).

The MRF likewise catalogues data on all releases and translocations of individual VIMs in the program. These data include the release location and date. The colony size at each site was estimated from this data as a count of the number of individuals (by name) that were sighted at each colony for annual inventory purposes per year. The region of release was used in this analysis as there was not enough data per release site to use. The MRF classifies regions as areas consisting of multiple sites/colonies, displayed in Figure 2.1. These two datasets (known mortalities and releases/translocations) were merged using the individual VIM name to associate releases and all known mortalities of VIMs in

the program. The resultant data frame contained entries for 412 released VIMs between the years of 1996 and 2015, 157 of which survived at least the first year, 156 had a hibernation-related mortality and 99 were catalogued as “other” mortality which is either unknown, or a different cause of death (e.g., predation). The 99 “other” mortalities were not included in this analysis.

Finally, to evaluate the N:L ratio as it pertains to hibernation-related mortality the last blood samples collected before release of each VIM was merged to this dataset using the name, the date of sample, and the date of release. As blood samples are not routinely collected from VIMs post-release, the sample from either the implant surgery or the pre-release exam was used. If an individual had a sample for both the implant surgery and the pre-release exam, the pre-release exam sample was used. The data frame which included pre-release samples had data for 139 VIMs, 53 samples from VIM that survived at least their first year, 49 experienced a hibernation-related mortality and 37 had an “other” mortality.

Environmental data

To evaluate environmental conditions and hibernation-related mortalities in VIMs, weather data was retrieved from automated snow weather stations (ASWS). These weather stations, operated by the government of B.C., use remote sensing technology to report meteorological data hourly (Government of British Columbia 2021). Jump Creek Weather Station (3B12P) is situated at an elevation of 1160m roughly 12km south of Nanaimo Lakes and was used to approximate conditions of the Nanaimo Lakes region. Wolf River Station (3B17P) is at an elevation of 1430m approximately 4km south-west

of Strathcona Provincial Park and was used to approximate conditions of the Clayoquot Plateau, Western Strathcona, Schoen Lake, and Forbidden Plateau regions. Data was assessed for the winter of each year of interest to the study. The “winter” season was considered to be between October 1st and April 31st. This date range was chosen as it reflects the common immergence and emergence dates of VIMs (Bryant and McAdie, 2003).

The environmental information collected from these two stations was minimum, maximum, and mean temperature and snowpack (measured in °C and snow water equivalent (SWE), respectively). In addition to minimum, maximum and mean temperature and snowpack, the standard deviation of each measure per year was calculated to represent seasonal variation. Finally, snow cover and snow melt dates were approximated from the measures of SWE. As there are no established criteria for determining snow cover or melt from SWE, for the purposes of this research snow cover was considered to be the second of two consecutive days above 10mm SWE, and snow melt was considered to be the second of two consecutive days below 10mm SWE. These dates were recoded as Julian days for each winter season over the period of interest.

Statistical analysis

All statistical analysis was conducted in R (R Core Team 2020). To evaluate the relative importance of each predictor, logistic regression models were created using the *glm2* package (Version 1.2.1; Marchner and Donoghoe, 2018). To avoid complications with multi-collinearity in the models, separate models were created to evaluate factors associated with release (henceforth release model), factors associated with the

environment (henceforth environment model), and intrinsic factors associated with individual VIMs (henceforth intrinsic model). The environmental model was further divided into a model that evaluated the mean factors (e.g., mean snowpack and mean temperature) and a model that evaluated the variability of these factors (e.g., standard deviation snowpack and temperature). A preliminary model with body condition (calculated as the residuals between body length and mass measurements (Schulte-Hostedde *et al.*, 2005)) was also evaluated, however this predictor was not significant and had a small effect size ($p = 0.42$; $\beta = 0.12$) and was thus excluded from the analysis. A full list of predictors evaluated in each model is outlined in Table 2.1.

For each model, the response was a binary predictor of 0 for survived the first year, or 1 for a hibernation-related mortality, therefore a binomial model family was used. Numeric predictors were scaled for comparison and data with missing values were removed from the dataset. The relative importance of each predictor was assessed using the p – value and a comparison of the estimate sizes. Visualization of these predictors was done using the *jtools* package (Version 2.1.0.; Long, 2020).

Results

Release factors

The model evaluating release factors included the fates of 293 VIMs between 1996 and 2015. The regions of release included Clayoquot Plateau ($n = 14$), Forbidden Plateau ($n = 90$), Nanaimo Lakes ($n = 74$), Schoen Lake ($n = 12$) and Western Strathcona ($n = 103$). The significant predictors in this model were colony size at release ($p = 0.01$, $\beta = 0.42$), the region of Western Strathcona ($p < 0.01$, $\beta = 1.02$) and the month of release ($p =$

0.05, $\beta = 0.25$; Table 2.1). Visualization of colony size indicates larger colony sizes experience a higher probability of hibernation – related mortality (Figure 2.2). Although not significant, all other regions had effect sizes over 0.5, which is considered reasonably strong (Sullivan and Feinn, 2012). Visualization of the regions of release indicate the highest probability of hibernation-related mortalities occurring in Western Strathcona and the lowest at Schoen Lake (Figure 2.3). Releases later in the active season resulted in higher instances of hibernation-related mortality.

Environmental factors

The model evaluating mean environmental factors included the fates of 304 VIMs between 1996 and 2015. None of the predictors in this model were statistically significant. The predictor with the largest estimate was mean snowpack ($\beta = -0.21$). Visualization of this predictor shows that higher mean snowpack throughout the winter trends with higher instances hibernation survival (Figure 2.4).

The model evaluating variability in environmental factors included the fates of 304 VIMs between 1996 and 2015. In this model, the standard deviation of snowpack was significant, with a moderate effect size ($p = 0.05$, $\beta = -0.27$). Visualization of this predictor shows that greater deviation in snowpack is associated with greater instances of hibernation survival (Figure 2.5).

Intrinsic factors

The model evaluating intrinsic factors included the fates of 88 VIM between the years of 2003 and 2015. This consisted of 4 VIMs originating in the wild, 29 from the TBMWMRC, 20 from MVF, 13 from CZ and 22 from TZ. The N:L ratio at release was

not a significant predictor in this model. The facilities all had very high effect sizes (> 2.3; Table 2.1) with TZ being significant ($p = 0.05$). Visualization indicated that VIMs originating at TZ experienced higher instances of hibernation-related mortality than those originating from the TBMWMRC and MVF (Figure 2.6).

Because the N:L ratio at release was insignificant in this model and was reducing the data due to missing values, another model was created without the N:L ratio to explore the other intrinsic factors with more statistical power. This model included the fates of 257 VIMs between 1996 and 2015. This dataset consisted of 31 VIMs originating in the wild, 65 from the TBMWMRC, 47 from MVF, 57 from CZ and 57 from TZ. This model suggests that VIMs originating from MVF and TZ experienced higher instances of hibernation related mortality (MVF: $p = 0.05$, $\beta = 0.92$ and TZ: $p < 0.01$, $\beta = 1.65$; Figure 2.7).

Discussion

Through analysis of historical records of mortality, both extrinsic environmental factors and intrinsic factors influenced first year hibernation-related mortality in VIMs. The retrospective approach in this study allowed for a robust investigation of hibernation-related mortality across a number of years in the program. In particular, the colony size at release and the region to which the individual is released had significant influence in the release model of hibernation-related mortality. Snowpack measures had the greatest influence in the environmental model, such that lower mean snowpack resulted in higher instances of hibernation-related mortality. Finally, the origin of the individual had

significant influence in the intrinsic model, with individuals originating from the TBMWMRC having the fewest instances of hibernation-related mortality. These results support the assertion that both extrinsic and intrinsic factors should be considered for this species to have a comprehensive picture of hibernation success.

Site-specific survival has been a recent research focus of the Marmot Recovery Foundation, exemplified by the implementation of the “stepping-stone” reintroduction strategy in which VIMs are released to Mt. Washington for one season, before being translocated to more remote sites (Lloyd *et al.*, 2019). The results of the current study support the importance of considering location for hibernation survival, as there were large differences in mortality among all the regions of release. The region of Western Strathcona had significantly higher hibernation-related mortalities than the other regions. This finding echoes the results of Lloyd *et al.* (2019) which found lower first year survival of VIMs released directly to sites in Strathcona. It has been suggested that Strathcona sites are a more “challenging” environment due to extreme terrain, higher elevation, and lack of available burrows (Aaltonen *et al.*, 2009; Lloyd *et al.*, 2019). It is possible that these characteristics are contributing to hibernation-related mortalities as well, as there might be differences in soil quality or vegetation at these sites (Buck and Barnes, 1999; Williams *et al.*, 2012). While the Schoen Lake region had the lowest overall first year hibernation-related mortality, it is important to note that this consisted of only 12 releases, all to Mount Seth. It is possible that this is not a sufficiently representative sample to accurately reflect hibernation survival in this region. Focusing on site-specific characteristics such as burrow quality, vegetation cover, and terrain in the

future will help to fully understand the mechanisms behind site-specific hibernation-related mortality.

Colony size at release was evaluated in this study as there are potential implications of social thermo-regulation on hibernation survival in VIMs (Bryant and McAdie, 2003). The release model suggests that larger colonies experience more hibernation-related mortality. This may be due to limitations on higher quality burrows in larger groups. It has been proposed existing burrow infrastructure may be vital to newly released VIMs that have only hibernated in nest-boxes in managed care (Lloyd *et al.*, 2019). It is possible that limited burrows in larger groups are contributing to poor overwinter survival. Although this result was significant, it had a moderate effect size which may be due to large variation in the colony size data. Due to dispersal in the active season, it is difficult to have a definitive count of colony size from year to year (M. McAdie; pers. comm. 2019), thus the estimate of colony size used for this analysis should be considered a proxy and may not reflect actual colony numbers. However, these results suggest that the stepping-stone approach is likely leading to higher overall hibernation survival, as it ensures existing burrow infrastructure for newly released VIMs. Moreover, releases should continue to be done with an emphasis on previously occupied sites or sites that currently have populations of VIMs rather than re-colonizing new sites.

We likewise found releases later in the active season were significantly more likely to result in a hibernation-related mortality, confirming the findings of Jackson *et al.* 2016. Although the exact mechanisms behind this result are yet unknown, it was proposed this

may be due to a longer acclimation period in the wild for newly released VIMs before hibernation (Jackson *et al.* 2016).

The impacts of weather conditions on hibernation survival have been investigated in many species (Johnstone *et al.*, 2012; Thogmartin and McKann, 2014; Turbill and Prior, 2015; Johnston *et al.*, 2021). For the two models in this study that evaluated weather, mean snowpack and variability in snowpack had the greatest overall influence on hibernation-related mortality. Winters with higher mean snowpack and greater standard deviation in snowpack resulted in higher overwinter survival for VIMs. Higher mean snowpack leading to increased survival was expected, as snow is an insulator and is vital for thermoregulation in burrows (Johnston *et al.*, 2021). Similar results were found in a study of hoary marmots (*Marmota caligata*), in which juvenile overwinter survival was highest in years with greater mean snow depth, and winter weather conditions were the most accurate predictor of overwinter survival (Patil *et al.*, 2013). This finding may be relevant in the immediate future, as some projections have snowpack reducing significantly due to warming temperatures over the next 20 years (Rhoades *et al.*, 2018). Closely monitoring snowpack trends may help predict hibernation survival for released VIMs.

The significance of the variability in snowpack was likely driven by overall greater mean snowpack in those winters, as years with higher mean snowpack would also have greater deviation. However, it might be possible that this effect was a result of changes in thermoregulation because of fluctuating temperatures. Internal burrow temperature has

been shown to fluctuate for most hibernators and can affect metabolic rate, torpor bout length, and body weight during hibernation (Buck and Barnes, 1999; Barnes and Buck, 2000; Belovezhets and Nikol'skii, 2012). It has also been suggested that variations in ambient temperature can increase efficient use of energy during hibernation, by limiting energetic investment in thermoregulatory processes (Dausmann *et al.*, 2020). As snowpack has an insulating effect, ambient air temperatures are not an accurate reflection of the internal burrow temperatures, so it may be the case that snowpack variation is also representing variation in burrow temperature. It is important to consider burrow temperatures for this conservation breeding program, as VIMs in managed care hibernate at a controlled temperature (Aymen *et al.*, 2021). Given the importance of this predictor for hibernation-related mortality in this species, and closely related species, future studies should focus on how hibernation cycles differ among VIMs in managed care under constant temperature, and wild VIMs with fluctuating temperatures. For example, bouts of torpor were found to be significantly different for captive alpine marmots (*Marmota marmota*) compared to their wild conspecifics (Geiser *et al.*, 2000). It is possible that VIMs in managed care are physiologically unequipped to cope with variations in temperature once in the wild and this may be contributing to poor overwinter survival in the first year.

The care facility of origin had the greatest influence on hibernation-related mortality of all the intrinsic factors. Predictably, VIMs that originated in the wild had the highest overwinter survival. This supports previous research that found wild VIMs to be efficient hibernators, and typically do not experience hibernation-related mortality (Bryant and

McAdie, 2003; Bryant and Page, 2005; Jackson *et al.*, 2016). Interestingly, recent research found that wild-born VIMs in managed care had significantly longer hibernation periods than captive-bred individuals, which might suggest that circannual cycles are acquired in the wild (Aymen *et al.*, 2021). Besides wild individuals, VIMs originating from the TBMWMRC had the next lowest instances of hibernation-related mortality. Studies investigating hibernation found VIMs housed on Vancouver Island to have hibernation cycles that most closely resemble those of wild VIMs (Bryant and McAdie, 2003; Aymen *et al.*, 2021). This has been attributed to similarities in photoperiod, elevation, and access to outdoor spaces within the natural habitat (Bryant and McAdie, 2003; Aymen *et al.*, 2021). Our results suggest that having a hibernation cycle in managed care more closely mirroring that of wild VIMs may be better preparing them for survival once released to the wild. This supports the notion that physiological adaptation as a result of managed care may be influencing hibernation survival in the species.

In both intrinsic models (with and without N:L ratio) VIMs originating from TZ had significantly higher instances of hibernation-related mortality. This is likely also a reflection of environmental differences in elevation, photoperiod, and climate due to location (Bryant and McAdie, 2003; Aymen *et al.*, 2021). However, somewhat contradictorily in the model without the N:L ratio, VIMs originating at MVF were found have significantly higher instances of first-year hibernation related mortality, which was not seen in the model including the N:L ratio. This is surprising as MVF is located in British Columbia, and hibernation cycles at this facility were close to that of wild individuals (Bryant and McAdie, 2003). These results suggest that proximity to the

natural habitat and hibernation duration are not suitable predictors of hibernation success alone, and a combination of intrinsic and extrinsic predictors should be considered to accurately understand overwinter survival for the species.

The N:L ratio at release was not significant in this analysis. However, this cannot rule out the possibility of increased HPA axis activity due to introduction to a novel environment contributing to hibernation mortality, as the samples used in this analysis would not have reflected the physiological condition of *in situ* VIMs. Hair cortisol has been found to be significantly elevated in newly released VIMs, suggesting there is a stress response as a result of introduction to the wild (Acker, 2017). Moreover, other studies in closely related species have found stress measures to be a reliable predictor of overwinter survival (Wheeler and Hik, 2014; Wey *et al.*, 2015). This represents a key area of further research in the program to identify a direct relationship between hibernation-related mortality and a stress. Sampling newly released VIMs will allow insights into physiological changes that occur immediately post-release. Moreover, a better understanding of how physiological parameters change seasonally in both captive and free-ranging VIMs will help determine whether there are physiological changes in the species as a result of managed care.

This study was the first to evaluate multiple factors on hibernation-related mortality for VIMs. While the retrospective analysis allowed robust sample sizes and to evaluate trends over many years in the program, not all the data used was available for the study period and had to be approximated, such as snow cover and snow melt dates. This

analysis provided insights into a previous unknown subject for this conservation breeding and release program, however future studies should focus on targeted data collection in order to validate these results. Moreover, missing data resulted in limitations in the number of observations for some models, which highlights the need for consistent and standardized data collection throughout the program to maximize the utility of retrospective investigations.

Conclusion

There has been significant population recovery for VIMs due to the efforts of the conservation breeding and release program, however hibernation-related mortality in newly released VIMs remains an area of concern (Jackson *et al.*, 2020; Aymen *et al.*, 2021). This study emphasizes the need to consider both extrinsic environmental factors and intrinsic factors to maximize overwinter survival in the species. In particular, releases to less severe sites with existing colonies should continue to be prioritized for VIMs in managed care. Further investigation should focus on differences in thermoregulation in individuals in managed care that hibernate under controlled temperatures and compare this to wild individuals. As studies have shown that hibernation cycles in VIMs are different not only between wild VIMs and VIMs in managed care but even among VIMs in managed care at different facilities it is important to consider these effects holistically, as individuals are often moved among these locations in the program.

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Table 2.1 Summary of all estimates and p-values for each predictor and each model used for this analysis. For all models, a binary response of 0 hibernation-mortality and 1 survived was used. Bolded values indicate a significance level less than 0.05

Model	Predictor	Estimate	P-Value
Release Model	Colony size at release	0.429	0.01*
	Year	0.059	0.72
	Month	0.258	0.05*
	Marmots on site	-0.418	0.24
	Clayoquot Plateau	0.577	0.37
	Nanaimo Lakes	0.567	0.19
	Schoen Lake	-0.756	0.37
	Western Strathcona	1.027	0.003**
Mean Environmental Model	Mean Temperature (°C)	-0.127	0.41
	Mean Snowpack (SWE)	-0.219	0.24
	Min. Temperature (°C)	0.116	0.50
	Max. Temperature (°C)	0.122	0.34
	Melt Date	-0.131	0.46
	Cover Date	-0.027	0.84
Variability Environmental Model	Standard Deviation Snowpack (SWE)	-0.275	0.05*
	Standard Deviation Temperature (°C)	-0.002	0.98
	Melt Date	-0.182	0.24
	Cover Date	-0.024	0.84
Intrinsic Model (With N:L ratio)	Origin (TBMWMRC)	2.366	0.17
	Origin (MVF)	2.939	0.08
	Origin (CZ)	3.250	0.06
	Origin (TZ)	3.285	0.05*
	Sex	0.715	0.16
	Age at release	0.295	0.21
	N : L Ratio at release	0.206	0.62
Intrinsic Model (without N:L ratio)	Sex	0.043	0.86
	Age at release	0.123	0.34
	Origin (TBMWMRC)	0.539	0.23
	Origin (MVF)	0.928	0.05*
	Origin (CZ)	0.328	0.48
	Origin (TZ)	1.657	> 0.001***

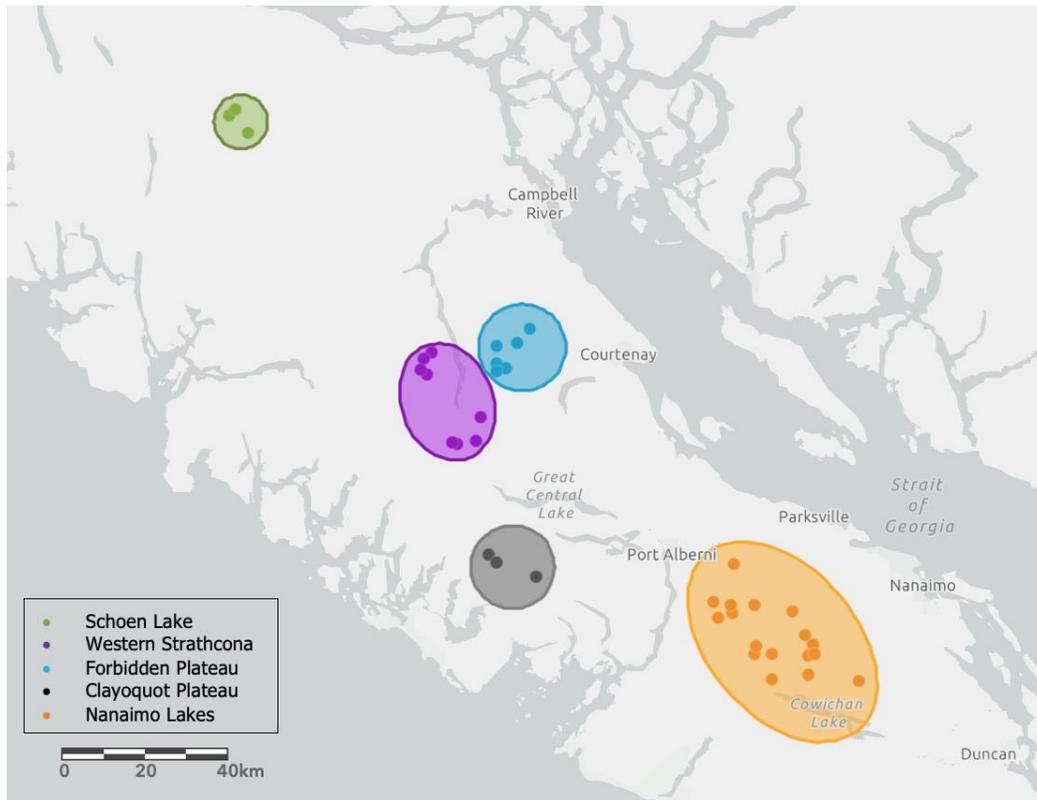


Figure 2.1 Map of regions (shaded) and sites (points) of release used in this study. Regions of use were designated by the Marmot Recovery Foundation.

¹ Sites in Schoen Lake include: Cain, Kunnum Slide, Mount Seth

² Sites in Western Strathcona include: Drinkwater, Flower Ridge, Greig Ridge, Henshaw, Marble Meadows, Morrison Spire, Phillips, Tibetan

³ Sites in Forbidden Plateau include: Albert Edwards, Allan Brooks, Castlecrag, Frink, Sunrise, Washington

⁴ Sites in Clayoquot Plateau include: 5040, Lions North, Steamboat

⁵ Sites in Nanaimo Lakes include: Arrowsmith, Bell Creek, Big Ugly, Butler, Buttle, Douglas Peak, Gemini, Green, Haley, Heather, Hooper, Hooper North, Limestone, McQuillan, McQuillan North, P Mountain, Tangle Mountain, Whymp

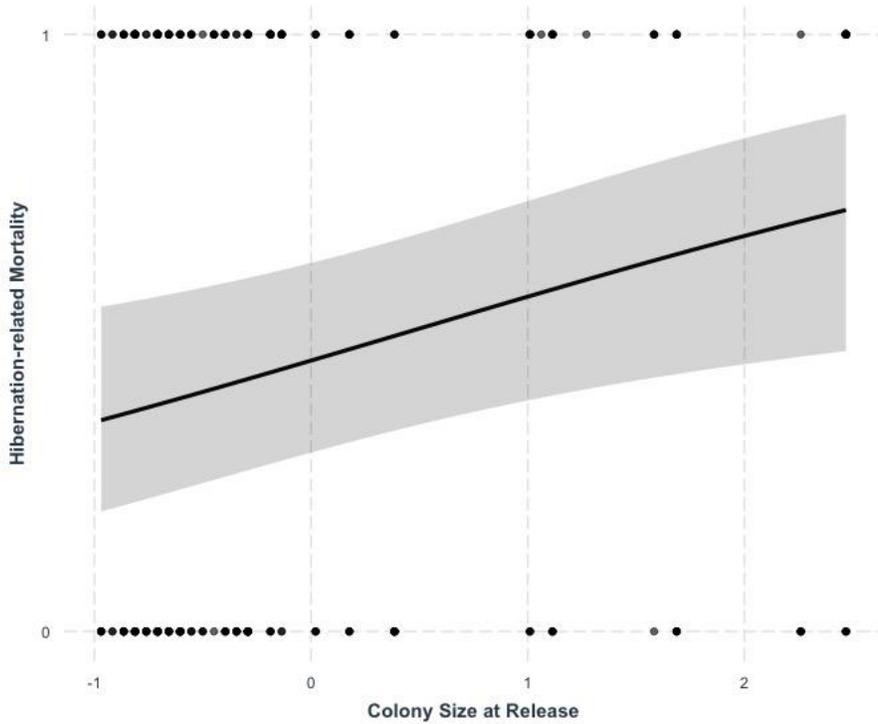


Figure 2.2 Visualization of colony size predictor from generalized linear mixed model. Hibernation-related mortality response is binary, where 1 is hibernation mortality and 0 is survived. Colony size was estimated as a count of unique individuals at each site per year for inventory purposes.

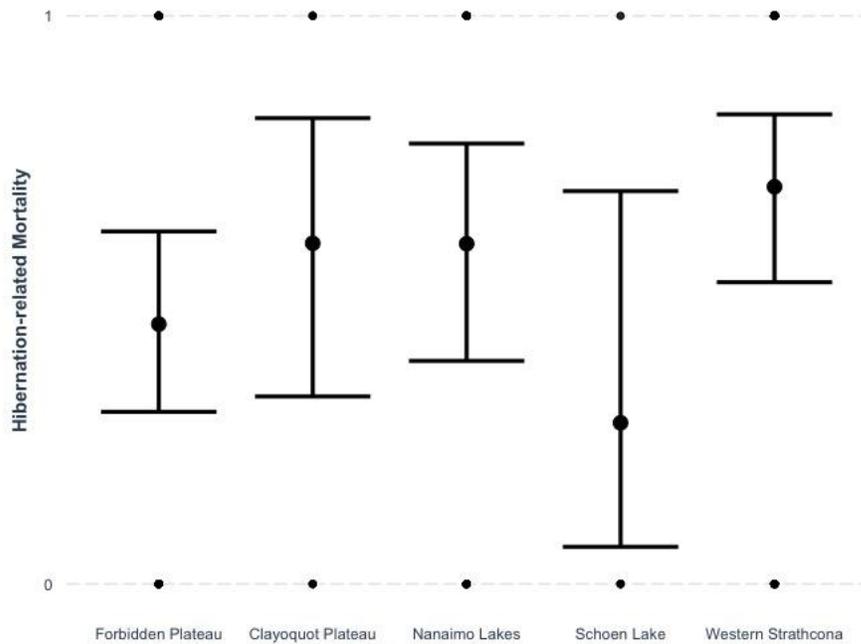


Figure 2.3 Visualization of region of release predictor from release factor generalized linear mixed model. Hibernation-related mortality response is binary, where 1 is hibernation mortality and 0 is survived.

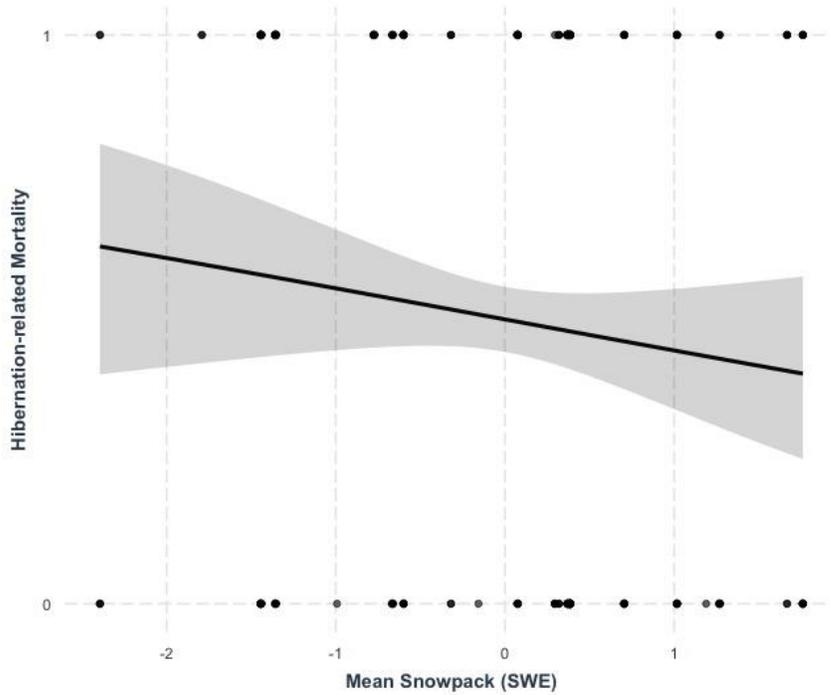


Figure 2.4 Visualization of average snowpack predictor from averages environmental factor generalized linear mixed model. Hibernation-related mortality response is binary, where 1 is hibernation mortality and 0 is survived. Numeric predictor was scaled around 0 for comparison.

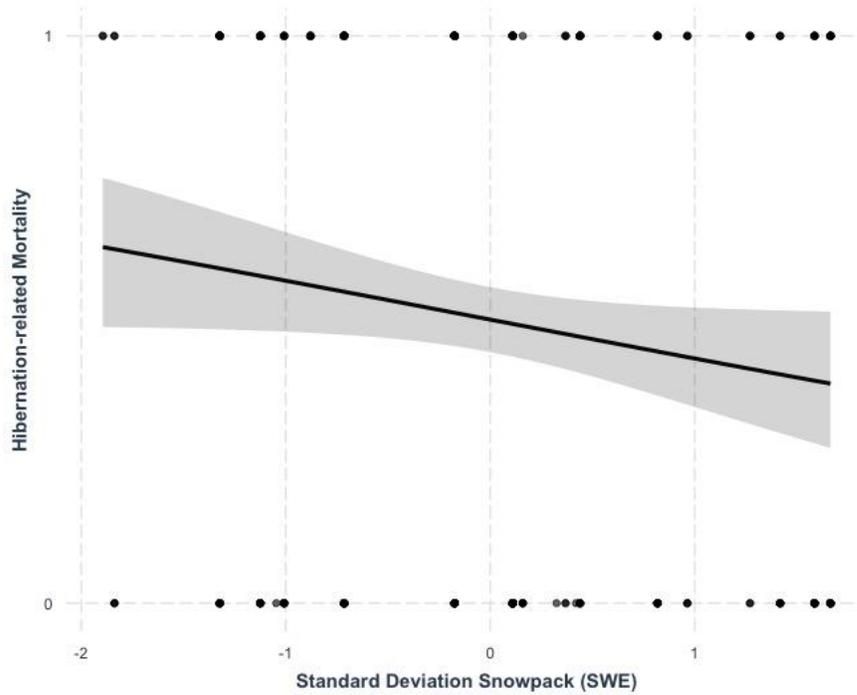


Figure 2.5 Visualization of standard deviation snowpack predictor from deviation environmental factor generalized linear mixed model. Hibernation-related mortality response is binary, where 1 is hibernation mortality and 0 is survived. Numeric predictor was scaled around 0 for comparison.

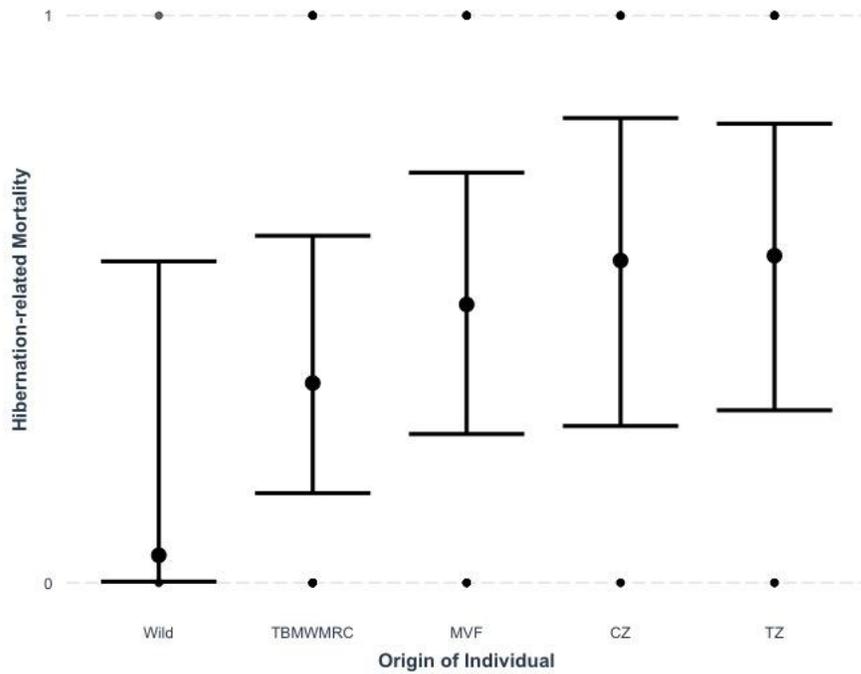


Figure 2.6 Visualization of origin predictor from intrinsic factor generalized linear mixed model (including N:L ratio). Hibernation-related mortality response is binary, where 1 is hibernation mortality and 0 is survived. Levels were compared against wild individuals.

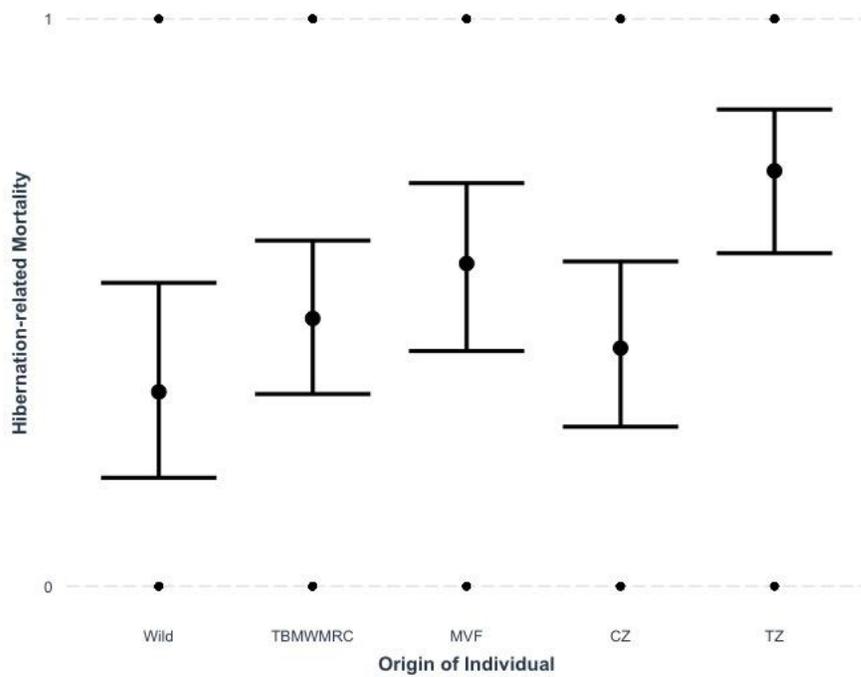


Figure 2.7 Visualization of origin predictor from intrinsic factor generalized linear mixed model (excluding N:L ratio). Hibernation-related mortality response is binary, where 1 is hibernation mortality and 0 is survived. Levels were compared against wild individuals.

Appendix

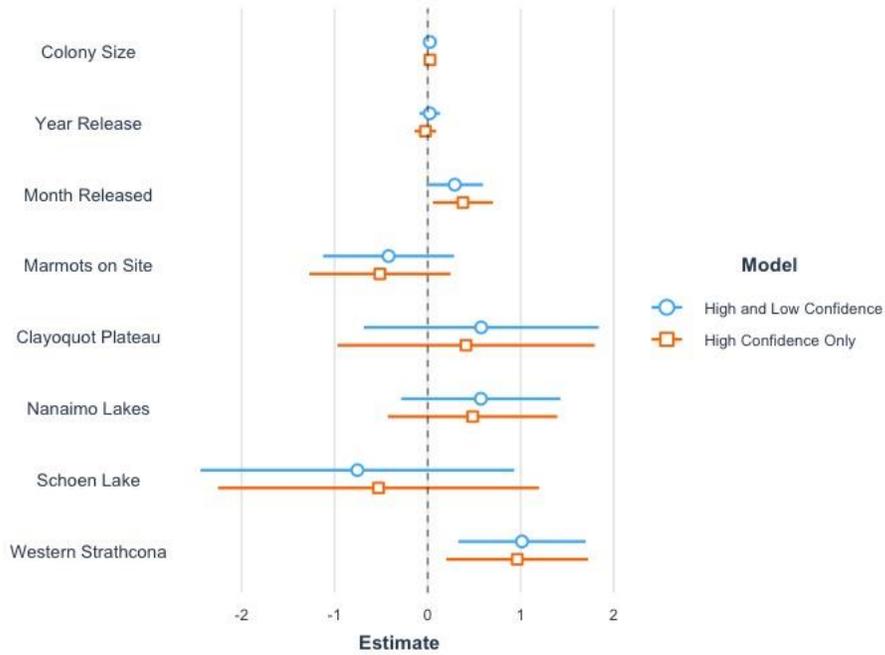


Figure A2.1 Comparison of effect sizes of high and mixed confidence models for release factors.

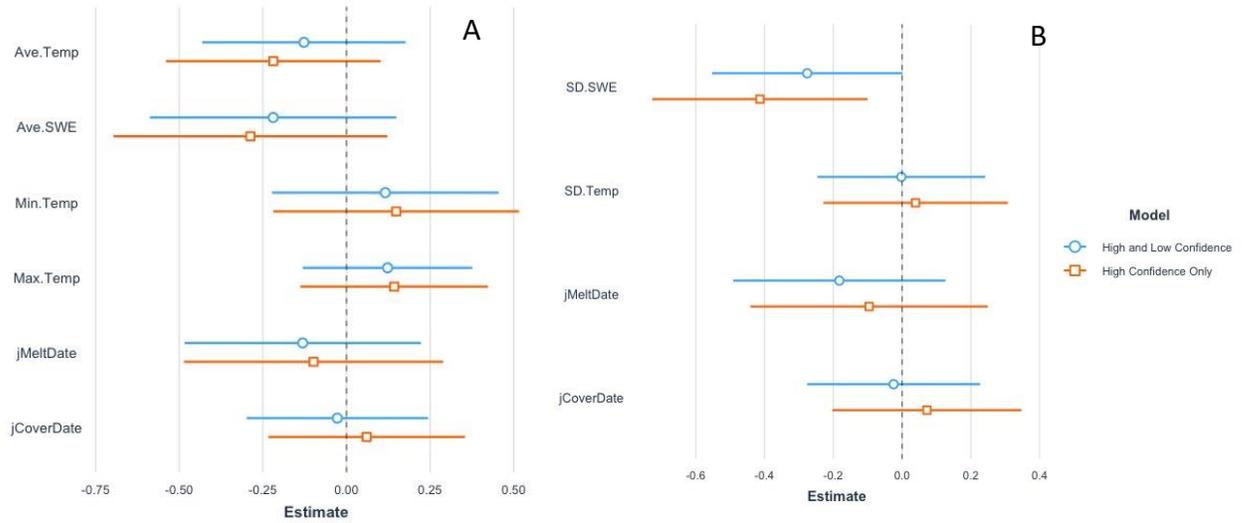


Figure A2.2 Comparison of effect sizes of high and mixed confidence models for mean environmental factors (A) and deviation in environmental factors (B)

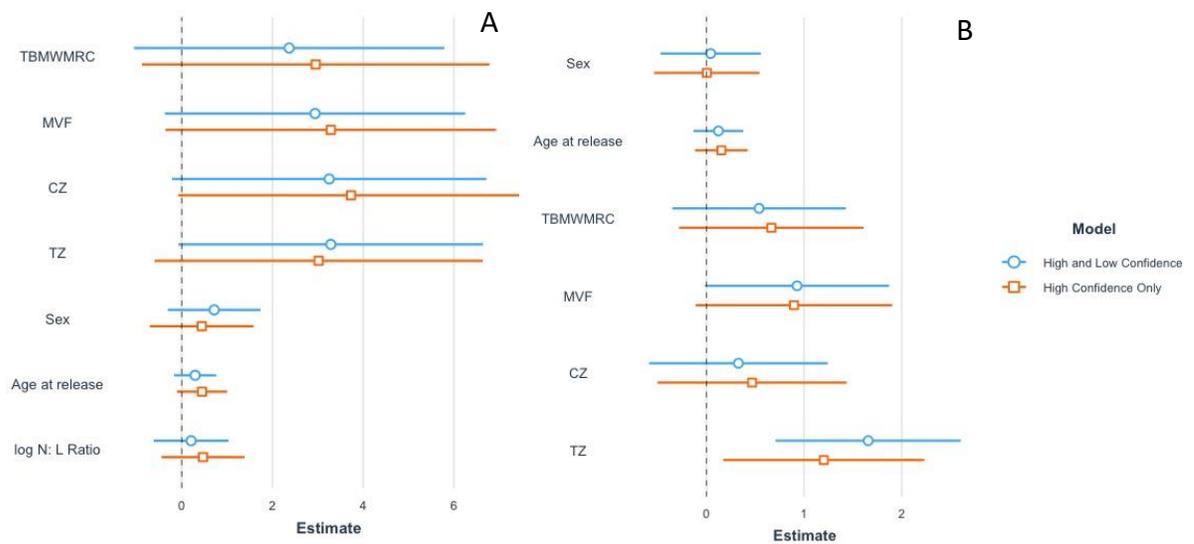


Figure A2.3 Comparison of effect sizes of high and low confidence models for intrinsic factors including the N:L ratio (A) and excluding the N:L ratio (B).

General Discussion

Although the value of reintroduction biology in recovering at-risk species cannot be understated, it is vital that programs are being managed with careful consideration of the species biology in mind to maximize program outcomes. Recently, researchers have advocated for the One Plan Approach (OPA) in conservation initiatives. The OPA supports integrated management, where individuals in the population both *in* and *ex situ* are considered holistically (Byers *et al.*, 2013; Traylor-Holzer *et al.*, 2019). The OPA is particularly applicable to conservation breeding and reintroduction/release programs, where some individuals are released to the wild. As individuals in such programs are being moved between *in* and *ex situ* environments, it becomes crucial to understand how changes in their physiology may affect their fitness in either context.

The Vancouver Island marmot (VIM; *Marmota vancouverensis*) conservation breeding and release program provides a somewhat unique opportunity to study the same species, and often the same individual, in a variety of contexts both *in* and *ex situ*. While extensive recovery efforts from a variety of partners including the Government of Canada, multiple zoos, and non-profit organizations have grown the *in situ* population substantially since its initial decline, there is still the need for supplementation from individuals bred in managed care. This provides the opportunity for further research using the OPA to identify possible changes in these individuals as a product of their environment.

The research in this thesis aimed to fit the OPA, as it considered individuals throughout the program to compare their physiology and survival. First, I compared the physiology of VIMs in different environments in the program using the neutrophil to lymphocyte (N:L) ratio to represent hypothalamus-pituitary-adrenal (HPA) axis activity. This included among captive, pre-release, post-release and wild populations. Finding a significantly lower N:L ratio between captive and pre-release VIMs, compared to post-release and wild VIMs exemplified the importance of considering the OPA for the conservation breeding and release program for this species as this suggests the physiology of this species is not conserved throughout the program.

There were also differences in the N:L ratio among the care facilities, with the highest N:L ratios in individuals at the Mountain View Conservation and Breeding Society (MVF) and Calgary Zoo (CZ), and the lowest at the Tony Barrett Mount Washington Marmot Recovery Centre (TBMWMRC) and Toronto Zoo (TZ). This result suggests a combination of factors, including access to the natural environment and the quality of care and management practices within the facilities may be contributing to the N:L ratio in VIMs.

Additionally, an analysis of three possible sources of variation in the N:L ratio, age, sex, and month of sampling, revealed that certain populations were affected differently in respect to these parameters. Specifically, age was found to have the greatest effect in wild VIMs, such that the N:L ratio increased with age. Sex was found to have a significant effect on the N:L ratio for captive and pre-release populations, but not for post-release or

wild populations. While the differences in N:L ratio is valuable information to know, one hinderance of using historic blood samples is that this provides only a static point of the physiological conditions at the time of the sample and cannot give us information about the individuals ability to cope with a stressor. This is crucial to understanding if there are any fitness consequences because of this physiological difference, especially upon release to Vancouver Island.

Hibernation in VIMs remains an area of research focus in the program (Aymen *et al.*, 2021). Particularly, there is concern over higher than expected rates of hibernation-related mortality in newly released VIMs (Jackson *et al.*, 2016). This concern also represents an area where the OPA is relevant, as this involves a period where *ex situ* VIMs are transitioned to the *in situ* environment. By investigating factors in three key areas relating to hibernation: release factors, environmental factors and intrinsic factors, this study confirmed that it is relevant to investigate a variety of parameters – not just physiological or environmental exclusively, to have a comprehensive picture of what is affecting hibernation survival.

Previously, the importance of site-specific survival for VIMs was highlighted through the adoption of the “stepping-stone” release method (Lloyd *et al.*, 2019). Our results support this method, as hibernation-related mortality was found to be significantly influenced by the region of release, with VIMs released to the Western Strathcona region exhibiting higher hibernation-related mortality. Snowpack parameters including mean snowpack and the standard deviation in snowpack (SWE) had the greatest influence for

hibernation-related mortality for the environmental models. There is extensive evidence to suggest that snowpack is important to hibernating animals as it provides insulation for burrows (Belovezhets and Nikol'skii, 2012; Patil *et al.*, 2013; Johnston *et al.*, 2021). Future studies should investigate burrow temperatures and thermo-energetics for *in situ* and *ex situ* VIMs as they experience different temperatures and exposure to snow as a product of their environments. Finally, differences in the hibernation-related mortality based on the care facility of origin suggests that a combination of location, access to the natural environment, and animal husbandry practices within those facilities may be contributing to hibernation-related mortality.

This analysis was the first to use a physiological metric of stress and compare it to hibernation-related mortality. However, the N:L ratio at release was not a significant predictor in the model. This cannot rule out the possibility of HPA axis activity contributing to hibernation-related mortality however, as the samples used in this analysis were taken pre-release and would not have been reflective of the physiological state of a newly released VIM. Previous research for VIMs found hair cortisol is significantly elevated in these individuals (Acker, 2017), and in free-ranging yellow-bellied marmots (*Marmota flaviventris*) elevated glucocorticoid levels were associated with lower overwinter survival (Wey *et al.*, 2015). Therefore, future studies should focus on physiology in the novel environment to fully understand if there are significant changes and how these might affect their fitness.

Although it is difficult to compare the results of the two chapters directly because they evaluated different responses, it is interesting to consider the results from the two studies as they pertain to the care facilities. Individuals at the TBMWMRC had the lowest N:L ratios, and the lowest instances of hibernation-related mortality from VIMs originating from that facility. Likewise, individuals at MVF had high N:L ratios and high instances of hibernation-related mortality. This might support the idea that differences in the physiological state of VIMs because of housing facilities may be affecting fitness post-release. However, individuals originating from TZ had significantly higher instances of hibernation-related mortality, but the N:L ratio was not significantly different at TZ compared to the TBMWMRC. Therefore, further research into the physiological condition of VIMs from each facility, directly compared to survival post-release would be needed to determine if there were any conclusive effects.

A challenge conservation breeding and release programs face is effective post-release monitoring (Armstrong and Seddon, 2008; Bubac *et al.*, 2019). Moreover, to apply the OPA, is it vital that all individuals can be monitored, as there needs to be a consideration of the population as a whole. For VIMs, post-release monitoring is difficult due to accessing remote release sites, the costs and personnel associated with monitoring, as well as allowing the released individuals to acclimatize with minimal disturbance. However, this study demonstrated it is crucial to understand if or why changes occur in VIMs during the critical post-release stage. Although our results suggest that long-term released VIMs appear to be in a similar physiological state to wild VIMs in respect to the N:L ratio, we do not know how long it takes for this to occur, nor what happens to

released VIMs immediately upon introduction to the novel environment. This is particularly relevant because it may be affecting their ability to survive hibernation in their first winter.

Once thought to be one of the rarest animals in the world, the Vancouver Island marmot population has recovered significantly due to the incredible efforts of the conservation breeding and release program (Jackson *et al.*, 2020). Taken together, the results of this thesis demonstrate the importance of considering both *in* and *ex situ* VIM populations as a whole, as there were significant differences in physiology between these groups. Moreover, both extrinsic and intrinsic factors were shown to affect hibernation-related mortality. The research presented in this thesis aimed to contribute to the incredible recovery efforts already in place, and ultimately provide greater insights to the management of animals in other conservation breeding programs. By growing our understanding of these management practices, these programs can continue to be effective in preventing the extinction of at-risk species.

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