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RESEARCH ARTICLE

First Record on Body Morphometrics and Chemical Immobilization of Wolves from Pakistan

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A B S T R A C T

The gray wolf (*Canis lupus*) is a less studied wide-ranging endangered carnivore in Pakistan. The current investigation is the first to report their body morphometrics and chemical immobilization in Pakistan. Body morphometrics was examined for 12 wolves by measuring 15 variables. The majority of the 12 wolves had body weights that were more similar or slightly higher than the weights of Indian wolves from Central India. Principal Component Analysis (PCA) revealed that the wolves from the southern lowland region have differing morphology, independent of body size compared to the wolves from other regions of Pakistan. To record the body morphometrics, wolves were immobilized using Zoletil™-50 (Z) (n=6) and Xylazine-Ketamine hydrochloride (X-K) combination (n=3). The wolves were immobilized by using drug doses 5-6 mg/kg for Z, and 1.25 mg/kg for X and 2-3 mg/kg for K. The first sign (minutes) of anesthesia was noted after 3.15±1.9 for Z and 4.97±2.3 for X-K combination. The recumbency time was 7.7±2.5 for Z and 11.7±3 for X-K combination. The sign of recovery was recorded at 40.4±13.5 for Z and 34.1±2.4 for X-K combination, while the sedation duration was recorded at 45.3±12.5 for Z and 39.6±3.5 for X-K combination. These results suggest that Z induced quicker induction, more profound recumbency and swifter recovery than X-K combination. Additionally, physiological parameters including rectal temperature, respiration, heart rate and palpebral and capillary reflexes with both combinations remained within the safe ranges.

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INTRODUCTION

Gray wolves (*Canis lupus*) were once widely distributed throughout the Holarctic biome, however, its current range has been restricted to about two-thirds, globally (Boitani *et al.,* 2018). In Pakistan, gray wolves are considered endangered and are among the last few large carnivores left in the country. Recently, there have been a few studies conducted in Pakistan on wolves, which discuss the distribution, population estimates, and human-wildlife conflict (Hamid *et al.,* 2019). The recorded gray wolf distribution in Pakistan ranges from northern mountain ranges to the lowland deserts – thus inhabiting almost all terrestrial ecological zones. However, significant gaps about the country-wide population size, distribution, and types of these endangered wolves are to be addressed. Additionally, and more strikingly, there is no scientific study on the husbandry and management of this endangered species in Pakistan.

A recent estimate concluded that ~400 individuals are present in Gilgit Baltistan, with around 70 individuals killed during a single year. Numerous factors are thought to be responsible for their decline which includes persecution, poaching and the expansion of agricultural practices resulting in habitat loss (Abbas *et al.,* 2013; Hinton *et al.,* 2016). Furthermore, the decline in natural prey and increased livestock rearing in its ranges has increased gray wolf reliance on domestic prey and reduced available habitat, thereby enhancing wolf-human conflict (Khan *et al.,* 2019).

Historically, two wolf subspecies have been described in Pakistan: The Tibetan wolf (C*anis lupus chanco*) found in Himalayan, Hindukush and Karakoram regions, and the Indian or peninsular wolf (*Canis lupus pallipes*) occupying southern arid regions from the Sulieman mountain region, including Kashmir valley. However, recent mitochondrial DNA work revealed South and Central Asian wolves to have a complicated phylogeographic history and, consequently, taxonomic uncertainty regarding their status (Shrotriya *et al.,* 2012). Based on morphological and molecular data, it has been clarified that wolves show considerable variations across South and Central Asia. The Indian peninsular gray wolf is estimated to have diverged from the wolf-dog maternal clade approximately 200,000-400,000 years ago (Aggarwal *et al.*, 2007). The Indian wolf is one of the smallest wolf subspecies with adult male and female wolves weighing between 19-25kg and 17-22kg, respectively. There is enough genomic evidence supporting that the high-altitude Tibetan gray wolf (*C. l chanco*) is an evolutionarily distinct lineage, which maternally diverged 800,000 years ago (Wang *et al.*, 2020). Tibetan wolves are larger, weighing around 35kg, and are adapted to high-altitudes (Shrotriya *et al.,* 2012; Werhahn *et al.*, 2018).

Understanding the morphological variation across wolf populations in Pakistan may provide complementary insight into the ecological and evolutionarily distinctiveness of different types of wolves in the country. Generally, gray wolf body size and skull size increases with latitude according to Bergmann's rule (O'Keefe *et al.*, 2013). Morphological differences have also been documented for ecologically different wolves (Munoz-Fuentes *et al.,* 2009). Among the statistical tools applied in morphometrics and craniometrical analyses, multivariate analysis is an efficient approach used to interpret the complex data for a large number of variables (Khosravi *et al.,* 2012). PCA has been extensively employed to help resolve the complete relationships of a large set of variables through extracting linearly uncorrelated variables from a suite of potentially correlated variables.

There are only a few studies in Central Asia involving husbandry management interventions on wolves, such as chemical immobilization. Keeping into consideration the welfare of the animal and personnel involved, chemical immobilization can be employed to restrain and capture many species, without capture myopathies and the risk of injuries associated with other restraint methods (Muliya *et al.*, 2016). In Pakistan, unpublished data and media reports that witnessed wolf deaths in captivity were primarily due to a lack of skilled managers and veterinarians, as well as complications for husbandry by the non-availability of potent and costly sedatives. Hence, there is an urgent need to document the safe immobilization protocols for wolves using drug combination techniques and standard operating procedures.

Ketamine–xylazine (KX) combinations have been widely used to chemically restraint wild canids (Muliya *et al.,* 2016). Ketamine is a dissociative anesthetic agent that is used either alone or in combination with α-2 adrenergic agonists. Xylazine is a potent α-2 central nervous system depressant with anxiolytic, muscle relaxant and analgesic properties that help counteract the undesirable side-effects of ketamine such as convulsions and catalepsy. The combination of tiletamine and zolazepam anesthesia is characterized by retention of cranial, spinal, laryngeal, and pharyngeal reflexes. Zoletil has been used successfully to immobilize a wide variety of wild and captive animals.

For this study, we assessed two chemical immobilization treatments, specifically Zoletil™-50 and X-K for 9 wolves housed in different zoos in Pakistan. In addition, we investigated the morphological variations for 12 wolves from Pakistan.

MATERIALS AND METHODS

Out of thirteen (13) wolves, nine (09; 03 females and 06 males) were housed at different zoos while four were collected as road kills (free-ranging) during the study period 2016-19 (Table 1 and Fig. 1). Data on wolves' origin, approximate age and status as wild-caught or captive-born was collected from Zoo authorities.

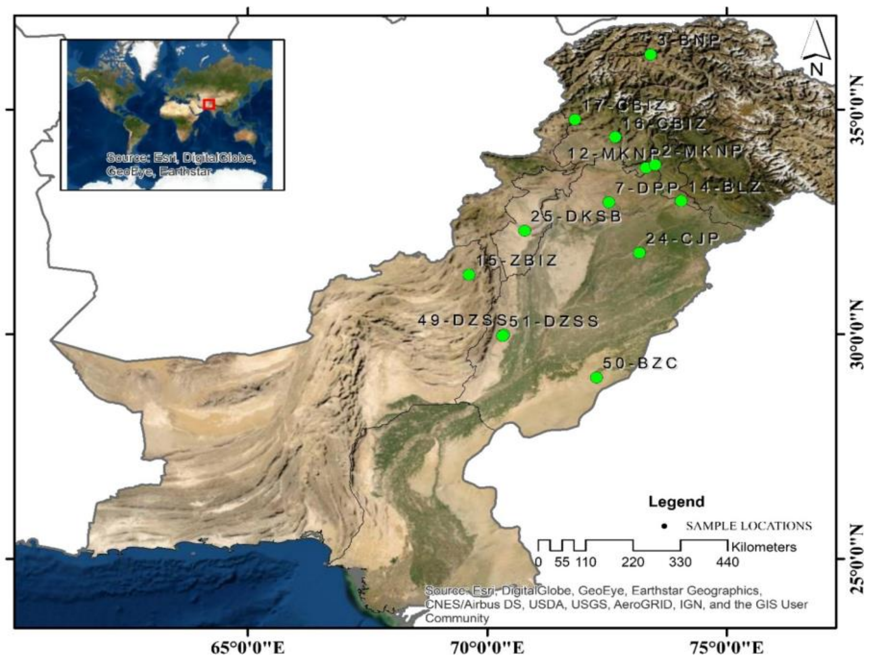

Fig. 1: Map of the study area. Green dots represent the geographical locations of wolf sample sites across Pakistan.

Table 1: Locality of origin, captive location, status and sex of 13 gray wolves

Locality of Origin and Coordinates	Status	Sex	Code#
1. Chitral-Broghil (Buffer Zone), KPK* 36°13'25.90"N; 73°24'30.38"E	Wild-Caught	Male	3-BNP
2. Zhob, Baluchistan (1) 31°19'27.65"N; 69°36'57.92"E	Wild-Caught	Female	15-ZBIZ
3. Shair Garh-Buner, KPK (2) 34°23'20.23"N; 72°40'17.61"E	Wild-Caught	Female	16-CBIZ
4. Kamrani Game Reserve Lower Dir, KPK (3) 34°46'42.48"N; 71°49'46.03"E	Wild-Caught	Male	17-CBIZ
5. Bimbhar, AJK (4) 32°57'58.16"N; 74° 2'53.54"E	Wild-Caught	Male	14-BLZ
6. Muree-Kahuta Kotli Satian (MKNP), Punjab (5) 33°42'9.94"N; 73°18'55.88"E	Road Kill	Male	12-MKNP
7. Dharabi-Talagang (Potohar), Punjab (6) 32°56'11.10"N; 72°32'21.23"E	Road Kill	Male	7-DPP
8. Lehtrar, MKNP, Punjab (7) 33°46'8.20"N; 73°30'18.98"E	Road Kill	Male	2-MKNP
9. Chiniot-Jhang Road, Punjab (8) 31°48'46.22"N; 73°10'33.71"E	Road Kill	Male	24-CJP
10. Dera Ismaeal Khan, KPK (9) 32°18'16.33"N; 70°46'37.35"E	Wild-Caught	Male	25-DKSB
11. Cholistan Desert, Punjab (10) 29° 2'12.33"N; 72°16'42.66"E	Wild-Caught	Male	50-BZC
12. Sakhi Sarwar (Koh e Suleman), Punjab (11) 29°59'34.01"N; 70°20'30.65"E	Wild-Caught	Female	49-DZSS
13. Sakhi Sarwar (Koh e Suleman), Punjab (12) 29°57'59.53"N; 70°19'13.09"E	Wild-Caught	Male	51-DZSS

*Sub adult was not included in the morphometric study.

Chemical immobilization: Before immobilization, the wolves were brought into night dens or closed alleyways to get closer access. Based on estimated body weight, each animal was injected using a pressurized plastic dart (3 cc or 5 cc dart syringe, TELINJECT U.S.A, Inc.), with an intended dose of (treatment 1: T1) 5-6 $mgkg^{-1}$ for Zoletil™-50 (Z) (tiletamine-zolazepam) (Zoletil®, Virbac U.S.A.,), and (treatment 2: T2) 1.25 $mgkg^{-1}$ for Xylazine hydrochloride (X) (Xylaz® 20mg/ml, Farvet Pvt. Ltd.) and 2-3 $mgkg^{-1}$ for Ketamine hydrochloride (K) (Ketanil, 100 mg/ml; Wildlife Pharmaceuticals, Inc. Fort Collins 80524, U.S.A), projected using a blowpipe (B31.C TELINECT). The needle used is K1138B (TELINJECT) for large dogs with collar of 1, 1 x 38 mm (Ø x Length).

Once the animal was approachable after lateral recumbency, it was blind folded and ears plugged with cotton balls. In order to moisten the eyelids, we applied eye-ointment and maintained the head in an upright position to ensure air way remains open. Animals were weighed by using a manual hand-held spring weighing scale. Body morphometric observations were made with a measurement tape and biological samples including blood, feces, and ectoparasites were taken for future genetic and medical studies.

During immobilization, physiological parameters including cardiac rate (beats/min.), respiratory rate (breaths/min.), and rectal temperature (°F) were recorded. Physiological parameters were observed and noted at the onset, 5, 15 and 25 minutes of the sedation. The effectiveness of both treatments was examined in terms of induction, recovery and physical reaction (i.e. excessive salivation, licking, vomiting, mucosal membrane color, muscle twitching and pedal withdrawal reflex) were also noted.

The first sign of each drug effect, to sternal and lateral recumbency, and induction (recumbency with eye closed) were recorded from time when wolf was administered with anesthetic treatment. Similarly, the first sign of recovery (return to normal motor function), time to head up, time to sternal posture and time to standing position and anesthetic duration for each anesthetic treatment was also recorded. Statistical analysis was performed for repeatedly collected data on different stages of anesthesia. Means were reported with standard deviation (SD). In addition, physiological measurements were tested with online post-hoc Tukey's HSD test at a significance level of $P<0.05$.

Morphometric Variables and Analysis: Morphometric measurements were taken from wolves (immobilized chemically or road kills with intact bodies) and were followed as closely to standard anatomical reference points as possible (Fig. 2 modified from Wiwchar and Mallory (2012)).

To evaluate the correlation between body size and elevation, we recorded the elevation (m) at each location of the road kill or site at which a captive wolf was caught from Google Earth. PCA was conducted on the morphological variables. To conduct the PCA, we first log-transformed all morphological variables to normalize the data. Subsequently, we regressed each of the 14 log-transformed variables against body size to calculate the residual, which allowed us to control for the effect of body size. To test for differences in shape that is independent of body size, we conducted a PCA using R (version 3.5.1) with the 14 morphological variables. We excluded the wolf (3-BNP) from morphometric analysis because it was recorded as sub-adult.

RESULTS

Chemical Immobilization: Twelve wolves were classified as adults, with mean body weight of 23.1±5.8 kg and one as sub-adult with body weight of 13.76 kg. The doses selected for the study induced the sedation/anesthesia without any uneventful medical situation. Only one wolf under T1 with Zoletil™-50 (Z) required supplemental Z dose of 25mg (0.5ml) and similarly one under T2 with X-K combination required 25mg of Ketamin (K). Onset of anesthesia was characterized by circular movements, slowing pace and unbalanced gait with mentation and enhanced ataxia followed by sternal to lateral recumbency. Table 2A present the time interval for different stages of sedation for both treatments T1 with Zoletil™-50 (Z) and T2 with Xylazine-Ketamine combination. Whereas, the time interval for different stages of recovery from anesthesia for both treatments Zoletil™-50 and X-K combination are given in Table 2B. A comparison of physiological parameters observed for both anesthetic treatments are given in Table 2C.

Morphometric Analysis: Descriptive statistics of different body morphometric variables are presented in Table 3B. The largest (BL=1276.8 and BW=37.0) and smallest (BL=950.7 and BW=18.0) individual belonged to a male from Murree-Kotli Satian National Park (MKNP) and a female from Sakhi Sarwar (Dera Ghazi Khan), respectively.

The Principal Component Analysis showed that the 12 individual wolves have body size-independent of morphological variations with clusters loosely associated with their respective elevation (Fig. 3). The first principal component (PC1) and second principal component (PC2) explained 60.3 and 16.2% of variations in our morphological traits (Fig. 3 and Table 3A). PC1 was dominated by chest girth and shoulder height, whereas PC2 was dominated by other variables, such as ulna and femur length (Fig. 3 and Table 3B). Generally, three wolves from lower elevations (51-DZSS, 49-DZSS, 50-BZC) were clustered together and showed larger PC1 and PC2 values (Fig. 3).

Table 2 A: Mean ± SD and range (minutes) for different stages of sedation for Treatment 1 with Zoletil™-50 (T1) and Treatment 2 with Xylazine-Ketamine Combination (T2)

	T 1 (n=6)		T 2 (n=3)	
Parameters	Mean ± SD	Range	Mean ± SD	Range
First sign (FS)	3.15±1.9	1.3-7	4.97±2.3	2.3-6.3
Sternal recumbency (SR)	4.82±1.9	3.3-8	6.87±2.38	4.3-9
Lateral recumbency (LR)	5.72±1.8	5-8	9.3±2.9	6-11
First approach (FA)	7.27±2.3	5-11	10.8±3.1	7.3-13.3
Induction complete (IC)	7.7±2.5	5-11.3	11.7±3	8.3-14

Table 2B: Mean ± SD and range (minutes) for different stages of recovery for Treatment 1 with Zoletil™-50 (T1) and Treatment 2 with Xylazine-Ketamine Combination (T2).

	T1 (n=6)		T2 (n=3)	
Parameters	Mean ± SD	Range	Mean ± SD	Range
First sign of recovery (Re)	40.4±13.5	26.3-61.3	34.1±2.4	31.3-36
Head up (HU)	45.3±12.5	32-64.3	39.6±3.5	36.3-43.3
Sternal posture (St)	51.2±10.7	41-66.3	43.3±4.1	40-48
Standing position (S)	59.03±10.7	48-74.3	56.9±0.6	56.3-57.3
Full recovery (FR)	57.47±12.3	53.3-86.3	72.7±2.25	71-75.3

Table 2C: A comparison of physiological parameters observed for Treatment 1 with Zoletil™-50 (T1) and Treatment 2 with Xylazine-Ketamine Combination (T2). No significant differences found amongst the physiological parameters *viz* rectal temperature (P=0.67), heart rate (P=0.13), and respiratory rate (P=0.73) amongst both treatments.

Parameters	Rectal Temp. (°F)		Cardiac Rate		Respiration Rate	
Intervals	T1 (n=6)	T2 (n=3)	T1 (n=6)	T2 (n=3)	T1 (n=6)	T2 (n=3)
At approach	102.8±1.7	103±3.1	88±4.2	82.7±7.0	23.6±2.5	23.6±1.5
After 5 min.	101.1±1.2	101.5±1.8	84±4.2	82.7±4.2	20.8±2.9	21±1
After 15 min.	100.3±1.5	101±2	82.7±5.3	80±3.5	19.1±2.5	20.3±0.58
After 25 min.	99±1.1	99.6±1.5	81.7±3.7	75.3±3.1	18.6±0.8	19.3±1.5

Table 3A: Percentage of explained variance for the first nine principal components of the PCA using 14 size-standardized morphological variables.

Percent of explained	PC1	PC2	PC3	PC4	PC5	PC6	PC7	PC8	PC9
variance	0.603	0.162	0.070	0.065	0.04	0.028	0.016	0.010	0.005

Table 3B: Descriptive Statistics and PCA loadings of the first four principal components (PCs) of the PCA using 14 size-standardized morphological variables across 12 Pakistani Gray Wolves (*C. lupus*)

	Descriptive Statistics			PCA loadings of the first four PCs			
Morphometric Variables	Max.	Min.	Mean ± SD	PC1	PC2	PC3	PC4
1. Head Contour length (HL)	260.3	365.9	313.7±32.1	-0.14	0.07	-0.63	0.64
2. Body Contour Length (BL)	950.7	1276.8	1046.8±92.0	-0.25	-0.26	0.41	0.20
3. Neck girth (NG)	348.9	495.6	404.7±47.8	-0.23	0.23	0.20	0.50
4. Chest girth (CG)	526.8	666.1	581.0±37.6	-0.31	-0.20	0.13	0.07
5. Humerus length (HumL)	218.9	267.9	238.3±17.2	-0.29	0.16	-0.16	-0.35
6. Ulna length (UL)	239.9	318.4	274.9±24.6	-0.21	0.48	-0.15	-0.23
7. Femur length (FL)	219.6	299.2	258.4±23.4	-0.26	0.38	-0.06	-0.09
8. Tibia length (TiL)	218.4	273.7	246.4±14.5	-0.32	0.07	-0.03	0.00
9. Tarsal length (TaL)	158.9	228.9	204.1±17.4	-0.33	-0.14	-0.05	-0.01
10. Tail length (TL)	365.3	446.6	401.9±26.4	-0.21	0.33	0.40	-0.01
11. Front paw length (FpL)	72.7	108.7	91.4±8.7	-0.30	-0.27	0.03	0.02
12. Hind paw length (HpL)	67.8	92.7	78.4±7.4	-0.31	-0.04	-0.21	-0.21
13. Shoulder height (SH)	496.6	637.6	564.5±34.7	-0.31	-0.19	0.15	0.03
14. Ear Length (EL)	96.2	122.6	109.8±7.7	-0.18	-0.43	-0.32	-0.24
15. Weight (BW) kg	18.0	37.0	23.9±5.5	-	-	-	-

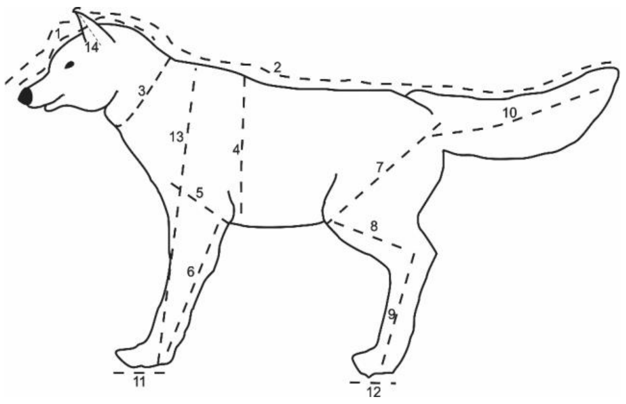

Fig. 2: Morphological variables (1-14) of gray wolf corresponding to table 3B.

Furthermore, with the exception of the large wolf from MKNP, the rest of the 11 gray wolves have body weights more similar to lowland Indian peninsular wolves (*C. l. pallipes*), rather than highland Tibetan wolves (*C. l. chanco*) with some wolves a little heavier than the heaviest Indian wolves (Indian wolf males reach only around 25kg). By including the largest wolf from MKNP, we found a positive and not significant relationship (p = 0.18, adjusted R^2 = 0.087) between body weight and elevation (Fig. 4A). While, excluding the largest gray wolf (MKNP-2), we find a positive, yet less significant, relationship (p=0.5, adjusted R^2 = -0.05) between body weight and elevation across 11 gray wolves in Pakistan (Fig. 4B).

The Linear regression between morphological variables and body weight showed that all 14 morphological variables are influenced by body weight (Fig. 5). Hence, the residual of each morphometric variable was taken, where the residual represents each morphological variable as relative in relation to body size and used in PCA as relative (e.g. relative neck girth).

The morphometric analysis of 15 measured variables through PCA and in the dendrogram (Fig. 6) showed the differences among the wolves collected from different regions.

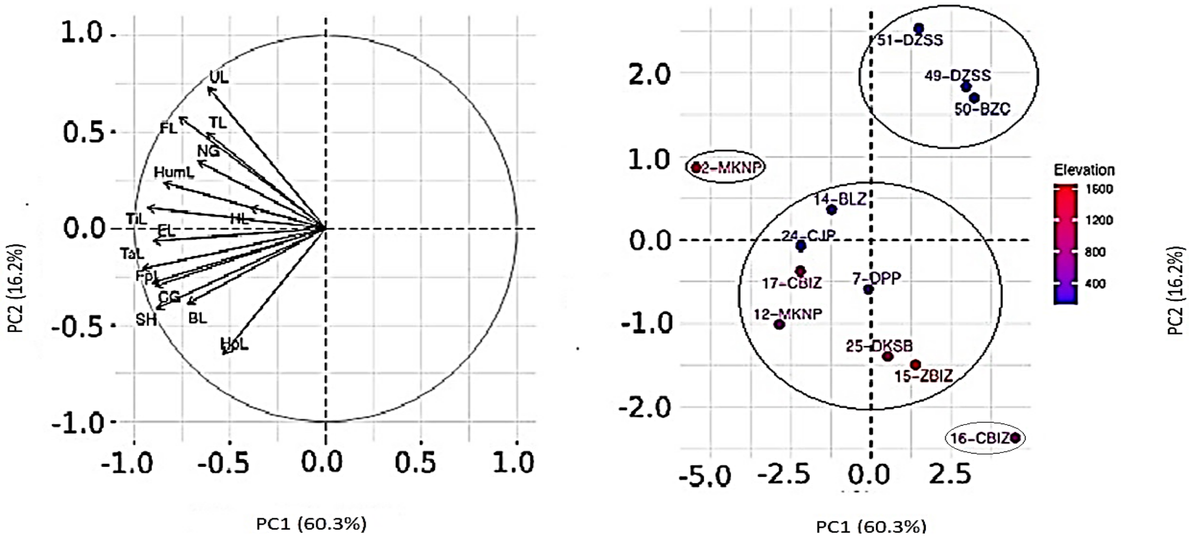

Fig. 3: PC1–PC2 plane of the PCA performed on 14 morphological characteristics of the 12 Pakistani Gray Wolves. The PCA showed the 12 individual wolves with colors of the dots corresponding to respective elevation and information on each gray wolf. See Table 1 and 3B for sample abbreviation and variable abbreviation, respectively.

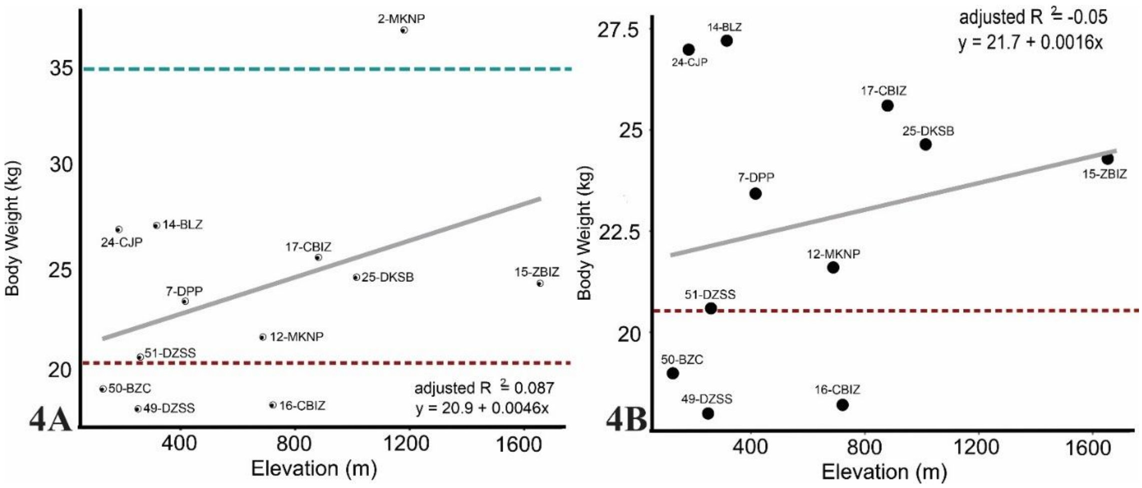

Fig. 4: Linear regression relationship between body weight (kg) and elevation (m) of the 12 (with heaviest 2MKNP) (5A) and 11 (without 2MKNP) (5B) Pakistani gray wolves. The blue dotted line at 35kg shows the average weight of Tibetan wolves, and the red dotted line at around 20kg shows the average weight of an Indian wolf (Shrotriya *et al.,* 2012).

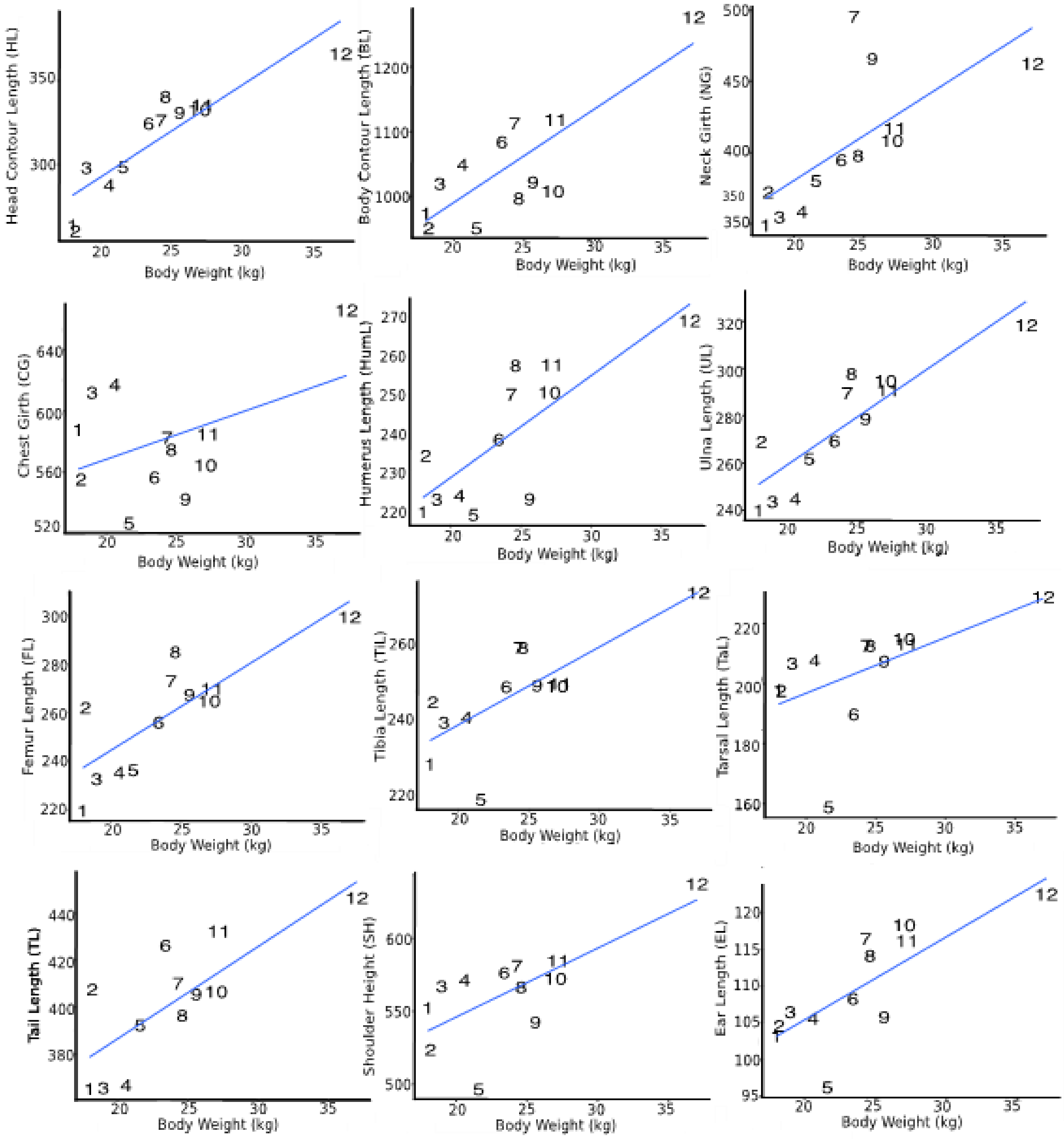

Fig. 5: Linear regression relationship between each body morphometric variable (mm) and body weight (kg) of the 12 Pakistani gray wolves indicated on regression line corresponds to numbers in parenthesis after locality name in Table 1.

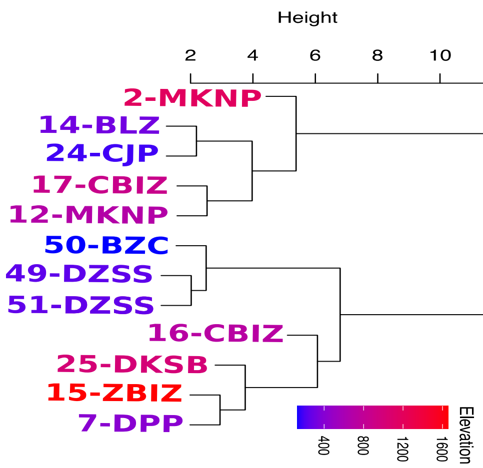

Fig. 6: Dendrogram based on morphometrical distances.

DISCUSSION

In Pakistan, wolves are endangered and persecution remains one of the biggest hurdles to recovery because of livestock depredation and its associated impact on livelihoods (Khan *et al.*, 2019). Hence, public awareness, strong legal protection, supportive media coverage and furthered ecological research in Pakistan could aid in supporting the conservation of this keystone species (Imbert *et al.*, 2016). In consideration of the lack of knowledge on wolves in Pakistan, we undertook this project to further research on these unique wolves' biology and ecology across Pakistan.

Our results based on multivariate analyses suggest considerable morphological variation across the gray wolves in our study. Among the 12 wolves in our study, we found that individuals inhabiting higher elevations were generally heavier than individuals inhabiting the plains. However, the main driver of this relationship was an individual from Muree Kolti Sattain Kahuta National Park, (2-MKNP), which is the only wolf that fell within the body size range of the larger Tibetan wolf (~35kg). This region has a contrasting ecosystem compared to the plains with subtropical broad-leaf forests, steep terrain, and is connected to the Himalayan mountain range (Khatoon *et al.,* 2019). It is possible that this individual may have originated and dispersed from higher elevations regions of Azad Jammu and Kashmir or Gilgit Baltistan. Additionally, the lowest weighted wolves were from the most Southern and arid regions, including one of the wolves inhabiting the outskirts of the Cholistan desert. In the neighboring counterpart populations in India, Shrotriya *et al.* (2012) noted that heavier wolves reside at higher elevations (i.e. in mountainous Himalaya) while wolves in plains are smaller in size. There are no published records for the body weights of gray wolves from Gilgit Baltistan in Pakistan, where the elevation is much higher and the climate colder than the regions that were included in our study. If the wolves are similar to Ladakh, where the landscape is continuous with Gilgit Baltistan, then we would expect the wolves inhabiting the highest elevations in Pakistan to be ~35kg, similar to the individual in our study in MKNP. Our study's elevation range (129-1655m) was much less than the higher elevations across Gilgit Baltistan and further sampling within Giglit Baltistan would provide stronger insight into the relationship between wolf body size and elevation.

Additionally, the smallest head contour length of wolves in our study was recorded from peninsular parts of the Pakistan while the largest belongs to the wolves from mountainous regions i.e. Himalayan foothills around Margallah hills of Muree Kotli Satian National Park bordering with Azad Jammu and Kashmir. Similar results have been reported by Shrotriya *et al.* (2012) that the skulls of wolves from Himalayan Ladakh (Chumar) were considerably larger (234 and 236 mm) than peninsular wolves (220mm). Previous work from Europe and Southwest Asia have also shown wolves inhabiting mountainous regions had larger skulls than wolves living in the lowland areas (Khosravi *et al.,* 2012). More broadly, differences in body size and shape of gray wolves have been shown to be associated with differences in ecology (O'Keefe *et al.*, 2013). It is then plausible that the differences in body size and morphological variation of the gray wolves in Pakistan may be influenced by ecological and environmental differences associated with different prey types, climate, and evolutionary history. Findings of the current study regarding morphometric variation among the Pakistani wolf population advocate that further research with more geographic coverage and with the addition of some suitable genetic markers will aid in resolving long debated wolves' taxonomic anomaly not only in Pakistan but also contribute to the understanding of wolves in whole South Asia.

Another finding of the morphometric study was indicated body size and mass specific sexual dimorphism that males are with larger body morphometric and weigh more than female wolves. However, our sample size is very small and further sampling can provide more robust conclusions. It has been recorded that male wolves' larger body size is strongly supported by the natural selection processes in relation to specialize prey availability, gender and dimorphism (Munoz-Fuentes *et al.,* 2009). This hypothesis that wolves prey selection though relates partially with abundance of prey species, but other factors also contribute such as its social behavior, adaptability to the habitat and body size (Newsome *et al.,* 2016).

For almost two centuries, wolf taxonomy in South and Central Asia has remained a challenge for wildlife biologists and ecologists until the application of recent molecular technologies. Initially, Sharma *et al.* (2004) described the two divergent maternal lineages of wolves from central India and Tibetan Plateau i.e. Indian peninsular wolf and Himalayan wolf. More recent studies further confirmed the genomic uniqueness of Himalayan wolves and its adaptation to hypoxic conditions of the high altitude Himalayan and Tibetan plateau landscapes (Werhahn *et al.,* 2020; Wang *et al.,* 2020). However, delineation of these lineages in Pakistan remains uncertain due to data deficiencies and the existence of gray wolves throughout the mountain ranges and lowlands directly adjunct to the Himalayas. Most of the gray wolves in our study have similar body weights to the lowland Central Indian wolf, rather than the Tibetan wolf. Correspondingly, previous research based on museum specimens has shown that gray wolves from southern Punjab and the Potohar plateau are part of the smaller-bodied and arid adapted Indian wolf maternal lineage. Therefore, our limited morphological data is consistent with gray wolves of southern Punjab and the Potohar plateau being of a similar type to the Indian wolf, rather than the Tibetan population. Overall, results of the current study based on morphometric analysis identified differences among lowland and high altitude wolves, thus a broader study involving genetic analysis along with ecological and behavioral insights is highly recommended with a larger sample size to illuminate the taxonomic status and evolutionary history of wolves from Pakistan.

Both anesthetic combinations Xylazine-Ketamine and Zoletil™-50 (tiletamine-zolazepam) (X-K and Z) appear to be safe and effective for immobilizing Pakistani gray wolves and has been used for others canids (Furtado *et al.,* 2006; Heerden *et al.,* 1991; Travaini and Delibes, 1994). The effectiveness of lesser doses (5-6 mg/kg) of the tiletamine-zolazepam combination used in this study than those recommended in the literature (7.0 mg/kg and 10 mg/kg) was in accordance with the previous studies of (Furtado *et al.,* 2006). Whereas, with the Xylazine-Ketamine (X-K) combination, the doses in our study for X was slightly more (1.25mg/kg versus 1mg/kg) and for K was lesser (2-3 mg/kg versus 8mg/kg) than those recommended for wolves in captivity. Meanwhile, Larsen and Kreeger (2007) used 2.2 mg/kg xylazine and 6.6 mg/kg ketamine dose to chemically restraint the wolves, as well as used yohimbine hydrochloride (0.15 mg/kg) as reversal agent. The doses selected for both anesthetics in this study supported the strategy to minimize the odds of rigidity, excitement, poor thermoregulatory ability and other adverse residual ketamine related effects during recovery.

The time (minutes) to get recumbent was quicker with Zoletil™-50 than Xylazine-Ketamine combination while the time required for the appearance of the first sign of recovery is more with Zoletil™-50 than Xylazine-Ketamine combination. The prolonged duration to get the wolves fully recovered from anesthesia under Xylazine-Ketamine combination than Zoletil™-50 indicates the residual ketamine related effects plus the bradycardic and hypotensive properties of xylazine are in agreement with the findings of (Muliya *et al.*, 2016).

The tiletamin-zolazepam combination induced an elevated, but stable cardiac rate than the Xylazine-Ketamine combination. In carnivores, the effects of tiletamine-zolazepam on heart rate have been inconclusive, apparently depending on a variety of variables, including the species involved (Selmi *et al.*, 2004). A gradual reduction in rectal temperature has been noticed over time in all immobilized wolves in both combinations of X-K and tiletamin-zolazepam (Sladky *et al.*, 2000). This gradual decrease in rectal temperature is due to the reduced muscular activity and depressive effect of the alfa$_2$-adrenoceptor agonist drugs on the central nervous system (Acosta-Jemmete *et al.,* 2010). The respiratory rate in our study does not vary significantly over time which is in accordance with previous studies of Larsen and Kreeger (2007).

The dose regimen used in this study for Zoletil™-50 and Xylazine-Ketamine combinations were adequate enough to immobilize the Pakistani wolves with sufficient analgesia and good muscular relaxation to carry out the routine management interventions. For the xylazine-ketamine combination, lower dose of ketamine than previously reported resulted in smooth recoveries without ataxia or disorientation. In addition, we recommend monitoring breathing, rectal temperature, cardiac rate along with a careful eye on secondary reflexes i.e. mucosal membrane color (pink to pale but not yellow or white), capillary refill time (CRT should be $\leq$ 3seconds) and keeping air passage open. Because large canids are more prone to hyperthermia and stress, we also recommend cooling pads, lactated ringer's solution with intravenous line maintained, ice packs, or ethanol be made available before chemical immobilization. An easily controlled sleeping den or availability of a night quarter with lesser disturbance (i.e. excessive light and noise) is ideal to allow the darted wolf to get in and sleep. This arrangement results in smooth and quick induction and is helpful for safe and uneventful recoveries. Overall, our study will facilitate wildlife managers and veterinarians in captive management of wolves and also serve as a baseline for research and medical interventions in other canid counterparts.

Authors contribution: FIA, AMK and GS contributed to the conception of the research. FIA, AMK and GS helped in study execution. GS, MTW and LMH contributed to data analysis and interpretation. GS, MTW and LMH prepared the manuscript. This manuscript is from the GS PhD thesis project.

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