

INFLUENCE OF ENVIRONMENTAL FACTORS ON RED PANDA WELFARE

by

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Influence of Environmental Factors on Red Panda Welfare

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at George Mason University

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## LIST OF ABBREVIATIONS

Adrenocorticotrophic hormone .....	ACTH
Akaike information criterion (corrected for small sample sizes).....	AICc
Analysis of variance.....	ANOVA
Association of Zoo & Aquariums .....	AZA
Celsius.....	°C
Conservation on International Trade in Endangered Species of Wild Fauna and Flora .....	CITES
Correlation coefficient .....	r
Corticotropin releasing hormone .....	CRH
Daily Average Temperature.....	T <sup>A</sup>
Daily Maximum Temperature .....	T <sup>Min</sup>
Daily Minimum Temperature .....	T <sup>Max</sup>
Decibel .....	dB
Enzyme Immunoassay .....	EIA
Fahrenheit .....	°F
Friends of the National Zoo .....	FONZ
Generalized Linear Mixed Models .....	GLMM
Hypothalamic-pituitary-adrenal.....	HPA
International Union for the Conservation of Nature .....	IUCN
Local Climatological Data .....	LCD
Mesker Park Zoo & Botanic Garden.....	MPZ
Miles per hour .....	mph
National Ecological Observatory Network.....	NEON
National Oceanic and Atmospheric Association .....	NOAA
Non-Specific Binding .....	NSB
Population and Habitat Viability Analysis.....	PHVA
Proportion of time active .....	PA
Proportion of time panting .....	PP
Proportion of time in straddle .....	PS
Relative centrifugal force.....	g
Room Temperature .....	RT
Rotations per minute .....	RPM
Seneca Park Zoo .....	SPZ
Smithsonian Conservation Biology Institute .....	SCBI
Smithsonian's National Zoological Park .....	NZP
Standard error of the mean.....	SE
Studbook .....	SB
Taxonomic Advisory Group .....	TAG
3,3',5,5'-Tetramethylbenzidine .....	TMB
Tukey's Honest Significant Difference.....	Tukey'sHSD
Variance Inflation Factor .....	VIF



Visitor Number ..... VN

## ABSTRACT

### INFLUENCE OF ENVIRONMENTAL FACTORS ON RED PANDA WELFARE

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Animals in zoological institutions are exposed to a multitude of environmental elements daily. Understanding which factors may invoke a stress response and be indicators of negative well-being is important to improve captive management of species and enhance reproductive success. Red pandas (*Ailurus fulgens*) are an endangered species facing multiple threats in the wild including habitat destruction, forest fragmentation and poaching. Captive populations are vital for longevity of the species, as wild populations continue to decline. Red pandas are commonly housed in zoos across the United States, yet growth of the *ex-situ* population is hindered by high cub mortality. To assess their well-being, behavioral and physiological responses of red pandas were assessed in relation to climate (temperature, humidity and windspeed), zoo visitors, and noise. From June 2018 to June 2019, behavioral observations were conducted 1-2 times per week between 0800 and 1000 h on red pandas housed at the Smithsonian's National Zoological Park and Conservation Biology Institute. Fecal samples were collected 3-7 days per week from ten individuals housed at four U.S. zoos. Fecal glucocorticoid metabolites (fGM) were measured with a corticosterone enzyme immunoassay and then analyzed

in relation to environmental conditions, such as ambient temperature and ambient noise.

Increased ambient temperature was associated with decreased activity and increased coping behaviors, including panting and straddle. Month of the year and the number of zoo visitors from the previous day also affected fGM concentrations such that red pandas had lower glucocorticoid concentrations May through August compared to other months. Increased zoo visitor numbers led to lower fGM concentrations the following day. Results suggest that environmental temperature impacts red panda behavior and the species displays seasonal variability in fecal glucocorticoid metabolites. Findings from the study may prove useful in improving *ex-situ* management and enhancing welfare by providing evidence that red pandas show the effects of temperature starting at 20°C, which is lower than threshold temperatures described in current care manual recommendations.

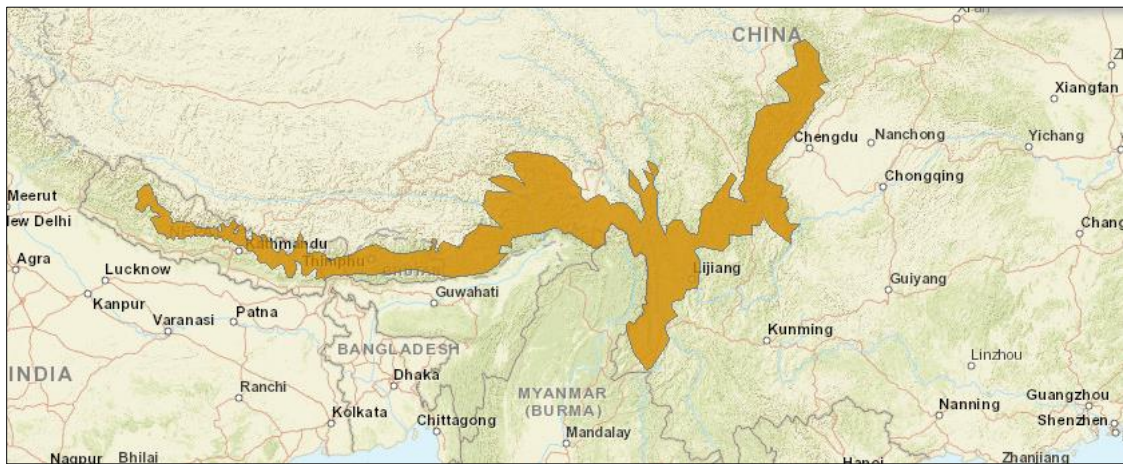
## CHAPTER ONE: BACKGROUND INFORMATION

### **1. The Red Panda**

The Himalayan Mountains are home to the endemic red panda (*Ailurus fulgens*). This species is the only living member of the Ailuridae family (Choudhury 2001), making it taxonomically unique. Red pandas are classified under the order Carnivora, yet bamboo leaves and shoots are their main food source (Yonzon and Hunter 1991). There are two subspecies, *A. fulgens fulgens* and *A. fulgens styani*, distinguished by their range as well as appearance. *A. fulgens fulgens* are native to Nepal, India, Bhutan, Northern Myanmar as well as southern Tibet and Western Yunnan areas of China (Dorji et al. 2012), and have a lighter colored pelt, most notably on the face, and is smaller in size (AZA Small Carnivore TAG 2012). *A. f. styani* in contrast have a stockier body type and are darker in color. They are confined to a smaller geographic range by the Salween River, occurring in south-central China within the Sichuan and Yunnan provinces (Choudhury 2001; Dorji et al. 2012). A recent study investigating the genetics of wild red pandas has disputed the geographic distribution of the two subspecies. According to Hu et al. (2020), populations of pandas in Myanmar and west of the Salween river in China tend to be more genetically similar to pandas in Eastern China than those found in Nepal. Rather than the Salween river as previously thought, this study suggest that the Yalu Zangbu River is the barrier between the subspecies and genetic evidence suggests that red pandas may instead be two distinct species, though samples from India and Bhutan are necessary to confirm (Hu et al. 2020).

As previously described, the current geographic range of the red panda spans five countries (Fig. 1). They are found in 23 districts in Nepal, three states in India, 13 districts in

Bhutan, one state in Myanmar, and three provinces of China (Dorji et al. 2012; Glatston et al. 2015). Red pandas occupy elevations from 2,400 to 3,900m in evergreen, deciduous, and mixed conifer-broadleaf, old growth forests with bamboo understory (CEPF 2005; Sharma et al. 2014; Yonzon and Hunter 1991). Bamboo is highly important because the woody grass is the main food source for red pandas. Even though they are classified as carnivores, over 90% of red panda diet can consist of bamboo (Wei et al. 1999), with opportunistic consumption of fruits, small birds, eggs, insects and other grasses (Dorji et al. 2012; Yonzon and Hunter 1991). Red pandas are primarily an arboreal species (Choudhury 2001) that prefers habitat typically less than 100m from water (Bhatta et al. 2014; Sharma et al. 2014). Downed or fallen logs also are necessary in panda habitat for resting, feeding, defecation, and movement between trees (Bhatta et al. 2014; Dendup et al. 2017; Glatston et al. 2015).



**Figure 1.** Red panda distribution range (IUCN, 2015)

Wild red panda populations are in decline. The International Union for Conservation of Nature (IUCN) Red List classifies the species as Endangered. Red pandas have undergone a 50% population decline over the last three generations or approximately 20 years (Glatston et al.

2015). This evidence of declining population, as well as decreasing habitat, led the IUCN to change the classification of red pandas from Vulnerable to Endangered in 2015. The exact number of wild red pandas is unknown, but it is estimated there are less than 10,000 mature individuals (Glatston et al. 2015). In order to better understand how many red pandas could remain in the wild, researchers have conducted multiple Population and Habitat Viability Analyses (PHVA). Estimated occupied habitat across the entire geographic range varies from as little as 18,000 km<sup>2</sup> up to 142,000 km<sup>2</sup> (Choudhury 2001; Glatston et al. 2015; Kandel et al. 2015; Panthi et al. 2019). It is difficult to know for certain how much habitat is currently suitable for the species and how many individuals are in the wild.

The red panda currently faces many viability concerns across its range, which place species survival at great risk. All the threats are directly or indirectly related to anthropogenic actions. Increasing human population causes more people, and their activities, to encroach on red panda habitat. Expanding infrastructure, habitat loss and degradation, poaching (Thapa et al. 2018), and lack of knowledge about the species including the inability of locals to identify red pandas (Dorji et al. 2012), are all major issues facing the species. Other dangers to red panda populations include climate change, forest fire, small population size, and high cub mortality (Hu et al. 2020; Thapa et al. 2018). A fundamental threat in China is deforestation, causing fragmentation of essential forest habitat (Wei et al. 1999). Most habitat destruction that has already taken place is irreversible for the needs of red pandas. Forest restoration, through planting new trees, would take decades or even centuries for the forest to return to ideal red panda habitat (CEPF 2005; Choudhury 2001). In addition to reduced habitat available to red pandas, forest fragmentation can also limit movement of individuals for successful food foraging and reproduction. For small populations that have already undergone bottlenecks (Hu et al. 2020) it is imperative to keep genetics moving across red panda ranges.

Agriculture expansion is also posing risks to red pandas. Cow-herders as well as their livestock and dogs disturb pandas in their native area. Cows consume bamboo leaves and trample stalks, possibly impeding plant growth which the pandas rely on as their predominant food source (Yonzon and Hunter 1991). Herders use guard dogs in order to protect livestock from predators. These dogs directly kill red pandas and carry disease (Glatston et al. 2015; Thapa et al. 2018) such as canine distemper virus, for which there is lack of wide-spread vaccination. Distemper has the potential to spread from domestic animals to wildlife and is fatal to red pandas if they contract the disease (Glatston et al. 2015). Red panda presence is lower in areas with livestock use (Sharma et al. 2014), and red pandas tend to avoid areas where livestock disturbances and timber harvesting occurs (Dendup et al. 2017). However, the ability to avoid these disturbances may become less possible as humans continue to encroach upon and fragment red panda habitat. When anthropogenic factors (human population density, livestock density, distance to roads/paths, and distance to human settlement) are included in PHVA, suitable habitat in Nepal decreases by almost 4,500km<sup>2</sup> and becomes more fragmented.

Poaching and illegal wildlife trade are also a threat, causing a decline in panda populations (Glatston et al. 2015; Thapa et al. 2018). Historically, red pandas were killed by people for their fur to make clothing and hats (Wei et al. 1999; Dorji et al. 2012). The Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) lists the red panda under Appendix I. Species under this appendix are at great risk for extinction and trade is only permitted in specific circumstances. Even with this distinction and protection, recent poaching and illegal trade has increased (Glatston et al. 2015). A recent investigation of what may be causing the demand to poach pandas for their pelt was not able to identify the drivers for increased illegal trade in Nepal (Bista et al. 2020). The authors suggest possible drivers for increased pelt demand may be due to miscommunication at awareness events, investigator's

influence indicating pelt demand, and insufficient knowledge about red panda related crime (Bista et al. 2020).

In addition to the direct anthropogenic threats described above, the growth of wild populations is limited by high cub mortality. Red pandas reach sexual maturity around 18 months of age, allowing them to breed during their second winter (Glatston et al. 2015). Breeding season for red pandas in the northern hemisphere is January-March, with females giving birth to a single litter of cubs in the summer (AZA Small Carnivore TAG 2012; Glatston et al. 2015). Most litters in the wild have either one or two cubs (Yonzon and Hunter 1991) but in captivity pandas can have up to four in a litter (Glatston et al. 2015). Cub mortality as high as 85% has been documented in the wild. Although some of these deaths are due to predation by snow leopards, disturbance from humans is considered a key factor (Yonzon and Hunter 1991). Low reproductive rate and high cub mortality, in addition to the multitude of anthropogenic threats, place red pandas at a greater risk for extinction in the wild.

## **2. Natural History & Behavior**

Red pandas are arboreal animals that spend most of their time in the forest canopy (Dorji et al. 2012), resting and sleeping in trees or other elevated surfaces. Temperature and humidity can influence the posture of pandas during these activities. In the winter, red pandas are often curled up with their tails covering their face, but during warm summer times pandas can be seen in stretched out positions or lying with their limbs hanging off logs as they rest (personal observation; Roberts and Gittleman 1984). Activity budgets of red pandas are not well characterized and there is conflicting evidence in the literature with regards to both daily and seasonal activity. Red pandas are generally considered to be crepuscular animals, most often active at dusk and dawn (Johnson et al. 1988; Roberts and Gittleman 1984). Pandas in the wild



rest multiple times throughout the day and are only active 36.5% of the daytime period, with consistent activity across months (Johnson et al. 1988). Roberts and Gittleman (1984) state activity patterns change throughout the year based on temperature and if a female has young. Radio-telemetry of red pandas in China show they are active 48.6% of the day and individuals were more active during the daytime specifically between 0700-1000 h and 1700-1800 h, than at dawn/dusk or nighttime (Zhang et al. 2011). Zhang et al (2011) also found that activity patterns in red pandas differ based on season.

Red pandas are believed to be a solitary species in the wild; they are typically seen alone except during mating season and when females are raising young (Roberts and Gittleman 1984; Yonzon and Hunter 1991). They communicate through scent marking and the use of common latrine sites (Yonzon and Hunter 1991) as well as many vocalizations including twitters, squeals, huffs, and grunts. Complete understanding of vocalizations is not known. Twitters are often only heard during the breeding season, when they increase in frequency (Roberts and Gittleman 1984) while grunts and huffs are associated more with aggressive interactions between pandas (personal observation). Even though they are solitary in the wild, *ex-situ* red pandas are housed together as a companion pair of females or a male and female together. In the wild and captivity, red pandas display a host of conspecific interactions that range from aggressive to more playful and social behaviors (Roberts and Gittleman 1984). Many of these same behaviors, such as play, grooming and scent marking, can be seen in individuals housed alone (personal observation).

### **3. Ex-Situ Population**

Protecting wild red panda populations and their habitat is vital for long term survival of the species. However, the number of threats facing *in-situ* populations makes successful protection challenging. Developing a genetically diverse *ex-situ* population is highly important

for the long-term survival of the species (Glatston et al. 2015) by creating a hedge-fund for the future. Captive pandas also serve as ambassadors for the species, promoting interest and inspiring conservation for the future. In 2017, there were 217 red pandas, 151 *A.f. fulgens* and 66 *A.f. styani*, located in over 90 Association of Zoo & Aquariums (AZA) accredited facilities (Glass et al. 2017). Currently, there are 154 *A.f. fulgens* in 59 AZA facilities and 86 *A.f. styani* in 30 facilities, with a population growth rate of 1% for *A.f. fulgens* and 6% for *A.f. styani* (Glass et al. 2020). Although population growth occurs, *ex-situ* populations have their own hurdles to surmount. Like wild populations, high cub mortality occurs in captivity. Maternal cannibalism and poor maternal care cause the death of 50% of cubs in the first year after birth (Glass et al. 2011). In the United States, the largest proportion (40.2%) of captive red panda deaths occur in the neonatal age group when cubs are less than 30 days old (Delaski et al. 2015). Pneumonia and trauma are the two leading causes of death for that age group, 29% and 20% of deaths respectively (Delaski et al. 2015). Disturbance by people will cause mothers to move their cubs and can cause bite wounds (Preece 2011; Yonzon and Hunter 1991), which may be the cause of trauma-related deaths. High cub mortality could be mitigated if zoological institutions and animal managers are better informed on what environmental factors could disturb or cause stress in red pandas. Beyond reproductive considerations, understanding which environmental stressors impact red pandas is also important for overall welfare of individuals. Therefore, reducing such factors could result in all-encompassing improvements of individual welfare, breeding, and survival for future generations of captive pandas. To date, possible stressors have not been systematically studied.

#### **4. HPA Axis & Stress**

Homeostasis is the tendency to maintain a steady state, which is achieved through multiple physiological processes in the body. A stressor is any change that occurs which alters homeostasis. Animals have adapted by developing mechanisms for coping with stress. Not all stress experienced by an animal is bad for that individual, as stress plays a vital role to optimize behavior in response to environmental occurrences (Shirazi et al. 2015). In response to a potential stressor, the body activates the hypothalamic-pituitary-adrenal (HPA) axis as part of the neuroendocrine stress response. Activation of the HPA axis begins a cascade of events starting with corticotropin releasing hormone (CRH) secretion from the hypothalamus. CRH stimulates the anterior pituitary to produce adrenocorticotrophic hormone (ACTH). This hormone acts on the adrenal cortex, which in response produces glucocorticoids such as cortisol and corticosterone (Matteri et al. 2000). Glucocorticoids have a negative feedback on the hypothalamus and pituitary to inhibit secretion of ACTH and CRH, in order to terminate the stress response (Shirazi et al. 2015; Smith and Vale 2006). As the end-product of the HPA axis cascade, glucocorticoids are seen as a good physiological measurement for assessing stress in animals.

HPA axis activation in response to stress is important for the maintenance of homeostasis. Activation can cause physiological changes such as increased cardiovascular and respiratory rate (Smith and Vale 2006). Acute stress is often believed to be adaptive, creating changes in the body to improve an individual's chance of survival. Long-term or chronic stress has been shown to negatively influence reproduction, immunity to disease, appetite, and behavior (Matteri et al. 2000; Shirazi et al. 2015). Besides a physiological response, activation of the HPA axis can cause mental changes including increased awareness and improved cognition (Smith and Vale 2006), that help individuals to cope with stress. CRH may play a role in regulating behavioral responses by increasing activity and decreasing food intake (Wingfield 2005).

Increased activity budgets in animals may therefore indicate stress caused by elevated glucocorticoids. In addition, captive animals can develop stereotypies because of fear or stress that they are unable to avoid (Mason 1991). A stereotypy, defined by Mason (1991), is a repetitive behavior pattern with no obvious function, which can develop due to environmental forces placed upon the animal. Many factors, including temperature (Rees 2004) and enclosure size and design (Morgan and Tromborg 2007) can lead to the formation of stereotypies in captive animals. With so many factors which could lead to stress, altered activity budgets, or the formation of stereotypic behaviors in zoo animals, it is important to research which specifically impact red pandas in order to optimize their welfare in captivity.

## **5. Zoo Visitors and Noise**

Captive animals housed in zoological institutions are exposed to people daily. Presence of the public in zoos creates a visual stimulant to animals as well as sound stimuli from people at or near the exhibits. The public and the noise they create can have a wide range of impacts on the welfare of captive animals. The environment outside an animal's exhibit, which may involve fluctuating visitor numbers or enclosures with greater exposure to the public, can negatively impact captive animals (Hosey 2008). Herbivores are more impacted by visitors than carnivores and arboreal species are less effected than terrestrial ones (Queiroz and Young 2018). Additionally, animals native to closed, forest habitats are more influenced by visitors than species from open, savannah type habitats (Queiroz and Young 2018). As the number of visitors increase, noise levels within the zoo increase (Morgan and Tromborg 2007; Quadros et al. 2014), which can impact animals both behaviorally, as they try to avoid people and/or the noise they make as well as physiologically, by causing an adrenal response.

Fecal glucocorticoid metabolite (fGM) concentrations in zoo species are commonly examined as a response to environmental stressors. Increased glucocorticoids have occurred in response to loud days at the zoo and with construction outside exhibits (Owen et al. 2004; Powell et al. 2006). Captive species can also display increased fGM concentrations in response to increased zoo visitor numbers (Pifarré et al. 2012; Scott et al. 2017).

Based on red panda management guidelines (Glatston 1989) and the AZA's Red Panda Care Manual (AZA Small Carnivore TAG 2012), visitors should only have viewing access to one or two sides of enclosures. Even though this is the standard for the species in captivity, more than half of zoo exhibits give visitors greater access to red pandas than management recommendations (Eriksson et al. 2010). Guidelines also state noise and vibration disturbances should be kept to a minimum (AZA Small Carnivore TAG 2012); however, it is unknown what level of noise red pandas can tolerate and what levels may compromise their welfare. More research is necessary to understand how noise and the number of public visitors at zoos impact red pandas.

## **6. Climate**

Species are well adapted for the climates they inhabit, and red pandas are no exception. They are highly adapted to live in the cold climate of the Himalayan Mountains. Dense fur coats extending to the soles of their feet, a thick bushy tail, the ability to reduce metabolic rate, and low skin temperature are some of the adaptations which allow them to live in a harsh environment (Princée and Glatston 2016; Yonzon and Hunter 1991). However, these adaptations to a specific ecological niche suggests that the red panda is more vulnerable to climate change than a generalist species. Red pandas exist in regions where the temperature ranges from -10°C to 28°C but the temperature often does not exceed 20°C (Johnson et al. 1988; Princée and Glatston 2016; Roberts and Gittleman 1984; Yonzon and Hunter 1991). Climate change in the Himalayan

Mountains is leading to a shift in the tree line, as climate sensitive tree species are restricted to higher altitudes, thereby decreasing forest size, and affecting water availability (Xu et al. 2009). As temperatures warm, pandas may be able to move up in elevation, but climate change could cause a decline in the distribution of bamboo species, due to slow dispersal rate (Li et al. 2015). Changing climate, in combination with habitat destruction, makes it vital to understand how species living in high risk areas such as the Himalayas will be impacted.

The captive red panda population can give us a glimpse into the possible behavioral and physiological impacts of climate. Many captive individuals are housed in zoos across the United States with warmer climates than native populations normally experience, which could be a stressor leading to abnormal behavior. The AZA recommends facilities with warm summers give pandas access to air-conditioned indoor areas, nest boxes, or other holding spaces when temperatures exceed 80°F or 26.6°C (AZA Small Carnivore TAG 2012). Increased temperatures in combination with high humidity could place animals at risk for experiencing heat related illness. Heat stress has been documented as a cause of death in red pandas and is aggravated by high humidity (Philippa and Ramsay 2011). Panting is a mechanism red pandas can use to cool their body temperature, but panting may be less effective with high humidity. Increased humidity reduces evaporative cooling, because the high moisture content of the ambient air decreases the amount of water that can evaporate from the body (McKinley et al. 2018), thereby reducing the animal's cooling efficiency. Enhancing knowledge of the relationship climate has with behavioral and physiological response of zoo animals is key for improving welfare. Although the impact of climate on red pandas is not well studied it is important to guide captive management. For instance, survival of red panda neonates is lowest for cubs born in sub-tropical climates that experience summer rain (Princée and Glatston 2016). Cub mortality during the first week after

birth is greater when ambient air temperature is warmer. The combination of high humidity and warm ambient temperatures decreases survivorship (Princée and Glatston 2016).

Fecal glucocorticoids may have seasonal patterns in red pandas and have been shown to vary based on month of the year (Freeman et al. 2012). In their native range in India, monthly mean fGM concentrations and ambient temperature have a negative relationship, with concentrations peaking in December and January when temperatures are lower (Budithi et al. 2016), possibly related to the breeding season. Further investigation into climatic influence on physiology and behavior in red pandas is needed. This information can help direct management of the captive collection housed in warmer climates, such as the Mid-Atlantic region where most of our study pandas are housed, to improve panda welfare.

## CHAPTER TWO: ENVIRONMENTAL IMPACTS ON RED PANDAS

### **1. Introduction**

Red pandas are a unique mammal and the sole living member of the Ailuridae family (Choudhury 2001). Endemic to the Himalayas, red panda habitat spans five countries, Nepal, India, Bhutan, Myanmar, and China (Dorji et al. 2012; Glatston et al. 2015). The species is arboreal and lives at high altitudes 2,400 - 3,900m, in mixed conifer and deciduous forests with a bamboo understory (Dorji et al. 2012; Sharma et al. 2014; Yonzon and Hunter 1991). Red pandas are classified as carnivores, but bamboo leaves and shoots are their main food source, comprising over 90% of their diet (Wei et al. 1999; Yonzon and Hunter 1991). Opportunistic consumption of fruits, small birds, eggs, insects and other grasses does occur (Dorji et al. 2012; Yonzon and Hunter 1991). The red panda is well adapted for the cold climate of their native habitat, where the temperature does not often exceed 20°C (Johnson et al. 1988; Roberts and Gittleman 1984; Yonzon and Hunter 1991). Dense fur coats, a thick bushy tail, and low metabolic rate are some of the adaptations red pandas have for their cold environment (Princée and Glatston 2016; Yonzon and Hunter 1991).

Red pandas are considered a solitary species in the wild, typically seen alone except during mating season and when females are raising young (Roberts and Gittleman 1984; Yonzon and Hunter 1991). Breeding season for red pandas in the northern hemisphere is January-March, with females giving birth to a single litter of cubs in the summer (Glatston et al. 2015). They communicate through vocalizations, that often increase in frequency during the breeding season (Roberts and Gittleman 1984), and scent marking using common latrine sites (Yonzon and Hunter



1991). Red pandas are generally considered crepuscular animals, most active at dusk and dawn (Johnson et al. 1988; Roberts and Gittleman 1984), but in China red pandas have the greatest activity between 0700-1000 h and 1700-1800 h (Zhang et al. 2011). They are only active 36.5% to 48.6% of the day (Johnson et al. 1988; Zhang et al. 2011), resting and sleeping in trees or other elevated surfaces when not moving. The posture of red pandas during these rest periods is influenced by temperature and humidity. During warm summer times pandas can be seen in stretched out positions or lying with their limbs hanging off logs as they rest, yet in the winter, red pandas are often curled up with their tails covering their face (Roberts and Gittleman 1984).

A vast amount of information is still unknown about the red panda which faces many threats in the wild today. Most have anthropogenic causes, as the human population increases and encroaches upon red panda habitat. Road development, timber harvesting, and conversion of land to agriculture causes deforestation and habitat degradation (Thapa et al. 2018; Wei et al. 1999). Livestock herding on the landscape causes habitat degradation as cows trample and consume bamboo (Yonzon and Hunter 1991), the primary food source of red pandas. Free roaming dogs, including herding dogs, feral, and hunting dogs, directly kill red pandas and carry disease such as canine distemper and rabies that are fatal to red pandas (Glatston et al. 2015; Thapa et al. 2018). Finally, red panda populations are threatened by rising poaching and the illegal wildlife trade. Due in large part to these anthropogenic threats, wild red panda populations have declined by 50% in the past 20 years (Glatston et al. 2015). It is estimated that less than 10,000 individuals are left in the wild and populations are declining, leading the red panda to be listed as Endangered under the IUCN Red List (Glatston et al. 2015).

With wild populations declining throughout their range, individuals in captivity play an important role in safeguarding the species against extinction, as well as a role in research and public education. However, red pandas in zoological institutions face issues which hinder

population growth. Cub mortality within the first year after birth is 50% in captivity (Glass et al. 2011), often occurring due to mothers moving their cubs, causing bite wounds, when disturbed by people (Preece 2011). Zoo visitors and noise at the zoo can cause stress in animals. Stress can alter homeostasis in the body and animals have adapted by developing mechanisms for coping with stress. In response to a potential stressor, activation of the hypothalamic-pituitary-adrenal (HPA) axis occurs as part of the neuroendocrine stress response. Activation of the HPA axis begins a cascade of events which ends in the release of glucocorticoids, including cortisol and corticosterone (Matteri et al. 2000). Release of these glucocorticoids can cause behavioral changes (Wingfield 2005) and as the end-product of the HPA axis cascade glucocorticoids are a measurable assessment of stress. It is not known how red pandas are directly impacted by factors such as zoo visitors, noise and climate, which can be potentially stressful. Noise levels within the zoo increase as the number of visitors increase (Morgan and Tromborg 2007; Quadros et al. 2014). Red panda management guidelines (Glatston 1989) and the AZA's Red Panda Care Manual (AZA Small Carnivore TAG 2012) state noise and vibration disturbances should be kept to a minimum and visitors should only have viewing access to one or two sides of enclosures. However, more than half of zoo exhibits give visitors greater access to red pandas than management recommendations (Eriksson et al. 2010) and it is unknown what level of noise red pandas can tolerate.

The species is well adapted to a cold climate, where temperatures do not exceed 20°C, but are housed in zoological facilities with warm climates and hot summers. The AZA recommends institutions with warm summers give red pandas access to air-conditioned indoor areas, nest boxes, or other holding spaces when temperatures exceed 80°F or 26.6°C (AZA Small Carnivore TAG 2012). High temperatures with high humidity can lead to heat-related illness such as heat stress, which is documented as a cause of adult death in the species (Philippa and Ramsay

2011). In addition, the combination of high temperature and high humidity increases cub mortality of red panda neonates during their first week of life (Princée and Glatston 2016). Temperature can also impact the physiological response in red pandas. Fecal glucocorticoid concentrations have been shown to vary based on month of the year (Freeman et al. 2012). Ambient temperature and monthly mean fGM concentrations have a negative relationship, with concentrations peaking in December and January when temperatures are lower (Budithi et al. 2016), possibly related to the breeding season. Despite previous knowledge, specific impacts of climate on red panda behavior and physiological response is necessary to guide captive management of red pandas housed in warm environments, for improved welfare.

Therefore, the aim of this study is to understand how environmental factors impact red panda behavior and physiology in order to guide management and optimize red panda welfare. Specifically, the influence of climate, number of zoo visitors, and noise levels outside enclosures on red panda behavior and fecal glucocorticoid metabolite concentrations were assessed. By understanding how these factors affect red pandas, and which may negatively impact welfare, management strategies can be implemented to potentially enhance well-being and population viability. Due to the species' adaptations for cold conditions it is hypothesized that warmer temperatures may be a potential stressor, and therefore increased glucocorticoids would be seen during times of elevated temperatures, such as the summer. In addition, warmer temperatures and humidity will result in a decreased activity budget and increased thermoregulatory coping behaviors as a mechanism for individuals to handle the environment. Third, larger numbers of visitors and noise would increase the number of coping behaviors red pandas perform. Finally, we hypothesized that fGM concentrations will increase with greater noise and number of zoo visitors as these also are potential stress inducers for the animals.

## **2. Research Methods**

### **2.1 Animals**

This study involved red pandas (n = 10) originally housed at the Smithsonian's National Zoological Park (NZP) and Conservation Biology Institute (SCBI), located in Washington D.C and Front Royal, VA respectively. During the project two individuals were moved between these institutions due to management decisions. In addition, three red pandas were transferred to two new institutions, Seneca Park Zoo (SPZ, n = 2) in Rochester, NY and Mesker Park Zoo & Botanic Garden (MPZ, n = 1) in Evansville, IN. There was no disruption in fecal sample collection or behavioral observations for red pandas moved between NZP and SCBI. For individuals transferred to SPZ and MPZ, collection of fecal samples and weather variables (temperature/humidity) continued after the move occurred, but no behavioral observations were conducted at the new facility. All red pandas were either housed in single animal enclosures or as male/female pairs, and were fed comparable diets of bamboo, leaf-eater biscuits, and fruit as well as given water *ad libidum* at their respective institutions. All individuals were *A.f. fulgens* subspecies and ranged in age from 1 to 6 years old at the beginning of the study (Table 1).

Table 1. Description of the red pandas (n = 10) involved in the study

<b>Studbook (SB) Number</b>	<b>Location</b>	<b>Sex</b>	<b>Date of Birth</b>	<b>Housed Single (S) or Pair (P)</b>	<b>Number of Behavioral Observations</b>	<b>Number of Fecal Samples Analyzed</b>
1235	SCBI	Male	7/11/2012	S	61	230
1304	SCBI/MPZ	Male	6/1/2013	S/P	37	257
1310	SCBI/NZP	Female	6/6/2013	S/P	43	316
1353	SCBI/SPZ	Male	7/19/2013	S/P	-	93
1407	NZP	Female	5/27/2014	P	27	415
1418	SCBI	Male	6/14/2014	S	35	211
1437	NZP	Male	6/27/2014	P	-	205
1513	SCBI	Female	6/14/2015	S/P	59	254
1705	SCBI/NZP	Male	6/14/2017	P	29	337
1713	SCBI/SPZ	Female	6/17/2017	S/P	-	97

## **2.2 Behavioral Methods**

### ***2.2.1 Behavioral Observations***

Behavioral observations were conducted on seven red pandas located at NZP and SCBI. SB# 1353, 1437, and 1713 did not have any behavioral observations conducted because they were transferred to another facility prior to behavioral data collection beginning. Behavior data were collected 1-3 times each week at both locations for one year, June 2018-2019. Recorded behaviors were scored using an ethogram (Appendix I) modified from Jule et al. (2009) and continuous scan sampling (Altmann 1974; Martin & Bateson 1993) for 30-minute sessions. Behavioral observations were conducted from the lower, public viewing area at NZP, and at SCBI directly outside the enclosure, 2 - 4 meters away from the perimeter mesh. All observations were conducted between 0800 and 1000 h to keep a standardized observation window between locations, and because this time was when animals were consistently outdoors with minimal keeper interference. The order in which animals were observed was randomized. Duration of state behaviors (e.g. locomotion, climbing, sleeping, eating; Appendix I) was measured to assess activity levels for each individual. Active behaviors included climbing, eating, grooming, locomotion, and pacing. Inactive behaviors included sleep, straddle and stationary. Panting is an important behavior for thermoregulation that can occur during multiple behavioral states (e.g. locomotion, stationary, climbing, or pacing). Thus, to fully assess how environmental factors impacted its frequency, the duration of panting was measured as a non-mutually exclusive behavior. In addition, the total number of event behaviors (e.g. head roll; Appendix I) were counted. Animals had to be visible for a minimum of 20 minutes during the 30-minute session for the observation to be included in further analysis. Additionally, observation periods with more than 10-minutes of keeper influence, or time animal care staff were present in the enclosure, were excluded.

At the start and end of each observation, temperature (°C), humidity (%), and windspeed (mph) were measured using a pocket weather meter (Kestrel 3000; Nielsen-Kellerman, Boothwyn, PA). The mean of these two measurements was calculated to determine the observational temperature, humidity and windspeed for each behavioral observation. Additionally, sound level (dB) outside of the panda enclosure was taken every five minutes during the 30-minute observation period using a digital sound level meter (Extech #407730, FLIR Commercial Systems Inc, Nashua, NH). At each five-minute measurement point, the maximum dB(A) over a 30-second window was recorded. The average noise level for each observation was achieved by calculating the mean from the seven maximum dB readings.

### ***2.2.2 Behavioral Statistical Analysis***

To determine the overall activity budget for red pandas in the study, the proportion of time spent conducting each behavioral state was calculated by dividing the duration of time the behavior was performed by the total time the panda was visible for that observation. State behaviors were analyzed with analysis of variance (ANOVA) and significant differences between the proportion of time red pandas conducted each state behavior was determined with TukeyHSD post-hoc analysis. In addition, three behavioral variables were used for further analysis. Environmental factors may impact the activity levels of red pandas, therefore all ‘active’ behaviors, the amount of time locomoting, climbing, pacing, grooming, and eating (browse and biscuit/fruit) were combined to calculate the *proportion of time spent active* (PA). In addition, the *proportion of time spent panting* (PP) and the *proportion in straddle position* (PS) was determined. The proportion of time pacing was also analyzed separately from PA to determine if pacing was influenced by environmental factors. All calculations resulted in a proportion between 0 and 1 for each observation. Event behaviors were analyzed as a rate of occurrence per hour visible.

Behavioral data were analyzed using generalized linear mixed models (GLMM), to account for non-independence of the data due to repeated measures on individual pandas. To avoid collinearity, Pearson correlation coefficients were calculated using the *cor* function in R (R Core Team 2019) between all behaviors and weather variables taken at the enclosure. Many environmental factors may be related to each other, therefore, correlation of variables in the study was conservatively set at a correlation coefficient ( $r$ ) of 0.50. Variables with a  $r > 0.5$  were not included together in models. Model selection was based on Akaike information criterion corrected for small sample size (AICc). The top model with the lowest AICc value was deemed the best model.

To test our hypothesis that environmental conditions impacted activity levels binomial GLMMs were used to determine if observational temperature, humidity, noise, and windspeed recorded at the exhibit affected the proportion of time pandas exhibited active (PA), pant (PP), and straddle (PS) behaviors. Visitor numbers were not used in behavioral models because visitor numbers around the exhibit at the time of behavioral observations were not counted. Red pandas were initially pooled, because the study primarily aimed to understand population level impacts over individual, and three models were run in which PA, PP, or PS was the response variable. Observational temperature, humidity, and noise were used as fixed effects with animal as the random effect, to control for inherent variability between individuals. An interaction term of noise and location also was included in the models because sound levels were significantly different between locations based on Wilcoxon Rank Sum ( $p < 0.001$ ). Due to consistently low wind speeds and multiple zero values, this factor was removed from all behavior models. Once a best model was ascertained, windspeed was added back to determine if it improved the model.

Models for individual pandas were analyzed to determine whether results from the individual models support the best model from the pooled panda data and explore any potential

individual differences. PA, PS, and PP were all investigated for pandas on an individual level, using the same model structures as described above for the pooled data. The interaction of sound and location was not used in individual models because six of the seven pandas only had behavioral observations at one location. Date was used as the random effect to account for repeated measures within each individual.

The odds ratio for each numeric variable in the final model was determined. Odds ratio is a measure of association between the response and predictor variables, that represents the odds of the outcome occurring based on one-unit change in the fixed effects (Szumilas 2010). In order to more easily interpret the results, the odds ratio value was subtracted from one and multiplied by 100 to give the percentage of change in active (PA), pant (PP), and straddle (PS) behavior based on a one unit increase in the fixed effect. All statistical analyses were performed using the lme4 package (Bates et al. 2015) in R version 3.6.2 (R Core Team 2019). Significance was determined as  $p < 0.05$  and all non-model results were reported as mean  $\pm$  standard error (SE).

## **2.3 Endocrine Methods**

### ***2.3.1 Fecal Sample Collection***

Fecal samples were collected non-invasively by animal care staff at each institution during routine, daily enclosure cleaning in the morning. When animals were housed with another individual, non-toxic glitter was used as a fecal marker, previously shown as an effective biomarker (Fuller et al. 2011), to differentiate samples among the red pandas. Glitter was placed on a food item that was hand-fed, the afternoon prior to collection, to ensure complete consumption by the correct individual. Only glitter marked feces were collected for multi-housed individuals in the study. At NZP, fecal samples were collected from May 2017 through June 2019 to ensure sample collection from two summers or high visitation seasons. At SCBI, samples were



collected from December 2017 through May 2019. Fecal collection continued for three months post-relocation for animals transferred to SPZ and MPZ. After collection, fecal samples were stored in the freezer (-20°C) until they were processed for hormone extraction. Samples from MPZ and SPZ were shipped overnight with ice to ensure optimal temperature was kept during transit.

### **2.3.2 Endocrine Analysis**

For each red panda, 3-4 fecal samples from each week were freeze-dried in a lyophilizer before being crushed and sifted to separate fecal material from undigested dry matter. No more than three days lagged between analyzed samples, when collection allowed. Hormones were extracted from dried and sifted fecal matter using a dry-weight shaking technique adapted from Edwards et al. (2019). In brief, 0.050g ( $\pm$  0.0050g) lyophilized fecal powder was added to 5.0ml of 80% methanol (Appendix II), samples were vortexed thoroughly to ensure the fecal powder freely mixed with the alcohol, and agitated on a multi-tube pulse shaker (Glas-Col, Terre Haute, IN) for 30 minutes. Samples were briefly vortexed again and then centrifuged at 1410g for 20 minutes, to separate the fecal material. Supernatant was poured off into new tubes before another 5.0ml of 80% methanol was added to the original tubes containing the fecal pellets. Tubes containing alcohol and fecal material were vortexed for 15 seconds and centrifuged a second time at 1410g for 15 minutes. Combined supernatants were dried down under air and once evaporated to dryness, resuspended in 1ml 100% methanol. After another dry down the extracts were brought up in a final 1ml phosphate buffer (0.039M NaH<sub>2</sub>PO<sub>4</sub>, 0.061M Na<sub>2</sub>HPO<sub>4</sub>, 0.15M NaCl; pH 7.0) and frozen at -20°C in preparation for hormone analysis. The average extraction efficiency of this process was 86.4% (range 70.0 – 99.9%) based on addition of <sup>3</sup>H-corticosterone to each sample prior to extraction.

### 2.3.3 Enzyme Immunoassay (EIA)

Concentrations of glucocorticoid metabolites were quantified using a double antibody corticosterone EIA incorporating a secondary goat-anti rabbit IgG antibody (A009, Arbor Assays, Ann Arbor, MI) and polyclonal rabbit anti-corticosterone antibody (CJM006, C. Munro, University of California, Davis, CA) adapted from Edwards et al. (2019). Corticosterone standards (50 $\mu$ l), controls (50 $\mu$ l), and samples (50 $\mu$ l; diluted 1:2 to 1:20 in phosphate buffer) were added in duplicate to 96-well plates pre-coated with anti-rabbit IgG. Corticosterone-horseradish peroxidase (25 $\mu$ l; 1:25,000; C. Munro, University of California, Davis, CA) was added to all wells. The primary anti-corticosterone antibody (25 $\mu$ l; CJM006; 1:60,000; C. Munro, University of California, Davis, CA) was added to every well except for the non-specific binding (NSB) wells, then incubated for 1 hour at room temperature (RT) on a shaker set to 500RPM. The plate was washed five times with wash buffer (X007, Arbor Assays) to remove unbound components, followed by the addition of a chromogen solution containing 3,3',5,5'-Tetramethylbenzidine (TMB; 100 $\mu$ l, X019, Arbor Assays) to all the wells. After incubation for 30 minutes at RT, the reaction was ceased by the addition of stop solution (50 $\mu$ l; X020 Arbor Assays) and optical densities were determined at 450nm with a reference of 630 nm. The inter-assay coefficient of variation (CV) of high- and low-value quality controls were 13.99% and 10.99% respectively. Intra-assay CV of high value quality control was 5.62% and low value quality control was 10.48%. The CV for 20 replicates of a pooled biological sample diluted at 50% binding was 5.67%.

This EIA was validated for male and female red pandas by demonstrating parallelism of the binding inhibition curve of pooled fecal extract dilutions (1:1 to 1:128) with the corticosterone standard (male:  $y = 1.117x - 1.882$ ,  $R^2 = 0.996$ ,  $F_{1,5} = 1125.369$ ,  $P < 0.001$ ; female:  $y = 1.118x - 1.084$ ,  $R^2 = 0.990$ ,  $F_{1,5} = 509.085$ ,  $P < 0.001$ ). There was no evidence of matrix interference on the

EIA, as addition of diluted fecal extract to synthetic standards did not alter the amount observed (male:  $y = 1.628x - 18.452$ ,  $R^2 = 0.9655$ ,  $F_{1,7} = 195.997$ ,  $P < 0.001$ ; female:  $y = 1.543x - 15.106$ ,  $R^2 = 0.979$ ,  $F_{1,7} = 329.201$ ,  $P < 0.001$ ). This assay was biologically validated for measuring adrenocortical activity in red pandas through demonstration of increased fGM concentrations following an adrenocorticotrophic hormone (ACTH) challenge (Appendix II).

#### ***2.3.4 Environmental Variables***

Fecal glucocorticoid metabolite concentrations represent an averaged measure over several hours, thus environmental variables taken during the 30-minute behavioral observation periods were not representative of conditions potentially contributing to fGM concentrations. Therefore, daily environmental variables for fGM analyses, including dry bulb temperature and humidity, were gathered from weather stations near each zoological facility. MPZ, NZP, and SPZ are all located within ten miles of an airport with a National Oceanic and Atmospheric Association (NOAA) station collecting Local Climatological Data (LCD), including daily average temperature ( $T^A$ ), daily minimum temperature ( $T^{\min}$ ), daily maximum temperature ( $T^{\max}$ ) and average humidity. Therefore, LCD was obtained from Evansville Regional Airport, Washington Regan National Airport, and Rochester Greater International for MPZ, NZP, and SPZ respectively (NOAA, 2020a). NOAA does not have a tower within a ten-mile radius of SCBI. Weather data for this location was collected from the National Ecological Observatory Network (NEON; 2020) tower on SCBI's campus. Temperature and humidity are recorded every 30 minutes by the tower, from which daily averages were calculated. In addition, daily minimum and maximum temperature were obtained for analyses. While windspeed was used as an environmental variable for behavioral analysis it was not used for fGM analysis due to the possibility that wind speeds at the airport, where the NOAA stations were present, may be significantly different than outside the exhibit because of design and nearby structures.

Visitor numbers (VN) reflected the possible number of public visitors the red pandas were exposed to daily. Animal care staff and maintenance personnel were not included. Visitor numbers for NZP were obtained from Friends of the National Zoo (FONZ), a nonprofit partner with the zoo. These data were a raw count of the total number of people entering the Asia Trail, the section of the zoo containing the red panda enclosure, each day. Visitor numbers over the study time period ranged widely (0 – 16,904), and thus were scaled for analysis by dividing by 100. SCBI is closed to the public and does not have visitors, therefore zero visitors were recorded for all days. Visitor numbers were not available for SPZ or MPZ for this project. Therefore, models that included VN as a fixed effect only contained data from pandas housed at NZP and SCBI. Visitor number was used for fGM models because the response and predictor variables were daily measures. To account for the 7-15 hour lag time between circulating hormones and excreted metabolites in feces (Appendix II), fGM concentrations were matched to LCD variables and visitor numbers from the previous day for analysis. Additionally, month that each fecal sample was collected was included to investigate possible seasonal differences in fGM concentrations.

### ***2.3.5 Endocrine Statistical Analysis***

To test our hypothesis that environmental factors impact fGM concentrations, GLMMs using a gamma distribution with log link were performed to assess possible relationships with month, visitor number, and LCD data. As with the behavioral analysis, correlation coefficients were determined for all environmental variables which included month, VN, daily average temperature, and humidity, prior to running models. Variables were deemed overly correlated if the Pearson correlation coefficient ( $r$ ) was greater than 0.50, and these variables were used separately in models. Model selection was based on Akaike information criterion corrected for small sample size (AICc) in which the top model was selected as the best model.

All pandas were combined, and animal assigned as a random effect. Due to a lack of visitor data for MPZ and SPZ, only data from SCBI and NZP was used to analyze models for pooled red panda data. SCBI had zero recorded for VN every day and NZP had fluctuating visitor numbers. Location was initially included as a nested random effect within animal, due to fGM collections on two individuals (SB# 1310 & 1705) at both NZP and SCBI. However, this nested effect of animal/location explained negligible additional variance in the models, so location was dropped as a random effect to ensure the most parsimonious model. One fGM outlier was removed from the data set because it was considered to have undue influence on the model. This glucocorticoid value was due to a sick animal which had a difficult catch-up process and veterinary exam. The fGM concentration was 1,896 ng/g and the animal did not have any other concentrations above 600ng/g feces, therefore the value was excluded. Removing this outlier did not affect the significant variables within the final model.

Individual models investigating the influence of month on glucocorticoid also were analyzed. These models included fGM levels from pandas housed at all four zoos in the study. Seven pandas had values spanning all 12 months. Two pandas, SB# 1353 and 1713, who were transferred to SPZ had fGM concentrations for seven months. One panda SB#1437 has fGM concentrations for nine months out of the year. Models had a response of fGM, month as the explanatory variable, and the random effect was date.

Multicollinearity of the variables in the final models were verified using variance inflation factor (VIF), with all VIF below 1.32. The odds ratio for each numeric variable in the final model was determined. As with behavioral analysis, all statistical fGM analysis was performed using the lme4 package (Bates et al. 2015) in R version 3.6.2 (R Core Team 2019) and significance was determined as  $p < 0.05$ .

### **3. Results**

During the one-year period a total of 291 acceptable behavioral observations, animal visibility for at least 20 out of the 30-minute observation period and less than 10 minutes of keeper influence, were recorded on seven individuals. In total, 2,415 fecal samples from ten red pandas were extracted and analyzed. Descriptive data of the number of behavioral observations conducted and fecal samples analyzed for each panda can be found in Table 1.

#### **3.1 Correlation of Variables**

##### ***3.1.1 Behavioral Data Correlations***

Observational temperature was moderately correlated to the mean sound (dB) and humidity (RH; Table 2) recordings taken during behavioral observations. Therefore, temperature was not used in models with these two variables for behavioral analysis.

Table 2: Correlation coefficients of the response variables (PA, PP, and PS) and environmental variables (sound(dB), Temperature, Humidity (RH), and windspeed (WS)) taken at the enclosure for behavioral analysis. Correlation coefficients greater than 0.5 are in bold.

	<b>PA</b>	<b>PP</b>	<b>PS</b>	<b>dB</b>	<b>Temp</b>	<b>RH</b>	<b>WS</b>
<b>PA</b>	<b>1</b>	0.19	-0.20	-0.22	-0.26	-0.10	0.14
<b>PP</b>		<b>1</b>	-0.01	0.23	0.27	0.12	-0.04
<b>PS</b>			<b>1</b>	0.16	0.22	0.08	-0.03
<b>dB</b>				<b>1</b>	<b>0.57</b>	0.24	-0.21
<b>Temp</b>					<b>1</b>	<b>0.51</b>	-0.28
<b>RH</b>						<b>1</b>	-0.46
<b>WS</b>							<b>1</b>

##### ***3.1.2 Endocrine Data Correlations***

Average daily temperature was highly correlated to minimum temperature and maximum temperature, as well as minimum and maximum temperature to each other (Table 3). To avoid collinearity, the three temperature values were never in models together and instead used separately. In addition, the correlation between visitor number (VN) and the average temperature

as well as VN and maximum temperature were greater than the  $r$  threshold (Table 3). VN and temperature variables were not used in models together.

Table 3: Correlation coefficients of fGM concentrations and environmental variables (Visitor Number (VN), Daily average temperature ( $T^A$ ), Humidity (RH), Daily maximum temperature ( $T^{Max}$ ), Daily minimum temperature ( $T^{Min}$ ), and month). Bold values have a  $r > 0.5$ .

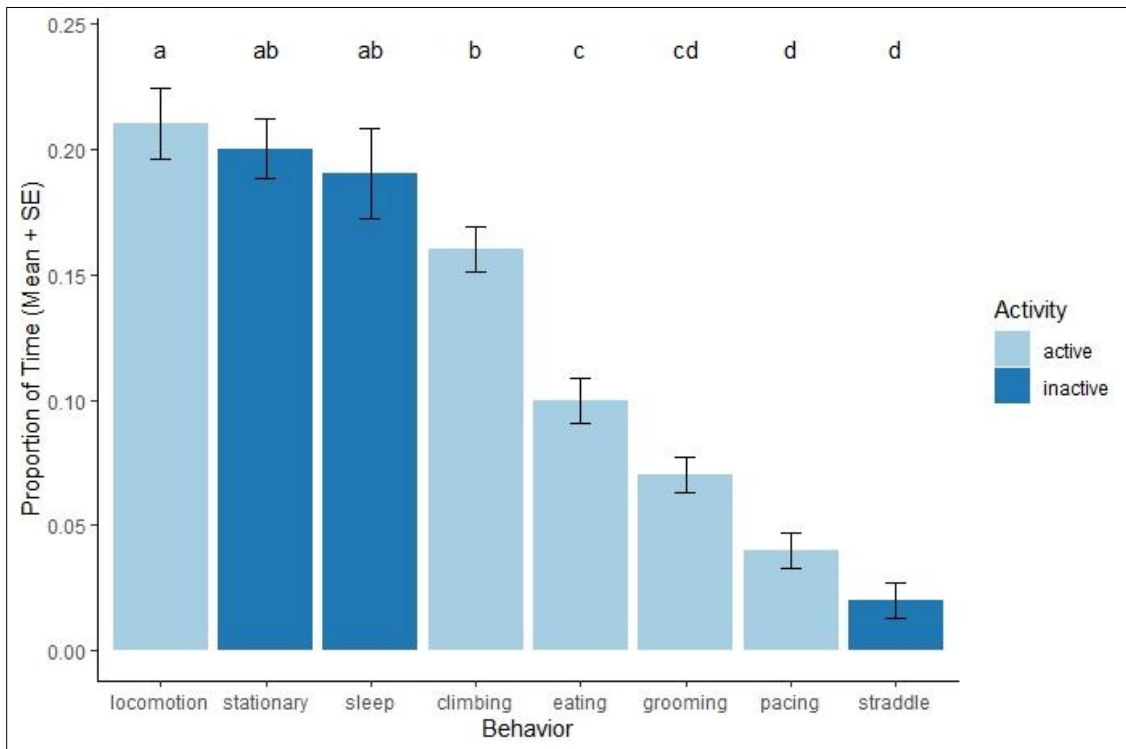
	fGM	VN	$T^A$	RH	$T^{Max}$	$T^{Min}$	Month
fGM	<b>1</b>	-0.13	-0.18	-0.06	-0.17	-0.19	-0.04
VN		<b>1</b>	<b>0.52</b>	0.04	<b>0.60</b>	0.38	0.10
$T^A$			<b>1</b>	0.37	<b>0.98</b>	<b>0.97</b>	0.28
RH				<b>1</b>	0.34	0.39	0.18
$T^{Max}$					<b>1</b>	<b>0.91</b>	0.26
$T^{Min}$						<b>1</b>	0.28
Month							<b>1</b>

### 3.2 Description of Activity Budgets

The proportion of time red pandas spent active was  $0.59 \pm 0.05$  (Fig. 2). Locomotion ( $0.21 \pm 0.01$ ), stationary ( $0.20 \pm 0.01$ ), and sleep ( $0.19 \pm 0.02$ ) behaviors were observed most frequently and in approximately equal proportions. Circle, fight, and conspecific grooming behavior were never observed during this study. The proportion of time drinking and playing occurred less than 0.01; thus, these two behaviors, as well as circle, fight, and conspecific grooming, were not depicted in Figure 2 or included in any models analyzing the effects of environmental variables on red pandas.

Head rolls occurred infrequently ( $0.68 \pm 0.19/\text{hr}$ ). Due to the infrequency or lack of stereotypic behaviors (e.g. circling, head roll), we did not investigate these behaviors further in comparison to environmental factors. Red pandas communicate chemically with anogenital scent marking ( $12.86 \pm 1.35/\text{hr}$ ), and lick/sniff ( $15.89 \pm 1.21/\text{hr}$ ) but vocalize infrequently ( $0.49 \pm 0.15/\text{hr}$ ). Because these communications were not related to our hypotheses about the effects of environmental factors they were not analyzed further. However, possible thermal coping

behaviors, such as panting and straddle, were observed in all pandas and not overly correlated ( $r < 0.5$ ) to other state or event behaviors. Therefore, straddle and panting were two behaviors analyzed further in models.



**Figure 2:** Mean  $\pm$  SE of proportion of time red pandas ( $n = 7$ ) at Smithsonian’s National Zoological Park and Conservation Biology Institute performed state behaviors. Light blue bars represent active behaviors and dark blue bars are inactive behaviors. Superscripts represent significant differences ( $p < 0.05$ ).

Proportion of time the red pandas in this study were active was related to environmental conditions at the time of the behavioral observation. The top model contained temperature and the second model was an interaction of sound (dB) with location (Table 4) but had a  $\Delta$  AICc greater than 2.0. The addition of windspeed to the top model did not lower the AICc value (Table 4) and windspeed was not significant in explaining active time. Therefore, the best model was



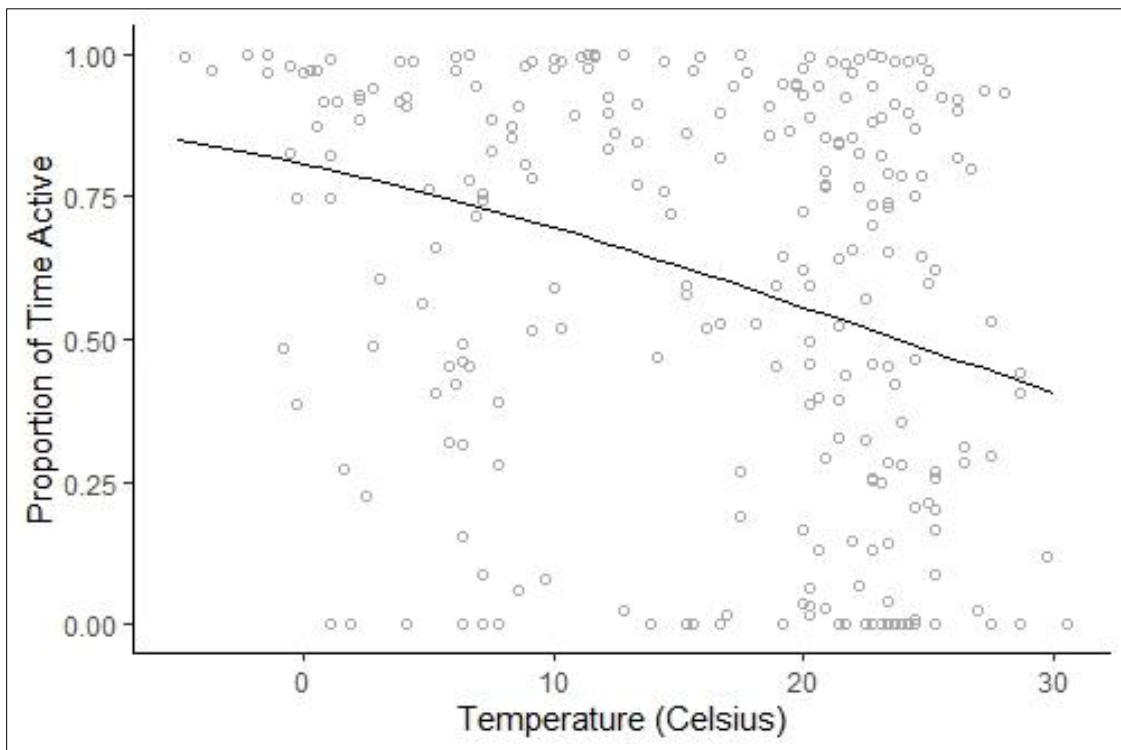
determined to be the top model which contained temperature ( $p < 0.001$ ). Based upon the odds ratio, every  $1^{\circ}\text{C}$  rise in temperature corresponded to a 5.86% decrease in the proportion of time spent active (Fig. 3).

The proportion of time individual red pandas were active was influenced by temperature, sound, and humidity. The top model for three individuals contained temperature as the fixed effect. For all three, activity decreased as temperature increased, but the relationship was not significant for any panda ( $p > 0.05$ ). Two individuals had a top model of sound and humidity. In one case, both sound and humidity were significant ( $p < 0.05$ ) and for the other individual only humidity was significant ( $p = 0.04$ ). The final two pandas had a top model containing only sound, but it was not a significant variable for either individual. For individuals with sound in the top model the relationship with activity was negative, activity decreased with increasing noise.

The time red pandas spent pacing was included in active (PA) behavior, but when the proportion of time pacing was analyzed separately, no variables were significantly associated with pacing. This held true for all red pandas combined and when investigating the two individuals (SB# 1310 and 1513) that paced most frequently.

Table 4. General Linear Mixed Model (GLMM) for analysis of environmental variables (temperature (°C), ambient noise (dB), humidity (RH; %) and windspeed (WS; mph)) that contributed to the proportion of time red pandas (n = 7) spent active (PA). Significant p-values are in bold.

<i>Model</i>	AICc	$\Delta$ AICc	Variable	Coefficient	Std Error	p-value
<i>Temp</i>	327.88	0.00	<i>Temp</i>	-0.060	0.017	<b>&lt;0.001</b>
<i>Temp + WS</i>	328.99	1.11	<i>Temp</i>	-0.056	0.018	<b>0.002</b>
			<i>WS</i>	0.297	0.312	0.340
<i>dB*location</i>	330.00	2.12	<i>dB</i>	-0.015	0.040	0.704
			<i>locationSCBI</i>	4.100	2.614	0.117
			<i>dB:locationSCBI</i>	-0.067	0.047	0.154
<i>dB*location + RH</i>	332.10	4.22	<i>dB</i>	-0.015	0.040	0.706
			<i>locationSCBI</i>	4.100	2.615	0.117
			<i>RH</i>	-6.8e <sup>-5</sup>	0.010	0.995
			<i>dB:locationSCBI</i>	-0.067	0.047	0.115

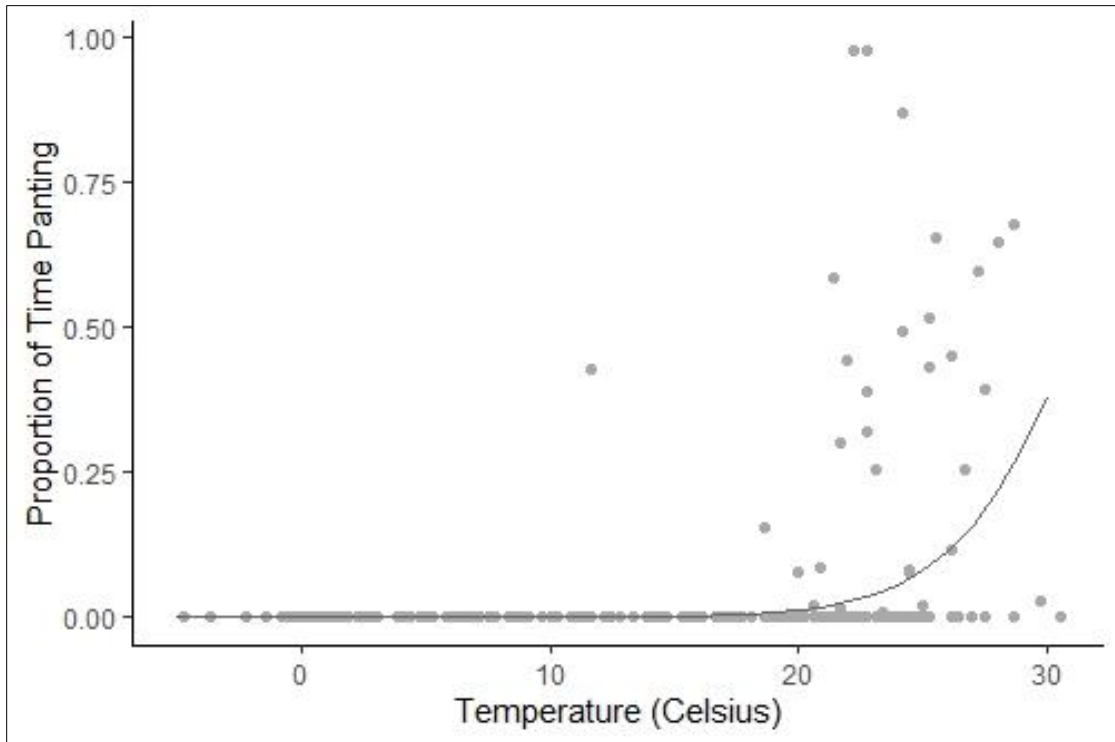


**Figure 3.** The effect of temperature on proportion of time spent active (PA) for red pandas (n = 7). Points represent PA for every behavioral observation and the black line is the predicted relationship between PA and temperature.

Proportion of time red pandas spent panting (PP) also was influenced by climate during observations. Temperature was the top model and the addition of windspeed did not improve it, as the  $\Delta$  AICc was greater than 1.0 (Table 5). Temperature had a positive relationship with, and was significant ( $p < 0.01$ ) in explaining PP. Each 1°C increase in temperature corresponded to a 47.67% increase in PP. Panting did not occur below 11°C and most frequently occurred over 20°C (Fig. 4).

Table 5: General Linear Mixed Model (GLMM) for analysis of environmental variables (temperature (°C), relative humidity (RH; %) and windspeed (WS; mph)) that contributed to the proportion of time red pandas ( $n = 7$ ) spent panting (PP). Significant p-values are in bold.

<i>Model</i>	AICc	$\Delta$ AICc	Variable	Coefficient	Std Error	p-value
<i>Temp</i>	65.89	0.00	<i>Temp</i>	0.390	0.147	<b>&lt;0.01</b>
<i>Temp + WS</i>	67.79	1.90	<i>Temp</i>	0.374	0.147	<b>0.011</b>
			<i>WS</i>	-0.746	1.968	0.705
<i>RH</i>	78.24	12.36	<i>RH</i>	0.068	0.035	0.052

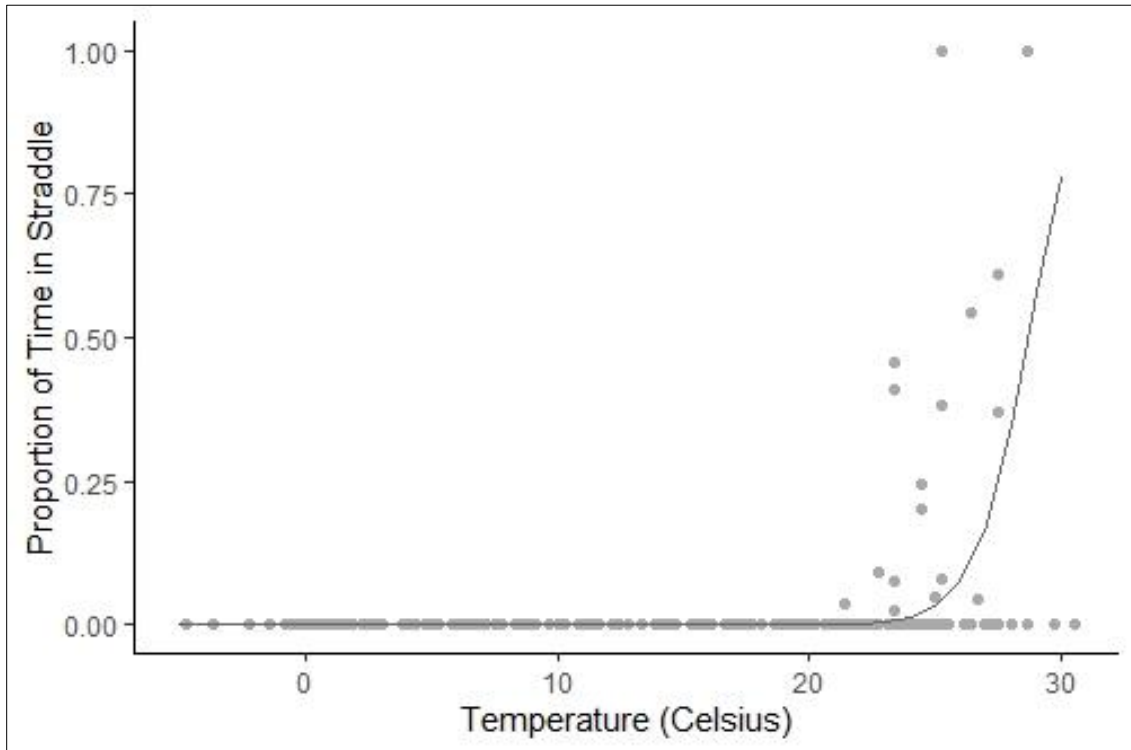


**Figure 4.** Proportion of time red pandas ( $n = 7$ ) spent panting (PP) as related to mean temperature during behavior observations. Light grey points represent PP for each observation and the darker solid line is the predicted relationship between PP and temperature.

Environmental conditions also impacted the proportion of time red pandas spent in the straddle position (PS). Only SCBI data was used to run PS model because all observations of straddle occurred at SCBI. Temperature alone was the top model followed by sound with a  $\Delta$  AICc of 9.81. Adding windspeed did not improve the model (Table 6). Pandas were only observed in straddle position when the ambient temperature was above 21°C (Fig. 5). A 1°C increase in temperature doubled the odds of straddle occurring during an observation ( $p < 0.01$ ). The proportion of time red pandas spent in straddle was also influenced by sex. Males spent significantly more time in straddle than females ( $p < 0.01$ ). The proportion of time individuals at SCBI straddled was influenced by temperature and was the top model for two pandas. The other individuals did not have enough data to determine a top model.

Table 6: General Linear Mixed Model (GLMM) for analysis of environmental variables (temperature (°C), sound level (dB), humidity (RH; %), and windspeed (WS; mph)) that contributed to the proportion of time red pandas (n = 7) spent in straddle position (PS). Significant values are in bold.

<i>Model</i>	AICc	$\Delta$ AICc	Variable	Coefficient	Std Error	p-value
<i>Temp</i>	27.12	0.00	<i>Temp</i>	0.941	0.351	<b>&lt;0.01</b>
<i>Temp + Windspeed</i>	29.05	1.93	<i>Temp</i>	0.974	0.370	<b>&lt;0.01</b>
			<i>WS</i>	-0.858	2.354	0.716
<i>dB</i>	36.93	9.81	<i>dB</i>	0.281	0.131	<b>0.032</b>
<i>dB + RH</i>	38.93	11.81	<i>dB</i>	0.288	0.132	<b>0.030</b>
			<i>RH</i>	-0.015	0.054	0.774



**Figure 5.** Proportion of time red pandas (n = 7) straddled (PS) based on mean observational temperature. Points denote PS for each observation and the line is the predicted relationship between PS and temperature.

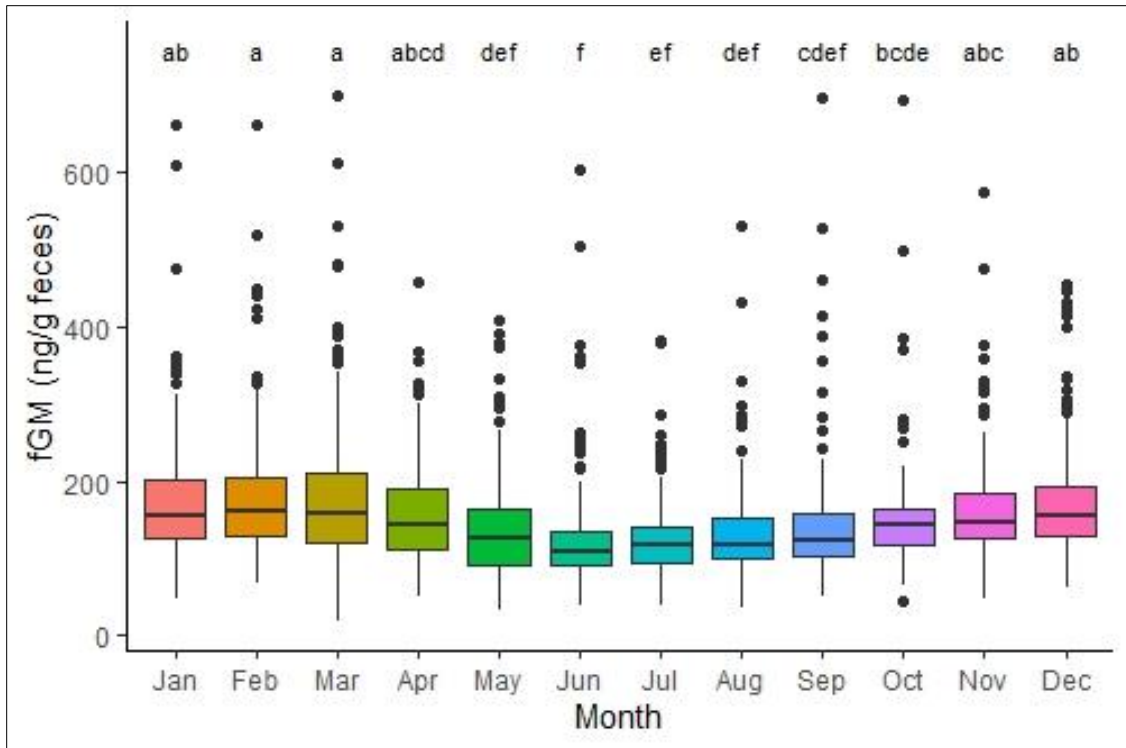
### 3.3 Glucocorticoids Results

Multiple environmental factors influenced fGM concentrations in red pandas. All ten red pandas were included in the study's glucocorticoid analysis while they were at NZP and/or SCBI.

The multi-variable model for predicting fGM concentrations consisted of two factors: month and visitor numbers (Table 7). The top suite of models included a combination of month, visitor number, and humidity. The best model (Month and VN) had the lowest AICc value and the next closest model had a  $\Delta$  AICc value close to 2.0, but humidity was not significant in this model (Table 7). Compared to January, glucocorticoid metabolites were significantly lower May through August ( $p < 0.01$ ), 14.1%, 20.8%, 16.4% 11.4% respectively (Fig. 6). As for the relationship with visitor numbers, 1000 fewer zoo visitors corresponded with a 2.1% increase in glucocorticoids ( $p < 0.01$ ; Fig. 7). Due to the difference in visitor numbers between NZP and SCBI, fGM concentrations also were analyzed separately on the pool of animals at each facility. Results in the top model for NZP was month and visitor number, while the top model for SCBI contained only month.

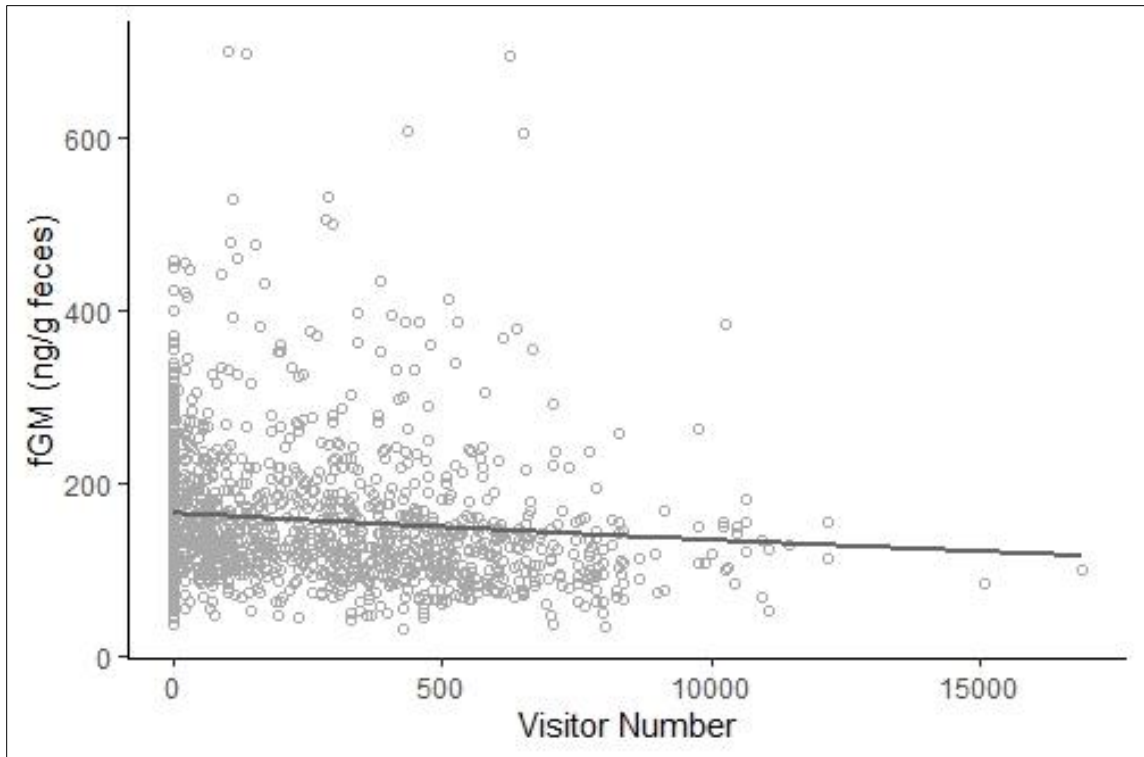
Table 7: General Linear Mixed Model (GLMM) for analysis of environmental variables (month, visitor numbers (VN), and relative humidity (RH; %)) that contributed to red panda (n = 10) fecal glucocorticoid metabolite (fGM) concentrations. Bold p-values are significant.

Model	AICc	Δ AICc	Variable	Coefficient	Std. Error	p-value
Month + VN	21089.65	0.00	Feb	0.0241	0.035	0.50
			Mar	0.0573	0.036	0.11
			Apr	0.0126	0.039	0.75
			May	-0.1515	0.037	<b>&lt;0.01</b>
			Jun	-0.2336	0.042	<b>&lt;0.01</b>
			Jul	-0.1794	0.040	<b>&lt;0.01</b>
			Aug	-0.1206	0.044	<b>&lt;0.01</b>
			Sep	-0.0698	0.042	0.09
			Oct	0.0031	0.043	0.94
			Nov	0.0717	0.045	0.11
			Dec	0.0361	0.036	0.32
			VN	-0.0021	0.0004	<b>&lt;0.01</b>
Month + VN + RH	21091.54	1.89	Feb	0.0228	0.036	0.52
			Mar	0.0589	0.036	0.11
			Apr	0.0130	0.040	0.74
			May	-0.1531	0.037	<b>&lt;0.01</b>
			Jun	-0.2354	0.043	<b>&lt;0.01</b>
			Jul	-0.1823	0.042	<b>&lt;0.01</b>
			Aug	-0.1234	0.044	<b>&lt;0.01</b>
			Sep	-0.074	0.044	0.08
			Oct	0.0001	0.043	0.99
			Nov	0.0708	0.045	0.11
			Dec	0.0362	0.036	0.31
			VN	-0.0021	0.0004	<b>&lt;0.01</b>
			RH	0.0002	0.0005	0.70



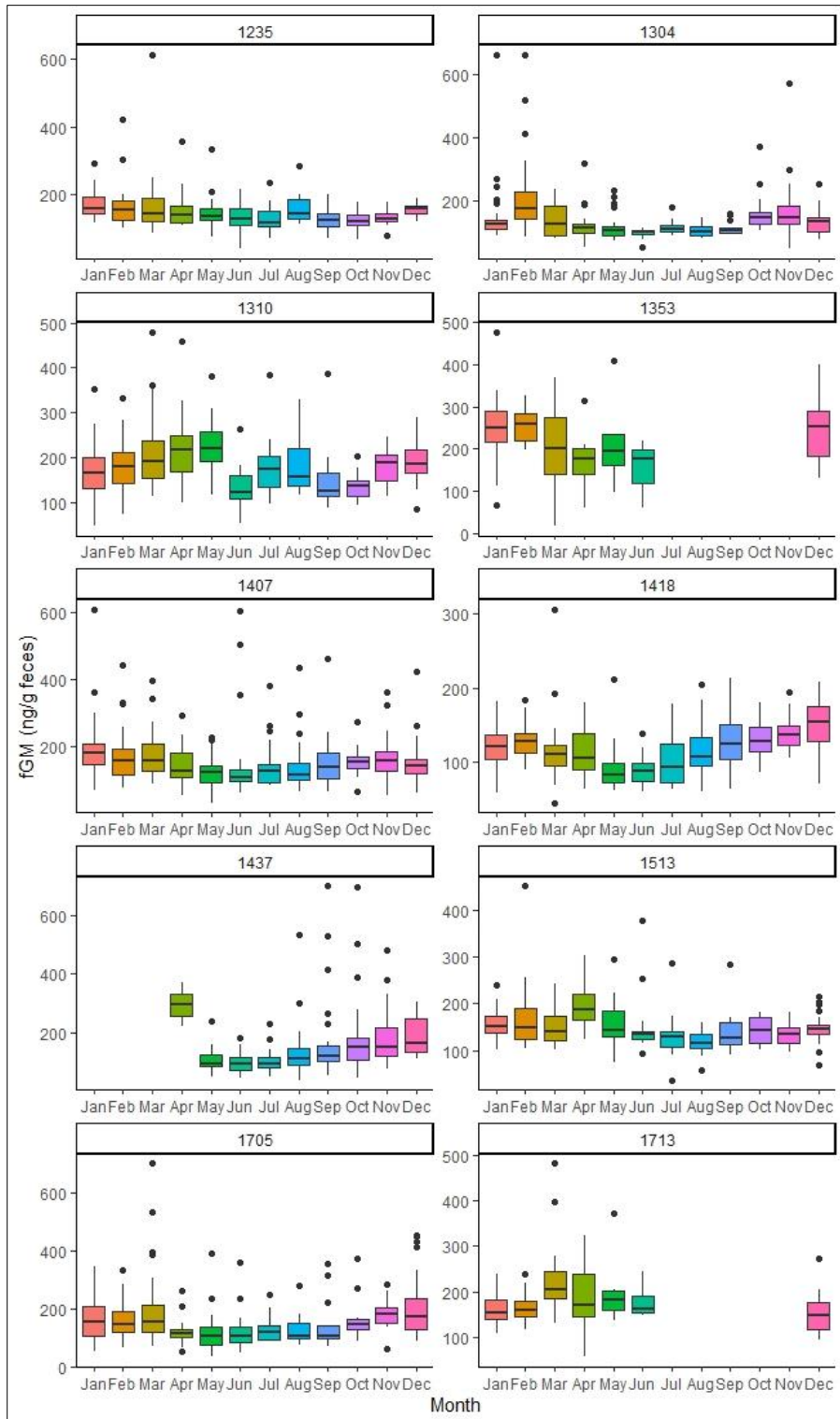
**Figure 6.** Boxplot showing the concentration of fecal glucocorticoid metabolites per month for red pandas ( $n = 10$ ) at four North American zoological facilities. Significant differences ( $p < 0.05$ ) are denoted with superscripts.





**Figure 7.** Scatterplot of the effect of zoo visitor numbers on fGM concentrations in red pandas ( $n = 10$ ). Every 1,000 fewer zoo visitors corresponded with a 2.4% increase in glucocorticoids ( $p < 0.001$ ) The dark line is the predicted relationship between visitor number and fGM concentration.

Individual red panda fGM concentrations were influenced by month (Fig. 8). In the majority of individuals ( $n = 8$ ) fGM concentrations were significantly lower in at least one month between May and August ( $p < 0.05$ ), similar to the pooled model. Six individuals had significantly lower concentrations in May and/or June. Five red pandas had significantly lower concentrations in July and four had significantly lower concentration in August. Over half of the individual models for red pandas ( $n = 6$ ) had fGM concentrations at the beginning of the year, in January and February that were not significantly different from November and/or December ( $p > 0.05$ ).



**Figure 8.** Concentrations of fecal glucocorticoid metabolites (fGM) varied by month for red pandas (n = 10; represented by studbook number). Not all red pandas had fecal samples collected every month.

#### **4. Discussion**

Red pandas are a popular and common species featured in zoological institutions around the world. It has long been suggested they are sensitive to environmental factors such as zoo visitors, noise, temperature and humidity (Glatston et al. 2015; Philippa and Ramsay 2011; Princée and Glatston 2016). However, the influence of the environment on captive red panda behavior and physiology has never been systematically tested. The present study demonstrated that temperature had a significant impact on the behavior displayed by red pandas. Higher temperatures decreased the proportion of time red pandas were active and increased the proportion of time they spent panting and in straddle position. fGM concentrations also varied by month in red pandas; with significantly lower concentrations often between May and August. Contrary to the hypothesized relationship, fGM concentrations in red pandas decreased with higher numbers of zoo visitors and temperature.

In this present study red pandas spent almost an equal proportion of time active and inactive. Wild red pandas are active 36.5% to 48.6% (Johnson et al. 1988; Zhang et al. 2011) of the time, specifically with high activity between 0700-1000 h (Zhang et al. 2011). Red pandas in the present study had behavioral observations conducted during that time frame, possibly accounting for the higher proportion of active time. Play, fight, and conspecific grooming were infrequently or never observed even though some individuals were housed together. This is consistent with research that states aggression is rare in red pandas and adults often do not interact unless it is breeding season (Roberts and Gittleman 1984). Only three individuals in our study were housed together outside of breeding season. Stereotypic behaviors, such as circling and head roll, had low occurrence rates and were only observed in three individuals, one at SCBI and two at NZP. This may indicate that head rolls are an individualistic behavior. Red pandas had high rates of scent marking and lick/sniff behavior compared to vocalizations in the present study,

supporting that the predominant means of communication for red pandas is chemical. The species is often seen scent marking and using common latrines to communicate (Yonzon and Hunter 1991).

Previous research on red panda activity predominantly has focused upon longitudinal patterns and circadian rhythm. For instance, red pandas demonstrate crepuscular and diurnal activity (Johnson et al. 1988; Reid et al. 1991). Wild red panda activity has no significant monthly variation (Johnson et al. 1988), but in captivity, activity changes throughout the year based on temperature (Roberts and Gittleman 1984). In contrast to our study results, which found higher activity at low temperatures, red pandas in China are most active in April and in the summer if a female is lactating (Reid et al. 1991). None of the females in the present study were lactating. Also, the present study had high summer temperatures, approaching 30°C, which may be higher than what pandas in the wild experience because the temperature does not often exceed 20°C in native habitat. This difference in temperature could explain our lower activity in the summer compared to previous research. Morgan and Tromborg (2007) describe how adverse temperature can change behavior in other species; for example, higher temperatures cause steer to increasingly mount their cohabitants and lower temperatures decrease play in pigs. Tigers in warm temperatures (>30°C) spend up to 75% of their time inactive but when given shade structures they increase activity (Stryker et al. 2019). Monitoring activity can be a way to evaluate adverse effects of temperature and ensure that optimal welfare occurs throughout temperature ranges experienced in captivity.

None of the variables measured during behavioral observations explained the time red pandas spent pacing, when the behavior was analyzed separately from the proportion of time spent active (PA). Pacing is a stereotypic or repetitive behavior pattern that often has no purpose or function (Mason 1991). Therefore, the stereotypy is not often due to immediate changes in the

environment, such as the weather variables or noise from zoo visitors examine in the present study. Pacing in captive leopards (*Panthera pardus*) is not influenced by the presence of zoo visitors, but instead by enclosure features, such as the complexity of the exhibit (Mallapur and Chellam 2002). Social grouping impacts locomotor stereotypes in captive carnivores and elephants (Clubb and Vickery 2006; Greco et al. 2017). Stereotypic pacing also can be anticipatory to the arrival of animal care staff, food, or enrichment (Bennett et al. 2015; Cless and Lukas 2017). Further investigation is needed to understand what factors influence pacing in red pandas and specifically for the individuals who performed most of the pacing in the present study.

The proportion of time pandas in the present study spent panting increased significantly with increasing temperature. Panting was often seen at temperatures above 20°C, which may mean that this temperature is a threshold for red pandas because panting is a physiological response to overheating in mammals (McKinley et al. 2018). Panting is an important method to dissipate heat, particularly in smaller mammalian species (Robertshaw 2006). Once the internal body temperature gets too warm, sweating and panting is initiated in mammals. Red pandas have a dense fur coat that insulates them well in the winter but can be a detriment in warmer weather. They do not have large areas of exposed skin to sweat from, which means panting is their most effective means of cooling their core temperature, like dogs and sheep (Robertshaw 2006). However, panting can lead to dehydration from loss of bodily fluids and prolonged panting can decrease the effectiveness of the process to cool the body (McKinley et al. 2018), which can be fatal. While panting is a process that red pandas use to cool off, it is important that they are not exposed to high temperatures and humid environments for long periods of time in order to prevent adverse consequences, such as heat stress which is documented in captive pandas (Philippa and Ramsay 2011).

Straddle was more prevalent with increasing temperatures. For an animal suited for colder climates, straddle position - lying on a structure or tree branch with 3 or 4 legs hanging on either side - may be another way to release heat on warmer days by not having a compact body form. The position can be seen by captive pandas in warmer climates as well as being documented in the wild, where red pandas lay with limbs hanging from branches in warmer summer months (Roberts and Gittleman 1984). As temperatures continue to increase around the world this stationary behavior may become even more common. This position is one way an animal can cope with warmer weather without the negative physiological effect panting can create. *Colobus* monkeys frequently sprawl during the hottest periods of the day (McGraw 1999). Squirrel monkeys (*Saimiri sciureus*) exhibit straddle, known as sprawl for their species, which is commonly seen in the summer and has a positive relationship with temperature (DuMond 1968). Koalas (*Phascolarctos cinereus*) are another arboreal species which lay in or hug trees in a similar straddle position more often as ambient temperature increases (Briscoe et al. 2014). This position enhances conducted heat loss by increasing the surface area of their body that is associated with the tree. This behavior means less heat needs to be lost through evaporative cooling (Briscoe et al. 2014). Straddle was only observed at SCBI, where animals do not have the ability to enter a climate-controlled area and have no trees in their enclosure for shade. Straddle behavior may be driven by differences in enclosure design. It would be beneficial to investigate if there are features that can be added to enclosures, such as shade or cooling devices, to decrease panting and time spent in straddle, while possibly increasing activity on warm days.

In the wild, red panda habitat does not exceed 20°C (Johnson et al. 1988; Roberts and Gittleman 1984; Yonzon and Hunter 1991) and optimal habitat occurs in areas with an annual mean temperature ranging from 5°C to 10°C (Panthi et al. 2019). Captive pandas are frequently kept in zoos with warmer climates than those in which they have evolved. The average

temperature during our yearlong behavioral observation period was 15.9°C, which is over five degrees higher than the described optimal temperature. Our study was conducted in the morning hours when temperatures had not reached their maximum, especially during the summer. The maximum temperature recorded during behavioral observations was 30.5°C in July 2018. Thus, we can expect the behavioral trends observed in the present study would be even stronger later in the day. Red pandas housed at NZP were provided indoor access when temperatures exceeded 21°C during our study period and therefore may be even less acclimated to the average ambient temperatures during the summer in Washington D.C. The ability to go into a climate-controlled building when temperatures were higher may mean we observed fewer coping behaviors than what would have been displayed if the pandas did not have indoor access. Thus, the positive relationship temperature has with panting and straddle might be stronger than reported in this present study. The Red Panda Care Manual states pandas should have access to air-conditioned indoor areas or other holding spaces when temperatures exceed 26.6°C (AZA Small Carnivore TAG 2012). Our results showed that behaviorally, pandas probably were impacted by temperatures lower than 26°C as panting and time in straddle position were most frequently observed when temperatures were over 20°C and time active was significantly lower. Zoological institutions that house red pandas may need to consider providing access to cooler environments beginning when temperatures are lower than those stated in the animal care manual for the species.

We can expect that temperatures at these zoological institutions, and native habitats, are rising. For instance, the average annual temperature for the state of Virginia, appears to be increasing, from 12.7°C (1901 to 2010) to 13°C (1981-2010; NOAA, 2020c). Washington D.C, where NZP is located with annual temperatures changing from 14.5°C (1947-2010) to 14.8°C (1981-2010; NOAA, 2020b). Based on these trends, climate change will continue to increase

mean temperatures experienced at these two zoological facilities and likely worldwide. Captive managers need to consider the impact temperature may have on red panda behavior, as behavior is often the first indicator of animal discomfort. During low temperatures (9°C), a higher frequency of stereotypic behaviors is seen in Asian elephants (*Elephas maximus*), which are adapted to a warm climate in the wild (Rees 2004). From other species, it is clear heat stress has the potential to have subsequent impacts on health and reproduction (Nardone et al. 2010). If we ignore the impacts of climate, and the change it is undergoing, on captive red pandas we could contribute to long-term stress from exposure to these increasing temperatures.

Fecal glucocorticoid metabolite concentrations were impacted by month and the number of zoo visitors. However, contrary to our hypothesis, fGM concentrations were lower May through August when temperatures are warmer. Multiple mammalian species frequently show increased glucocorticoids when exposed to increased temperatures, particularly for short periods of time (de Bruijn and Romero 2018). Pigs have increased salivary corticosterone when exposed to temperatures above 17°C and cows have increased plasma cortisol during acute exposure to heat stress (de Bruijn and Romero 2018; Morgan and Tromborg 2007). African penguins (*Spheniscus demersus*) have a significant increase in adrenal cortical activity when daily mean temperatures increase (Ozella et al. 2017). Therefore, we anticipated a positive relationship in red pandas, but found the opposite. The relationship between temperature and glucocorticoids in red pandas may be primarily driven by season and breeding. Our results are supported by previous research in red pandas. Budithi et al. (2016) also describe red pandas having higher fecal glucocorticoids in December and January when ambient temperatures are lower, which they believe is related to breeding.

In the present study month had a significant impact on adrenal activity. May through August had lower fGM concentrations compared to January, while November and December



concentrations were similar. Individual models were consistent with this population level model, showing significantly lower fGM concentrations in the summer months and not statistically different fall season concentrations. Results support findings from Freeman et al. (2012) that red pandas of both subspecies have lower concentrations in spring compared to other seasons. The same study noted consistent seasonal patterns of glucocorticoids in all individuals. Mammals, birds, reptiles, and amphibian species seasonally modulate glucocorticoid concentrations (Romero 2002). Red squirrels (*Sciurus vulgaris*) have the lowest fGM concentrations in the summer and highest values in the winter, when they are engaged in mating and sometimes lactating already (Dantzer et al. 2016). Wild bison (*Bison bison*) have low fGM concentrations in January–February and high values in April–July, which coincides with the calving season and lactation (Metrione et al. 2020). Monthly influence on fGM concentrations in red pandas may be related to reproduction, but we do not know if this relationship may be due to physiological processes or to physical activity during the breeding season. Red panda breeding season in the northern hemisphere is January through March (Glatston et al. 2015). No animals in the present study were in a breeding pair during the 2018 breeding season. While most animals were housed individually during the 2019 breeding season, one female (SB# 1513) successfully bred with a male who was not included in the study. In addition, SB# 1705 and 1407 were kept together during the 2019 breeding season, but the female was contracepted to prevent reproduction. Even though most animals in the present study were not in breeding pairs, their fGM concentrations were still elevated during the breeding season, therefore driving monthly concentrations. Breeding is not the only possible reason for a seasonal response in fGM concentrations. Banded mongoose (*Mungos mungo*) exhibit seasonality in glucocorticoid production associated with food limitation; glucocorticoid concentrations are lower when food is abundant, including access to anthropogenic food resources (Laver et al. 2020). Food sources of red pandas change seasonally

and accessibility to these sources influence behavior of red pandas in the wild (Zhang et al. 2009). Activity is high during the day in summer compared to night, coinciding with arboreal foraging (Reid et al. 1991). In a zoo setting, food availability is consistent year-round possibly explaining the opposite results found in our current study.

Red panda fecal glucocorticoid metabolite concentrations declined with increasing zoo visitor numbers; although only the fGM values from NZP had a visitor number above zero associated with them. When data from NZP and SCBI was split the top model for NZP was the same, containing month and VN, while the top model for SCBI was just month. Also, fGM concentrations were not significantly different between NZP and SCBI, which is closed to visitors, demonstrating that zoo visitors might not have as much of an impact on fGM concentrations as originally hypothesized. Visitor numbers at NZP demonstrate an oscillating pattern with high visitation in the summer and low visitor numbers in the winter. Most captive mammal research demonstrates that an increase in visitors causes an adrenal response raising glucocorticoid levels (Morgan and Tromborg 2007). Fecal glucocorticoids increase in meerkats (*Suricata suricatta*) when the median number of visitors from the previous day increase (Scott et al. 2017). Giant pandas have significantly higher urinary cortisol on loud days compared to quiet ones at the San Diego Zoo (Owen et al. 2004) and with construction noise present near their exhibits at the National Zoo (Powell et al. 2006). Mexican wolves (*Canis lupus baileyi*) have higher fecal cortisol levels on Sundays, when they were exposed to more zoo visitors (Pifarré et al. 2012). Yet our results from the present study show the opposite relationship. Arboreal species and carnivores are less impacted by visitors than ground dwelling species and herbivores (Queiroz and Young 2018). Red pandas in the present study may not respond physiologically to visitors because they possibly have become adapted to the zoo environment or the enclosure at NZP provides enough enrichment and places to retreat from people that the red pandas are not

greatly impacted. In addition, red pandas at NZP have the option to be inside during the summer months when visitor numbers are at their highest and therefore the public may have less effect on the NZP red pandas than other zoos. Similar to temperature, the results from visitor numbers may be overshadowed by seasonality. Red pandas have higher fGM concentrations in the fall and winter seasons when temperatures are lower and fewer people visit the zoo. It is possible that the negative relationship between zoo visitors and fGM concentrations occur in other species, but these results are not published because a classic stress response to visitors is not demonstrated or the response to zoo visitors may not be studied in animals that seem well adapted to the captive environment.

Rather than focusing on the adrenal response to visitors, some studies have found a relationship to behavior. For instance, flamingos (*Phoenicopteridae*) do not change their activity pattern or how they use their enclosure based on visitor numbers (Rose et al. 2018). Ring tail lemurs (*Lemur catta*) respond to more visitors by increasing locomotion, but it has a weak association with changing behavioral diversity of the lemurs (Collins et al. 2017). Koalas increase vigilance due to nearby visitors (Larsen et al. 2014). Bush dog (*Speothos venaticus*), golden-headed lion tamarin (*Leontopithecus chrysomelas*), and giraffe (*Giraffa Camelopardalis*) increase movement with higher numbers of zoo visitors and loud noise (Quadros et al. 2014). The present study did not investigate how zoo visitors impact red panda behavior because the number of people outside the exhibit was not counted during the behavioral observations. Complexity of the exhibit and other study design elements meant it was not feasible to record visitor numbers while conducting behavioral observations. In the present study, activity increased with decreasing noise. A few individual pandas had top models which had noise as a variable with a negative relationship, though it was not significant. Previous research shows visitor numbers and noise have a positive relationship (Quadros et al. 2014); therefore, red pandas may be more active when

there are fewer zoo visitors, but the relationship needs further investigation. Increasing the sample size of zoos which have visitors present may give a clearer picture as to the impact public visitor numbers has on red pandas in captive institutions.

## **5. Conclusions**

Results of the present study confirm that *ex-situ* red pandas are sensitive to their environment. Stereotypic behaviors, besides pacing, were not found to be present often during behavioral observations. Coping behaviors such as panting and straddle were displayed and positively related to temperature. Decreased activity and coping behaviors can be good indicators to captive managers that temperatures are impacting red pandas and/or that these animals may be at risk for serious health issues such as heat stress. Alternatively, humidity and noise around the exhibit have lesser effects on behavior.

Contrary to our hypothesis, fGM concentrations did not increase with increasing temperature, but instead were best explained by monthly variation with lower concentration May through August. Red pandas should be given access to climate-controlled areas when housed in zoological facilities with warm climates, where temperatures often exceed those seen in their native habitat. More research is needed to understand the potential short and long-term consequences of elevated temperatures on red panda health, to build upon heat stress and causes of death in the species as discussed by Philippa and Ramsay (2011) and Delaski et al. (2015). Not all zoos can house red pandas in climate-controlled areas where they are viewable to the public. If features, such as shade or cooling devices, can be found that decrease coping behaviors (e.g. panting, straddle) it could allow the animals to stay on exhibit while also maintaining optimal welfare of the individual. Although fGM concentrations were not significantly correlated with temperature, this study confirms that red pandas demonstrate monthly variation, with lower

concentrations in summer months, May through August, compared to winter months, November through February. Seasonal adrenal activity could be related to reproduction and should be investigated further to understand potential welfare indicators for this endangered species.

Self-sustaining captive populations, with strong reproductive success and cub survivorship, are important for the long-term survival of endangered species. Our study was able to identify environmental factors such as temperature and season that impact behavior and physiological responses in red pandas. This information can guide management of the species, specifically in areas with warm climates, to ensure optimal welfare of individuals and safeguard future generations of red pandas.

## APPENDIX I

### Red Panda Behavior Ethogram

Behavior	Description	Modifier
<b>State Behaviors (Activity Budget) – record duration**</b>		
Circling	Walking in a consistent, repeated path through the enclosure more than one time	
Climbing	Walking, running or climbing along a tree trunk, branch or elevated surface (horizontal or vertical)	
Drinking	Drinking water in the enclosure	
Eating	Consuming a food item	Browse vs. Biscuit/fruit
Fight	Make aggressive physical contact with another conspecific, typically including arching of the tail and back, hitting or biting. Note identity of conspecific	Conspecific
Grooming	Scratching or licking the fur or skin in a consistent repetitive action on self or conspecific. Note identity of conspecific	Self vs. Conspecific
Locomotion	Walking or running along the ground	
Pacing	Locomoting at a steady and consistent speed, back and forth within a small area. Must occur twice before marking as behavior and recording time	
Play	Rolling, playing with tail, wrestling, stand/climb on, tickle, grab, kick, ambush, tag, nudge, swat or mouth. Either with self or conspecific. Note identity of conspecific	Self vs. Conspecific
Sleep	Lying still (curled in a ball, on back or stomach), in a non-straddle position, eyes closed	
Stationary	Standing on all fours, sitting on back legs or lying on the ground, structure or tree in a non-straddle position with no forward movement. Eyes Open	
Straddle	Lying on a structure or tree branch with legs (3+) dangling on either side of the branch or structure and head resting on branch/structure. Or lying flat on a level surface with all four legs stretched out from the body. Eyes open or closed.	Normal vs. pancake
<b>Event Behaviors – record each occurrence</b>		
Anogenital scent marking	Lifting tail and rubbing anus or genital area along the ground or an object; either in a sideways or front to back motion	
Head Roll	Moving the head in a circular motion	

Lick/Sniff	Sniffing and/or licking non-animal object or lifting head and sniffing the air	
Grunt	Short, deep vocalization	
Huff-Quack	Harsh, broad-band, polysyllabic. Discreet bursts of sound in low frequencies	
Squeal	Long, high pitched cry or noise	
Twitter/ Chirp	Compound, high frequency, modulated call	
<b>Miscellaneous – record duration</b>		
Panting	Rapid, shallow breathing with the mouth open. Can occur simultaneous with another state behavior	
Indoor area <sup>++</sup>	Occupying the indoor building	
Keeper influence	Keeper interacting with individual either by entering enclosure, giving food, speaking to or having physical contact with the animal	
Not Visible	Not visible to observer	
Outdoor exhibit <sup>++</sup>	Occupying the outdoor area	

\*\*State behaviors (those with duration) were considered continuous unless stopped or not visible for  $\geq 3$  seconds. Exception when transitioning from locomotion to climbing.

++ For pandas at NZP with indoor/outdoor access

## APPENDIX II

### Method Validation

#### **Fecal Extraction Alcohol**

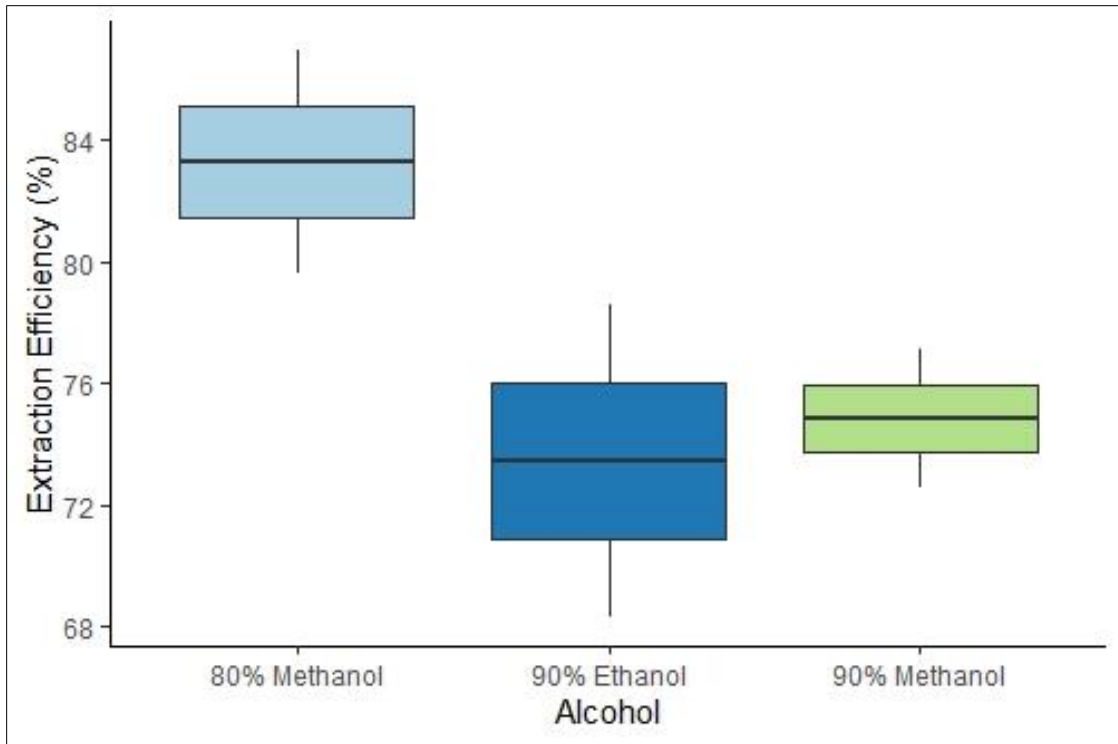
##### **Methods**

Three different extraction methods were used on red panda fecal samples to determine the type and percentage of alcohol (90% methanol, 80% methanol, and 90% ethanol) that was best for hormone extraction. Twenty samples of lyophilized feces were extracted using a modified shaking technique adapted from Edwards et al. (2019), and the three different alcohol solutions. The average extraction efficiency of this process was determined with the addition of <sup>3</sup>H-corticosterone to each sample prior to extraction.

##### **Results**

Average extraction efficiencies for the three different alcohol solutions ranged from 73.45% to 83.3% (Fig. 9). The best alcohol solution for red panda fecal samples was determined to be 80% methanol because it had the highest extraction efficiency of the three tested.





**Figure 9:** Extraction efficiencies for red panda fecal samples using three different alcohol solutions: 80 methanol, 90% ethanol, and 90% methanol.

### **ACTH Corticosterone Validation**

#### **Methods**

To validate the corticosterone EIA, ACTH (Acthar gel, Wedgewood Pharmacy, Swedboro, NJ, USA) was administered intramuscularly (10 IU/kg) to 4 individuals (1 male, 3 females). Fecal samples were collected for 3 days prior and 6 days after ACTH administration (Freeman et al. 2012).

#### ***Corticosterone***

Hormones were extracted from lyophilized feces using a modified shaking technique and 80% methanol. Concentrations of fGM were quantified using a double antibody corticosterone EIA incorporating a secondary goat-anti rabbit IgG antibody (A009, Arbor Assays, Ann Arbor,

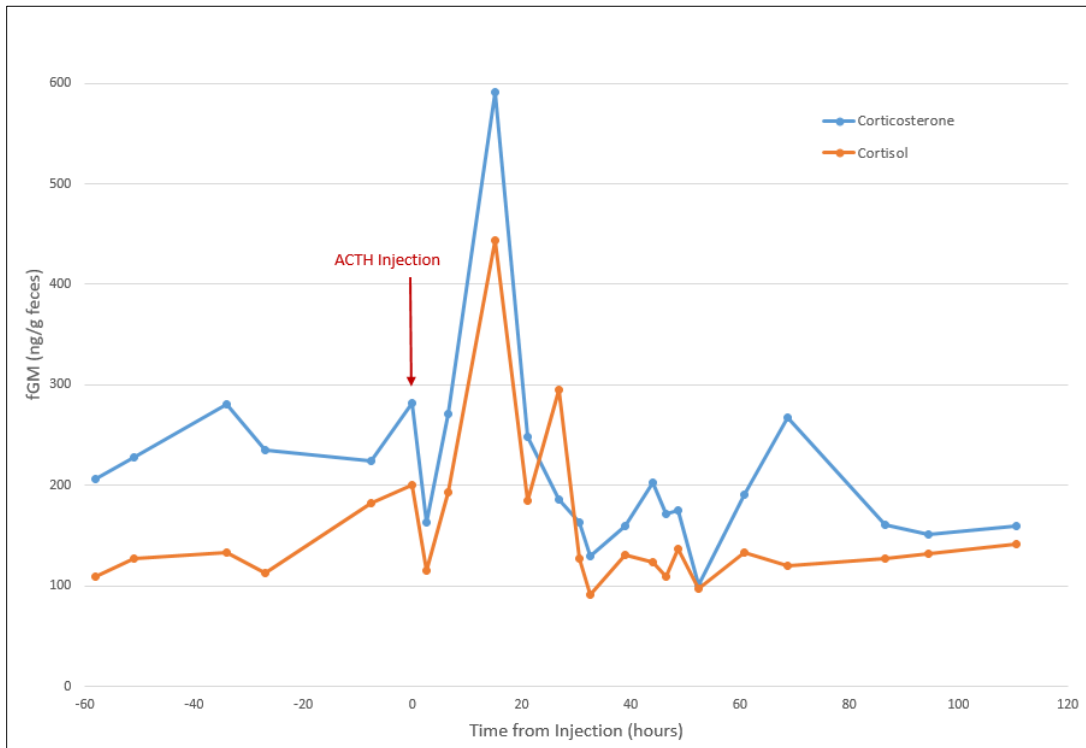
MI) and polyclonal rabbit anti-corticosterone antibody (CJM006, C. Munro, University of California, Davis, CA).

### **Cortisol**

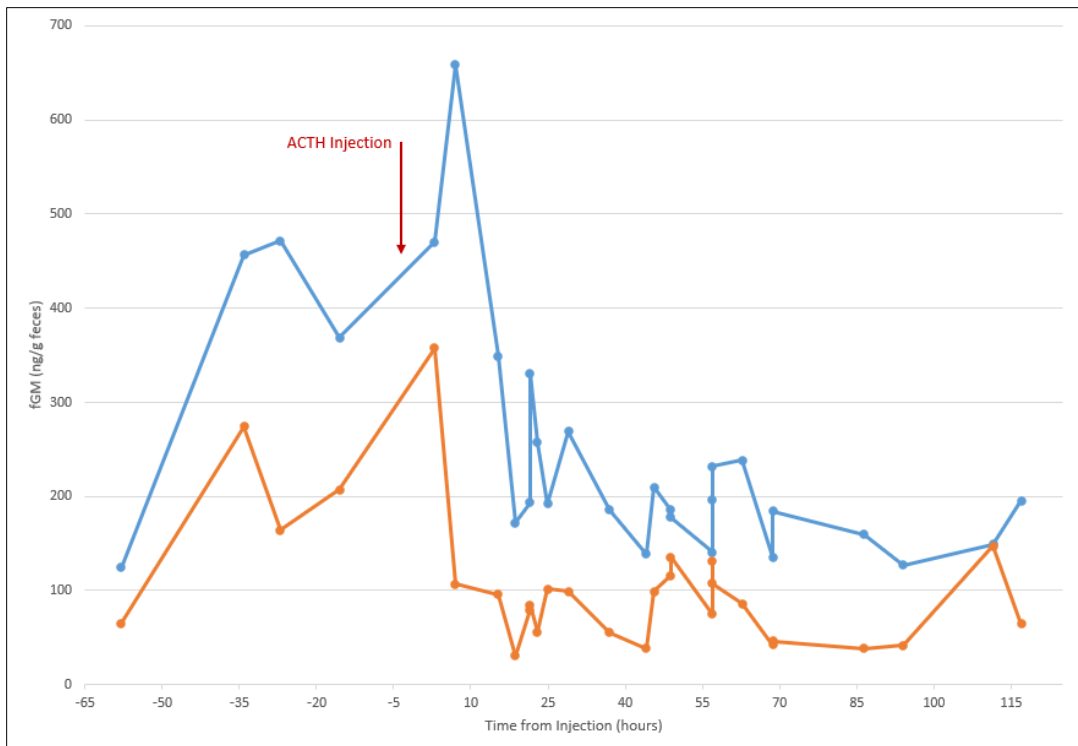
Hormones were extracted from lyophilized feces using a shaking protocol and 90% ethanol. Concentrations of fGM were quantified using a single antibody cortisol EIA (R4866) and cortisol-horseradish peroxidase (Coralie Murano, University of California, Davis, CA).

### **Results**

Peak fGM concentrations following the ACTH challenge were 7-15.25 hours post injection. Fig. 10 displays the response to ACTH injection in a female red panda and Fig. 11 shows the response in the male red panda.



**Figure 10:** ACTH response for corticosterone (blue line) and cortisol (orange line) enzyme immunoassay (EIA) in a female red panda with peak fGM concentrations 15.25 hours post-injection.



**Figure 11:** ACTH response for corticosterone (blue line) and cortisol (orange line) enzyme immunoassay (EIA) in a male red panda with peak fGM concentrations 7 hours after injection.

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