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Feathers, eggs, and blood as bioindicators of heavy metals and their impact on DNA damage in captive *Pavo cristatus*

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ABSTRACT

Heavy metals and their genotoxic effects in captive Indian Peafowl (*Pavo cristatus*) residing in various regions of Punjab, Pakistan, specifically, Wildlife Park Bahawalpur (WPB), Jallo Wildlife Park Lahore (JWPL), and Wildlife Park Murree (WPM) were evaluated in blood, feathers, eggshell and egg content samples. The Single-cell gel electrophoresis (Comet) assay was performed to evaluate DNA damage. The results showed that the concentration of Cr was significantly high ($P < 0.05$) in Blood (3.79 µg/g), Feather (4.87 µg/g), Egg shell (51.02 µg/g) and Egg Content (13.59 µg/g) samples of Jallo Wildlife Park Lahore followed by Pb, Mn, Ni and Co. The highest ($P < 0.05$) metal accumulation was found in eggshell samples due to its porous structure as compared to other samples. Likewise region-wise analysis showed that Jallo Wildlife Park Lahore appeared to be more polluted than WPB and WPM. Indian Peafowl kept at WPM and JWPL exhibited higher levels of genotoxicity compared to the birds kept at WPB. This disparity can be attributed to the increased exposure to pollution and heightened stress experienced by the peafowl in the former two locations. This study concluded that among all the three study sites of Punjab, the WPB is most suitable for housing captive animals and birds.

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Pavo cristatus; captivity; heavy metals pollution; bioaccumulation; genotoxic; DNA damage

Introduction

Pheasants are widely regarded as one of the most distinctive avian species due to their remarkable characteristics and significant ecological contributions. Their visual appeal and pivotal role within ecosystems are primary factors that underline their importance. They serve as valuable indicators for assessing the environmental quality of their respective habitats, as highlighted by Ali et al. (1987). *Pavo cristatus*, commonly referred to as the common or blue peafowl, is a species belonging to the pheasant family. It is recognized as the largest flying bird within the pheasant family and is classified under the order Galliformes and family Pheasianidae, as noted by Shahbaz (2020).

In Pakistan, *Pavo cristatus* is predominantly found in the extreme of Sindh province and the north-eastern border areas of the Punjab province, which include the border belt in district Narowal and northern Punjab (Roberts 1991; Azam and Shafique 2005). However, its population has experienced local extinction in certain areas within this historical distribution range, and it has become rare in the wild due to several threats to its survival. The primary factor posing risk to the existing peafowl population include habitat loss and degradation (Anwar et al. 2015; Jose and Nameer 2020). To overcome these threats, measures have been taken to protect the Indian Peafowl, and hunting of *P. cristatus* is explicitly prohibited under the Punjab Wildlife Act-1974 (Mushtaq-ul-Hassan et al. 2012).

For the conservation of *P. cristatus*, some individuals are maintained in captivity, which serves as a preventive measure against extinction (El-Shahawy 2010). Captivity, in this context, refers to the confinement of both domestically bred and wild-caught birds in cages and enclosures (Akram et al. 2019). While captivity plays a crucial role in animal conservation, it also entails significant consequences. The restricted living spaces subject the birds to stress, leading to a decline in their overall fitness (Parveen and Sidra 2018). Additionally, captivity-induced stress can exacerbate the problem of parasitic infections, posing a serious threat to this endangered species. Moreover, captivity also decrease the resistance of species to disease (Akram et al. 2019).

The zoos, which were constructed outside the cities now come in the center of the towns and cities due to the unplanned expansion of towns with increase in the human population. In addition to experiencing captivity-induced stress, wild animals kept in protected zones also are confronted with the mounting problem of environmental pollution, which poses adverse effects to their health and well-being. The increasing levels of pollution in these environments disrupt the natural ecological balance and can compromise the overall health and comfort of the captive wildlife (Gupta and Bakre 2013). Especially, heavy metal pollution is one of the major threats to health, since these are particularly hazardous and dangerous elements of contamination (Gworek et al. 2016). A small amount of specific metals such as Co, Cu, Fe,

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Mn and Zn plays an important role for the normal metabolism of organisms. On the other hand, their excessive amount leads to toxicity by bio-accumulation (Lenntech 2012). Organisms show variations in the accumulation of heavy metals, influenced by factors such as size, dietary preferences, age, gender, and other internal or external parameters. These diverse characteristics can lead to differential levels of heavy metal uptake and storage in different species or individuals within a population (Anbazhagan et al. 2021). Some metals are highly toxic like Pd. Cd, Hg and As. Even their small dose can lead to serious problems (Aragay and Merkoçi 2012; Yasmeen et al. 2020). Heavy metal contamination from anthropogenic activities displays a possible risk to health through bio-accumulation in the food chain (Sheppard et al. 2009).

Heavy metals can induce various toxic effects in birds, such as reduced reproductive rates, eggshell thinning, slowed growth, compromised immunocompetence, and developmental deformities and malformations. Consequently, the bird population experiences declines (Dauwe et al. 2006). As a result, public concern has escalated concerning heavy metal contamination. It is now imperative to monitor, assess, manage, and remediate the biological and ecological damage caused by heavy metal pollution (Movalli 2000; Naccari et al. 2009). Numerous bird tissues have been used for monitoring bird's exposure to heavy metals and evaluating risk mainly feathers, liver and more recently, blood is used. Feathers, blood, and eggs can be obtained without any difficulty and repetitively from the same individual if required, and without losing the bird therefore they are preferable (Alvárez et al. 2013). Furthermore, this study aims to evaluate whether the combination of captivity-related stresses and excessive metal contamination can negatively impact the DNA of *P. cristatus*. Understanding the potential impacts on their DNA will contribute to a comprehensive assessment of the risks faced by these birds in captive environments and aid in the development of appropriate conservation strategies.

This research aimed to evaluate and contrast the buildup of metallic elements and their impacts on DNA damage in captive *P. cristatus*.

Materials and methods

Study area

This study was conducted at three geographically different sites of Punjab, Pakistan that are Wildlife Park Bahawalnagar (WPB), Jallo Wildlife Park Lahore (JWPB) and Wildlife Park Murree (WPM). These sites have different environmental and management conditions. Jallo Wildlife Park was established in 1978. It is situated 20 km east to Lahore city which is a semi-aired hot plane area (Sarfaraz et al. 2014) and have an elevation of 217 m with average annual rain fall 628 mm. The minimum temperature of Lahore is 20 °C in winter while in summer it exceeds up to 40 °C. It covers an area of 461 acres or 187 ha. Wildlife Park Bahawalnagar was established in 1986–1988 under the developmental program of Captive Breeding of Blackbuck at Bahawalnagar. It has an area of fifteen acre. It is 4 Km from Railway station, Bahawalnagar which has semi-arid climate (Sarfaraz et al. 2014). The annual rainfall is 194.4 mm. The minimum temperature recorded is 7-12°C and 38-42°C is the maximum range. Wildlife Park Murree was established in 1986–1992 under the developmental program of 'Development of Wildlife Park, Bansra Gali, Murree'. It is 70 Km from Rawalpindi close to Lawrence College, Bansra Gali, Murree. It has an area of two hundred forty acres. The annual rainfall is 1789.3 mm. The climate remains charming throughout the year having four seasons of spring, summer, autumn and winter. The minimum temperature recorded is −10 °C and 22–25 °C is the maximum range (Sarfaraz et al. 2014, Figure 1).

Species selection

P. cristatus is a common species that may be found in parks and zoos throughout the Pakistan. It has a high capacity to adapt to changing environments in a wide range of habitats. The number of selected birds were counted and recorded from all the selected sites. Selected number of birds were used to compare the heavy metal accumulation and DNA damage.

Collection of samples

Blood samples

About 2-3 ml of blood sample (20 samples from each site) were collected from the avian brachial vein into EDTA tubes (10 samples for Single-cell gel electrophoresis) and serum separator tubes (10 samples from each site for screening of heavy metal bio-accumulation). These tubes were wrapped in a cloth towel and placed in bags of handling with ice packs (Samour et al. 2010).

Feather samples

The tail feathers were collected from the peafowls' cages of each site because these feathers have constant supply of blood and are also in direct contact with the environment contaminants. Only molten feathers of birds were collected, with no interaction or injury to the birds. The feathers were properly packed in sterile ziplock bags with proper labelling (Anbazhagan et al. 2021).

Egg samples

During the breeding season of *P. cristatus* from April to May (Naseer et al. 2018),10 eggs were collected from each site. The collected eggs were labelled with nest numbers before being put in glass jars washed with acetone (Ashkoo et al. 2020)

Screening for heavy metal concentration

Digestion of samples

Blood samples in serum vacutainers were collected and allowed to clot in plain bottles. The clotted blood samples were subjected to centrifugation at 2000 rpm for 10 min to separate the serum from the unwanted red blood cells, as this method efficiently increases the volume of serum per unit of blood. Subsequently, one millilitre of serum was diluted with ten millilitres of de-ionized water (Akan et al. 2014).

The feather samples were washed with acetone and then rinsed three times with de-ionized water. Then the feathers

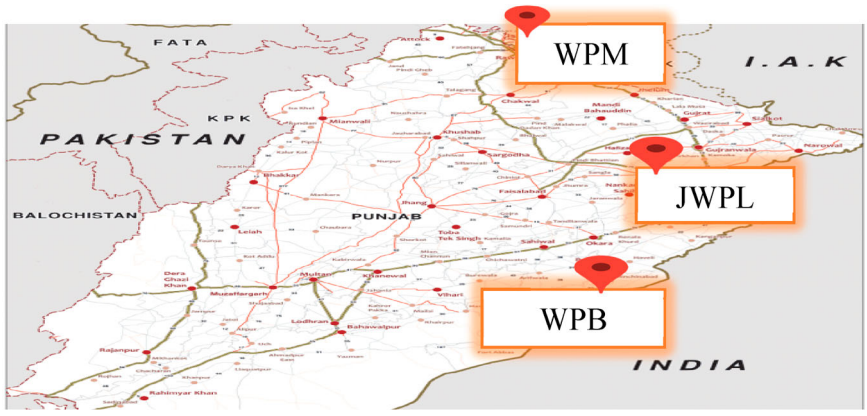

Figure 1. Map of Jallo Wildlife Park Lahore, Wildlife Park Murree and Wildlife Park Bahawalnagar.

were dried in the oven for 48 h at 60 0 C and cut into smaller pieces. One gram of crushed feathers from each sample underwent treatment with a reagent consisting of 5 ml of nitric acid and 5 ml of hydrogen peroxide on a hot plate set at 70 °C until the acid digestion process was fully completed. The resulting extract was then allowed to gradually reach room temperature and then filtered afterwards using Whatman filter paper. The filtered solution was further diluted with 25 ml of de-ionized water (Anbazhagan et al. 2021).

In order to eliminate the adhering exterior pollutants, eggs were washed with acetone and then with de-ionized water. Then eggs' content was removed with the help of toothpick and poured in petri dishes, while the egg shells were placed in other petri dishes. The samples were dried in an oven to obtain a consistent dry weight. The dried samples of eggshells and egg contents were homogenized separately. Erlenmeyer flasks were used and 0.5 g of homogenized powder from each egg sample was added in the flasks together with 10 ml of nitric acid. Erlenmeyer flasks were heated at 140°C until a clear solution was obtained (Miri et al. 2017). After cooling at room temperature, the digested acidic samples were filtered by using Whatman filter paper and the solution was diluted with 25 ml of de-ionized water into 25 ml polypropylene volumetric flask. All digested samples were maintained in the refrigerator at 4 °C before the chemical analysis of heavy metals by Atomic Absorption Spectrophotometry (Ashkoo et al. 2020).

Chemical analysis of samples

All the prepared solutions of feather, blood, and egg samples were subjected to chemical analysis for heavy metals using Atomic Absorption Spectrometry (AAS). The concentraitons of, Pb, Cr, Co, Ni and Mn were measured at different wavelengths using this analytical technique at 357.9, 217.0, 232.0, 240.7 and 403.0 nm, respectively (Yasmeen et al. 2020).

Calculation of metal concentration

The concentrations of metals were calculated by using the following formula:

In current study, the dilution factor was 25 ml for 1 g feather, egg shell and egg content samples and 10 ml for 1 ml blood serum as mentioned above in the digestion of samples.

DNA Damage examination

The Single-Cell gel electrophoresis (SCGE) or Comet assay determines the DNA damage caused by heavy metals contamination. The assay was conducted following the protocols recommended by Singh et al. (1988). DNA damage in different parts of the cells were analyzed and recorded.

Statistical analysis

All statistical analyses were conducted using IBM SPSS Statistics 21 and Statistics 8.1 software. ANOVA (Analysis of variance) was employed to compare the concentrations of heavy metals and DNA damage based on different sites (Steel et al. 1997). Post hoc Tukey's range test was utilized to compare means among different groups.

Results

Table 1 demonstrates that the mean concentration of heavy metals significantly varied ($P < 0.05$) in blood, feather, eggshell and egg content samples of *P. cristatus* kept at WPM, JWPL and WPB. The Cr contamination was significantly higher in blood, feather, eggshell and egg content samples of all locations followed by Mn, Pb and finally Ni and Co.

Overall, heavy metals accumulation pattern in samples of all sites were egg shells > Egg contents > Feathers > Blood. JWPL was polluted as indicated by the levels of all heavy metals compared to WPM and WPB (Figure 2).

The results obtained from the single-cell gel electrophoresis indicated significant differences in the mean values of LHead, LTail, LComet, Head DNA, and Tail DNA at a significance level of $P < 0.05$. However, OTM and TM values were observed to be consistent across all sites, displaying only minimal deviations. Values for LHead, LTail, LComet, Head DNA, and Tail DNA were the highest in WPM followed by JWPL, and lowest in WPB (Table 2).

The Post Hoc Tukey test for between-group comparison revealed that DNA mutations in *P. cristatus* kept at WPM and JWPL were nearly similar. However, the DNA variations in *P. cristatus* kept at WPB differed significantly from the previous groups (Figure 3).

Table 1. Comparisons of Means ± SD, (µg/g) of heavy metals in the blood and feather samples of *P. cristatus* kept at three wildlife parks on wet weight basis.

Metals	Samples	Jallo Wildlife Park Lahore Means ± SD	Wildlife Park Murree Means ± SD	Wildlife Park Bahawalnagar Means ± SD	*P*-value
Cr	Blood	3.79 ± 0.23 [a]	2.02 ± 0.37 [b]	0.20 ± 0.02 [c]	0.000*
	Feather	4.87 ± 1.22 [a]	0.74 ± 0.27 [b]	0.47 ± 0.08 [c]	
	Eggshell	51.02 ± 19.05[a]	13.05 ± 4.66[b]	6.62 ± 1.29[c]	
	Egg content	13.59 ± 0.83 [a]	11.69 ± 1.88 [b]	0.78 ± 0.07 [c]	
Mn	Blood	0.30 ± 0.01 [a]	0.25 ± 0.11 [b]	0.14 ± 0.06 [c]	0.000*
	Feather	0.49 ± 0.20 [a]	0.421 ± 0.06 [b]	0.30 ± 0.06 [c]	
	Eggshell	1.32 ± 0.10[a]	0.99 ± 0.12[b]	0.85 ± 0.02[c]	
	Egg content	1.21 ± 0.16[a]	0.70 ± 0.15[b]	0.31 ± 0.08[c]	
Pb	Blood	0.18 ± 0.02[a]	0.19 ± 0.02[a]	0.13 ± 0.02[b]	0.000*
	Feather	0.43 ± 0.03[a]	0.420 ± 0.05[a]	0.38 ± 0.03[b]	
	Eggshell	3.60 ± 1.48[a]	0.79 ± 0.13[b]	0.69 ± 0.04[c]	
	Egg content	1.30 ± 0.12[a]	0.71 ± 0.22[b]	0.53 ± 0.07[c]	
Ni	Blood	0.19 ± 0.02[a]	0.15 ± 0.01[b]	0.11 ± 0.02[c]	0.000*
	Feather	0.50 ± 0.08[a]	0.31 ± 0.03[b]	0.21 ± 0.02[c]	
	Eggshell	1.03 ± 0.06[a]	0.78 ± 0.06[b]	0.51 ± 0.04[c]	
	Egg content	0.87 ± 0.17[a]	0.62 ± 0.05[b]	0.46 ± 0.11[c]	
Co	Blood	0.08 ± 0.03[a]	0.06 ± 0.03[b]	0.06 ± 0.03[b]	0.000*
	Feather	0.40 ± 0.08[a]	0.28 ± 0.04[b]	0.13 ± 0.02[c]	
	Eggshell	0.43 ± 0.11[a]	0.29 ± 0.13[b]	0.14 ± 0.02[c]	
	Egg content	0.30 ± 0.04[a]	0.23 ± 0.03[b]	0.11 ± 0.06[c]	

* = $P < 0.01$ = highly significant difference.

Discussion

In this research work, blood, feathers and egg samples of *P. cristatus* were utilized as a non-destructive technique to investigate the concentration of heavy metal bio-accumulation. Actually, heavy metal concentrations in blood samples were correlated to the distance from source of pollution (Berglund 2018) and captivity stress (Yasmeen and Asif 2022). Lahore is densely populated, has a heavy traffic load, and is more industrialized as compared to Murree and Bahawalnagar therefore the concentration of Cr is higher in Lahore. The mean concentration of Cr (0.20–3.78 µg/g) in blood was below the threshold level in all the sites as compared to the study reported by Riaz et al. (2021). In the latter study, the mean concentration of Chromium (Cr) in the blood samples of scavenger birds from three waste disposal sites, namely Lakhodair landfill Lahore, Mehmood booti waste dumping site Lahore, and

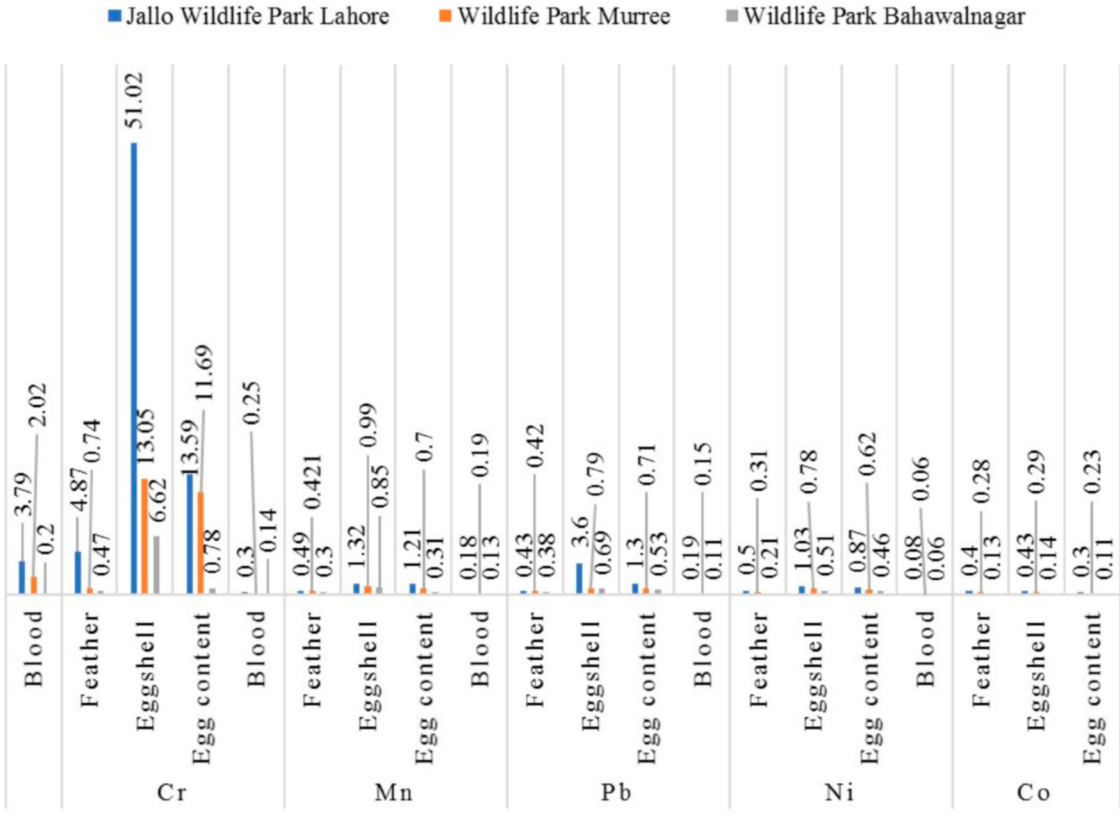

Figure 2. Comparative concentration of all selected heavy metals(µg/g) in the blood, feather and egg samples of *P. cristatus* kept at three wildlife parks on wet weight basis.

Table 2. Assessment of Comet parameters in blood of captive *P. cristatus* at three wilflife parks.

Factors	Jallo Wildlife Park Lahore Mean ± S.D	Wildlife Park Bahawalnagar Mean ± S.D	Wildlife Park Murree Mean ± S.D	*P*-value
LHead	22.20 ± 4.61^{a}	16.36 ± 4.99^{b}	23.88 ± 17.67^{a}	0.00**
LTail	4.96 ± 3.01^{a}	3.28 ± 1.05^{b}	4.44 ± 2.177^{a}	0.00**
LComet	27.16 ± 6.04^{a}	19.64 ± 4.89^{b}	28.32 ± 7.791^{a}	0.00**
HeadDNA	94.79 ± 7.78^{a}	92.53 ± 20.35^{b}	87.36 ± 11.91^{ab}	0.03*
TailDNA	5.21 ± 7.78^{b}	7.47 ± 11.91^{ab}	12.64 ± 20.35^{a}	0.03*
TM	0.41 ± 0.84^{a}	0.34 ± 1.02^{a}	0.76 ± 1.43^{a}	0.15
OTM	0.64 ± 1.17^{a}	0.50 ± 0.75^{a}	0.80 ± 1.37^{a}	0.39

* = $P < 0.05$ = Significant difference; ** = $P < 0.01$ = highly significant difference.

Muhammad wala Faisalabad waste dumping site, was found to be 25 µg/g. The mean concentration of Mn was lower in blood samples in our study as compared to the study of Van Wyk et al. (2001). Khan et al. (2015) reported the mean concentrations of Ni in blood samples of poultry chicken gallus domesticus in three selected cities of Pakistan and showed that Ni concentration in blood samples of Karrachi, Hyderabad and Thatta were 0.543, 0.885 µg/g and 0.402 µg/g, respectively. And this concentration was higher than the concentration determined in our study and its possible reason might be site and species differences. In contrast, the concentration of Co that was measured in our research work was higher compared to that found in the research work of Van Wyk et al. (2001). Cr concentration determined in feather samples of JWPL exceeded the threshold level (2.8 µg/g) similar to work reported by Burger and Gochfeld (2000) and it might be linked to the anthropogenic source of contamination. In current study the mean concentration of Mn was very low in feather samples of *P. cristatus* kept at WPM (0.42 µg/g), JWPL (0.49 µg/g) and WPB (0.30 µg/g) as compared to the study of Malik and Zeb (2009), in which the mean concentration of Mn in the urban and rural feather samples of Bubulcus ibis was 9.65 and 8.79 µg/g respectively. The mean concentration of Pb (0.38 µg/g −0.43 µg/g) measured in the feather samples of *P. cristatus* in study area were much lower than that reported by Tasneem et al. Tasneem et al. who reported concentrations of Pb within 31.62 ± 9.80 µg/g in the tail feathers of *A. grayii* in a research work performed in the outskirts of Lahore, Pakistan and its possible reason might be the type of feather. Ni concentrations in the current research work in feather samples of *P. cristatus* kept at WPM, JWPL and WPB were 0.50, 0.31, and 0.21 µg/g respectively, which were much lower than those found in previous studies, in Pakistan. For example, Abdullah et al. (2015) reported that the concentration of Ni in feathers of birds was 30-47.5 µg/g in Lahore and 77–89 µg/g in Sialkot. In current study work the mean concentration of Cr was very high in eggshell samples of *P. cristatus*, kept at WPM (13.05 µg/g), JWPL (51.01 µg/g) and WPB (6.62 µg/g). Mn concentration (0.84 µg/g −1.32 µg/g) and Pb concentration (0.69 µg/g −3.61 µg/g) was lower and concentration of Ni (0.51 µg/g −1.02 µg/g) was higher than the concentrations reported by Hashmi et al. (2013), who found that the mean concentration of Cr, Mn, Pb and Ni concentration in the eggshell samples of cattle egret (*Bubulcus ibis*) and little egret (*Egretta garzetta*) from the Punjab province, Pakistan ranged from 0.35-0.8µg/g; 0.47-3.98µg/g; 1.05-5.45µg/g and 0.01-0.08µg/g, respectively. For both species, variations in heavy metal concentrations at different sites might represent different exposures due to local variances in pollutant concentrations (Burger et al. 2009), distance from the contaminants source, and habitat characteristics. In the current study, the concentration of Cr in egg content was 13.59 µg/g at JWPL,11.69 µg/g at WPM and 0.79 µg/g at WPB that was much higher than the study reported by Ashkoo et al. (2020), where the concentration of Cr in egg content of seabirds Lesser (*Thalasseus bengalensis*) and Greater Crested Tern (*Thalasseus bergii*) were below the detection limit. In our study the concentration range of Pb and Mn in egg content samples of *P. cristatus* kept at JWPL, WPM and WPB were 0.53 µg/g −1.30 µg/g and 0.31–1.21 µg/g, respectively, similar to the study reported by Kim and Oh (2014), who measured the concentration of heavy metals in egg content samples of black-tailed gull (*Larus crassirostris*) from Korea and found that concentration of lead and Mn were 0.92 µg/g and 1.99 µg/g, respectively. These values were within the range of our research work. The concentration of Ni and Co in egg content samples of *P. cristatus* in our research work were 0.87 and 0.29 µg/g at Jallo Wildlife Park Lahore,0.62 µg/g and0.23 µg/g at WPM and 0.46 and 0.11 µg/g at WPB. The only possible reason might be Lahore is more industrial area as compared to Murree and Bahawalnagar.

The Comet Assay has proven to be a sensitive and efficient technique for assessing DNA damage in birds and animals. This study aimed to measure the relationship between ecological damage and genotoxicity, with a particular focus on heavy metal exposure in captive *P. cristatus* at JWPL, WPB, and WPM.

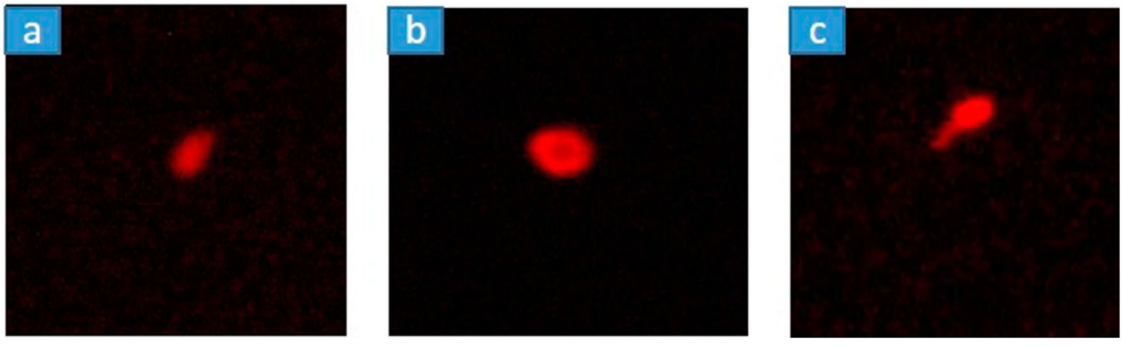

Figure 3. Microscopic examination of blood specimens from captive *P. cristatus* following Comet Assay kept at (a) JWPL (b) WPB (c) WPM.

The results of the current study indicated that Lahore and Murree exhibited higher levels of heavy metal pollution compared to Bahawalnagar. Additionally, the DNA damage was greatest in the birds of WPM and JWPL. These findings are supported by Bonisoli-Alquati et al. (2010), who studied DNA damage in *Hirundo rustica* exposed to low-level radioactive contamination in the Chernobyl region. Their results suggested that birds in contaminated areas exhibited more DNA damage than those in distant regions, which aligns with our study's findings.

A similar study by Gomes et al. (2018) assessed the impact of pollution on DNA impairment in *Geophagus brasiliensis* fish before and after a mining company's tailings influx disaster in Mariana. Indications of genetic harm in fish were also observed previously due to the presence of local industries discharging effluents containing toxic heavy metals. Similarly, Naz et al. (2020) reported DNA damage in captive *P. cristatus* species due to long-term captivity, which is consistent with our study's findings.

In the present study, captive *P. cristatus* experienced stress from captivity, and exposure to pollutants like heavy metals, which could lead to DNA damage. The results demonstrated that DNA damage increased with increasing levels of heavy metal exposure in captive *P. cristatus*. As Lahore exhibited the highest level of heavy metal exposure, the DNA damage was higher in the captive birds kept at WPM and JWPL.

Conclusions

This study demonstrates that captivity plays a significant role in conserving *P. cristatus*; however, environmental pollution have detrimental effects on captive animals. Among the three wildlife parks studied, Wildlife Park Bahawalnagar appears to be the most suitable for captivity, as it exhibits the least accumulation of heavy metals. Chromium was found to be the most accumulated metal compared to others. DNA damage was observed in *P. cristatus* kept at Wildlife Park Murree and Jallo Wildlife Park Lahore, which can be attributed to their increased exposure to pollution and stress. To safeguard this species, proper management and control of environmental pollutants are imperative. Such investigations will aid in implementing effective conservation strategies and mitigating potential threats to wildlife in captivity.

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Ethical statement

The Committee on Animal Rights and Welfare, GC University Faisalabad, Pakistan approved this study (DZ/122/2019).

Disclosure statement

No potential conflict of interest was reported by the author(s).

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