

# Dietary analysis of snow leopards (*Panther uncia*) in the Gilget-Baltistan region of Pakistan using next-generation sequencing

PIs:

Matthew J. Jevit<sup>1</sup>, Jan E. Janecka<sup>1</sup>

Collaborators:

Shafqat Hussain<sup>2,3</sup>, Ghulam Muhammad<sup>3</sup>,

1. Duquesne University, Pittsburgh PA, 15203
2. Trinity College, Hartford CT, 06106
3. Project Snow Leopard, Skardu, Pakistan

## Executive Summary

In recent years, it has been shown that some species have adapted to climate change by shifting their ranges causing competition for resources between native species and invasive species (Ruggiero et al. 1999). Some species have greater ability to adapt to anthropogenic changes. For example, in North America with humans encroaching on black bear (*Ursus americanus*) habitat, bears are now concentrated around settlements due to their increasing reliance on anthropogenic food sources (Beckman et al. 2003). This has largely led to a ~1,000% increase in bear-human interactions/altercations over the last 10 years. Other species, such as the ocelot (*Leopardus pardalis*) are very sensitive to human alterations (Janecka et al. 2011). Effects from human activities are compounded by impacts of global climate change. Together, these pressures can lead to ecosystem-level change in not only habitat but also the distribution of food resources. As a result, predators may be forced to utilize different prey resources and/or habitat.

Several important prey species in Central Asia, such as the markhor (*Capra falconeri*) and argali (*Ovis ammon*), are listed as endangered or near threatened by the IUCN. Climate change and increases in human activity may cause further reductions in these prey species, or changes in their distributions, in turn causing major shifts in prey use by snow leopards. If the diet and habitat use of snow leopards is altered then current management practices may no longer be effective. Furthermore, if their use of human occupied and areas dependence on livestock increases, it is likely there will be an increase in human-snow leopard conflicts. This will place an even greater strain on snow leopard populations and may cause changes in the diet habits of these animals.

Though there have been studies that have looked into the diet of snow leopards there is one distinct problem with these studies. They provide only a snap shot in the dietary habits of the animals from data that is collected from one time point. The areas and ecosystems that snow leopards inhabit are dynamic and change which may cause the diet to change. Due to the speed of which these ecosystems are changing it may mean that data published in the past is no longer relevant for use in management decisions. Thus it is more important to recognize changes in trends of the diet in order to understand what may be effecting these changes. In order to accomplish an area of known inhabitation where a previous diet study (Anwar et al. 2011) had been conducted in the Gilget-Baltistan region of Northern Pakistan has been resurveyed and another dietary analysis has been performed.

(Anwar et al. 2011) was conducted on a morphological basis by identifying hairs in scat. This is a qualitative method that is subject to error due to phenotypic variation. Recently “Next-Generation” sequencing has been used to amplify prey DNA from scat to identify prey species to provide more definitive identification of prey items. (Anwar et al. 2011) identified caprids in

40.8% of scat, birds in 2.2%, and bovids in 8.6%. Recently, snow leopard scats (N=71) were sampled in the same area. These were genetically confirmed and individuals identified. Prey DNA from these samples and 8 controls of known composition, was amplified using general 12S primers and sequenced with an Illumina Miseq to an average of 70,239.9 reads/sample. This method consistently identified all species present in the controls.

Across all scat samples, the domestic bovids was the most prevalent, being identified in 42% of the scat samples of which prey species could be identified. Bovid was followed in frequency by domestic goat in 24% of the samples. The most abundant wild prey was pika which was identified in 18% of the scat samples. Overall domestic prey was identified in 94% of the scats that which prey species were identified in. Wild prey was only identified in 37% of the scat samples which prey species were identified. This is an increase over the baseline study, Anwar et al. 2011, which identified domestic prey in 36.5% of their scat samples. It is important to note that these two studies do use a different methodology. Additionally, where wild prey was identified it was generally small bodied, either the pika or civet, large bodied prey were only identified in 6 scat samples. This may indicate that large bodied wild prey are becoming more scarce thus causing the increase in the reliance of domestic and small bodied wild prey when compared to Anwar et al. 2011.

### **Objectives**

The major goal of this study are to understand the temporal variation in snow leopard prey use and compare it with available climatological data over a 7 year period. To meet this goal we will achieve the following objectives:

*Objective 1:* We will analyze prey remains in scats collected from the Baltistan region of northern Pakistan to determine prey use. We already have 205 scat samples from these areas in the Janecka laboratory. The first set (95 scats) was collected between April of 2007 and March of 2009 as part of a snow leopard diet study published in Anwar et al. (2011). In 2013 - 2014, Dr. Hussain and G. Mohammad resampled these field sites (110 scats) to examine any potential change in the snow leopard population. G. Mohammad will collect additional samples in 2015. These samples will be used to estimate the level of temporal variation in prey use.

*Objective 2:* We will generate estimates of the frequency of livestock depredation relative to wild ungulates. The percentage of scat with livestock will be examined to determine if reliance on domestic sheep, goats, or bovids has increased or decreased since 2009. In addition, individual identification of snow leopards will be used to explore whether livestock is routinely used as a prey source by the population as a whole, or if there are specific individuals that are responsible for most of the livestock depredation.

*Objective 3:* We will obtain available meteorological data from the study sites including average air temperature, snow cover, and severity of winters from G. Mohammad. This will be used to determine whether the climate in the Baltistan region of northern Pakistan has had fluctuated over the last 5-7 years. Any observed changes will be tested for correlations with the prey use data.

## **Materials and Methods**

DNA was extracted from scats using a Qiagen QIAamp DNA Stool Kit (Hilden, Germany). PCR and sequencing of a cytochrome b fragment were used to identify snow leopard scats following methods of Janecka et al. (2008). The *cytochrome b* sequences were edited in *Sequencher* and compared to reference sequences in *Blast* to determine which scat samples were snow leopard in origin. If the DNA obtained from the scats had a 97% or greater similarity with the reference sequence as well as a 97% coverage over the reference sequence it was determined to be a snow leopard. The scats that were confirmed to be snow leopard were then genotyped with 8 microsatellites and 1 sex marker to make individual and sex identification (Janecka et al. 2014). Analysis of microsatellites genotypes was performed in *Genemarkerx*. The DNA extracted from snow leopard scat was then PCR amplified using the 12SV51 mtDNA primer set, to amplify a 152 bp region of the mtDNA. This primer has been shown to amplify across mammals and can be used to obtain species level identification (Shehzad et al 2012). An additional primer set was developed using the program *primateclade* to produce a primer set for lagomorphs and rodents DNA obtained from GenBank (accession numbers; KF038213\_12S, AY012127\_12S, AB053258\_12S, AY227529\_12S). In Shehzad et al. (2012) rodents such as the marmot and lagomorphs such as the pika were not represented in the data. Because these animals are generally found in scat of snow leopards this may indicate that these species were not amplified by the 12SV5 primer set (Anwar et al. 2011). By using the developed marmot primer set it ensures that rodent and lagomorph DNA was amplified. The 12SV51 primer set was tested on DNA extracted from tissue samples of domestic rabbit (*Oryctolagus cuniculus*), a lagomorph closely related to the pika, as well as DNA from groundhog (*Marmota monax*) which is within the same genus as the marmot. Both the samples from the rabbit and the groundhog amplified showing that the 12SV51 primers are capable of amplifying both pika and marmota,

due to this a second amplification with rodent/lagomorph specific primers was unwarranted. Following the amplification of DNA using the 12SV5 primers the 16S Metagenomic Library Preparation protocol provided by Illumina was followed to prepare these samples for sequences. This included performing a PCR clean up using SPIRE beads and determining whether the product of the PCR was viable with the use of a 2% agarose gel and a Nano drop spectrophotometer. A second PCR was performed using these sample to incorporated barcodes on to the amplified fragments. A second SPIRE bead cleanup was preformed and the samples where pooled and analyzed using a bio-analyzer service at the University of Pittsburgh (Pittsburgh, Pennsylvania). Upon completion of the 16s protocol the samples were sent for sequencing on an Illumina miSeq platform. The 12S sequence reads from Illumina miSeq of PCR amplicons were analyzed in the *CLC Genomics Workbench*. The reads were demultiplexed, trimmed of barcodes, adapters, and primers, filtered reads by a quality score of Q30 and mapped to references obtained from NCBI GenBank to obtain prey species identification following methods based on Shehzad et al. (2012). A 97% similarity and a 97% coverage was required for a positive ID.

After determining the prey species that were present in the scat sample; the proportion of occurrence for each prey species, and proportion of occurrence of domestic livestock (domestic sheep, domestic goat, domestic cattle and yak) were estimated. These estimates were then compared between the populations of 2 time periods in Gilget-baltistan, and among the individual snow leopards of each spatial and temporal population. Animals that have a greater occurrence of domestic sheep, goat, and yak in scat may be ones that specialize on livestock. The differences in prey use across the two temporal periods (2007-2009 versus 2013-2015), and yearly difference.

During the initial analysis, it was discovered that many of the wild goat and sheep species in the area have a high sequence similarity in the amplified region. Though the sequence will often match 100% sequence identity with one particular species, often the domestic goat, it shares between 99% and 97% sequence identity with other caprid species as well and can technically provide a species level identification corresponding to more than one species. In order to increase the diagnostic power of this protocol, additional primers for the caprine control region were designed and are currently being tested. Once the efficacy of these primers are validated they will be used in the same protocol described above and re-sequenced. This will occur within the next couple of months and will serve to be able to definitively identify goat and sheep species in the scat.

Additionally the efficacy of blocking oligos as described in Shehzad et al 2012 was tested. The protocol described above was performed twice on a subset of 10 samples. One replicate used the blocking oligos published in Shehzad et al. 2012 in the initial amplification and the other replicate did not. The percentage of the reads that were identified as snow leopard in each sample were compared to the corresponding replicate to determine if there was a significant difference between the two methods.

Government agencies, administrative centers, communities, and conservation agencies will be contacted in order to obtain available contemporary and historical meteorological data for the study sites; this included air temperature, precipitation, snow cover, and winter severity. This data will be averaged and compared between sampling periods during which the snow leopard population were surveyed. This meteorological data will then be correlated with the diet data to determine whether meteorological conditions may have correlation with a change in diet as soon as the control region primers are used to validate the caprid species observed in our study.

## Results

The blocking oligos were determined to have no significant effect on relative proportion of reads occupied by snow leopards in any of the 8 samples that they had been tested. The difference in the percentage of the 8 samples were; 7.3%, 5.3%, 2.7%, 0.9%, 0.65%, 0.15%, 0.39%, and 0.09% respectively. Of these the greatest change occurred in the two replicates of samples MJ03 with a difference of 7.3%. Functionally these difference in percentages are negligible and could easily be explained by random variations in equipment or reagents.

Utilizing the microsatellite data, a total of 5 individual leopards had been identified and labeled PUN1-5. 6 scats were identified to have come from pun1, 13 scats from pun 2, 9 scats from pun 3, as well as 2 scats from both pun 4 and pun5. For a prey species to be considered present, reads mapping to the particular prey species would need to account for at least 2% of the total reads per sample. The MiSeq produced a mean of 70,239.9 reads per sample with a standard distribution of 38,424.3. For PUN1, domestic goat was identified in 3 scat samples and domestic bovid was identified in 1 scat. For PUN 2 domestic goat was identified in 2 samples, domestic bovid in 4 samples, pika and civet were both identified in 1 sample. For PUN3 domestic goat was identified in 2 samples and red deer was identified in 1 sample. For PUN4 domestic bovid and pika were each identified in 1 sample. Finally, for PUN 5 domestic goat was identified in 2 samples.

Across all scat samples, the domestic bovids was the most prevalent, being identified in 42% of the scat samples of which prey species could be identified. Bovids were followed in frequency by domestic goat in 24% of the samples. The most abundant wild prey was pika

which was identified in 18% of the scat samples. Overall domestic prey was identified in 94% of the scats that which prey species were identified in. Wild prey was only identified in 37% of the scat samples which prey species were identified. This is an increase over the baseline study, Anwar et al. 2011, which identified domestic prey in 36.5% of their scat samples. It is important to note that these two studies do use a different methodology. Additionally, where wild prey was identified it was generally small bodied, either the pika or civet, large bodied prey were only identified in 6 scat samples.

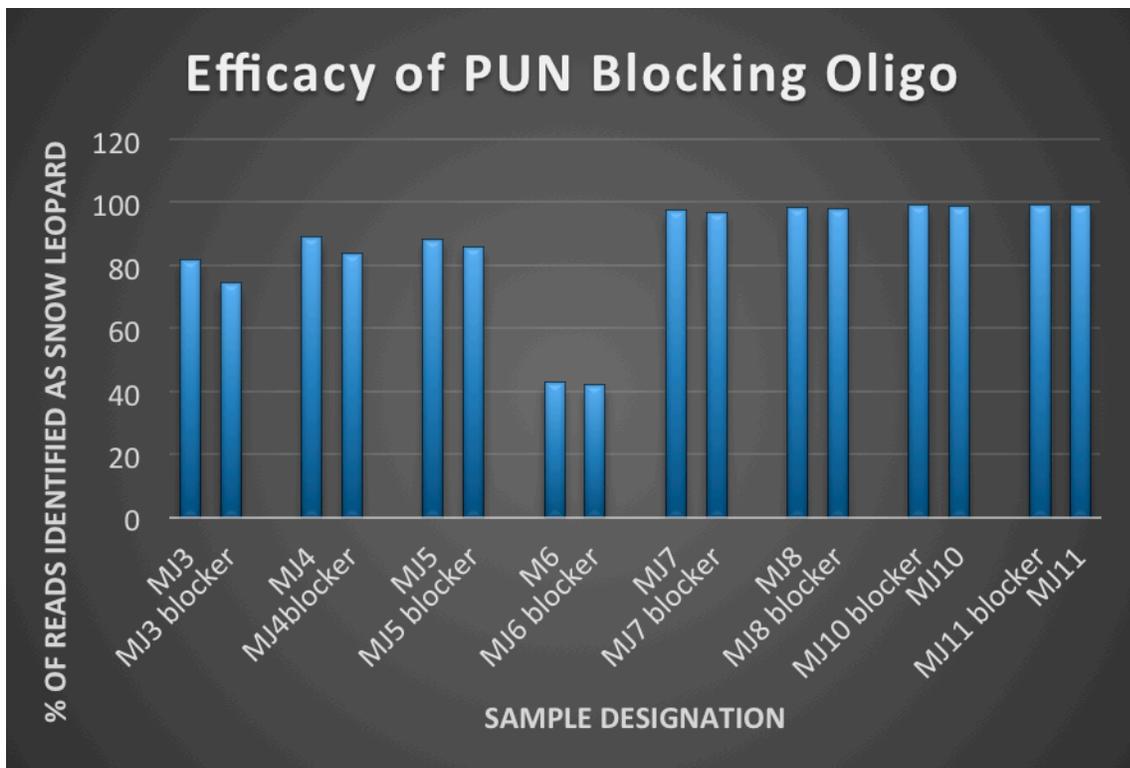


Figure 1; shows the relative change in replicate samples that either included a blocking oligo in the protocol, denoted by the sample name followed by blocker, or did not utilize a blocking oligo. There was not functional difference.

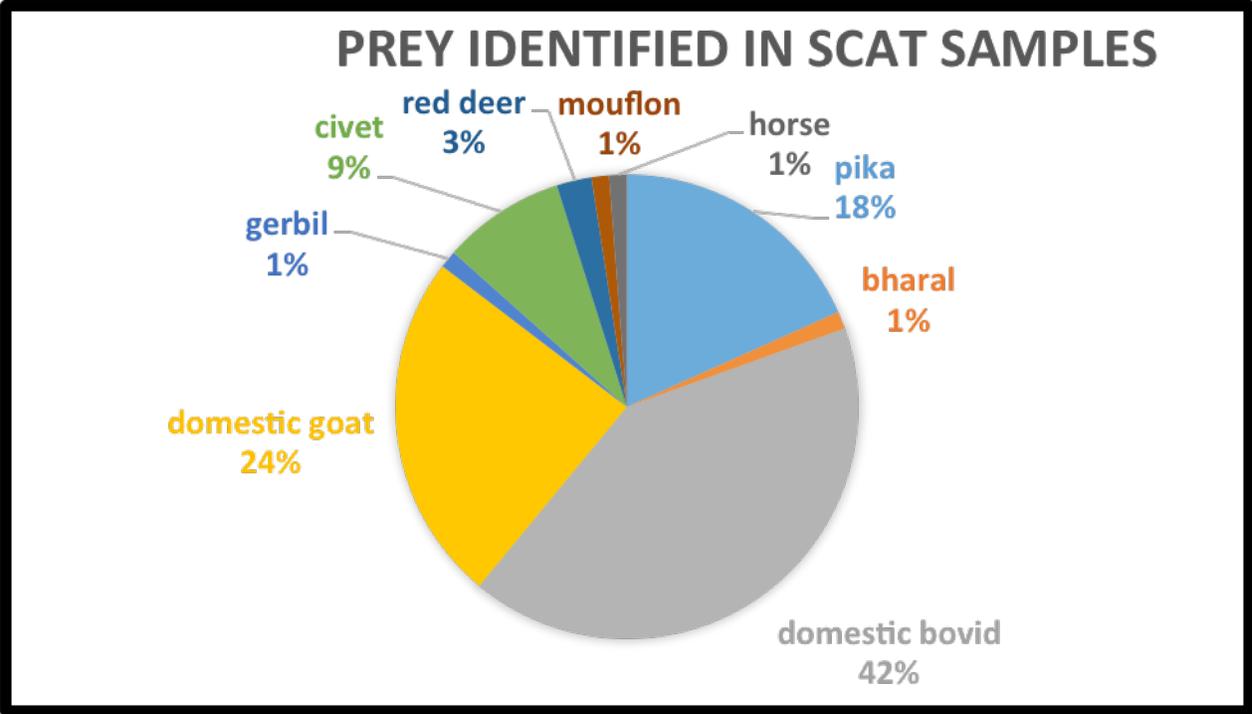


Figure 2; shows identified prey species and which percentage of scat samples that each had occurred in. Prey was identified in 51 scat samples, which is approximately 61% of the scat samples that were processed as part of this study.

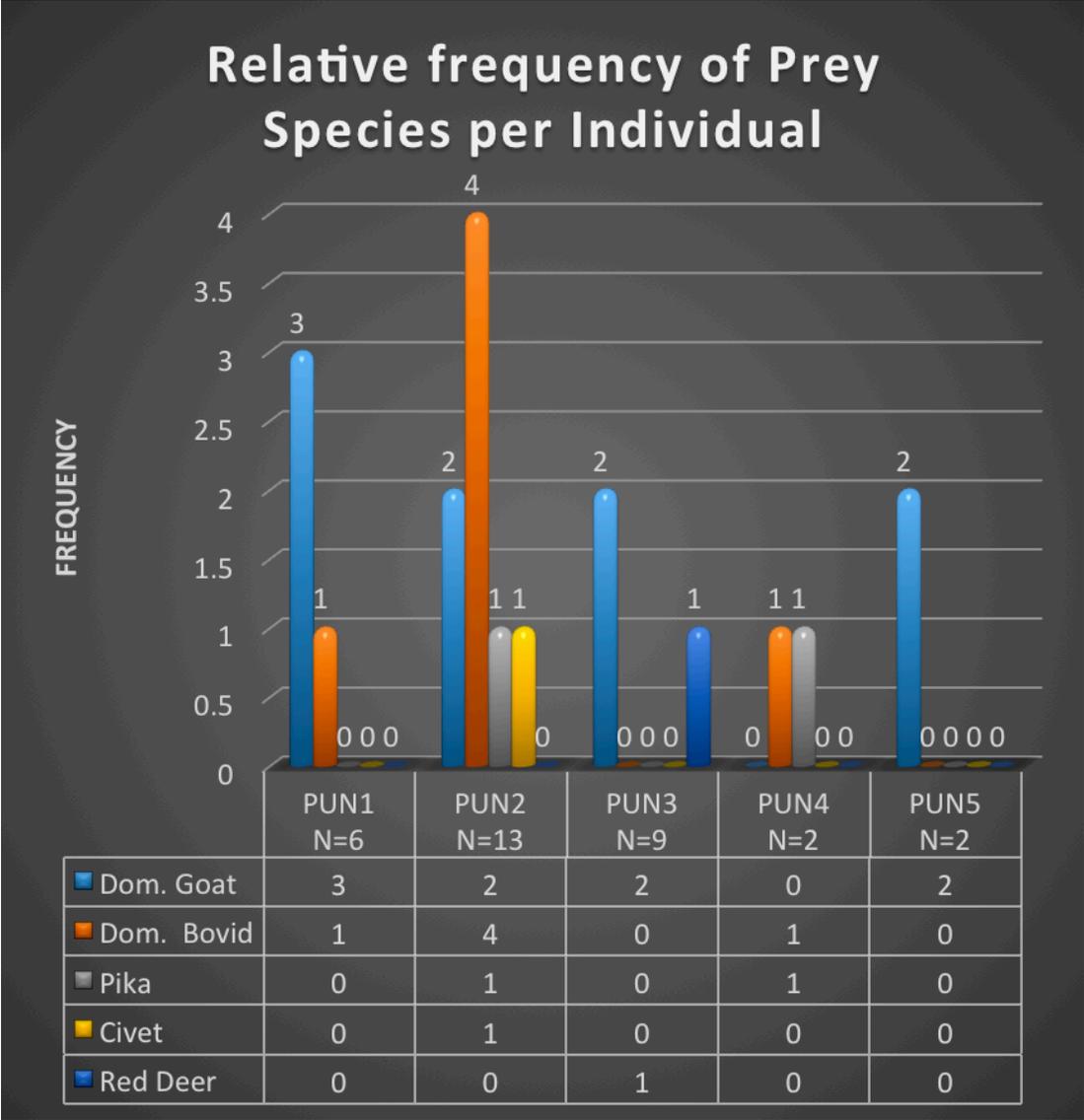


Figure 3; shows the prey species identified per individual. It includes the amount of scats that were identified to have come from a specific individual. The prey species that had been identified from scats of each individual are included and the number of scats that each species was I identified in are also denoted in this figure.

## Discussion

The blocking oligos have not had any effect on the study. The greatest difference in the abundance of snow leopard reads was identified in PUN1 with a 7% difference between the two replicates. Furthermore it appears as if the total reads per sample were lower in some replicates that had used the blocking oligos but higher in others. The data presented in this study suggests that the presence of the blocking oligos can either disrupt both the amplification of snow leopards as well as the amplification of prey DNA. It was also observed to have increased the number of reads per sample. Though these fluctuations may be caused by variations in the equipment or the reagents that we used, the blocking oligos did not change the relative abundance of snow leopard reads per sample and may be correlated with an increase in the variability of the data obtained by our protocol. Due to this it was determined that it would be more beneficial to obtain as many reads per sample without effecting the consistency of our protocol rather than attempt to use the oligos to filter out snow leopard reads. The blocking oligos were not used on the remainder of the samples present in this study.

For all of the prey species identified in the 61 scat samples that were able to have prey identified in the around 66% of the prey identified belonged to domestic prey. These included domestic bovids, which include both yak (*Bos grunniens*) and cattle (*Bos taurus*). Of the remaining 34% the most identified species were pika (*Ochonta ssp.*) and civet (*Vivericula ssp.*). The only large bodied wild prey that were identified were red deer (*Cervus elaphus*), mouflon (*Ovis aries orientalis*), and bharal (*Pseudois nayaur*) which were collectively account for only 5% of the prey species identified.

The outputs from our entire sample is supported by the data received from analyzing individual snow leopards. Once again the two most commonly identified prey species were

domestic goat and domestic bovid, which were identified in 15 times and were present in all animals. The next most commonly identified prey species was the pika which was identified a total of two times in two different animals. The civet was identified only once in one animal. Finally, the red deer was identified once in one animal.

Overall, 94% of the scats that prey was identified in contained domestic prey, this includes both domestic bovids and caprids. Wild prey was identified in 37% of the scats which prey could be identified.

In conclusion, this particular population of snow leopards are highly reliant on domestic prey sources. 94% of the scat samples which prey was identified in, contained domestic prey. This is a relative increase from Anwar et al. 2011, where wild prey was identified in 36.5% of the scat samples. It is important to note that this may be due to the difference in methodology. This study uses a genetic approach, whereas Anwar et al. 2011 utilized a morphological approach. In Anwar et al. 2011, 20 hairs were randomly selected and identified based off of their physical characteristics. It is possible that due to phenotypic variation among animal population that some of the domestic goats and sheep that were present in reality may have been misidentified as wild species, decreasing the actual amount of scats that contained wild prey. Additionally it is possible that when selecting 20 random hairs from each sample, the hairs were not representative of the sample due to random chance. Both of which could have decreased the observed domestic prey matter in the previous study.

One of the most important discoveries of this study is that even when wild prey is consumed, leopards appear to be preferentially consuming small bodied animals such as civet and pika. Large bodied prey was only identified 5% of the time. This may suggest that large bodied prey population are decreasing to the point snow leopards cannot find enough food to

sustain themselves and must rely on small bodied or domestic prey. This has been a concern of the IUCN in the management of these animals. Further sampling on ungulate population will be needed to confirm this and identify a cause. Cause could include either a degradation of natural prey populations due to poaching by an increasing human population, or due to competition with domestic animal populations which may be increasing along with the human populations.

### Works Cited

- Anwar, M. B., Jackson, R., Nadeem, M. S., Janecka, J. E., Hussain, S., Beg, M. A., & Qayyum, M. (2011). Food habits of the snow leopard *Panthera uncia* (Schreber, 1775) in Baltistan, Northern Pakistan. *European Journal of Wildlife Research*, 57, 1-7.
- Beckmann, J. P., & Berger, J. (2003). Rapid ecological and behavioral changes in carnivores: the responses of black bears (*Ursus americanus*) to altered food. *Journal of Zoology*, 261, 207-212.
- Begg, C. M., Begg, K. S., Du Toit, J. T., & Mills, M. G. L. (2003). Sexual and seasonal variation in the diet and foraging behavior of a sexually dimorphic carnivore, the honey badger (*Mellivora capensis*). *Journal of Zoology*, 260, 301-316.
- Jackson, R. M., Roe, J. D., Wangchuk, R., & Hunter, D. O. (2006) Estimating snow leopard population abundance using photography and capture-recapture techniques. *Wildlife Society Bulletin* 34, 772 – 781.
- Janecka, J.E., Jackson, R., Munkhtsog, B., & Murphy, W.J. (2014). Characterization of 9 microsatellites and primers in snow leopards and a species-specific PCR assay for identifying noninvasive samples. *Conservation Genetic Resources* 6, 369–373.
- Janecka, J.E., Jackson, R., Zhang, Y., Li, D., Munkhtsog, B., Buckley-Beason, V., & Murphy, W.J. (2008). Population monitoring of snow leopards using noninvasive collection of scat samples: A pilot study. *Animal Conservation* 11, 401–411
- Janecka, J.E., Munkhtsog, B., Jackson, R.M., Mallon, D.P., Naranbaatar, G., Gerelmaa, K., & Murphy, W.J. (2011). Comparison of noninvasive genetic and camera-trapping techniques for surveying snow leopards. *Journal of Mammalogy* 92, 771–783.
- Janecka, J.E., Tewes, M.E., Laack, L., Caso, A., Grassman, L.I., & Honeycutt, R.L. (2014). Loss of genetic diversity among ocelots in the United States during the 20th century linked to human induced population reductions. *Plos One* 9, e89384
- Ikeda, N. (2004). Economic impacts of livestock depredation by snow leopard *Uncia uncia* in the Kanchenjunga Conservation Area, Nepal Himalaya. *Environmental Conservation*, 31(4), 322-330.
- McCarthy, K. P., Fuller, T. K., Ming, M., McCarthy, T. M., Waits, L., Jumabaev, K. (2008) Assessing estimators of snow leopard abundance. *Journal of Wildlife Management* 72, 1826-1833.

- Mishra C. (1997). Livestock depredation by large carnivores in the Indian trans-Himalaya: conflict perceptions and conservation prospects. *Environmental Conservation* 24, 338-343.
- Parmesan, C. (2006). Ecological and evolutionary responses to recent climate change. *Annual Review of Ecology, Evolution, and Systematics*, 37, 637-669.
- Sharma, K., Bayrakkismith, R., Tumursukh, L, Johansson, O., Sevger, P., McCarthy, T., Mishra, C. (2014). Vigorous Dynamics Underlie a Stable Population of the Endangered Snow Leopard *Panthera uncia* in Tost Mountains, South Gobi, Mongolia. *Plos One* 9, e101319.
- Shehzad, W., McCarthy, T. M., Pompanon, F., Purevjav, L., Coissac, E., Riaz, T., & Tabarlet, P. (2012). Prey preference of snow leopard (*Panthera uncia*) in south Gobi, Mongolia. *PLoS One*, 7(2), e32104.
- Ruggeiro, L.F., K. B. Aubry, S. W. Buskirk, G. M. Koehler, C. J. Krebs, K. S. McKelvey, & J. R. Squires (1999) *Ecology and Conservation of Lynx in the United States*. United States Department of Agriculture Forest Service, Technical Report No. RMRS-GTR-30WWW.
- Wang, J., Laguardia, A., Damerell, P. J., Riordan, P., Shi, K. (2014) Dietary overlap of snow leopard and other carnivores in the Pamirs of Northwestern China. *Chinese Science Bulletin* DOI 10.1007/s11434-014-0370-y.