SPECIES RICHNESS PATTERNS AND MORPHOLOGICAL VARIATIONS IN ANURANS ALONG AN ELEVATIONAL GRADIENT OF ANNAPURNA CONSERVATION AREA, NEPAL

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Submitted to

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Institute of Science and Technology

Tribhuvan University,

Kirtipur, Kathmandu,

Nepal

May 2023

DECLARATION

I hereby declare that the work presented in this thesis has been done by myself and has not been submitted elsewhere for the award of any degree. All sources of information have been specifically acknowledged by reference to the author(s) or institution(s).

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Date: 14th May 2023

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RECOMMENDATION

This is to recommend that the thesis entitled **"SPECIES RICHNESS PATTERNS AND MORPHOLOGICAL VARIATIONS IN ANURANS ALONG AN ELEVATIONAL GRADIENT OF ANNAPURNA CONSERVATION AREA, NEPAL"** has been carried out by Miss Mamata Thapa for the partial fulfillment of Master's Degree of Science in Zoology with special paper Ecology and Environment. This is her original work and has been carried out under my supervision. To the best of my knowledge, this thesis work has not been submitted for any other degree in any institution.

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LETTER OF APPROVAL

On the recommendation of supervisor **Assistant Professor** Dr. Bishnu Prasad Bhattarai, **Central Department of Zoology, Tribhuvan University,** this thesis submitted by Miss Mamata Thapa entitled **"SPECIES RICHNESS PATTERNS AND MORPHOLOGICAL VARIATIONS IN ANURANS ALONG AN ELEVATIONAL GRADIENT OF ANNAPURNA CONSERVATION AREA, NEPAL"** is approved for the examination and submitted to the Tribhuvan University in partial fulfillment of the requirements for Master's Degree of Science in Zoology with special paper Ecology and Environment.

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This thesis work submitted by Miss Mamata Thapa entitled **"SPECIES RICHNESS PATTERNS AND MORPHOLOGICAL VARIATIONS IN ANURANS ALONG AN ELEVATIONAL GRADIENT OF ANNAPURNA CONSERVATION AREA, NEPAL"** has been accepted as a partial fulfillment for the requirement of Master's Degree of Science in Zoology with special paper Ecology.

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LIST OF ABBREVIATIONS

Abbreviated form	Details of abbreviations
ACAP	Annapurna Conservation Area Project
Asl	Above sea level
DEM	Digital Elevational Model
DNPWC	Department of National Parks and Wildlife Conservation
GPS	Global Positioning System
GLM	Generalized Linear Model
NDVI	Normalized Difference Vegetation Index
TRI	Terrain Ruggedness Index

ABSTRACT

Anurans are a group of amphibians widely distributed worldwide. Body size and extremities of amphibians along an elevational gradient have always been a subject of curiosity. To study the species richness and morphological variation of anurans live samples were measured. An intensive field survey was conducted in August 2022, from Phedi (1150 m) to Mardi (4000 m) following the trekking trail. The amphibian survey was done by using the nocturnal time-constrained visual encounter method along the transects (100m × 4m). The survey was conducted between 7:00 p.m. and 9:00 p.m. Different morphological variables were measured by vernier caliper and environmental variables such as elevation were measured by using GPS. However, QGIS extracted slope, aspect, and TRI from DEM and NDVI from satellite images. Linear regression showed the decline of species richness as elevation increases. Similarly, linear regression showed that the body size of anurans increases as elevation increases i.e., it follows Bergmann's rule. However, multiple linear regression showed that the length of the metatarsus of the family Bufonidae decreases as elevation increases i.e., it follows Allen's rule but extremities of family Dicroglossidae does not follow. Hierarchical partitioning showed that temperature and NDVI were the predictors that influence species richness, body size and extremities of anurans.

1. INTRODUCTION

1.1 Background

Anurans are a group of tailless amphibians that belongs to the order Anura, which includes frogs and toads. There are more than 7,584 species worldwide (Frost 2023) and 55 species in Nepal (Rai et al. 2022). They mostly live in aquatic environments and show a wide range of habitats at different elevations (Khatiwada and Haugaasen 2015). They play a vital ecological role as both predators as well as prey.

Species richness patterns of any organism are determined by elevational gradients and environments (Körner 2000). Earlier, species richness was expected to decrease as elevation increases (Sanders and Rahbek 2012) but many studies have also declined this pattern (Naniwadekar and Vasudevan 2007, Khatiwada and Haugaasen 2015). Several studies have also demonstrated that the species richness pattern along an elevational gradient is influenced by biotic and abiotic factors including climate, topography, and human activities (Rahbek 1995, Khatiwada and Haugaasen 2015, Khatiwada et al. 2019). Factors such as temperature, rainfall (Pyron 2014), precipitation (Chettri and Acharya 2020), elevation, and other environmental elements like habitat type, humidity, and canopy cover may be considered as initial filters that influence the species richness of amphibians (Khatiwada et al. 2019). Rapid environmental changes can greatly affect anurans due to their permeable skin (Parmesan 2007, Blaustein et al. 2010). Hence, two well-known eco-geographical rules have linked environmental conditions with body size and extremities i.e. Bergmann's Rule and Allen's Rule (Mayr 1956, James 1970).

Bergmann’s rule states that endothermic organisms living in cold climates tend to have larger body sizes compared to those in warmer climates. Bergmann (1847) observed this pattern could be attributed to a mechanism of heat conservation since larger animals can tolerate cold temperatures as they need to produce less heat relative to their size to maintain their internal temperature above the surrounding environment. However, smaller animals are better adapted to warmer climates because their smaller size results in a higher surface area to volume ratio and hence more heat is removed from the body (Mayr 1956, Olalla-Tárraga and Rodríguez 2007). This rule has been formulated for endothermic animals and has been proven to be true for mammals (Meiri and Dayan 2003, Blackburn and Hawkins 2004) and birds (Ashton 2002, Blackburn et al. 2018, Romano et al. 2020). Empirical studies have demonstrated that this rule has predicted body size patterns in numerous amphibians (Lu et al. 2006, Liu et al. 2012, Baraquet et al. 2018, Rivas et al. 2018, Yu et al. 2019). However, it has also been reported that some species do not follow the trend or show the opposite pattern (Adams and Church 2008, Hsu et al. 2014, Liu et al. 2018). Among the amphibians studied, anurans such as *Rana temporaria* (Laugen et al. 2005), *Pelophylax pleuraden* (Lou et al. 2012) and spiny frogs of subfamily Painae (Hu et al. 2011) did not follow the trend. Similarly, anurans such as *Nanorana parkeri* (Ma et al. 2009), *Rana sauteri* (Hsu et al. 2014) and *Rana kukunoris* (Yu et al. 2022) converse the trend. Therefore, this rule has been controversial in the case of ectothermic animals such as amphibians.

Furthermore, Allen's rule states that endothermic organisms living in cold climates tend to have shorter appendages (e.g., limbs, ears, or tails) compared to those in warmer climates. (Allen 1877) observed that short extremities in cold climates minimize heat loss by decreasing the surface area-to-volume ratio of extremities (Ray 1960). This rule is also formulated for endothermic animals and has been proven true in mammals (Griffing and P. 1974, Lindsay 1987) and birds (Danner and Greenberg 2014). Since only a few studies have been done so far, it has been reported that this rule also applies to amphibians (Alho et al. 2011).

In ecogeographical studies of anurans, the body size is the most studied trait as compared to body extremities (Leung et al. 2021). It is not only influenced by climatic conditions (Reading 2007) such as temperature and precipitation (Martínez-Monzón et al. 2018) but also by other physiological and ecological restrictions (Tracy et al. 2010, Jeckel et al. 2015). However, studies of anurans extremities (e.g., forelimb and hind limb) have frequently been overshadowed by those of body length (Alho et al. 2011). Previous research has demonstrated that anurans morphology can differ significantly within and between species due to environmental effects like temperature and precipitation (Olalla-Tárraga and Rodríguez 2007, Olalla-Tárraga et al. 2009, Martínez Monzón et al. 2018).

The Annapurna Conservation Area (ACA) region offers a significant elevation gradient ranging from 1000 m to 8000 m. This presents a distinctive opportunity to study how ecological rules, such as Bergmann's rule and Allen's rule, impact anurans in this region, as this aspect has not been explored yet. Thus, the present study explores the species richness patterns and morphological variation along an elevational gradient and the factors that are affecting them.

1.2 Rationale of the study

Nepal possesses approximately 1% of the total species of amphibians found in the world but there has been a lack of studies on them. Understanding species richness patterns and morphological variations of anurans is important for understanding their relationship with the environment (Sanders and Rahbek 2012). However, the validity of Bergmann's rule has not been sufficiently tested in Nepal while Allen's rule has not been examined yet. Thus, this study explores the factors influencing species richness patterns and morphological variations and also provides the validity of Bergmann's and Allen's rule in Nepal.

1.3 Objectives

1.3.1 General objective

The general objective of this study was to explore the species richness patterns and morphological variations of anurans along an elevational gradient in Annapurna Conservation Area, Nepal.

1.3.2 Specific objectives

The specific objectives were to:-

1. To evaluate factors affecting species richness patterns and morphological variations among anurans in Annapurna Conservation Area, Nepal

2. To examine the validity of Bergmann's and Allen's rule for anurans in Nepal

1.4 Limitations

There are a few limitations during the study. They are:

i. High altitude survey requires an extensive survey period, due to the limited academic research period this study covers the range of the Mardi trekking route only.

ii. There was a possibility of natural hazards due to extreme rainfall in monsoon therefore data were collected during the late monsoon period.

2. LITERATURE REVIEW

2.1 Species richness pattern of anurans

According to Rahbek (1995), three patterns of species richness along elevational gradient were identified i.e., a monotonic decline in species richness as elevation increases, a hump-shaped pattern with the highest richness at mid-elevations, and a relatively stable richness from lowlands to mid-elevations followed by a sharp decline at higher elevations. Rahbek (2005) revealed that decreasing or a hump-shaped pattern along an altitudinal gradient are depending on the primary scale factors (i.e., the sample unit and the geographic area covered).

Naniwadekar and Vasudevan (2007) studied patterns of diversity in Western Ghats, India along an elevational gradient of 40- 1260 m asl. Findings reveal that there was a decrease in species richness as elevation increases and it can be attributed to factors such as decreasing temperature, habitat structure changes and limited resources available at higher elevations.

The study conducted on species richness patterns along elevation in spiny frogs which was conducted in China (Hu et al. 2011), found that the number of species decreased with increasing elevation and suggested that these patterns could be due to various factors such as changes in temperature, moisture as well as resource availability as elevation increases. Similarly, species richness declines as elevation increases in the study conducted at Gunung Raya in Malaysia (Corak et al. 2018) and suggested it can be due to a lack of water – availability at a higher elevation.

Nepal has a diverse group of anurans but most of the studies were conducted on distribution and diversity. However, there are not enough studies on the species richness patterns of anurans along elevational gradients. Khatiwada and Haugaasen (2015) studied species richness in Chitwan, Nepal along an elevational gradient of 200-1600 m asl, on both the southern and northern slopes of Siraichuli Hill. The result reveals that there was no significant difference in frog species richness between the southern and northern slopes. Similarly, when data from both slopes were combined, the result found a declining trend in species richness with increasing elevation. However, this relationship lost significance when the two slopes were analyzed separately.

To examine the effects of elevation and environmental factors on species richness, Khatiwada et al. (2019) used a combination of statistical methods, including polynomial regression, generalized linear models, hierarchical partitioning and canonical correspondence analysis. The findings indicate a consistent decrease in species richness with increasing elevation. A similar result was found in a study conducted in Ghandruk of Annapurna Conservation Area (Gautam et al. 2020). Elevation, surface area and humidity were found to be the environmental variables affecting species richness (Khatiwada et al. 2019).

2.2 Body size variation along elevational gradients of anurans

Bergmann's Rule is one of the most studied ecogeographical rules which is still controversial for anurans. However, some of the regional species of amphibians have followed Bergmann's rule (Ashton 2002, Olalla-Tárraga and Rodríguez 2007), whereas others did not (Laugen et al. 2005, Adams and Church 2008). *Rana swinhoana* (Lai et al. 2005), *Rana chensinensis* (Lu et al. 2006, Ma et al. 2009), *Pleurodema thaul* (Iturra-Cid et al. 2010), *Hyla annectans chuanxiensis* (Liao and Lu 2010), and *Rana limnocharis* (Liu et al. 2012) shows a positive correlation between body size and elevation whereas *Rana muscosa* (Matthews and Miaud 2007) did not. However, *Nanorana parkeri* (Ma et al. 2009), *Rana sauteri* (Hsu et al. 2014) and *Rana kukunoris* (Yu et al. 2022) reported a converse the Bergmann's rule. On the other hand, *Nanorana parkeri* showed a higher elevational decline in female size, although no clear elevational relationship was seen in males (Zhang et al. 2012). *Rana temporaria* (Laugen et al. 2005) and spiny frogs of subfamily Painae (Hu et al. 2011) *Rana sauteri* (Hsu et al. 2014), *Feirana taihangnica* (Fu et al. 2022) do not follow the Bergmann's rule. Cvetković et al. (2009) revealed that the temperature gradient was not only the factor affecting the body size variation in amphibians. The study of altitudinal variation in age and body size in Yunnan Pond frogs (*Pelophylax pleuraden*) by (Lou et al. 2012) did not follow Bergmann's rule and the SVL of males differed significantly but not in females. However, Chen and Lu (2011) study reveals that male and female *Rana amurensis* show different trends of body size about elevations, female *Rana amurensis* follows Bergmann's rule but male doesn't.

Ashton (2002) used meta-analytic techniques and find out that opposite patterns and the relationship between environmental temperature and body size changes were less clear. The availability of water (e.g., precipitation and humidity) is more strongly related to the pattern of body size variation in amphibians than other environmental factors (Ashton 2002) whereas according to Angilletta and Dunham (2003), body size is a complicated trait and its variation is influenced by several factors. Laugen et al. (2005) suggested that genetic factors may also play a role in determining body size behind environmental factors.

Delgado-Acevedo and Restrepo (2008) investigated in two *Eleutherodactylus* frogs from Puerto Rico how habitat loss affected changes in body size, allometry, and bilateral asymmetry. The findings showed that frog body size decreases as habitats are destroyed. The size of the body is reduced from a forest with low to high levels of disturbance.

Leung et al. (2021) discussed that *Rana kukunoris* does not follow Bergmann's rule and biological factors such as age and environmental factors such as NDVI, precipitation and temperature are responsible for it. Similarly, Fu et al. (2022) studied that morphological traits in *Feirana taihangnica* differed among ages and temperature seasonality is responsible for variation in body sizes but annual precipitation does not have any relation with body size.

In the context of Nepal, Khatiwada et al. (2019) studied in the eastern Himalayas and found that amphibian species show an increasing trend of body size with increasing elevation along an elevation gradient.

2.3 Body extremities variation along an elevational gradient of anurans

Alho et al. (2011) examined the leg length of common frogs (*Rana temporaria*) using both wild and common garden data. They discovered that in the wild, the relationship between femur and tibia length did not follow Allen's rule but instead peaked at mid-latitudes. However, the femur-to-tibia ratio increased towards the north, and the common garden data showed a genetic pattern consistent with Allen's rule for some trait and treatment combinations. The authors suggest that environmental effects may partially mask the genetic trend. Additionally, the researchers found that Allen's rule applies to turtles and amphibians among terrestrial ectothermic vertebrates. Rivas et al. (2018) studied the extremities of the *Pleurodema thaul* but found no variation, which means the result was still controversial.

Leung et al. (2021) studied LAHL (lower arm and hand length) and HLL (hindlimb length) of *Rana kukunoris* in Qinghai–Tibetan Plateau of China and found that they follow Allen's rule and environmental factors such as NDVI, precipitation and temperature are responsible for it. Allen's rule has gotten less attention in the literature than Bergmann's rule (Symonds and Tattersall 2010).

This literature review shows that still there is a lack of information and studies about the species richness pattern of anurans in Nepal. Since there is only one study so far on body size along an elevational gradient of anurans. However, on the body extremities of anurans, no study conducted so far in Nepal.

3. MATERIALS AND METHODS

3.1 Study area

Annapurna Conservation Area which extends over 7,629 km² in the Annapurna range, is the largest protected area of Nepal (DNPWC 2022). The area is recognized as a global biodiversity hotspot which represents mountain ecosystems and covers tropical, temperate, alpine, and nival climatic regions (Appel et al. 2013). It harbours 22 different forest types and 23 species of amphibians (NTNC-ACAP 2022). The conservation area span across the districts of Manang, Mustang, Kaski, Myagdi, and Lamjung.

This study was conducted from an elevational range of 1150 m (Phedi) to 4000 m (viewpoint of Mardi) following the trekking trail. These areas are distinguished by the presence of agricultural fields, forests, and marshy grassland (Pandey et al. 2020). The path passes through several popular tourist locations, including Dhampus, Pitam Deurali, Forest Camp, Rest Camp and Low Camp. An upper sub-tropical bioclimatic zone is found which is characterized by the presence of vegetation like *Schima wallichii*, *Castanopsis indica*, *Alnus nepalensis*, *Holarrhena antidysenterica*, *Quercus semecarpifolia*, *Rhododendron arboretum, Juniperus squamat* etc. (Pandey et al. 2020). Rainfall is heavier in the study area as it is located in the southern part of Annapurna Mountain (DNPWC 2022). Annual precipitation is the highest during monsoon between mid-June to mid-September, ranging from 5,032 mm at 2950m elevation (Putkonen 2004).

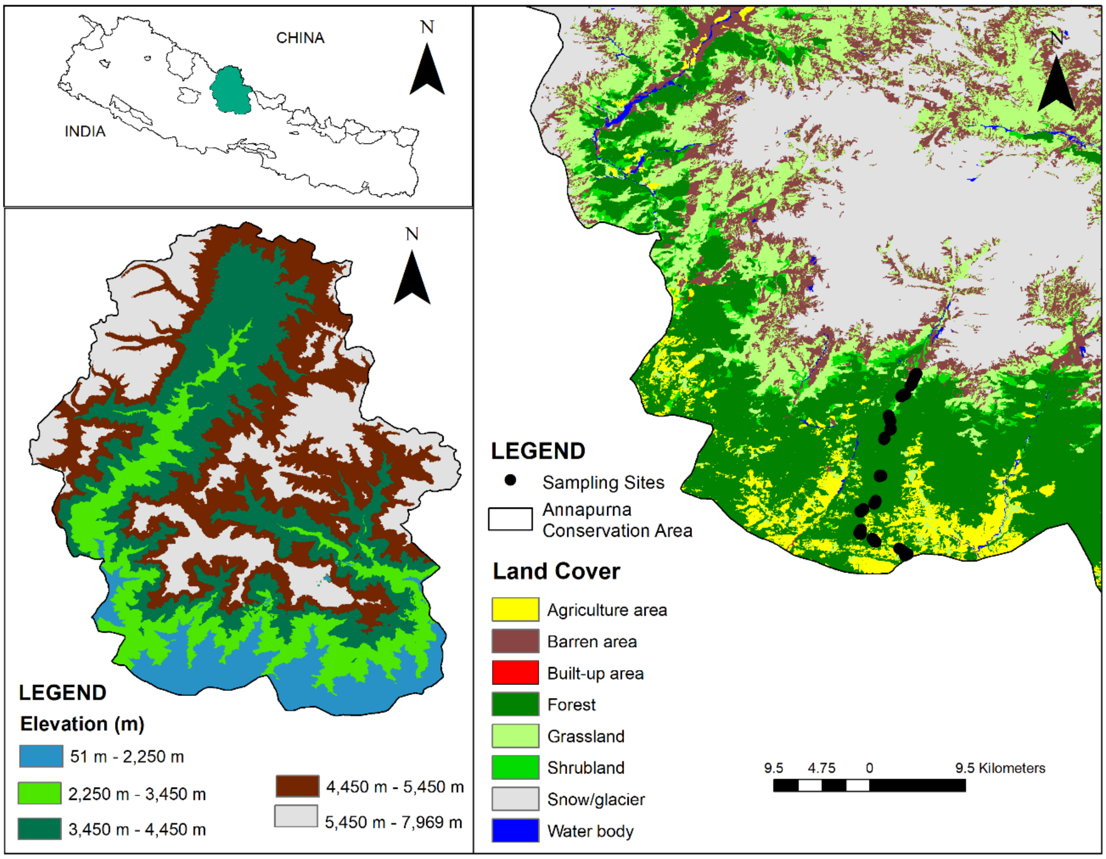

Figure 1. Map of study area showing elevation, types of land cover and sampling sites.

3.2 Research design

The field research design was created using Google Earth. Sampling sites were designed wherever it was possible at different habitats and elevations. The line transects (100 m × 4 m) method was used to survey the study area. Three transects each were made at intervals of 200 m- 250 m in elevation, starting from an elevation of 1150 m and continuing up to 4000 m (Fig.1). A total of 39 transects were made in the study area.

3.3 Methods

3.3.1 Line transects

An anuran survey was carried out during monsoon in different elevations using the nocturnal time-constrained visual encounter method (Campbell and Christman 1982). It effectively covers the entire community of amphibians, including terrestrial, arboreal, aquatic, fossorial, and even well-camouflaged species (Keller et al. 2009). Data were collected in August 2022. The searches were conducted every night along transects (100 m ×4 m) between 7:00 p.m. to 9:00 p.m. by using torches/ headlights. Transects were searched by two people for an hour, walking at a slow pace. Each site was sampled just once during the sampling period. Transects were placed at least 200 m- 250 m elevation intervals from each other. A total of 20 transects had the presence of anurans, however, none were found over 3000 m, hence transects above this elevation were excluded from the data analysis.

All captured individuals in each transect were taken to a nearby dry place where they were photographed and identified to species and their sex by field guidebooks such as (Schleich and Kästle 2002) and (Shah and Tiwari 2004). Body size and extremities were measured by using the vernier calliper following (Olalla-Tárraga and Rodríguez 2007). During the measurement, new latex gloves were used for each individual to prevent the transmission of diseases. All the captured individuals were released back to their capture location once the sampling was completed.

3.3.2 Measurements of body size and extremities

To determine the body size along the elevational gradient, the snout-vent length (SVL) was measured of all adult individuals by using a vernier caliper (DIN862) with a precision of 0.1mm (Olalla-Tárraga and Rodríguez 2007).

Additionally, to determine the body extremities along an elevational gradient, other morphological variables were measured such as length of forelimb (FLL), length of tarsus (LT), length of femur (LF), length of tibia-fibula (LtF), length of meta-tarsus (LMT), etc. However, other variables were measured to identify the species.

Table 1. Morphological variables measured by vernier caliper

S.No.	**Abbreviation**	**Morphology (in mm)**
1.	SVL	Snout-vent length
2	HL	Head length
3	HW	Head Width
4	SL	Snout length
5	ED	Eye diameter
6	NoE	Nostril to eye
7	WUE	Width of upper eyelid
8	IOW	Inter-orbital width
9	INW	Inter-Narial width
10	TD	Tympanum diameter
11	LA	Length of arm
12	FLL	Length of hand (forelimb)
13	L1F	Length of 1st finger
14	L2F	Length of 2nd finger
15	L3F	Length of 3rd finger

16	L4F	Length of 4th finger
17	LF	Length of femur
18	LtF	Length of tibia-fibula
19	LT	Length of tarsus
20	LMT	Length of meta-tarsus
21	L1T	Length of 1st toe
22	L2T	Length of 2nd toe
23	L3T	Length of 3rd toe
24	L4T	Length of 4th toe
25	L5T	Length of 5th toe
26	NMD	Nose- to- mouth distance
27	LAHL	Lower arm and Hand length
28	BW	Body weight (gm)

3.3.3 Environmental variables and collection methods

Table 2. Environmental variables and their description

Parameters	**Variables**	**Description**
Topographic Variables	Elevation	Measured by using GPS (Garmin Etrex 10)
	Slope	Measured by using QGIS Desktop 3.16.16, extracted from Digital Elevational Model (DEM)
	Aspect	
	Terrain Ruggedness Index	
Climatic Variables	Temperature	Measured by thermometer; HTC-2
Bioclimatic Variables	BIO2 = Mean Diurnal Range (Mean of monthly (max temp–min temp))	19 Bioclimatic Variables were obtained from World Clim (https://www.worldclim.org/). To avoid high collinearity, VIF (Variance Inflation Factor) was calculated among 19
	BIO4 = Temperature Seasonality (standard deviation × 100)	
	BIO9 = Mean Temperature of Driest Quarter	

	BIO17 = Precipitation of Driest Quarter	bioclimatic variables by using the 'car' package in R (Fox and Weisberg 2019). 5 bioclimatic variables were retained by using VIF.
	BIO19 = Precipitation of Coldest Quarter	
Disturbance Variables	Distance to nearest settlement	Distance measured from sampling points to nearest settlement with the help of Google Earth Pro
	Distance to Road	Distance measured from sampling points to nearest track or road with the help of Google Earth Pro
Land Cover Variables	NDVI (Normalized Difference Vegetation Index)	It was obtained from (https://earthexplorer.usgs.gov/) By using Landsat 8-9 OLI/TIRS C2L2
	NDBI (Normalized Difference Build up Index)	
	NDWI (Normalized Difference Water Index)	
Habitat	Types of Habitats	Agricultural field, Grassland, Forest, and Settlement

3.4 Data Analysis

Data were extracted in Excel 2019. Species richness was determined by the number of species. All the statistical analysis were carried out in R 4.3.0 (R Development Core Team 2023).

3.4.1 Linear regression model

The linear regression model used R package 'stats' to describe the species richness pattern along an elevational gradient. Elevation was taken as an independent variable whereas species richness was taken as dependent variable. Similarly, a linear regression model was also used to describe the relationship between body size (SVL) and elevations (i.e., test for Bergmann's rule). Elevation was taken as independent variable whereas SVL was taken as the dependent variable. 'ggplot2' package was used to generate the plot (Wickham 2009).

3.4.2 Multicollinearity

R package 'corrplot' was used to find the correlation to check the multicollinearity between environmental variables (Wei and Simko 2021). Since, high collinearity was detected between elevation, temperature and 5 bioclimatic variables. Elevation and 5 bioclimatic variables were excluded.

3.4.3 Multiple linear regression

Similarly, using the R package 'stats' was used for a multiple linear regression model to describe the relationship between body extremities (Forelimb and Hindlimb) and elevations (i.e., test for Allen's rule). Here, elevation was also taken as an independent variable whereas body extremities (LA, LAHL, FLL, LF, LtF and LT) were taken as a dependent variable. 'ggplot2' package was used to generate the plot. These analyses were limited to adults.

It was also used to determine the effect of environmental variables (Distance to Road, Distance to settlement, Slope, Aspect, Terrain Ruggedness Index, Temperature and NDVI) on species richness. Species richness was taken as a dependent variable and environmental variables were considered as independent variable.

Multiple linear regression was used to determine the effect of environmental variables on body size (SVL). SVL was taken as dependent variable and environmental variable as independent variable.

Similarly, it was also used to determine the effect of environmental variables on different variables of body extremities. Body extremities were taken as dependent variable and environmental variable were taken as independent variable.

3.4.4 Generalized linear model (GLM)

A generalized linear model (GLM) was used to determine the effect of environmental variables (Distance to Road, Distance to settlement, Temperature, Slope, Aspect, TRI and NDVI) on species richness. GLM was performed by using the R package 'vegan' community ecology with Poisson distribution (Oksanen et al. 2017). The species richness was taken as a response variable, and all other environmental variables were considered predictors.

3.4.5 Hierarchical partitioning analyses

R package hier.part was used to determine the independent linear contributions of each environmental variable on species richness as well as body size and extremities (Walsh 2013). Environmental variables were predictors or explanatory variable and species richness, body size and extremities were response variable.

4. RESULTS

4.1 Documentation of anurans

A total of 10 species were recorded from the study area. The anurans belong to five families; the family Dicroglossidae (4 species), Bufonidae (2 species), Ranidae (2 species), Microhylidae (1 species) and Rhacophoridae (1 species).

Table 3. List of amphibians at different habitats

S.N.	Family Name	Species Name	Habitats
1.	Bufonidae	*Duttaphrynus melanostictus*	Agricultural Field, Settlement
		Duttaphrynus himalaynus	Agricultural Field, Settlement, Grassland
2.	Dicroglossidae	*Euphlyctis cyanophlyctis*	Forest, Wetland
		Minervarya syhadrensis	Forest, Wetland
		Minervarya nepalensis	Agricultural Field, Forest, Wetland
		Nanorana liebigii	Wetland
3.	Microhylidae	*Microhyla nilphamariensis*	Agricultural Field
4.	Ranidae	*Amolops marmoratus*	Wetland
		Amolops mahabharatensis	Wetland
5.	Rhacophoridae	*Polypedates maculatus*	Forest

4.2 Species richness along an elevational gradient

Species richness gradually followed the declining trend as elevation increased. Elevation was negatively correlated with species richness and their linear relationship was also significant (Figure 2). Four species were recorded from the lowest elevation. *Amolops marmoratu*s and *Duttaphrynus melanostictus* were dominant species at lower elevations (i.e., 1155 m). Five species were recorded from 1424m elevation, seven species were recorded from 1756m elevation. Three species were recorded from 1950m elevation, and two species were recorded from 2152m of elevation. *Duttaphrynus himalaynus* and *Minervaya nepalensis* were dominant species at mid-elevation. Only one species i.e., *Duttaphrynus himalaynus* was recorded from 2420m and 2550m of elevation. No species were recorded from 2797m of elevation. However, one species i.e., *Nanorana liebigii* was recorded from 2998m of elevation. After that, there was no presence of anurans above 3000m of elevation.

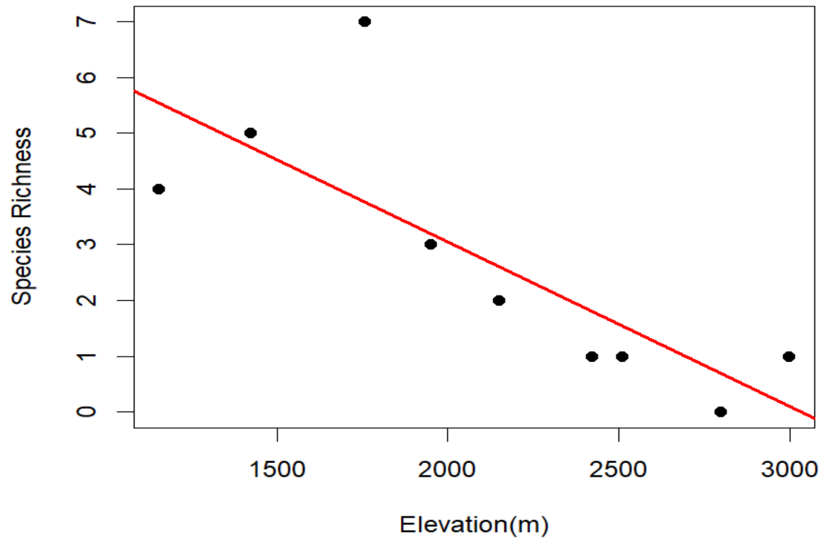

Figure 2. Linear regression model showing the effect of elevation on anurans **($y = -0.002947x + 8.94$, $R^2 = 0.6303$, $P < 0.01$)**

4.3 Body size variation along an elevational gradient

Body size or Snout-vent length (SVL) was analyzed of all individuals from family Bufonidae and family Dicroglossidae by linear regression to find the effect of elevation on the body size of anurans. Individuals of these families were selected from various bands of elevation. From family Bufonidae *Duttaphrynus melanostictus* and *Duttaphrynus himalaynus* and family Dicroglossidae *Euphlyctis cyanophlyctis*, *Minervarya syhadrensis* and *Minervarya nepalensis* were used for the analysis. However, *Nanorana liebigii* was not concluded as only one individual was found in the study area.

In the other three remaining families, species and individuals were not enough. Family Microhylidae had only one species i.e., *Microhyla nilphamariensis* and 3 individuals. Family Ranidae had two species i.e., *Amolops mahabharatensis* and *Amolops marmoratus* and 10 individuals but they were collected from only one band of elevation. Similarly, Family Rhacophoridae had only one species and 1 individual. Therefore, the body size variation along elevation gradients was not examined in individuals of these families.

The linear regression model showed that the body size (SVL) of all individuals belonging to family Bufonidae and family Dicroglossidae have significantly increased along an elevation (Fig.3, Fig.4, Table 4). Hence, Species of family Bufonidae and Dicroglossidae followed Bergmann's rule.

Table 4. Relationship between body size and elevation

Morphological Variable	Family Bufonidae	Family Dicroglossiade
SVL	$R^2 = 0.1959$	$R^2 = 0.241$
	p-value = **<0.01**	p-value = **<0.01**

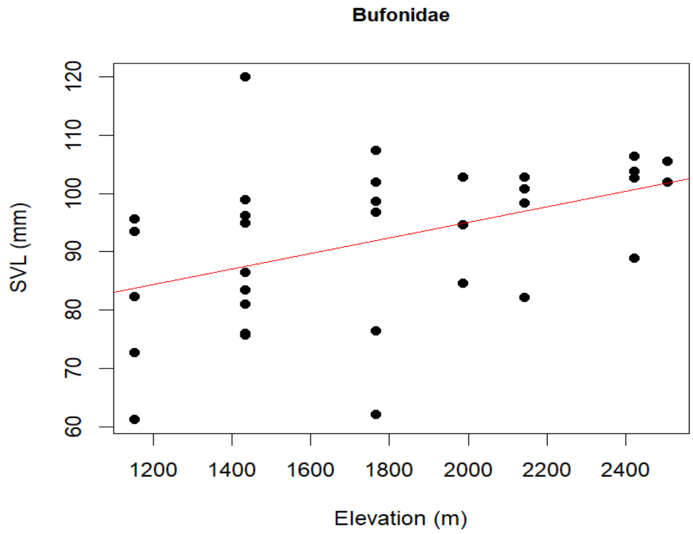

Figure 3. Linear regression model showing relationship between family Bufonidae and elevation

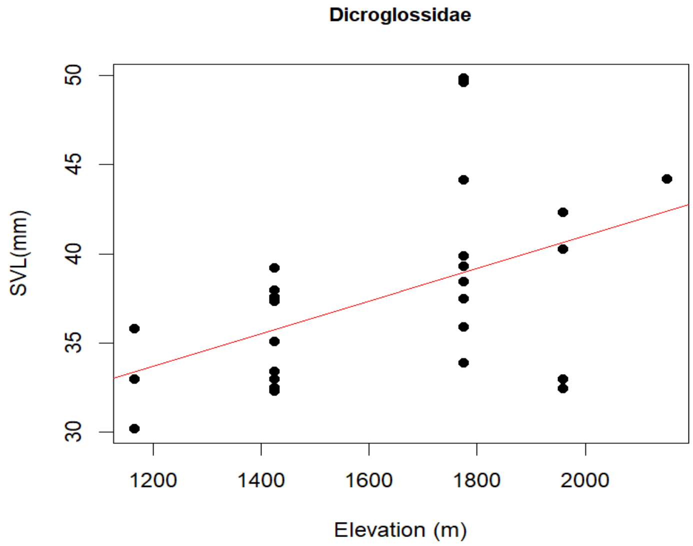

Figure 4. Linear regression model showing relationship between family Dicroglossidae and elevation

4.4 Body extremities variation along an elevational gradient

Body extremities were analyzed of all individuals belonging to family Bufonidae and family Dicroglossidae by multiple linear regression to find the effect of body extremities of anurans. The multiple linear regression model shows that LA and LtF of all individuals from family Bufonidae did not show any significant towards body extremities along elevation (Table 5). However, LAHL, FLL and LF show significantly increased body extremities along elevation (Fig.5, Fig.6, Fig.7). But LMT shows significantly decreased body extremities along elevation (Fig.8). It shows that the metatarsus of species of family Bufonidae follows Allen’s rule.

Table 5. Relationship between measurements of extremities of anurans and elevation

Morphological Variables	Family Bufonidae	Family Dicroglossidae
LA	R^2=0.007	R^2=0.06
	p-value=0.6	p-value=0.2
LAHL	R^2=0.47	R^2=0.0079
	p-value=<**0.01**	p-value=0.6
FLL	R^2=0.19	R^2=0.058
	p-value=<**0.01**	p-value=0.23
LF	R^2=0.144	R^2=0.151
	p-value=**0.02**	p-value=**0.04**
LtF	R^2=0.00004	R^2=0.33
	p-value=0.97	p-value=<**0.01**
LT	R^2=0.025	R^2=0.018
	p-value=0.3	p-value=0.5
LMT	R^2=0.184	R^2=0.011
	p-value=<**0.01**	p-value=0.6

Similarly, multiple linear regression model shows that LA, LAHL, FLL, LT and LMT from family Dicroglossidae did not show any significance towards body extremities along elevation (Table 5). However, LF and LtF shows significantly increased body extremities along elevation (Fig.9, Fig. 10). It shows that species of the family Dicroglossidae don’t follow Allen’s Rule.

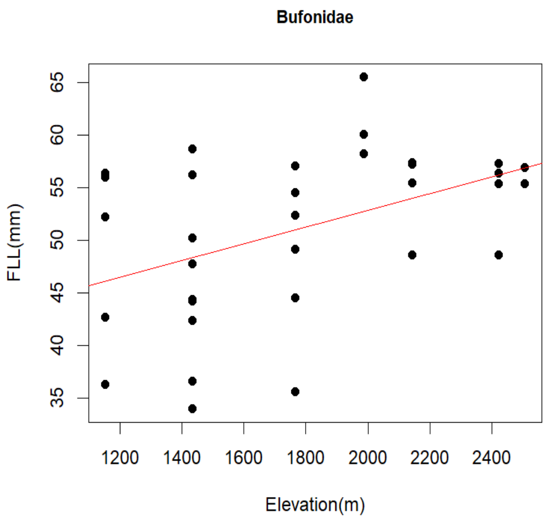

Figure 5. Relationship between FLL of family Bufonidae and elevation

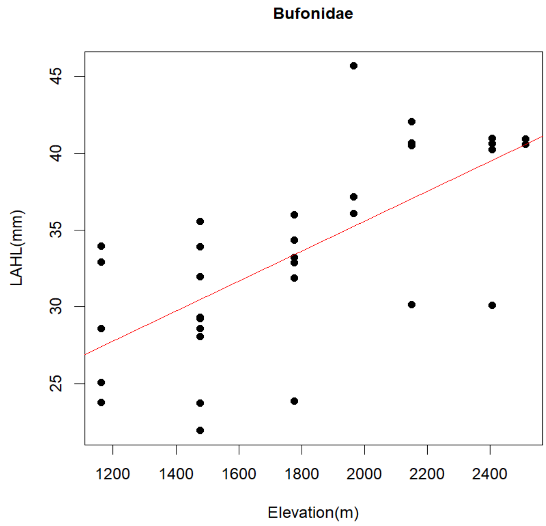

Figure 6. Relationship between LAHL of family Bufonidae and elevation

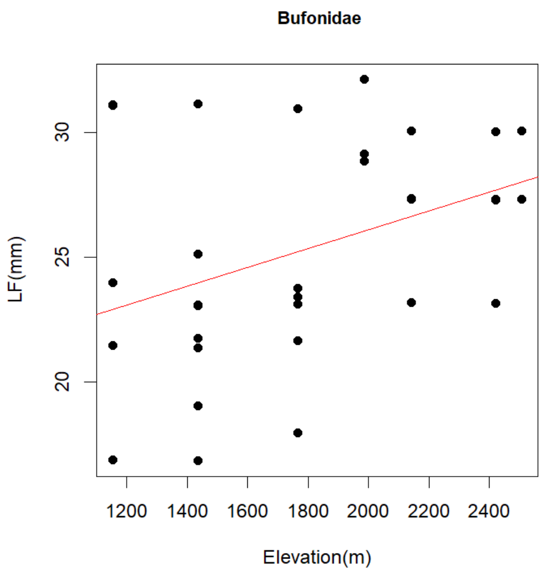

Figure 7. Relationship between LF of family Bufonidae and elevation

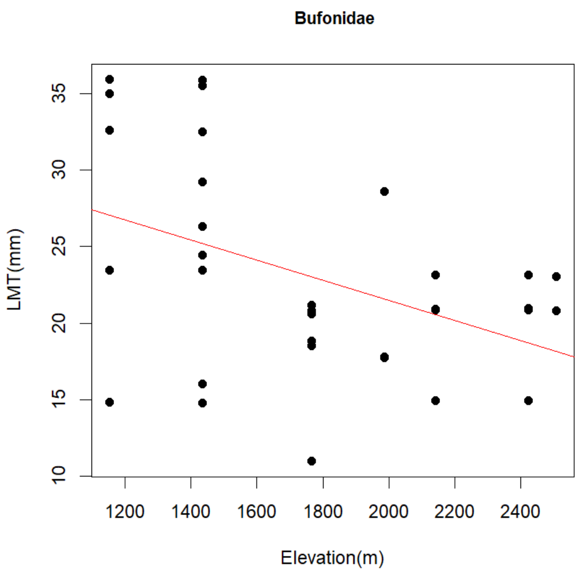

Figure 8. Relationship between LMT of family Bufonidae and elevation

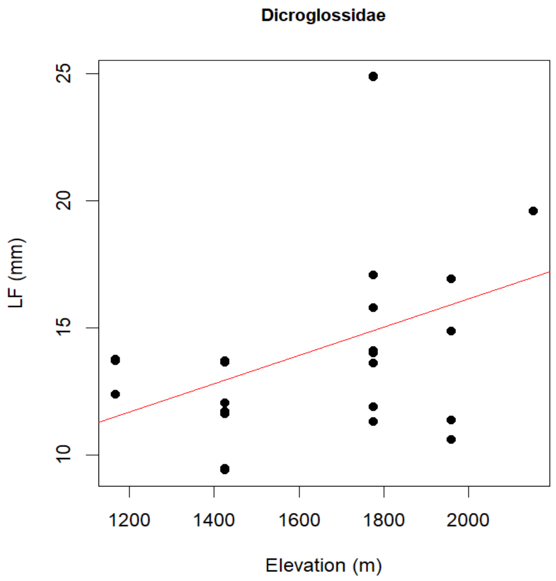

Figure 9. Relationship between LF of family Dicroglossidae and elevation

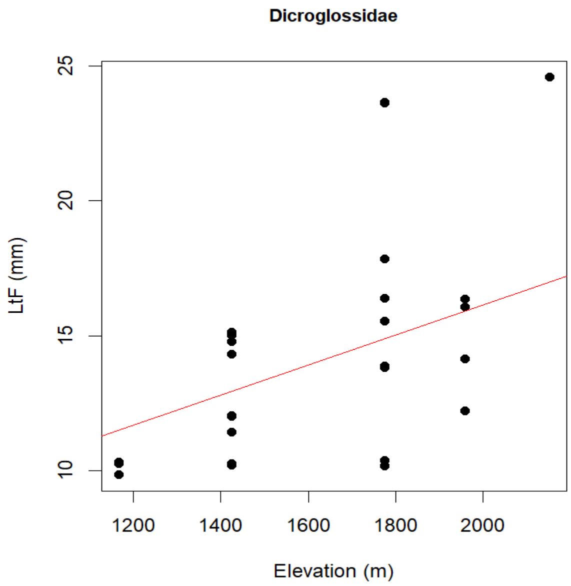

Figure 10. Relationship between LtF of family Dicroglossidae and elevation

4.5 Factors affecting species richness along elevation

Generalized linear model (GLMs) explained that temperature had a positive significant relationship with species richness along elevation. However, GLM does not predict any significant relation of species richness with distance to road, distance to settlement, slope, aspect, terrain ruggedness index (TRI) and NDVI.

Species richness of anurans showed a negative association with distance to road, aspect and NDVI but their role on species richness was not significant. It was found that distance to settlement, slope and TRI were positively associated with species richness of anurans but their role was also insignificant.

Table 6. GLM result of species richness of anurans with different environmental factors

	Estimate	Std. Error	z value	Pr (>|z|)
(Intercept)	-2.2403353	2.5312641	-0.885	0.3761
Distance to Road	-0.0515607	0.0427807	-1.205	0.2281
Distance to Settlement	0.0044059	0.0138335	0.318	0.7501
Temperature	0.1677525	0.0824433	2.035	**0.0419***
Slope	0.0266338	0.0302206	0.881	0.3781
Aspect	-0.0091471	0.0117576	-0.778	0.4366

TRI	0.0004021	0.0341404	0.012	0.9906
NDVI	-0.7434595	4.7421253	-0.157	0.8754

Hierarchical partitioning analysis was performed to evaluate the effect of different environmental variables on species richness. Explanatory variable like temperature was the most contributed variable which affect species richness by 39.23%. The second most contributor was NDVI, which affect species richness by 25.93%. Similarly, the third most contributor was Slope which explained 10.59%. Aspect, Distance to Settlement and Distance to Road were explained by (6.76%, 6.57%, and 6.03%). Terrain Ruggedness Index (TRI) was the least contributed variable which affect species richness by 4.91%.

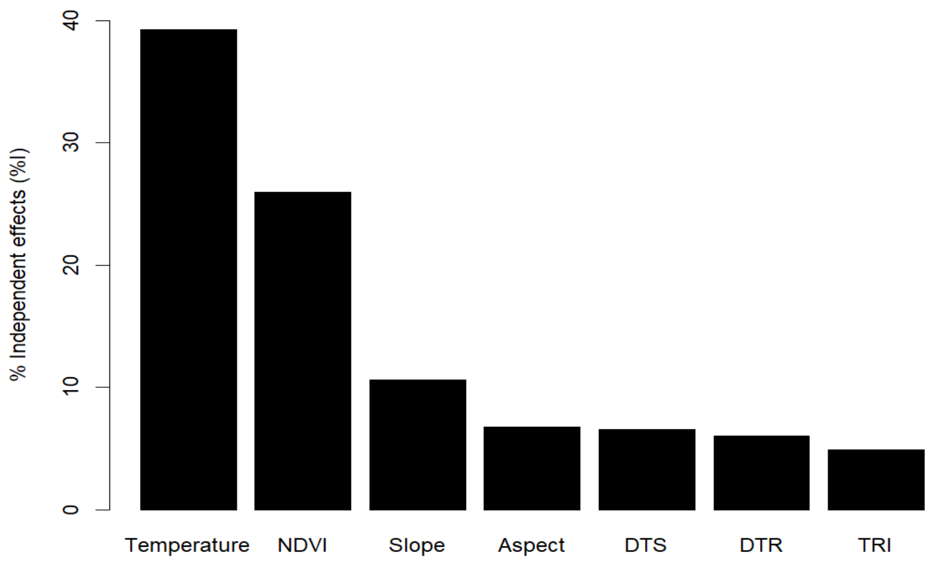

Figure 11. Hierarchical partitioning showing the percentage of independent effects of different variables on species richness

4.6 Factors affecting body size and extremities along elevation

Multiple linear regression model and hierarchical partitioning were used to determine the factors that affect body size and extremities of anurans along elevation. Multiple linear regression model showed that SVL, LA, FLL, LF, LtF, LT and LMT of individuals belonging to family Bufonidae were not significant to the environmental variables. However, LAHL showed significance to the environmental variables (Table 7).

Table 7. Multiple regression model and hierarchical partitioning showing effect of environmental variables on body size and extremities on family Bufonidae

Family: Bufonidae

Morphometric Variable	Multiple Regression Model	Full Model Variables (Independent explained variation %)						
		DTR	DTS	Temp.	Slope	Aspect	T.R. I	NDVI
SVL	R^2= 0.2741 p-value= 0.27	1.21 %	6.36%	62.04 %	3.7%	2.66%	11.08 %	12.95 %
LA	R^2= 0.2035 p-value= 0.51	4.65 %	44.65 %	11.77 %	8.92%	5.68%	23.82 %	0.48 %
LAHL	R^2=0.5303 p-value= **<0.01**	3.43 %	7.8%	71.55 %	2.43%	2.58%	10.08 %	2.11 %
FLL	R^2= 0.2819 p-value= 0.24	3.09 %	18.55 %	54.25 %	2.97%	3.11%	16.33 %	1.66 %
LF	R^2= 0.3479 p-value= 0.11	3.47 %	17.36 %	33.45 %	3.60%	2.61%	10.71 %	28.77 %
LtF	R^2= 0.223 p-value= 0.43	2.17 %	73.06 %	5.73%	7.29%	5.82%	3.90 %	2.006 %
LT	R^2= 0.35 p-value= 0.1	1.52 %	13.64 %	19.006 %	9.94%	27.94 %	3.11 %	24.81 %
LMT	R^2= 0.32 p-value= 0.14	3.20 %	31.45 %	31.66 %	2.75%	25.63 %	4.39 %	0.89 %

Hierarchical partitioning showed that temperature and Terrain Ruggedness Index (T.R.I.) was the most contributed variable which affect body size and extremities of individuals belonging to the family Bufonidae by 71.55% and 10.08% (Fig.12). Distance to settlement, Distance to road contributes by 7.80% and 3.43%. However, aspect, slope and NDVI contribute only 2.58%, 2.43% and 2.11%.

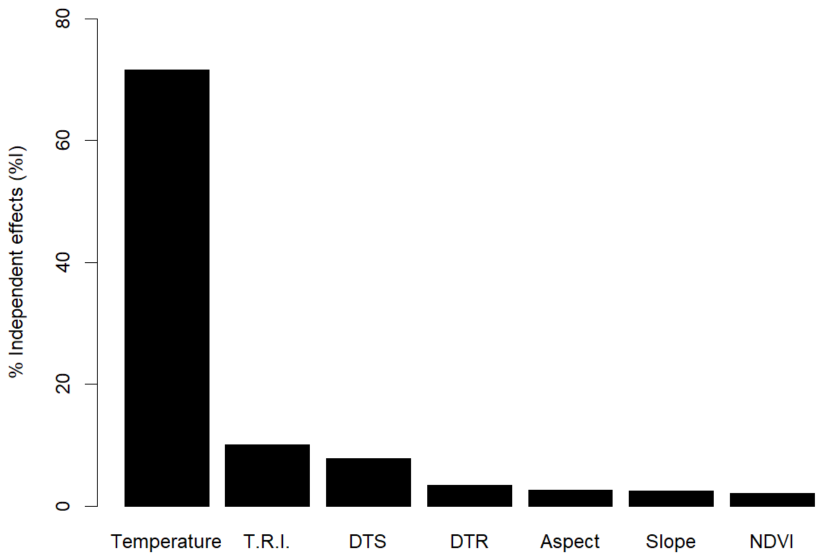

Figure 12. Hierarchical partitioning showing the percentage of independent effects in LHAL of family Bufonidae

Similarly, Multiple linear regression model also showed that Snout- Vent Length (SVL), Length of Arm (LA), Lower arm and Hand Length (LAHL), Length of Forelimb (FLL), Length of Femur (LF), Length of tibia- Fibula (LtF), Length of Tarsus (LT) of individuals belonging to family Dicroglossidae were significant with environmental variables. However, Length of Meta-tarsus (LMT) was not significant with environmental variables.

Table 8. Multiple regression model and hierarchical partitioning showing effect of environmental variables on body size and extremities in family Dicroglossidae

Family: Dicroglossidae

Morphometric Variable	Multiple Regression Model	Full Model Variables (Independent explained variation %)						
		DTR	DTS	Temp.	Slope	Aspect	T.R. I	NDVI
SVL	R^2= 0.6236	3.49%	13.47 %	42.79 %	10.25 %	16.63 %	3.35 %	9.99%
	p-value= **<0.01**							
LA	R^2= 0.546							6.58%

	p-value= **0.02**	24.41 %	19.55 %	24.62 %	10.04 %	12.47 %	2.29 %	
LAHL	R^2= 0.5137 p-value= **0.04**	7.33%	7.25 %	22.18 %	40.05 %	9.77%	12.17 %	1.21%
FLL	R^2= 0.5867 p-value= **<0.01**	11.9%	8.81 %	31.12 %	25.79 %	12.11 %	8.36 %	1.86%
LF	R^2=0.668 p-value= **<0.01**	17.34 %	12.12 %	32.51 %	13.27 %	13.001 %	4.27 %	7.45%
LtF	R^2= 0.7136 p-value= **<0.01**	6.69%	14.09 %	47.58 %	9.37 %	16.24 %	1.91 %	4.09%
LT	R^2= 0.596 p-value= **<0.01**	29.22 %	5.61 %	14.98 %	19.01 %	5.86%	19.05 %	6.25%
LMT	R^2= 0.3126 p-value= 0.36	59.43 %	14.81 %	9.47%	2.18 %	10.47 %	1.79 %	1.81%

Hierarchical partitioning showed that temperature was the most contributed variable as compared to other variables which affect body size and extremities of individuals belonging to the family Dicroglossidae (Table 8, Fig.13).

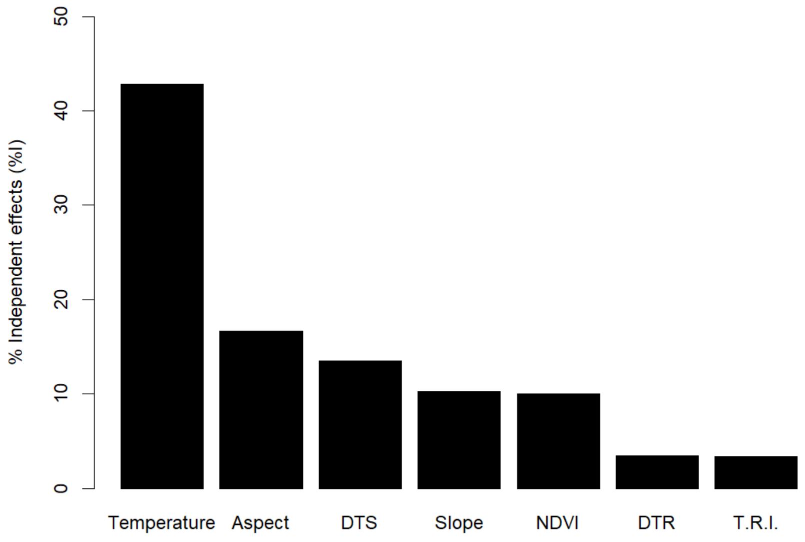

Figure 13. Hierarchical partitioning showing Percentage of independent effects in SVL of family Dicroglossidae

5. DISCUSSION

The study was conducted in the Mardi trekking route of Annapurna Conservation Area. This study explored the species richness pattern and morphological (body size and extremities) variations of anurans along elevational gradient.

5.1 Species richness pattern of anurans

It is important to understand the relationships between species richness and elevation for the development of a basic theory on species diversity (Rowe 2009). From the previous studies on the species richness pattern of amphibians, some of the studies observed a monotonic decline in species richness as elevation increases along a gradient (Malonza and Veith 2011, Zancolli et al. 2014), some suggest a humped-shaped pattern (Fu et al. 2006, Hu et al. 2011), while some indicate a declining trend with increasing elevation (Naniwadekar and Vasudevan 2007, Khatiwada and Haugaasen 2015). In this study, findings showed a declining trend of species richness with increasing elevation. Similar trends were observed in amphibians along elevational gradient, including in the Western Ghats in India (Naniwadekar and Vasudevan 2007), Jigme National Park in the western part of Bhutan (Koirala 2019), Gunung Raya in Malaysia (Corak et al. 2018), Eastern Himalayas (Khatiwada et al. 2019), Chitwan in Nepal (Khatiwada and Haugaasen 2015), Ghandruk of Annapurna Conservation Area (Gautam et al. 2020), etc.

Since species richness was observed in lower elevations as compared to higher elevations. Based on generalized linear regression, temperature was found to be the major factor that influenced the species richness pattern. It indicates that anurans prefer a higher average temperature (Hu et al. 2011) as it has a variety of effects on their physiology, behaviour, and ecological role (Navas et al. 2008). Previous studies have also observed the significant effects of NDVI on the species richness pattern (Chettri and Acharya 2020) as lower elevation can provide a better habitat for anurans but this study didn't find any significant effects of NDVI. Other studies have also mentioned that the decreasing trend of species richness can be due to the larger land surface area (Khatiwada et al. 2019), the optimum interaction of energy (temperature, PET and solar radiation) (Manish et al. 2017) as well as humidity (Khatiwada et al. 2019), water availability (annual precipitation) found in the lower elevation (Chettri and Acharya 2020).

5.2 Body size variation along an elevational gradient

The body size of anurans is an important life trait that affects fitness and is thus subject to natural selection (Lou et al. 2012). In the case of herpetofauna, Bergmann's rule has been widely controversial (Adams and Church 2008) as this rule had been made for endothermic animals such as mammals and birds as these large animals can adjust to changes in their environment (Hu et al. 2011, Khatiwada et al. 2019). Previous studies have claimed that amphibians generally follow Bergmann's rule (Ashton 2002, Olalla-Tárraga and Rodríguez 2007). Some studies have also provided evidence supporting the rule for specific regional anuran species such as *Rana limnocharis* (also known as *Fejervarya limnocharis*) (Liu et al. 2012), *Bufo andrewsi* (Liao and Lu 2014), *Bufo minshanicus* (Yu et al. 2019) and *Rana chensinensis* (Li et al. 2006). However, some other studies suggested that not all amphibians such as *Nanorana parkeri* (Ma et al. 2009), *Pelophylax pleuraden* (Lou et al. 2012), *Rana sauteri* (Hsu et al. 2014), *Fejervarya limnocharis* (Liu et al. 2018), *Rana kukunoris* (Yu et al. 2022) adhere to it. The findings of this study observed that the body size of anurans from family Bufonidae and family Dicroglossidae tended to increase as elevation increased. A similar type of result was found in Eastern Nepal Himalayas (Khatiwada et al. 2019). In addition, the body size of male and female anurans from lower elevations had smaller body sizes compared to those from higher elevations. It is generally known that endothermic species may have a smaller body size in warmer regions as compared to colder environments due to thermoregulation (Bergmann 1847, Ashton 2002).

According to Laugen et al. (2005), body size variation may be influenced by environmental factors as well as biological factors. Previous studies have majorly focused on age and growth rate and observed that frogs from higher altitudes have a faster growth rate which affects their body size (Li et al. 2006, Baraquet et al. 2018, Yu et al. 2022). However, some of the studies observed temperature to be the major factor responsible for body size variation because higher temperatures accelerate the growth rate and development which leads to early maturity and reduces the body size (Ma et al. 2009). Similarly, based on hierarchical partitioning, this study reveals that the temperature and NDVI influence the body size variation. It means that regions with higher productivity are likely to have a larger body size. This indicates that a sufficient food supply is necessary to sustain the body size (Yom-Tov and Geffen 2006, Leung et al. 2021). Previous studies have also revealed that the availability of water such as precipitation and humidity (Liu et al. 2018, Rivas et al. 2018) can also be a major environmental factor than temperature as they need to maintain the moisture of the skin to allow respiration (Ashton 2002).

5.3 Body extremities variation along elevational gradient

Body extremities haven't got much attention as body size. But all the studies that have been done so far show that amphibians such as *Rana temporia* (Alho et al. 2011) and *Rana kukunoris* (Leung et al. 2021) follow Allen's rule. However, there is no study conducted on the species belonging to the family Bufonidae and family Dicroglossidae. The findings of this study reveal that in the family Bufoniade, length of meta-tarsus is the only body extremities variable that complies with Allen's rule. However, it is unclear for the family Dicroglossiade, if they follow Allen's rule or not. Since there is a lack of studies on body extremities Allen's rule is still controversial in the case of amphibians (Alho et al. 2011).

In a similar manner to body size variation, Leung et al. (2021) reveal that body extremities may be influenced by environmental factors as well as biological factors. A study conducted on the Plateau brown frog reveals precipitation as the factor that affects body extremities (Leung et al. 2021) because precipitation can reduce the air temperature and delay sexual maturity, which in turn slows down growth rates that are typically accelerated by higher temperatures (Atkinson et al. 1996). However, this study shows an association of body extremities with temperature only. A similar result was observed in the common frog (*Rana temporia*) conducted by (Alho et al. 2011) in their common garden and discovered that genetic variation in extremities showed an Allenian pattern in response to specific environmental conditions, even though the primary function of legs is not related to thermoregulation.

6. CONCLUSION AND RECOMMENDATION

6.1 Conclusion

There was a decreasing trend of species richness with an increase in elevation. The species richness of anurans was significantly found to be affected by temperature. This pattern may also be influenced by the presence of more favourable habitats and food and water resources available at lower elevations than at higher elevations.

The study also examined how body size and extremities of two families, Bufonidae and Dicroglossidae, along an elevational gradient. The result indicated that body size tended to increase with higher elevation, consistent with Bergmann's rule. However, the length of metatarsus in the Bufonidae family follows Allen's rule while no significant relationship was observed for extremities in the Dicroglossidae family. Hierarchical partitioning shows that temperature and NDVI were the predictors influencing anurans' body size and extremities.

6.2 Recommendation

- Local communities were found to be exploiting the Paha frog species (especially *Nanorana* spp.) throughout the survey. To effectively protect and conserve these frogs, it is essential to conduct awareness programs that educate and engage the local people.

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APPENDICES

Appendix 1. Datasheet for morphometric measurement of anurans

S.N.	Abbreviation	Morphology (in mm)	Measurement description
1.	SVL	Snout-vent length	Distance from the tip of the snout to the posterior edge of the vent
2	HL	Head length	Distance from the angle of jaws and snout-tip
3	HW	Head Width	Measured at a posterior angle of jaws
4	SL	Snout length	From the tip of the snout to the anterior corner of the eye
5	ED	Eye diameter	Horizontal diameter of eye
6	NoE	Nostril to eye	Distance from nostril to eye
7	WUE	Width of upper eyelid	The greatest width of upper eyelid
8	IOW	Inter-orbital width	The minimum distance between upper eyelids
9	INW	Inter-Narial width	The minimum distance between external nares
10	TD	Tympanum diameter	Largest tympanum diameter
11	LA	Length of arm	Distance from elbow to the base of outer metacarpal tubercle
12	FLL	Length of hand (forelimb)	Measured from the base of the outer metacarpal tubercle to the tip of the third finger
13	LHAL	Lower arm and Hand length	Distance from elbow to the tip of the third finger
14	L1F	Length of 1st finger	Distance from the base of the second finger to the tip of the first finger
15	L2F	Length of 2nd finger	Distance from the base of the first finger to the tip of the second finger

16	L3F	Length of 3rd finger	Distance from the base of the second finger to the tip of the third finger
17	L4F	Length of 4th finger	Distance from the base of the third finger to the tip of the fourth finger
18	LF	Length of femur	Distance from groin to knee
19	LtF	Length of tibia-fibula	Distance from knee to heel
20	LT	Length of tarsus	Distance from heel to inner metatarsal tubercle
21	LMT	Length of meta-tarsus	Distance from inner metatarsal tubercle to tip of the fourth toe
22	L1T	Length of 1st toe	The maximum length from the base of the first subarticular tubercle first toe tip
23	L2T	Length of 2nd toe	The maximum length from the base of the first subarticular tubercle second toe tip
24	L3T	Length of 3rd toe	The maximum length from the base of the first subarticular tubercle third toe tip
25	L4T	Length of 4th toe	The maximum length from the base of the first subarticular tubercle fourth toe tip
26	L5T	Length of 5th toe	The maximum length from the base of the first subarticular tubercle fifth toe tip
27	NMD	Nose- to- mouth distance	Distance from nose to mouth
28	BW	Body weight (gm)	

Appendix 2. List of Photographs

Picture 1. *Duttaphrynus melanostictus*

Picture 2. *Duttaphrynus himalaynus*

Picture 3. *Minervarya nepalensis*

Picture 4. *Minervarya syhadrensis*

Picture 5. *Euphlyctis cyanophlyctis*

Picture 6. *Nanorana liebigii*

Picture 7. *Amolops mahabharatensis*

Picture 8. *Amolops marmoratus*

Picture 9. *Microhyla nilphamariensis*

Picture 10. *Polypedates maculatus*