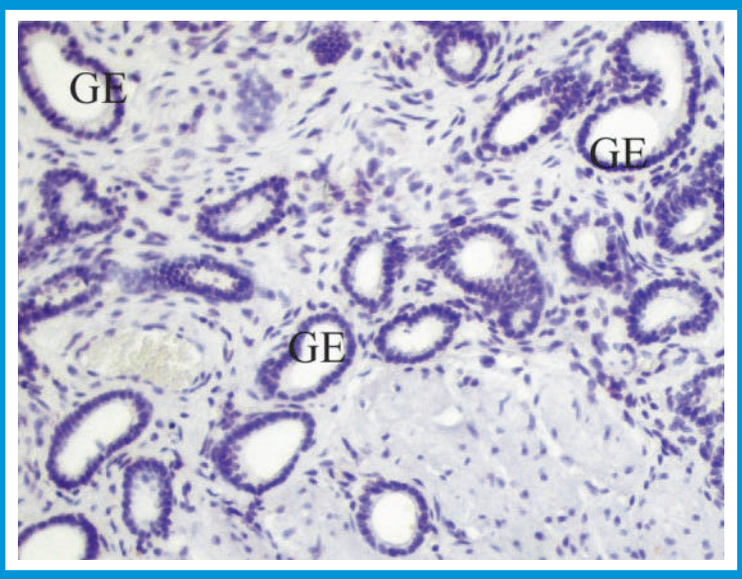
ISSN 1580-4003

THE SCIENTIFIC JOURNAL OF THE VETERINARY FACULTY UNIVERSITY OF LJUBLJANA

SLOVENIAN VETERINARY RESEARCH

SLOVENSKI VETERINARSKI ZBORNIK



Slov Vet Res 2022; 59 (2): 71–124

Volume 59 2

Slov Vet Res • Ljubljana • 2022 • Volume 59 • Number 2 • 71–124

THE SCIENTIFIC JOURNAL OF THE VETERINARY FACULTY UNIVERSITY OF LJUBLJANA

SLOVENIAN VETERINARY RESEARCH

SLOVENSKI VETERINARSKI ZBORNIK

Volume 59 2

Slov Vet Res • Ljubljana • 2022 • Volume 59 • Number 2 • 71–124

The Scientific Journal of the Veterinary Faculty University of Ljubljana

SLOVENIAN VETERINARY RESEARCH
SLOVENSKI VETERINARSKI ZBORNIK

Previously: RESEARCH REPORTS OF THE VETERINARY FACULTY UNIVERSITY OF LJUBLJANA
Prej: ZBORNIK VETERINARSKE FAKULTETE UNIVERZA V LJUBLJANI

4 issues per year / izhaja štirikrat letno
Volume 59, Number 2 / Letnik 59, Številka 2

Editor in Chief / glavna in odgovorna urednica: Klementina Fon Tacer

Co-Editor / sourednik: Modest Vengušt
Technical Editor / tehnični urednik: Matjaž Uršič
Assistant to Editor / pomočnica urednice: Valentina Kubale Dvojmoč

Published by / Založila: University of Ljubljana Press / Založba Univerze v Ljubljani
For the Publisher / Za založbo: Gregor Majdič, the Rector of the University of Ljubljana / rektor Univerze v Ljubljani

Issued by / Izdala: Veterinary Faculty University of Ljubljana / Veterinarska fakulteta Univerze v Ljubljani
For the Issuer / Za izdajatelja: Breda Jakovac Strajn, the Dean of the Veterinary Faculty / dekanja Veterinarske fakultete

Address: Veterinary Faculty, Gerbičeva 60, 1000 Ljubljana, Slovenia
Naslov: Veterinarska fakulteta, Gerbičeva 60, 1000 Ljubljana, Slovenija
Tel.: +386 (0)1 47 79 100, Fax: +386 (0)1 28 32 243
E-mail: slovetres@vf.uni-lj.si

Sponsored by the Slovenian Research Agency
Sofinancira: Javna agencija za raziskovalno dejavnost Republike Slovenije

ISSN 1580-4003
Printed by/tisk: DZS, d.d., Ljubljana, June 2022
Number of copies printed / Naklada: 220
Indexed in/indeksirano v: Agris, Biomedicina Slovenica, CAB Abstracts, IVSI
Urlich's International Periodicals Directory, Science Citation Index Expanded,
Journal Citation Reports – Science Edition
https://www.slovetres.si/

SLOVENIAN VETERINARY RESEARCH
SLOVENSKI VETERINARSKI ZBORNIK

Slov Vet Res 2022; 59 (2)

Slov Vet Res 2022; 59 (2): 75–87
DOI 10.26873/SVR-1464-2022
UDC 636.3.09:578.833.3:616-036.22(292.5)

Review Article

REGIONAL EPIDEMIOLOGY AND ASSOCIATED RISK FATORS OF PESTE DES PETITS RUMINANTS IN ASIA – A REVIEW

Munibullah Munibullah[1, 2, 3], Yanmin Li[2]*, Kainat Munib[4], Zhidong Zhang[1, 2]

[1]State Key Laboratory of Veterinary Etiological Biology, Lanzhou Veterinary Research Institute, Chinese Academy of Agricultural Sciences, Lanzhou, China, [2]College of Animal Husbandry and Veterinary Medicine, Southwest Minzu University, Chengdu, Sichuan, China, [3]Department of Clinical Studies, Faculty of Veterinary and Animal Sciences, PMAS-Arid Agriculture University Rawalpindi, Pakistan, [4]Department of Sociology, Allama Iqbal Open University, Islamabad, Pakistan

Email: drlym@163.com

Abstract: Peste des petits ruminants (PPR) is *World Organization* for Animal Health (OIE) notifiable, economically important transboundary, highly contagious, and an acute viral disease of small ruminants. The disease is caused by the PPR virus (PPRV). PPRV belongs to the genus *morbillivirus* of the family *paramyxoviridae*. The recent epidemiological and molecular characterization of PPR virus isolates subdivides them into four genetically distinct lineages (I, II, III and IV). The disease is endemic across Asia, the Middle East and African regions and is considered a major obstacle to the development of sustainable agriculture across the developing world due to its huge burden on the economy and development of the affected countries and has recently been targeted by the Food and Agriculture Organization (FAO) and the OIE for global eradication by 2030. PPR-endemic countries should join the regional force, and implement regional road maps for the progressive and successful control and elimination of PPRV. In this review, the regional epidemiology of PPR outbreaks and associated risk factors, including animal factors (age, species and sex), environmental factors (season, spatial distribution of disease in various locations) and trade associated factors with special reference to the PPR affected countries in South, Central and East Asia are comprehensively discussed.

Key words: epidemiology; PPR-virus; risk factors; control; eradication; Asia

Introduction

Peste des Petits Ruminants (PPR) are a worldwide prevalent infectious disease of domestic and wild small ruminants and a threat to food safety, prosperity of humans and animals across Asia, the Middle East and African regions (1, 2, 3). PPR infection was first described in Côte d'Ivoire, West Africa in 1942. In 1987, PPR appeared in the Middle East and has since then been confirmed in Saudi Arabia (4), Jordan (5), India (6), Bangladesh (7), Pakistan (8), Iraq (9), Afghanistan (10), Turkey (11, 12, 13, 14), Kazakhstan (15), Tajikistan (16), China (18) Nepal and Bhutan (17). PPR has received a growing attention because of its continuing spread and economic impacts (19). Phylogenetically, PPRV can be classified into four distinct lineages based on the fusion (F) and nucleocapsid (N) genes. PPRV lineages I and II are confined to West Africa. Lineage III is limited to East Africa and Middle East. Lineage IV was found only in Asia, but has spread into African territories over the past double decades (3, 17, 20, 21, 22, 23, 24). *The* disease *is characterized* by fever, stomatitis, oculonasal discharges pneumonia and diarrhea. With different outbreaks condition morbidity & mortality rates *vary and can reach* up to 100% (19). In acute cases of PPR mortality fluctuates from 70-80% with duration between ten and twelve days. The postmortem inspection revealed dark red parts (congestion) in

Received: 24 November 2021
Accepted for publication: 11 April 2022

different lobes of the lungs, small and large intestines (25). On behalf of existing epidemiological data, the virus exists in 65 countries in the world, with more than 20 countries additionally categorized as "at risk". A recent cost-benefit investigation, determined that the worldwide eradication of PPR will provide $74 billion profit over fifteen years (26). Developing countries have more than 90% of the world's small ruminant population, providing food, trade revenue from animals and animal goods; also improve economic strength and livelihood of smallholder holdings (27). Mass vaccination of sheep and goats in endemic countries might be a sensible tactic to control PPR in the first phase of disease eradication (3, 28, 29).

Succeeding Rinderpest eradication in 2011, the FAO & OIE authoritatively stated PPR as next targeted disease, to be controlled, eliminated and eradicated. Although there are some tasks completed for the progressive control of PPR in different countries of the region, however it is essential to understand regional disease epidemiology and to establish a countrywide control and eradication program for PPR, which will link with the regional control and eradication setup. Similarly, it is essential to have broad socioeconomic assessments, disease mapping (hot spot) and documentation of the role of buffalo, cattle, yak, camel, wildlife and other species in the transmission dynamics of the disease. Through collective struggles of indigenous, national and international experts and political will, there is high possibility that this shocking disease can be controlled and finally eradicated in the near future (30). Epidemiological approaches concerning PPR eradication will provide significant welfare and sustainability to vulnerable populations of Africa, Middle East and Asia. This review will provide a regional road map for the eradication of PPR in Asia through a clear regional definition of the epidemiology and associated risk factors of PPR for scientists, policymakers and stakeholders of FAO/OIE.

Regional PPR status in Asia

Throughout the developing world, predominantly in Asia PPR is a major risk for the development of sustainable sheep and goat production. The infection in Asia was first described in southern India and causes significant economic losses. Like, in South Asia Association for Regional Cooperation (SAARC) countries, the overall annual estimated losses by PPR in small ruminants were about 3,012.59 million US$ (31, 32). PPR remains endemic in most of the SAARC-countries of the region except Sri Lanka. Maldives and Bhutan had sporadic outbreaks. Recently in 2021, Chinese experts obtained a regional least cost path (LCP) of PPR trans-boundary spread by domestic and wild animal interface at natural grazing zone between China, India, Kazakhstan, Pakistan and Tajikistan (33). The countries in South Asian *region have varied* capacities, capabilities and facilities in the fields of epidemiology, diagnosis and vaccine production. Bangladesh, India, Nepal and Pakistan *have developed a national action plan* for the eradication of PPR in accordance with *the global eradication campaign* (32). Regional predictable pooled prevalence of PPR in small ruminants in Asia is shown in Table.1. A pooled prevalence (pooled analysis) is a statistical technique for combining the results of multiple epidemiological studies. It is often used when the results of individual studies do not allow for a firm conclusion to be drawn.

South Asia

Bangladesh

In Bangladesh, PPR was first reported in goats in 1993 and since then it has been endemically present in the country (35). Most of the previous PPR studies conducted in Bangladesh were based on either serology or clinical signs except a few recent publications with genetic characterization (36, 37). Phylogenetically, the Bangladesh PPRV strains belong to the PPRV lineage IV and formed a separate subgroup closely related to China-Tibet/07 and Indian/TN/VEL/2015 PPRV isolates proving the regional transmission dynamics of the disease. The overall PPR seroprevalence was 21% in 2008, ranging from 6% to 49% in different geographical locations/districts in Bangladesh (38). D*ue* to seasonal disparity the maximum prevalence observed in rainy period, 11.30%, in contrast to midsummer 6.40% also winter period 8.25%. This dissimilarity may be due to diverse topographical zones and study duration. The maximum frequency of PPR was observed in August, 13.75% and October, 11.51% in contrast with the results of Sarker and Islam (39) and Abubakar et al. (40) Who documented

Table 1: Regional predictable pooled prevalence of PPR in small ruminants in Asia (34)

Country	Study population	Pooled estimate prevalence (%)	95% CI (Confidence interval)	Heterogeneity (x2)	*p*-value
Bangladesh	41,418	31.02	26.60–35.45	7,945.5	<.0001
China	1632	42.93	0–88.72	1503.58	<.0001
India	43,838	39.7	33.73–45.68	15,824.5	<.0001
Kazakhstan	679	0.59	0.07–1.11	0.08	0.774
Mongolia	1950	0.81	0.41–1.21	0.18	0.674
Nepal	460	82.61	79.15–86.08	0	—
Pakistan	56,984	43.55	38–22–48.88	8,936.32	<.0001

the peak prevalence in December (31.68%) and 9.43% in April respectively. Outcomes shown that the lowermost incidence was in May (4.09%) in contrast to Abubakar et al. (41) And Sarker & Islam (39) who investigated the lowest incidence in June. This discrepancy may be due to diverse organizational practices, study period and diverse geographical situation, like in Bangladesh mild winter in October to March; humid summer in March to June; warm rainy monsoon from June to October, however Pakistan has four seasons: a cool, dry winter in December to February; a hot, dry spring in March to May; the summer rainy season in June to September; and the retreating monsoon from October to November, on the other hand, in India spring is from Feb to March, summer is from April to June, Monsoon is from July to mid-September, Autumn is from September to November, however, in Nepal, the warmest months are June-July, The winter month with the lowest temperature is January, spring is from Feb to March. Bangladesh faces analogous problems like India, Nepal and Pakistan regarding future control and eradication targets.

India

In India PPR is endemic and is a major threat to about 223 million small ruminants in the country, causing economic damage of about 247,542.3 US$ annually (21, 42). Animals' movement through the borders of states usually acts as a source of epizootic occurrence of PPR, particularly; unrestricted transportation during religious and social festivals pointedly contributes to disease epidemiology and associated risk factors in the region (44). Singh et al. (45) discussed the relationship between migration of animals and transmission of PPRV in nature. Movements of animals between various regions are common, particularly, in the sub-Himalaya, Gujarat and Rajasthan regions (44, 6). Hence, the close relationships between PPR epidemics and movement of small ruminants have been investigated (43, 45). Consistently according to Mahajan et al. (46), the risk of PPR is greater in nomadic animals than in local non-migratory animals. Furthermore, malnutrition, transportation stress and parasitic infestation are the main issues of nomadic flocks which lead towards immunosuppression and susceptibility of flocks to PPRV infection. These findings were also technically supported by the outcomes of other studies who reported PPR outbreaks in migratory and nomadic small ruminants of Himachal Pradesh (47), Rajasthan region (48) and Punjab province of Pakistan (49). In India maximum outbreaks are documented during the winter periods. Hence, immunization takes place prior to the onset of the winter season. The goat PPRV *strains* identified in the recent epidemic of Tripura indicated 99.2 to 99.6% nucleotide resemblances with the Bangladesh strains (50). The overall epidemiological investigation conforms the trans-boundary communication of PPRV with the adjacent neighboring countries.

Nepal

The First outbreak of PPR was investigated in Nepal during 1995 (17, 21) from the Bara, Mahottari, Dhanusha, Sarlahi, Gorkha and Rauthat regions (51) and about 68 districts of Nepal have documented PPR epidemics in all ecological zones with significant economic losses in small ruminant population (52, 53). The PPR virus circulating in different countries of Asia, including Nepal belongs to the lineage (IV) (24) however, the F and N gene sequencing based study

has shown that the virus found in Nepal, India and Bangladesh is more closely related than virus found in the rest of the country (21). According to Acharya et al. (53), eco-zone wise distribution of PPR showed that the PPR outbreaks were reported mostly in the mountain followed by hills and the least in Terai. High incidence of PPR in the mountains may be due to excessive movements of nomadic herd of sheep and goat, common pasture, low vaccine coverage in mountain as compared to other eco-zones. Hence, strategic vaccination campaigns, proper biosecurity, movement control, risk analysis and the early diagnosis need to be implemented for the control of PPR in the country and also the same strategies for the region. The above studies justify the regional links of virus transmission, temporal and spatial pattern of disease distribution in the region, virus lineage distribution and other complex phenomenon of the virus regionally.

Pakistan

In Pakistan PPR was documented in the commencement of 1990s, however, became important during Rinderpest (RP) Eradication Campaign in 1995. During the previous double decade, enormous investigations commenced towards epidemiology of PPR and its risk factor identification, laboratory analysis, immunization and progression in prevention and control strategies (54). The virus circulates endemically in small ruminants throughout Pakistan; PPR outbreaks have risen to terrible level connecting innovative zones (55, 56). Though both sheep and goats are prone to PPRV, however, according to 50 laboratories established outbreak investigations including Zahur et al. (56); PPR is more severe in caprine (goats) than ovine (sheep) which are in Contrast to African region where PPR commonly affects goats (57). A maximum number of PPR cases are recorded during the summer period with a peak during the period between April - July and then the frequency fall again indicating the temporal pattern of PPR (58). A local scarcity of fodder and poor nutritional position of the animals, possibly play a crucial role in the transmission of PPR (59). Current immunization is strongly recommended in certain zones of Pakistan. The vaccine used is created on Nig75/1 strain belonging to lineage II, while in Pakistan field isolates are belonged to lineage IV. Even if the PPR vaccine production capability is present in the country however there is no planned vaccination program going on and there are huge gaps exist between animal producers, livestock officials and policymakers (30, 62, 63, 64).

Central Asia

Afghanistan

PPR is endemic throughout Afghanistan. In Afghanistan, mostly small ruminants are kept by Kuchi nomadic pastoralists and represent the most significant economic strength of 75% rural population. These nomadic pastoralists cover huge ranges of the country, with interrupted stays (stops) at livestock markets, summer grazing zones, and then settling in rural points (villages) during the winter period. Epidemiological the Kuchi communities were identified as the primary sentinel group to be targeted for PPRV investigation because of their way of lifestyle and have great influence on the spread of the disease in Afghanistan and to neighboring countries of the region (63). Afghanistan was qualified in April 2016 for stage 1 of the FAO/OIE Global Strategy for the Control and Eradication of the PPR progressive pathway. In 2018, about 12.5 million small ruminants in Afghanistan were immunized since 2015. In addition to vaccination, between 2015 and 2017 about 3,004 serum samples in pre and post-vaccination stages were collected and tested in the Central Veterinary Diagnostic and Research Laboratory. The number of outbreaks in seven Asian regions increased between 2015 to 2019, including Afghanistan, which had 824 outbreaks reported (63, 64). Afghanistan is a conflict-hit country due to Taliban militancy having a lack of advanced diagnostic tools and research institutions, so poor data is available on PPR in the country; however, there is an animal movement from Afghanistan to Khyber Pakhtunkhwa and Baluchistan provinces of Pakistan; hence it is observed in these provinces that PPR is reported in large scale by Pakistanian veterinarians and researches (Personal Observation). It is necessary to investigate PPR from root and to know the epidemiology of disease for a better contribution in the region.

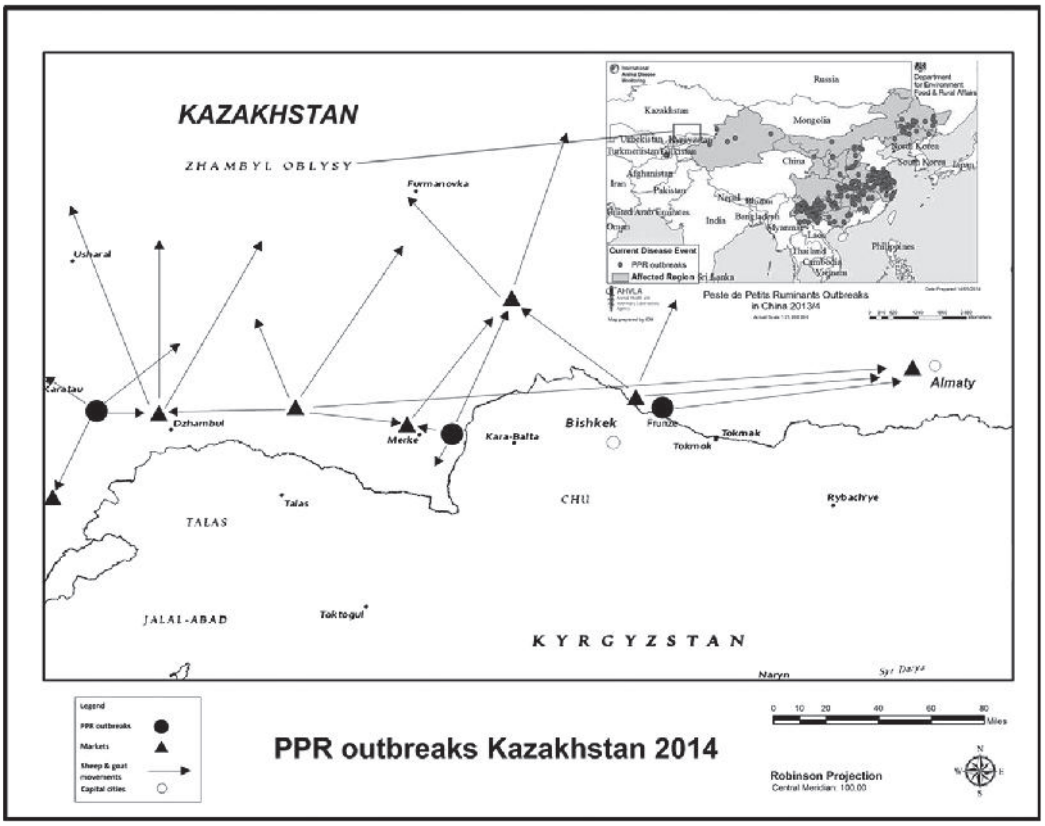


Figure 2: MMap of recent PPR outbreaks reported in Asia and Zhambyl oblast marked by the rectangular box on the conti-nental map (AHVLA 2014). The oblast map extracted to show the three outbreak sites (black dots) reported in this study in the Zhualy, Merke and Korday districts (rayon) and showing small ruminant movement and trade patterns (arrows) (65)

Kazakhstan

An epidemiological sero-survey of livestock in Kazakhstan was documented in the period between 1997-1998. In this survey several OIE List-A diseases in Central Kazakhstan were investigated. Kazakhstanian are nomadic livestock producers, because most of Kazakhstan is unsuitable for agriculture due to its semi-arid range land status. About 958 serum samples from different animals (sheep, cattle and goats) were tested for antibodies against different infections, including PPR with few reported seropositives for PPRV (15). Up to the end of 2014; no PPR cases were officially reported to the OIE from Kazakhstan. Kock et al. (65) Reported clinicopathological, epidemiological and genetic characterization of PPRV in three farm level outbreaks in Zhambyl region in southern Kazakhstan for the first time. Phylogenetic investigation based on partial N gene classification facts conorms the lineage IV PPRV spread, similar to the situation in China. The isolated viruses were 99. 5 - 99. 7% similar to the PPRV isolated in 2014 from Heilongjiang Province in China and therefore this investigation suggest the cross boundary transmission of PPRV. Despite the vaccination of an adult sheep and goats, there is a risk of further maintenance of virus in young stock. Along livestock trading and pastoral routes, threat to both small ruminants and endangered susceptible wildlife populations throughout Kazakhstan and to the neighboring regions.

Mongolia

Between 2016–2017 PPRV was introduced in livestock due to infection in Mongolian saiga antelope and other endangered wild ungulates (66). In fall 2016, PPRV outbreak among domestic small ruminants was confirmed in western region of Mongolia; (67, 68). A total, 83,889 sheep and goats from about 1,081 families were affected by PPR in 14 districts of 3 provinces, of which 12,976 small ruminants expired. The overall case-fatality was 15.5% (57). The saiga antelope

(*hereafter saiga*) occupies <20% of range area in 2 provinces (Khovd and Gobi-Altai) of Mongolia, this historic range representing 36,000 km^2 of desert steppe bordered by lakes, high mountain regions and sand dunes (68). In 8 soums sheep and goats are >1.5 million, dominated by livestock and covering both mountain and desert steppe areas which are considered as the saiga seasonal grazing range (69). Mapping of PPRV outbreak suggest that wildlife might be infected before (possibly in July 2016) the first case was confirmed in December 2016 and also that wildlife infections was closely following the temporal pattern of the livestock outbreak. Strong epidemiological investigations show that these cases were fragments of the same PPRV outbreaks, suggesting spillover of virus from livestock at multiple locations and time points and subsequent spread among wild ungulates with a decline of 80% saiga, raising substantial concerns for the species' survival. Consideration of the entire ungulate community (wild and domestic) is essential for elucidating the epidemiology of PPRV in Mongolia, addressing the threats to wild ungulate conservation, and achieving global PPRV eradication (66).

Tajikistan

PPR was reported in Tajikistan annually between 2005 and 2014. In 2005, samples from sick and dead goats from different farms in Tajikistan demonstrated the occurrence of PPR in Central Asia (70). In 2006, seroprevalence of PPR in small ruminants was reported in Tajikistan and Kyrgyzstan in samples taken from livestock before the vaccination campaign started in the Central Asian region; however, no virus was isolated (71). A study conducted by Kwiatek et al. (16) in which sporadic occurrence of PPR in three districts of Tajikistan was described. The causal strain (PPR Tajikistan) was characterized and the sequence of its N gene was compared with 43 other strains isolated since 1968 in Africa, the Middle East and Asia. The local veterinarians described the outbreaks first as *Pasteurellosis*, but later on it was described as the sporadic occurrence of PPR in Tajikistan. Tajikistan is deficient in research and innovation so limited work has been reported on PPR; however, the disease is endemic in neighboring countries like Afghanistan, Iran and Pakistan. It is important to include Tajikistan in the regional control and eradication program in the future to tear out the root of the disease (PPR) from the Geo-strategically and socioeconomically point of view.

East Asia

China

In 2007, the first epizootic of PPR reported in the Ngari region of Tibet in China, resulting 5751 deaths of sheep population (18). In October 2007, wild bharals (*pseudois nayaur*) were infected in Ge'gyai region. Specimen from domestic small ruminants and bharals were closely associated (72). PPR emerged in Xinjiang in China during 2013, and quickly spread to the rest of the country, including Anhui, Guangxi, Guizhou, Hunan, Hubei, Shanxi, Yunnan, Xinjiang, and Zhejiang Provinces by the first half of 2014 (73). Chinese isolates found these two epizootics were closely linked to isolate obtained from neighboring countries belonging to lineage IV, with diverse divisions (74). Interestingly, Xinjiang has borders with Afghanistan, India, Kazakhstan, Kyrgyzstan, Mongolia, Pakistan, Russia and Tajikistan. Several neighboring countries have reported PPRV infection. The PPRV strain recognized in Xinjiang during 2013–2014 showed a greater genomics resemblance to the strains obtained from Pakistan, Tajikistan and Tibet region (75, 16). In 2008, PPRV was controlled by using stamping-out procedures, animal movement control, and increased screening of herds, while the first vaccination was carried out in 2010 (76). In bird eye view, the details of PPRV transmission dynamics to China fully discovered and observed that the two outbreaks of PPR in China may be independent incidents originated by the communication of the virus from the neighboring endzootic states. Similarly, the threat of further spread from China to neighboring states cannot be ignored (77).

Regional distribution of host in the region

Sheep and goats are usually a host of PPRV, but goats are affected more rigorously as compared to sheep (19). Clinically sheep rarely suffer, even though high mortality and morbidity rate has

been documented, but it is anticipated that sheep embrace inborn resistance to clinical disease (78). PPR has also been investigated in farm / domesticated animals, i.e. buffaloes, cattle, goats, sheep, yaks and camels (59, 66, 79, 80). However, field outbreaks from a zoological group were also reported in Alain (81). PPRV also has been reported in gazelle world widely (4). Consistently the evidence of the disease also exists in Dorcas Gazelles (*gazella dorcas*), Antelope, Laristan sheep (*ovis orientalis laristani*), Nubian Ibex (*capra ibex nubiana*), Nigale (*tragelaphinae*) and gemsbok (*oryx gazella*) (82). PPR has been investigated, found in wild animals, including Saiga antelope (*saiga tatarica mongolica*) and Sindh Ibex, (*saiga tatarica mongolica*) (66, 60, 83).

Disease pattern and seasonal occurrence

Movement of animals within the same country or trans-boundary migration is responsible for the dynamic of the disease incidence. In dry season animal movement takes place in search of water and fodder (6). In tropical regions, PPR constantly appeared in an epidemic. PPR has been often fatal and typically occurs as a sub clinical in arid and semi-arid areas (19). PPRV is constantly circulating between age groups of 4-24 months. However, morbidity and mortality are observed high in all the age groups (4).

Climatic factors affect occurrence of PPR. PPR outbreaks, mainly decline in rainy season due to increased nutrition, health status, fodder availability and decreased animal movement. In dusty and dry season (Dec-Feb) there is poor nutritional status of animals that leads to disease spreading and cases reach peak in rainy season. Khan et al. (58) investigated the highest frequency of PPR in Dec-Feb & Sept-Oct while Abubakar et al. (40) identified maximum case reports in Jan-Apr and 33% of cases detected in March. In China, a study conducted regarding patterns of seasonality of the disease with a peak in April in 2014 (77). According to Abubakar et al. (76), most of PPR cases arise in the beginning of spring with a peak during the rainy period indicating the temporal pattern of disease. According to reported data, we could conclude that the disease is present throughout the year with the severe discrepancy in different weathers.

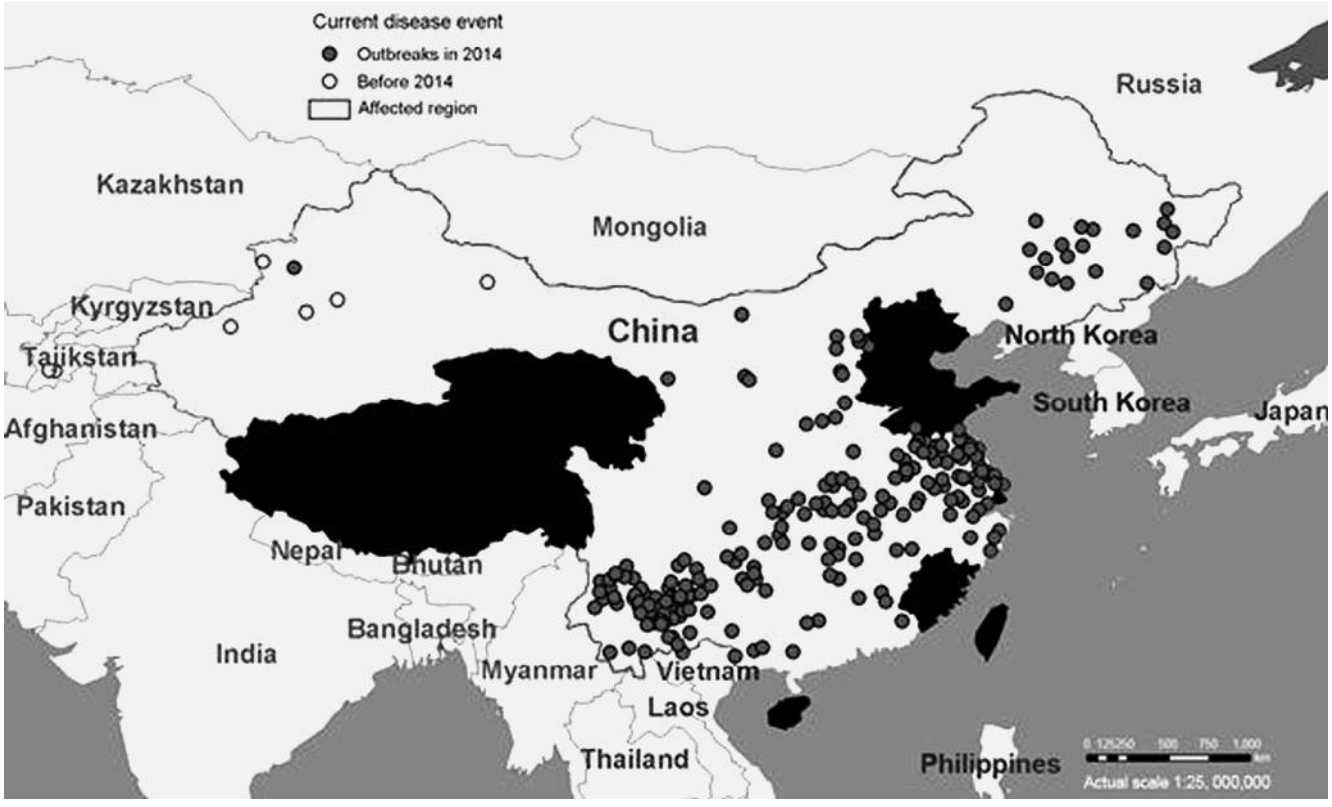


Figure 2: Outbreaks of peste des petits ruminants virus (PPRV) across China during December 2013–May 2014. Data are from ProMed alerts during the period described (76)

Associated-Risk Factors of PPR

Animal Factors

Age

Age is one of the main animals associated risk factors for PPR. The mature animals are more probable to be seropositive for PPR as compared to younger animals (56).

Species

Although PPR affects sheep, goats and as was recently reported that PPR affect also other species, including cattle, buffaloes, camel and wild small ruminants (Sindh ibex). PPR has diverse severity and prevalence among various species, even in goats and sheep's. Prevalence of PPR is higher in goats in comparison with sheep in numerous documentations of many regions (40, 45, 84, 85, 86, 87, 88). Transmission of PPRV amongst the wildlife population at risk significantly associated with closed contact rate among a population of diverse or similar species (72). China investigates PPRV in free-living bharals (pseudois nayaur) in the Tibet region (49). So it is investigated that PPRV, circulates in wild animals and acts as a potential source of virus for domestic species with the possibility of wild small ruminants serving as a reservoir of PPRV and playing an important role in PPR control strategies and could also act as a constraint to the region as well as worldwide eradication of PPR on the other hand in the future.

Sex

Jalees et al. (85) Described that sheep indicated greater sero-positivity in the female's than in males. Furthermore, this scene has been supported by Nanda et al. (6) who reported that male is generally slaughtered at younger stage and female goats and sheep are kept in flocks. These situations remain the same in neighbor countries like, India, Nepal, Afghanistan, and Kazakhstan etc.

Environmental Factors

Season

Seasonal animal movements can spread PPRV over significant distances across states and even continents. Though the disease is endemic in different countries of the region yet few case reports of its seasonal occurrence exist. The incidence of PPR is decreased in rainy season, due to sufficient amounts of forage accessibility leading to increased resistance against the disease (40). Seasonal movements, large flock size, inadequate veterinary services and mixing of animals that visit animal market are the main risk factors of PPR outbreaks in different regions (87). In dry season, small ruminants typical travel long distances in search of forage and water (19). In tropical regions, epidemic form of PPR occurs, however the disease is often lethal and typically occurs as a sub clinical in arid and semi-arid areas (57). So for control strategies zonal division with the countries of Asian region is essential to categorize regions with hot, humid, arid and semi-arid spots, as well as to categorize regions on the basis of sporadic, endemic and epidemic occurrence of PPR.

Spatial distribution of PPR in various locations

Although both sheep and goats are prone to PPRV and incidence of disease takes place yet the pattern of disease are not always the same, like in the African region PPR is observed mostly in goats, however in Southwestern Asia sheep are more affected by PPRV (89). In Pakistan, as compare to India, Bangladesh, Nepal, and Afghanistan, PPR affects both sheep and goats, but in many areas it is seen that only goats are affected (90) consistent with the results of Obi et al. (91).

Trade-associated factors

Trading of small ruminants and associated factors play an important role in the spread and propagation of PPR outbreaks (92). Trade associated consequences of PPR caused a numerous economic influence and the direct production losses in the region. For example, goat entrepreneurship in Laos is the smallest livestock sector trade, the current rise of mutton prices in Vietnam and China has pointed a "goat boom" in South East Asia. Laos is a landlocked country with "porous" borders now "at risk" of PPRV spread due to: closeness and trade with China and other Asian countries. Trade, travel or natural infection can transmit an organism into a

previously unaffected country or region. PPR free countries protect their native production system by excluding import of animal products from PPRV affected regions or import make conditional upon a series of counteractive actions. Overall local, national, and international efforts for the control of trans-boundary animal diseases, including PPR must be designed for achieving the "optimal" level of protection, where the marginal cost of control is equivalent to marginal benefit (93).

Conclusion

The Asian region has millions sheep and goat population and certain countries of the region have an endemic situation. Most of the countries have no organized epidemiological investigation and vaccination campaign of PPR in their territories; PPR is continuing threat to food safety and economy. Although various projects launched by the FAO & OIE for the progressive control and even for risk based eradication of PPR in different countries; however there is a need to understand, comprehensive regional epidemiology, associated risk factors and geostrategic importance of PPR endemic countries for progressive control strategies. This goal could only be achieved by the combined efforts of local, national and regional stakeholders as well as local, national and regional political will; along with continuous financial as well as strategic support and strengthening by international agencies and stakeholders.

Acknowledgments

This work was supported by the National Key R & D Program of China (2016YFD0500108; 2016YFE0204100) and Department of Clinical Studies (Epidemiology Section), Faculty of Veterinary and Animal Sciences, Pir Mehr Ali Shah Arid Agriculture University Rawalpindi, Pakistan.

The authors have declared no conflict of interest.

No ethical approval was required as this is a review article with no original research data.

References

1. Baron MD, Diop B, Njeumi F, Willett BJ, Bailey D. Future research to underpin successful peste des petits ruminants virus (PPRV) eradication. J Gen Virol 2017; 98(11): 2635–44.

2. Kumar N, Maherchandani S, Kashyap SK, et al. Peste des petits ruminants virus infection of small ruminants: a comprehensive review. Viruses 2014; 6: 2287–327.

3. OIE. Infection with Peste des petits ruminants. In: Terrestial animal health code. [online] Paris : Office International des Epizooties, 2016: chapter 14.7. https://www.woah.org/fileadmin/Home/eng/Health_standards/tahc/current/chapitre_ppr.pdf

4. Abu Elzein EME, Hassanien MM, Alfaleg AIA, Abd Elhadi MA, Housawi FMT. Isolation of PPR virus from goats in Saudi Arabia. Vet Rec 1990; 127: 309–10.

5. Lefevre PC, Diallo A, Schenkel F, Hussein S, Staak G. Serological evidence of Peste des petits ruminants in Jordan. Vet Rec 1991; 128: 110.

6. Nanda YP, Chatterjee A, Purohit AK, et al. The isolation of Peste des petits ruminants virus from northern India. Vet Microbiol 1996, 51(3/4): 207–16.

7. Sil BK, Rahman MM, Taimur MJFA, Sarker AJ. Observation of outbreaks of PPR in organized goat farms and its control strategy. In: Annual Conference of Bangladesh Society for Veterinary Education and Research. Dhaka : BARC, 1995.

8. Amjad, H, Muhammad QI, Forsyth AM, Barrett T, Rossiter PB. Peste des petits ruminants in goats. Pak Vet Rec 1996; 139: 118–9.

9. Barhoom SS, Hassan WA, Mohammed TAR. Peste des petits ruminants in sheep in Iraq. Iraq J Vet Sci 2000; 13: 381–5.

10. Abdollahpour GA, Najafi RJ, Sasani F, Sakhaie E. Clinical and paraclinical findings of a recent outbreak of Peste des petits ruminants in Iran. J Vet Med 2006; 53: 14-16.

11. Ozkul A, Akca Y, Alkan F, et al. Prevalence, distribution, and host range of Peste des petits ruminants virus, Turkey. Emerg Infect Dis 2002; 8: 708–12.

12. Toplu N. Characteristic and non-characteristic pathological findings in Peste des petits ruminants (PPR) of sheep in the Ege district of Turkey. J Comp Pathol 2004; 131: 135–41.

13. Anderson J, Sammin D. Peste des petits ruminants in the Thrace region of Turkey. EMPRES Transbound Anim Dis Bull 2005; 27: 12–5.

14. Yesilbag K, Yilmaz Z, Golcu E, Ozkul A. Peste des petits ruminants outbreak in western Turkey. Vet Rec 2005; 157: 260–1.

15. Lundervold M, Milner GEJ, O'Callaghan CJ, Hamblin C, Corteyn A, Macmillan A. A sero-

logical survey of ruminant livestock in Kazakhstan during post-soviet transitions in farming and disease control. Acta Vet Scand 2004; 45: 211–24.

16. Kwiatek O, Minet C, Grillet C, et al. Peste des petits ruminants (PPR) outbreak in Tajikistan. J Comp Pathol 2007; 136(2/3): 111–9.

17. Banyard AC, Parida S, Batten C, Oura C, Kwiatek O, Libeau G. Global distribution of Peste des petits ruminants virus and prospects for improved diagnosis and control. J Gen Virol 2010; 91: 2885–97.

18. Wang Z, Bao J, Wu X, et al. Peste des petits ruminants virus in Tibet, China. Emerg Infect Dis 2009; 15: 299–301.

19. Lefevre PC, Diallo A. Peste des petits ruminants. Rev Sci Tech Off Int Epiz 1990; 9: 951–65.

20. Munir M, Zohari S, Berg M. Molecular biology and pathogenesis of Peste des petits ruminants virus. Berlin : Springer, 2013: 69–104.

21. Dhar P, Sreenivasa BP, Barrett T, Singh RP, Bandyopadhyay SK. Recent epidemiology of peste des petits ruminants virus (PPRV). Vet Microbiol 2002; 8(2): 53–159.

22. Dundon WG, Adombi C, Waqas A, et al. Full genome sequence of a peste des petits ruminants virus (PPRV) from Ghana. Virus Genes 2014; 49(3): 497–501.

23. Muniraju M, Mahapatra M, Ayelet G, et al. Financial impacts of foot-and-mouth disease at villgage and national levels in lao PDR. Trans Emerg Dis 2016; 63(5): 403–11.

24. Parida S, Muniraju M, Mahapatra M, Muthuchelvan D, Buczkowski H, Banyard AC. Peste des petits ruminants. Vet Microbiol 2015; 181: 90–106.

25. Diallo AC, Minet CL, Goff G, Berhe E, Albina G, Barrett LT. The threat of peste des petits ruminants: progress in vaccine development for disease control. Vaccine 2007; 25: 5591–7.

26. Jones BA, Rich KM, Mariner JC, et al. The economic impact of eradicating peste des petits ruminants: a benefit-cost analysis. PLoS One 2016; 11(2): e0149982 doi: 10.1371/journal.pone.0149982

27. Herrero M, Grace D, Njuki J, et al. The roles of livestock in developing countries. Animal 2013; 7(suppl.1): 3–18.

28. Albina E, Kwiatek O, Minet C, Lancelot R, Almeida DRS, Libeau G. Peste des Petits Ruminants, the next eradicated animal disease? Vet Microbiol 2016; 165(1/2): 38–44.

29. OIE, FAO. Global strategy for the control and erradication of PPR. Paris : OIE; Rome : FAO, 2015 http://www.fao.org/3/a-i4460e.pdf

30. Abubakar M, Manzoor S, Ali Q. Evaluating the role of vaccine to combat peste des petits ruminants outbreaks in endemic disease situation. J Anim Sci Technol 2015; 57: art 2. doi: 10.1186/s40781-014-0036-y

31. Singh B, Bardhan D, Verma M, Prasad S, Sinha D. Estimation of economic losses due to Peste de petits ruminants in small ruminants in India. Vet Worl 2014; 7(4): 194–9.

32. Samad MA, Yousuf MA, Siddiky MNA, et al. SAARC Regional training on molecular diagnosis and laboratory surveillance of PPR. Savar, Dhaka : Bangladesh Livestock Research Institute, 2019.

33. Gao S, Xu G, Zeng Z, et al. Transboundary spread of peste des petits ruminants virus in western China: a prediction model. PLoS One 2021; 16(9): e0257898. doi: 10.1371/journal.pone.0257898

34. Ahaduzzaman M. Peste des petits ruminants (PPR) in Africa and Asia: a systematic review and meta-analysis of the prevalence in sheep and goats between 1969 and 2018. Vet Med Sci 2020; *6*(4): 813–33.

35. Islam MR, Shamsuddin M, Rahman MA, Das PM, Dewan ML. An outbreak of peste des petits ruminants in Black Bengal goats in Mymensingh, Bangladesh. Bangl Vet 2001; 18: 14–9.

36. Chowdhury EH, Bhuiyan AR, Rahman MM, Siddique MS, Islam MR. Natural peste des petits ruminants virus infection in Black Bengal goats: virological pathological and immunohistochemical investigation. BMC Vet Res 2014; 10: e263. doi: 10.1186/s12917-014-0263-y

37. Rahman MA, Shadmin I, Noor M, Parvin R, Chowdhury EH, Islam MR. Peste des petits ruminants virus infection of goats in Bangladesh: pathological investigation, molecular detection and isolation of the virus. Bangl Vet 2011; 28(1): 1 –7.

38. Bhuiyan AR. Epidemiology and pathology of peste des petits ruminants (PPR) in Bangladesh and molecular characterization of the virus. Mymensingh : Bangladesh Agricultural University Department of Pathology, 2012. PhD thesis

39. Sarker S, Islam MH. Prevalence and Risk Factor Assessment of Peste des petits ruminants in goats in Rajshahi, Bangladesh. Vet World 2011; 4(12): 546–9.

40. Abubakar M, Jamal SM, Arshed MJ, Hussain M, Ali Q. Peste des petits ruminants virus

(PPRV) infection: its association with species, seasonal variations and geography. Trop Anim Health Prod 2009; 41: 1197–202.

41. Government of India, Ministry of Agriculture, Department of Animal Husbandry, Dairying and Fisheries (DAHDF) (2003). Total number of livestock and poultry-2003-State-wise (provisional). Government of India, Ministry of Agriculture, DAHDF, New Delhi, India. www.dahd.nic.in/relcensus.htm accessed on (20. Aug. 2009)

42. Bhadyopadhyay SK. The economic appraisal of a PPR control pro-gramme in India. In: 14th Annual Conference and National Seminar on Management of Viral Diseases with Emphasis on Global Trade and WTO Regime. Hebbal Bangalore : Indan Virological Society, 2002.

43. Sudarshan KJ, Rajasekhar M, Upadhye AS. Prevalence of peste des petits ruminant and rinderpest antibodies in small ruminants. Ind Vet J 1995; 72(12): 1246–50.

44. Singh RP, Saravanan P, Sreenivasa B, Singh RK, Bandyopadhyay SK. Prevalence and distribution of Peste des petits ruminants virus infection in small ruminants in India. Rev Sci Tech OIE 2004; 23(3): 807–19.

45. Dhand NK, Sharma CS, Sandhu KS, Sharma DR, Singh J. Out-breaks of Peste des petits ruminants (PPR) in Punjab. Ind J Anim Sci 2002; 72(10): 853–4.

46. Mahajan S, Agrawal R, Kumar M, Mohan A, Pande N. Risk of seroconversion to Peste des petits ruminants (PPR) and its association with species, sex, age and migration. Small Ruminant Res 2012; 104(1/3): 195–200.

47. Joshi VB, Nagal KB, Sharma M, Katoch RC, Batta MK, Sharma AK. Pestedes-petits ruminants (PPR) among gaddi sheep and goats in Himachal Pardesh. Ind J Anim Sci 1996; 66(11): 1126–7.

48. Singh VP, Chum VK, Mondhe KS. Peste des petits ruminants: an outbreak in sheep in Rajasthan. Ind Vet J 1996; 73: 466–7.

49. Khan HA, Siddique M, Arshad MJ, Khan QM, Rahman SU. Seroprevalance of Peste des petitis ruminants (PPR) virus in sheep and goat in Punjab province of Pakistan. Pak Vet J 2007; 27: 109–12.

50. Muthuchelvan D, De A, Debnath B, et al. Molecular characterization of Peste-des-petits ruminant's virus (PPRV) isolated from an outbreak in the Indo-Bangladesh border of Tripura state of North-East India. Vet Microbiol 2014; 174(3/4): 591–5.

51. Jha VK, Singh DB, Thakuri KC, Gautam SP. Peste des petits ruminants: epidemiological status and control approaches adopted in Nepal. In: Gurung TB, Joshi BR, Singh UM, et al., eds. Proceedings of the National Workshop on Research and Development Strategies for Goat Enterprises in Nepal. Kathmandu, Nepal, 2013: 73. https://www.researchgate.net/publication/ 257143203_Proceedings_of_the_National_Workshop_on_Research_and_Development_Strategies_for_Goat_Enterprises_in_Nepal (9. Nov. 2017)

52. DoAH. Annual Epidem Biological Bulletin. Tripureshwor, Kathmandu: Veterinary Epidemiological Centre, Directorate of Animal Health, 2015.

53. Acharya N, Poudel SP, Acharya KP. Cross-sectional sero-prevalence study of Peste des petits ruminants (PPR) in goats of Syangja and Kaski districts of Nepal. VirusDiseases 2018; *29*(2): 173–9.

54. Abubakar M, Irfan M, Manzoor S. Peste des petits ruminants in Pakistan: past, present and future perspectives. J Anim Sci Technol 2015; 57(1): e32. doi: 10.1186/s40781-015-0066-0

55. Ali Q. National Policy for Control of Peste des Petits Ruminants in Pakistan. Islamabad: GCP/PAK/ 088-EC, FAO, 2004.

56. Zahur AB, Ullah A, Hussain M, Irshad H, Hameed A, Jahangir M. Sero-epidemiology of peste des petits ruminants (PPR) in Pakistan. Prev Vet Med 2011; 102: 87–92.

57. FAO. Recognizing Peste des petitis ruminants: a field manual. FAO Animal Health Manual No. 5. Rome : FAO UN 1999: 1–27.

58. Abubakar M, Ashiq S, Hussain Z, Hussain M, Saleha SH, Arshed MJ. Comparison of antigen detection methods of peste des petits ruminants virus in clinical samples of small ruminants. Bulg J Vet Med 2011; 14(2): 103–8.

59. Khan HA, Siddique M, Abubakar M, Javed MA, Hussain M. Prevalence and distribution of peste des petits ruminant's virus infection in small ruminants. Small Ruminant Res 2008; 79: 152–7.

60. Abubakar M, Arshed MJ, Hussain M, Ali Q. Evidence of Peste des petits ruminants in serology of sheep and goats from Sindh, Pakistan. Transbound Emerg Dis 2011; 58(2): 152–6.

61. Abubakar M, Arshed MJ, Zahur AB, Ali Q, Banyard AC. Natural infection with peste des petits ruminants virus: a pre and post vaccinal assessment following an outbreak scenario. Virus Res 2012; 167: 43–7.

62. Odo BI. Comparative study of some prevalent diseases of ecotype goats reared in southeast-

ern Nigeria. Small Ruminant Res 2003; 50(1/2): 203–7.

63. Azizi N, Farid A. Peste des petits ruminants in Afghanistan: the role of veterinary para-professionals in successful implementation Kabul : Ministry of Agriculture, Irrigation, and Livestock. https://oiebulletin.com/?panorama ppr control and eradication programme in afghanistan Bull OIE 2010(2) doi: 10.20506/bull.2018.2.2877

64. Zhao H, Njeumi F, Parida S, Benfield CTO. Progress towards eradication of Peste des petits ruminants through vaccination. Vir 2021; 13(1): e 59. doi: 10.3390/v13010059.

65. Kock RA, Orynbayev MB, Sultankulova KT, et al. Detection and genetic characterization of lineage IV peste des petits ruminant virus in Kazakhstan. Trans Eemerg Dis 2015; 62(5): 470-9.

66. Pruvot M, Fine AE, Hollinger C, et al. Mongolian saiga and other wild ungulates, Mongolia, 2016–2017. Emerg Infect Dis 2020; 26(1): e51–62. doi: 10.3201/eid2601.181998.

67. Shatar M, Khanui B, Purevtseren D, Khishgee B, Loitsch A, Unger H. First genetic characterization of peste des petits ruminants virus from Mongolia. Arch Virol 2017; 162: 3157–60.

68. Ts U, Uuganbayar E, Odbileg R. Sero-surveillance of "Peste des Petits Ruminants" PPR in Mongolia and development of recommendation. Mong J Agric Sci 2017; 19: 22–6.

69. Lkhagvadorj D, Hauck M, Dulamsuren C, Tsogtbaatar J. Pastoral nomadism in the forest-steppe of the Mongolian Altai under a changing economy and a warming climate. J Arid Environ 2013; 88: 82–9.

70. Orynbayev MB, Mamadaliyev SM, Koshemetov ZK, Nurabayev SS. Peste des petits ruminants in the Republic of Tajikistan. In: Urgent problems of veterinary medicine and agricultural biotechnology. Materials of the International Science and Practice Conference. Pavlodar, 2005: 66–71 (in Russian).

71. Orynbayev MB, Mamadaliyev SM, Khairullin BM, et al. Serological monitoring of PPR among animals in Central Asia. In: 3rd Science and Practice Conference "Problems of infectious pathology in the regions of Siberia, far east and extreme north". Novosibirsk, 2006: 176–7. (in Russian).

72. Bao J, Wang Z, Li L, et al. Detection and genetic characterization of Peste des petits ruminants virus in free-living bharals (*Pseudois nayaur*) in Tibet, China. Res Vet Sci 2011; 90(2): 238–40.

73. Bao J, Wang Q, Zhang Y, Liu C, Li L, Wang Z. Complete genome sequence of a novel variant strain of Peste des petits ruminants virus, China/XJYL/2013. Genome Announc 2014; 2(5): e00762–14. doi: 10.1128/genomeA.00762-14

74. Xia J, Zheng XG, Adili GZ, Wei YR, Huang J. Sequence analysis of peste des petits ruminants virus from ibexes in Xinjiang, China. Genet Mol Res 2016; 15(2): gmr7783. doi: 10.4238/gmr.15027783

75. Anees M, Shabbir MZ, Muhammad K, et al. Genetic analysis of peste des petits ruminants virus from Pakistan. BMC Vet Res 2013; 9: e60. doi: 10.1186/1746-6148-9-60

76. ProMedMail. Peste des petits ruminants-China (11): Sheep, goat, spread, OIE, request for information. ProMed Posts May 5, 2014. http://www.promedmail.org/promed-posts/ (archive no. 20140511.2465094).

77. Gao X, Liu T, Zheng K, Xiao J, Wang H. Spatio-temporal analysis of peste des petits ruminants outbreaks in PR China (2013–2018): updates based on the newest data. Trans Emer Dis 2019; *66*(5): 2163–70.

78. Shaila MS, Purashothaman V, Bhavsar D, Venugopal K, Venkatesan RA. Peste des petits ruminants of sheep in India. Vet Rec 1989; 125: 602.

79. Abubakar M, Rajput ZI, Arshed MJ, Sarwar G, Ali Q. Evidence of peste des petits ruminants virus (PPRV) infection in Sindh ibex (*Capra aegagrus blythi*) in Pakistan as confirmed by detection of antigen and antibody. Trop Anim Health Prod 2011; 43: 745–7.

80. Ismail TH, Yamanaka MK, Saliki JK, Elkhoy A, Mebus C, Yilma T. Cloning and expression of the nucleoprotein of PPR virus in baculovirus for use in serological diagnosis. Virology 1995; 208: 776–8.

81. Furley CW, Taylor WP, Obi TU. An outbreak of Peste des petits ruminants in a zoological collection. Vet Rec 1987; 121: 443–7.

82. Abu Elzein EME, Housawi FMT, Bashareek Y, Gameel AA, Al-Afaleq AI, Anderson ECEC. Severe PPR infection in Gazelles kept under semi-free range conditions in Saudi Arabia. J Vet Microbiol B 2004; 51(2): 68–71.

83. Office International des Epizooties (OIE). OIE Manual of Standards for Diagnostic Tests and Vaccines. List A and B Diseases of Mammals, Birds and Bees. Paris: Office International des Epizooties. 2000.

84. Durrani AZ, Kamal N, Mehmood N, Shakoori AR. Prevalence of peste des petits ruminants

(KATA) in sheep and goats of Punjab. Pak J Zool 2010; 42: 211–6.

85. Jalees MM, Hussain I, Arshad M, Muhammad G, Khan QM, Mahmood MS. Occurrence of peste des petitis ruminants in five districts of Punjab, Pakistan. Pak Vet J 2013; 33(2): 165–9.

86. Kumar P, Kumar R, Sharma A, Tripathi BN. Pathology of peste des petits ruminants (PPR) in goats and sheep: spontaneous study. Ind J Vet Pathol 2002; 26: 15–8.

87. Abubakar M, Arshed MJ, Zahur AB, Ullah F, Ishfaq F, Ali Q. Post outbreak profile of Peste des petits ruminants (PPR) virus antibodies in relation with vaccination in recovered goats. Pak J Life Soc Sci 2011; 9(2): 169–71.

88. Munir M. Role of wild small ruminants in the epidemiology of peste des petits ruminants. Transboun Emerg Dis 2014; 61.5: 411–24

89. Taylor WP, Ali Q. Recognizing PPR. In: A field manual of Pakistan. Government of Pakistan. Strengthening of Livestock Services Project, Federal Project management unit. EU/ GOP/ PAK/ RELEX/2001/0129. 2005.

90. Abubakar M, Jamal SM, Khan MA, Ali Q. Peste des petits ruminants outbreak in small ruminants of Northern areas of Pakistan. Res J Vet Sci 2008; 1(1): 56–61.

91. Obi TU, Ojo MO, Taylor WP, Rowe LW. Studies on the epidemiology of peste des petits ruminants in Southern Nigeria. Trop Vet 1983; 1(4): 209–17.

92. Balamurugan V, Hemadri D, Gajendragad MR, Singh RK, Rahman H. Diagnosis and control of peste des petits ruminants: a comprehensive review. VirusDiseases 2014; 25(1): 39–56.

93. Otte MJ, Rachel N, Anni McL. Transboundary animal diseases: assessment of socio-economic impacts and institutional responses.“ Rome, Italy : FAO, 2004: 119

REGIONALNA EPIDEMIOLOGIJA IN Z NJO POVEZANI DEJAVNIKI TVEGANJA ZA KUGO DROBNICE V AZIJI – PREGLED

M. Munibullah, Y. Li, K. Munib, Z. Zhang

Izvleček: Kuga drobnice (angl., Peste des petits ruminants, PPR) je gospodarsko pomembna, čezmejno nevarna, visoko nalezljiva, akutna virusna bolezen drobnice, ki jo je potrebno prijaviti Svetovni organizaciji za zdravje živali (OIE). Bolezen povzroča virus PPR (PPRV). PPRV spada v rod virusov *Morbillivirus* iz družine *Paramyxoviridae*. Nedavna epidemiološka in molekularna karakterizacija izolatov virusa PPRV deli izolate na štiri genetsko različne linije (I, II, III in IV). Bolezen je endemična v Aziji, na bližnjem vzhodu in v Afriki. Zaradi močnega bremena za gospodarstvo in razvoj prizadetih držav velja za glavno oviro pri razvoju trajnostnega kmetijstva v državah v razvoju. Organizacija Združenih narodov za prehrano in kmetijstvo (FAO) in OIE sta nedavno določili, da je bolezen potrebno do leta 2030 izkoreniniti po vsem svetu. Države z endemijo PPR bi se morale pridružiti regionalnim silam in izvajati regionalne načrte za postopen in uspešen nadzor in izkoreninjenje PPRV. V tem pregledu so izčrpno obravnavani regionalna epidemiologija izbruhov PPR in povezani dejavniki tveganja, vključno z dejavniki živali (starost, vrsta in spol), okolja (letni čas, prostorska razširjenost bolezni na različnih lokacijah) in trgovine, s posebnim poudarkom na državah s PPR v južni, osrednji in vzhodni Aziji.

Ključne besede: epidemiologija; virus PPR; dejavniki tveganja; nadzor; izkoreninjenje; Azija

Slov Vet Res 2022; 59 (2): 89–98
DOI 10.26873/SVR-1316-2022
UDC 636.082:637.54:598.261.7:577.171.6

Original Research Article

EFFECTS OF SELECTION IN TERMS OF MEAT YIELD TRAITS ON LEPTIN RECEPTOR GENE IN JAPANESE QUAIL LINES

Kemal Karabağ[1], Sezai Alkan[2]*, Taki Karslı[3], Cengiz İkten[4], İnci Şahin[5], Mehmet Mendeş[6]

[1]Department of Agricultural Biotechnology, [3]Department of Animal Science, [4]Department of Plant Protection, Faculty of Agriculture, Akdeniz University, 07058 Antalya, [2]Department of Animal Science, Faculty of Agriculture, Ordu University, 52200 Ordu, [5]Department of Plant Protection, Faculty of Agriculture, Selçuk University, 42030 Konya, [6]Department of Animal Science, Faculty of Agriculture, Çanakkale Onsekiz Mart University, Çanakkale, 17020, Turkey

*Corresponding author, E-mail: sezaialkan61@gmail.com

Abstract: This study was carried out to investigate the effects of selection on the single nucleotide polymorphisms (SNPs) in coding sequence of leptin receptor (*LEPR*) gene and possible associations between SNPs' and some meat yield traits of Japanese quail lines. Fifteen generations divergently selected two lines (HBW and LBW) for 5-weeks of age body weight and a control were used as materials for this study. A 348-bp part of the *LEPR* coding region (18th exon) were sequenced in a total of 113 individuals from the three quail lines and shown that the fragments contained four SNPs loci (T490C, C528T, G537A, T571C) and five haplotypes (TTGT, CTGT, TCGT, TCAT, TCAC). T490C replacement caused the missense mutation of phenylalanine to convert to leucine (Phe>Leu). However, other SNPs were synonymous and there were no changes in transcripts. It was determined that the quails with higher phenotypic values were in the TT genotype at the T390C locus. Statistical analyses showed that there were significant differences among the quail lines, SNP alleles and haplotypes in terms of interested phenotypic traits (P<0.05), and also SNP and haplotype distributions changed depending on quail lines (P<0.001). When all results were evaluated together, it was concluded that the fifteen generations of selection caused significant changes in the *LEPR* gene in terms of economically important traits in Japanese quail lines (*C. coturnix japonica*).

Key words: SNP; haplotype; leptin receptor; selection; Japanese quail

Introduction

The control of feed intake, energy balance and fat deposition have high economic importance in farm animals. The accumulation of excess fat affects meat quality, fertility, productivity, and whole-body metabolism (1, 2). Leptin activity at specific receptors in the hypothalamus suppresses feed intake, which increases the use of energy, and leptin is a polypeptide hormone that controls the body's energy balance (3, 4). Leptin receptor (*LEPR*) is a member of the class 1 cytokine receptor family that mediates most of LEP central and peripheral effects (5). The presence of leptin in laying and broiler poultry genotypes decreases feed intake (6, 7). Quail chicks that were given recombinant mouse leptin on the fifth embryonic day were removed from incubation earlier (5-24 hours) and reached a higher body weight than the control group (8). DNA sequence analysis of leptin from several mammalian and avian species, including livestock animals, showed that leptin is highly conserved among vertebrate species. Among vertebrates, the leptin amino acid sequence shows more than 80% homology (9). The entire coding region of the leptin receptor (*LEPR*) in Japanese quail (*C. coturnix japonica*) was sequenced by Wang et al. (10). Also, four alternative *LEPR* spliced variants (one long and three soluble) were identified in Japanese quails

Received: 17 March 2021
Accepted for publication: 23 May 2022

(11). DNA sequencing methods have focused on single nucleotide polymorphisms (SNP) in recent years. SNPs of leptin and its receptors in relation to livestock yields has accelerated research efforts. Researchers are actively investigating the relationship between leptin and traits of high economic importance, such as feed intake (12, 13), fertility (14), milk production (15, 16) and meat production (17, 18). Tarabany et al. (19) has reported SNP haplotypes significantly associated with growth and egg production in Japanese quails. Also, a SNP of *LEPR* gene was significantly associated with abdominal fat in chickens (20).

Statistical analysis is an important part of scientific studies and there are different statistical tests and approaches have been developed for analyzing data sets which have been obtained the studies carried out for the same purpose. However, it is extremely important to use a statistical test or methods that will be able to give more detailed information about the effect of interested factor(s). Due to its advantages over classical methods (i.e., ANOVA and its parametric and nonparametric counterparts) Analysis of Mean Technique (ANOM) was used for analyzing data sets.

ANOM is not only a powerful tool for comparing means but also for comparing variances, proportions and other location and scale measures. This procedure can also be used efficiently as a multiple comparison test especially when there are a large number of groups (21). ANOM is accepted as a graphical counterpart to ANOVA for comparing group means. Since it presents the comparisons graphically, the researchers can easily see which treatment mean (s) are different. This is a big advantage especially for non-statisticians (21 - 23).

The aim of this study is to investigate the relationships between SNPs in the coding sequences (18^{th} exon) of the leptin receptor (*LEPR*) gene, which may occur as a result of 15 generations of long-term selection in Japanese quail (*C. coturnix japonica*) lines, and some phenotypic features.

Material and methods

All the experimental procedures were reviewed and approved by Akdeniz University Local Committee on Animal Research Ethics (Protocol number: 2012.02.02).

Animal

The material of this study was consisted of three different Japanese quail (C. coturnix japonica) lines (C: control, HBW: high body weight and LBW: low body weight). Control group was not selected previously while the quails in the treatment groups were divergently selected according to their high and low body weight at the fifth week for 15 generations. Quail lines were obtained in previous project which was supported by the Scientific Research Projects Coordination Unit of Akdeniz University (Project ID: 2003.03.121.004). The data sets used in this study were obtained from the projects supported by the Scientific Research Projects Coordination Unit of Akdeniz University (Project ID: 2012.01.0104.002) and the Scientific and Technological Research Council of Turkey (Project ID: 114O047).

Raising of Material

Fertilized eggs were collected from the HBW, LBW and C lines for a week and stored at 15-20 °C with 75-80% humidity. These eggs were incubated at 36.5 °C with 65% humidity for the first 14 days and at 36.0 °C and 55% humidity for the last 4 days. Hatching weight (HW) of chicks were weighed individually using 0.01 g precision scales, and an aluminum ID number was attached to the left wings of chicks after incubation. These chicks were fed 24% crude protein and 2900 kcal/kg ME (metabolic energy) during the first four weeks in a breeding cage. Sex determination was performed by observing the cloaca and breast feather color at the end of the fourth week. 50 males and 50 females were selected randomly from each quail line and transferred to individual breeding cages for 10 weeks. All birds were fed with 21% crude protein and 2800 kcal/kg ME for ten weeks. Lighting was applied continuously for the first four weeks and then 16 hours a day.

Feed Intake (FI)

Since the animals were not fed on the day of weighing the differences due to feed intake was fixed. Thus, 10 weeks of individual feed intake (FI) were recorded as grams per day. A total of FI for per individual was calculated from these records.

Sexual Maturity

The first ovulation day and the first day of release foam were considered as sexual maturity for females and males, respectively. Sexual maturity weight (SMW) of males and females were determined on the same day.

Body Weight and Carcass Weight

A total of 50 quails (25 males and 25 females) were selected randomly from the HBW, LBW and C groups, respectively. Then were introduced to the cutting process which was performed at the end of the 15th week. The body weights (BW) of the birds were measured just before cutting using 0.01 g precision scales from hatching to the 15th week on the same day each week. Low-voltage electrical current (100 mA, 50 Hz) was used to stun animals as recommended in the relevant scientific literature (24, 25), and then the jugular vein was cut. After the blood flow was over, the feathers were cleaned, and the internal organs were removed. Eventually, the carcass weights (CW) were measured using a precision scale of 0.01 g.

Tissue Samples and Total RNA Extraction

Liver tissue samples from each individual were isolated using sterile forceps and scissors immediately after cutting and placed into numbered tubes (Corning, New York, USA) containing RNAsave. These tissues were stored at -80 °C until use. RNA extractions from liver tissue samples were performed using a commercial kit (Axygen) after cellular degradation of the liver tissues using a lyser with tungsten beads. The concentrations of isolated RNAs were measured using a biodrop. Also, RNA gel analysis (Reliant Gel System) was performed to determine RNA quality. For this purpose, 3µL of each Total RNA sample from each quail and 3µL of formaldehyde were added to a microcentrifuge tube and centrifuged for 10 seconds. Then, the samples were heated at 65 °C for 15 min. Total RNAs were loaded individually into the RNA gel and run on an electrophoresis device for 2 hours. For staining, 5µL of ethidium bromide was added into 50 mL TE Buffer. The running RNA gel was immersed in this buffer for about 10 hours at slow speed on a magnetic stirrer. UV imaging device was used to visualize RNA gel results. Finally, RNAs obtained from 150 quails were stored at –80 °C until use.

cDNA Synthesis and PCR Amplification

cDNA contains only exons and is representative of the expressed genes of the cell. cDNA sequencing results are clearer because primers do not bind non-specifically. A commercial kit (Thermo Scientific #K1621) was used to synthesize cDNAs from total RNAs using the following protocol: 60 min at 42 °C, 5 min at 70 °C. Primers (forward, gcttgctcaggtagctcctg and reverse, tgcggcacgta tggcacgat) based on the recommendations of Dridi et al. (26) were used to PCR amplify a 348bp LEPR coding region (18th exon), from cDNA. PCR products (15 µL) were evaluated for a 348 bp length using 2% agarose gels (electrophoresed at 80 V/2 h) and stained with ethidium bromide. Separated fragments in the electrophoresis gel were cut with a sterile scalpel under UV light and transferred to individual 1.5 mL pre-numbered tubes. The PCR reactions were performed in 20 µL reaction volumes with 2 µL of genomic DNA (20 ng) as a template, 2 µL of buffer (NH2SO4), 0.4 µL of a dNTP mix (2.5 mmol/L), 0.5 mL of each primer (20 nmol/ mL), 1.25 µL of MgCl2 (25 mM) and 0.15 µL of EX Taq polymerase (Takara Bio Inc. Shiga, Japan). Amplifications were performed using a thermal cycler (Thermo Arktik) with the following conditions: 3 min for an initial denaturation at 94 °C, 35 cycles at 94 °C for 30 s for denaturation, 30 s for annealing at 60 or 62 °C, 45 s for extension at 72 °C, and a final extension for 5 min at 72 °C. β-actin gene primers (F:caaggagaagctgtgctacgtgc and R: ttaatcctgagtcaagcgcc) were used to determine that the PCR protocol worked (13).

Sequence Analysis and SNP Determination

cDNA samples were concentrated in the PCR and sequenced directly in a sequencing instrument (ABI-3730) after being purified from a gel and denatured at 94°C. LEPR gene fragments were sequenced for total of 150 individuals from the HBW, LBW and C lines. Firstly, in order to confirm the accuracy of the readings obtained as a result of sequence analysis, the nucleotides‘ peaks were examined using Chromas Pro software (version 2.1.3). Eventually, a total of 113 DNA sequences (HBW=33, LBW=44, and C=36) were used in this study. The BLAST online software (http://blast.

ncbi.nlm.nih.gov/Blast.cgi) was used to determine the location of 348 bp long fragment in the in the 18th exon of LEPR gene whose sequence was determined in this study. After this examining, the DNA sequences of the individuals belonging to the quail lines were aligned by using Mega6.0 software (version 6.06). According to SNP points seen in individuals, haplotype distributions of populations were determined in DnaSP software (version 5.10).

Statistical Analyses

SNP alleles were detected in each individual from each quail population and SNP haplotypes were coded numerically and phenotypic measurements (HW, BW, SMW, CW and FI) of these individuals were matched to these codes. Results of Kolmogorov-Smirnov test showed that the normality assumption was not fulfilled in data sets. Analysis of Means (ANOM) technique was used to compare quail genotypes, SNPs and SNP haplotypes in terms of measured phenotypic traits. Although, the ANOM is accepted as a graphical alternative to ANOVA, it has two advantages over ANOVA especially when researchers are interested in studying main effects. The advantages of the ANOM over the ANOVA are: a) if any of the group mean is statistically different from the others, it enables the researchers to see exactly which one is different easily and b) since the ANOM is a graphical technique, it presents the results visually that provides a quick way for researchers and readers to evaluate both practical and statistical significant differences between the treatment groups and the overall mean (21-23).

The results of the ANOM technique are based on confidence interval or decision lines (upper decision line (UDL) and lower decision line (LDL). The computation of the upper and lower decision lines is given as below (18,19). Computation of UDL and LDL for equal sample size:

$$UDL = \overline{Y}_{..} + h(\alpha, k, N-k)\sqrt{MSE}\sqrt{\frac{(k-1)}{N}}$$

$$LDL = \overline{Y}_{..} - h(\alpha, k, N-k)\sqrt{MSE}\sqrt{\frac{(k-1)}{N}}$$

Computation of UDL and LDL for unequal sample size:

$$UDL = \overline{Y}_{..} + h(\alpha, k, N-k)\sqrt{MSE}\sqrt{\frac{(N-n_i)}{Nn_i}}$$

$$LDL = \overline{Y}_{..} - h(\alpha, k, N-k)\sqrt{MSE}\sqrt{\frac{(N-n_i)}{Nn_i}}$$

```
        10         20         30         40         50         60         70         80
GCTTGCTCAGGTAGCTCCTGGGAGCTGGGGAGCGAGGCATTCCTCCTGCTGCCTGACCAGCCTGACAGCCGGCCCTGCAG
GCTTGCTCAGGTAGCTCCCGGGAGCTGGGGAGCGAGGCATTCCTCCTGCTGCCTGACCAGCCTGACAGCCGGCCCTGCAG

        90        100        110        120        130        140        150        160
GACCCTTATATTTTCAGAGGGACTTTCAGAGCCTTCAGAGCAGGATGGTGCTTTCACAGCCGGAGGTCCGGAGCGAGGTC
GACCCTTATATTTTCAGAGGGACCTTCAGAGCCTTCAGAGCAGGATGGTGCTTTCACAGCTGGAGGTCCAGAGCGAGGTC

       170        180        190        200        210        220        230        240
TCTGTTACCTGGGGATGACATCATTGGGCAAAAGAGAAAATGGCATTTTTTTAACACAGAGCTCCAGACTGAGGTGCCAT
TCTGTTACCTGGGGATGACATCACTGGGCAAAAGAGAAAATGGCATTTTTTTAACACAGAGCTCCAGACTGAGGTGCCAT

       250        260        270        280        290        300        310        320
TTCCATACAGCTGATCTACTCAGAGGTGTGGGGTTTCTTCAGGATACACCTCCTAATTTAAATGCATTTATCCAGAGCAG
TTCCATACAGCTGATCTACTCAGAGGTGTGGGGTTTCTTCAGGATACACCTCCTAATTTAAATGCATTTATCCAGAGCAG

       330        340
CATTAAAGCCATCGTGCCATACATGCCG
CATTAAAGCCATCGTGCCATACATGCCG
```

Figure 1: Sequenced 18th exon region of LEPR. Bold letters are SNPs, Underlined sequences are primer binding sites

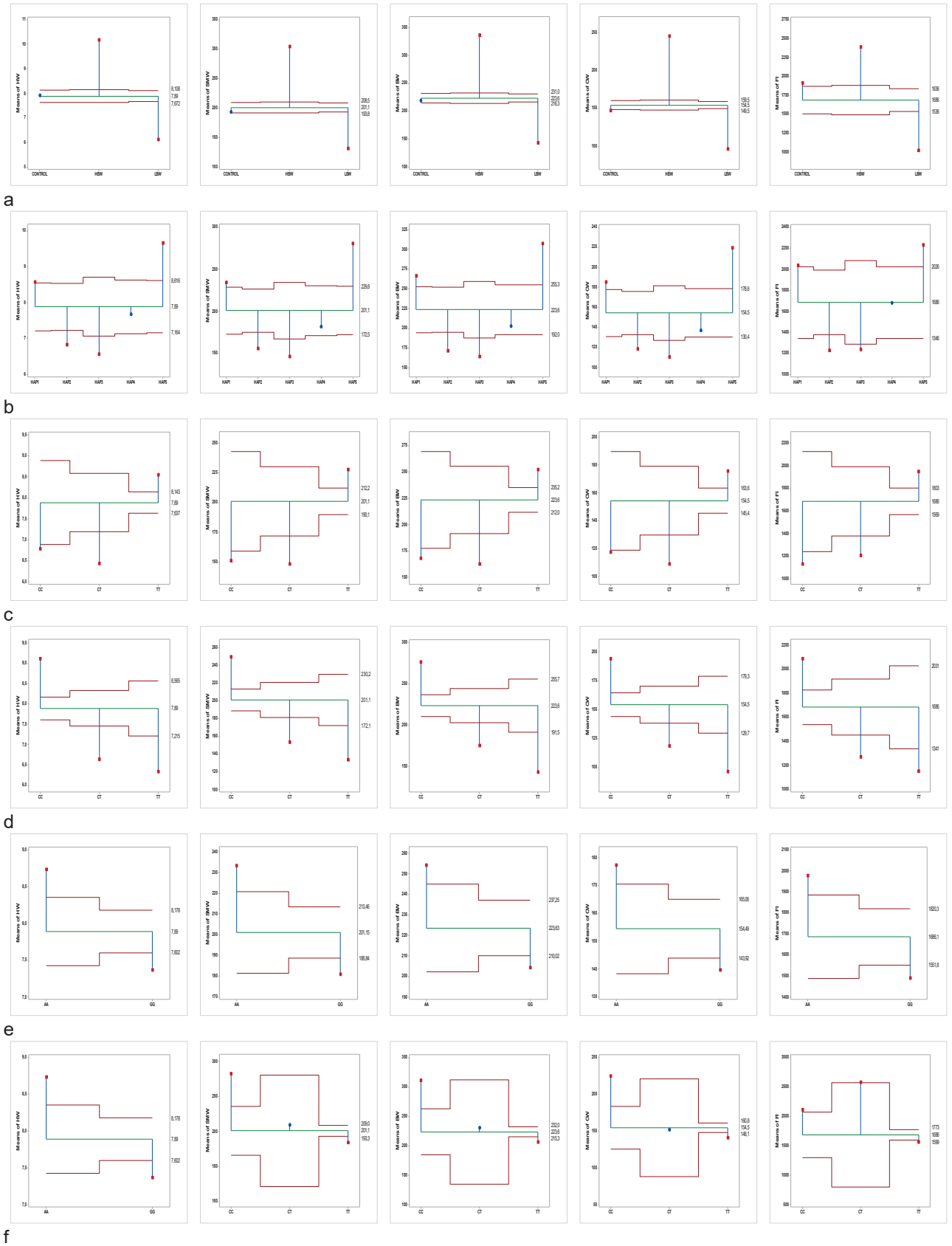


Figure 1: ANOM for comparing quail lines (a), haplotypes (b), SNP1 (c), SNP2 (d), SNP3 (e), and SNP4 (f) in terms of HW (Hatching Weight), SMW (Sexual Maturity Weight), BW (Body Weight), CW (Carcass Weight), and FI (Feed Intake), respectively

Where $\overline{Y}_{..}$, h, k, and MSE denote overall mean, critical table values for the ANOM technique based on α number of treatment group number and mean square error (21-23).

The decision is set as follows: if all means fall between the UDL and LDL, then the null hypothesis is accepted, and it is concluded that all means are equal. Any of the group mean, on the other hand, falls outside the decision lines, then the null hypothesis is rejected, and it is concluded that at least one group mean is significantly different from the overall or grand mean. In addition, Chi-square analysis was used on each of the haplotypes and SNPs to determine whether the ratio was due to genotype possession.

Results

A 348-bp fragment at the 18th exon of the *LEPR* gene was sequenced in 113 individuals from three quail lines. This fragment was BLAST searched against GenBank to confirm its identity as fragment of the *LEPR* gene. The 348 bp part of the sequence uploaded with KP674327.1 accession number that we identified exactly matched bases from 3163 to 3511 of the 3579 bp of whole *LEPR* sequence. Also, this region's position on the transcripts is between 811. codon and 926. codon in the entire gene (NCBI: XP_032302064.1). The sequenced fragments in this study were contained four SNPs loci (T490C, C528T, G537A, T571C) and five haplotypes (TTGT, CTGT, TCGT,TCAT, TCAC). When investigated the transcripts of the sequences, we found that the first SNP (T490C) caused with changing of phenylalanine to leucine (Phe-Leu) by occurring missense mutation. However, the other SNPs were synonyms and there were no changes on the transcripts. Loci of the four SNPs were marked in bold and locations of primers were underlined as shown Figure 1.

ANOM technique was used to investigate the effects of quail lines, SNPs and haplotypes on HW, SMW, BW, CW, and FI and the results have been presented in Figure 2a-f, respectively.

The results of the ANOM technique are based on confidence interval or decision lines (UDL: upper decision line and LDL: lower decision line). UDL and LDL are shown with red lines. Differences outside the UDL and LDL boundaries are indicated by the red dot.

When the results of ANOM for comparing quail lines in terms of HW, SMW, BW, CW, and FI were examined, it was clearly seen that the results were generally very similar for all traits except FI. As it can be seen from the Figure 2a, at least one mean falls outside the decision lines, that means there were statistical significance differences among the genotypes in terms of HW, SMW, BW, CW, and FI. The highest values for all traits have been observed for the HBW genotype while the least values observed for the LBW. The values of means from control population were generally located between decision lines. That means there were statistically significant differences among the quail lines in terms of studied phenotypic traits. Therefore, it was possible to conclude that there were statistically significant changes in the gene of the *LEPR* due to long-term selection. This suggests that long-term divergent selection for body weight in quail's results in different changes in the same locus of the *LEPR* gene. As seen in Figure 1b, all haplotypes except the fourth haplotype affected HW, BW, SMW, CW, and FI. The Hap1 and the Hap5 have positive affect while the Hap2 and the Hap3 have negative affect on interested traits in this study. The highest and the lowest HW values were obtained for the Hap5 and the Hap3, respectively.

For the effect of SNP1, obvious differences have been observed among the CC, CT, and TT genotypes. As it is seen from the Figure 2c, the HW, SMW, BW, CW and FI values for the TT were obviously higher than that of the CC and CT. Therefore, it can be concluded that the TT has a positive impact on the phenotypic traits while the CC and CT have negative. When the effect of SNP2 was examined, it was not difficult to observe that the CC genotype has positive impact, and the highest phenotypic values were obtained for the CC. The CT and TT genotypes, on the other hand, have negative affect and the lowest values were observed for the TT (Figure 2d). When Figure 1e is examined, it can be seen that the AA genotype has obviously positive impact while the GG genotype has negative affect on the phenotypic traits. The CC genotype at the SNP4 loci has positive affect on the HW, SMW, BW, CW and FI, while the TT has negative (Figure 2f). All means fall between lower and upper decision lines for the CT genotype that just has positive affect on the FI.

The distribution of the SNP genotypes and the haplotypes in quail lines are not the same

(Table 1, P<0.01). The allele numbers were found as almost same for the SNP1 locus in the LBW quails. However, this case was not valid for the control and HBW quail lines. It is possible to say that the selection results in favor of the TT at SNP1 locus in LBW because of there were no CC and CT genotypes in control line, and just 2 individuals in HBW (Table 1).

Table 1: Results of Pearson chi-square test of distributions of SNP genotypes in quail lines

SNPs LBW		Quail Lines C	HBW		Chi-square
T	CC	11	0	2	
103	CT	22	0	2	0.000*
C	TT	11	36	29	
C	CC	5	23	32	
141	CT	22	12	1	0.000*
T	TT	17	1	0	
G					
150	AA	5	23	16	0.000*
A	GG	39	13	17	
T	CC	0	4	14	
184	CT	0	4	0	0.000*
C	TT	44	28	19	

*p<0.01

Absence of the CC and CT and present of the TT in all individuals in LBW quail line suggests that negative selection in terms of low body weight effective in favor of the TT for SNP4. It was possible to claim that similar situation may be valid for the GG genotype at SNP3 locus. However, the numbers of TT at SNP1 and CC at SNP2 have increased by long term selection in terms of high body weight. The hap1 and hap5 were mostly observed in the HBW genotype while the hap 2 and hap 3 were mostly observed in the LBW. The most commonly observed haplotype for the control group was hap 4 (Table 2). As in the SNP alleles the haplotypes have also been formed in different quail lines resulting in long-term selection.

Table 2: Results of Pearson chi-square test of distributions of haplotypes in quail lines

	Quail Lines			
Haplotypes	LBW	C	HBW	Chi-square
Hap-1	5	7	13	
Hap-2	22	0	4	
Hap-3	12	6	0	0.000*
Hap-4	5	17	0	
Hap-5	0	6	16	

*p<0.01

Discussion

LEP and *LEPR* are excellent candidate genes for livestock production, as it is associated with features of economic importance. Indeed, in recent years, leptin gene polymorphism studies of several single nucleotide polymorphisms (SNPs) have been identified in cows and pigs, and several SNPs have been identified that are associated with important economic traits, such as milk yield, feed intake, adiposity, growth, and carcass quality (12-18, 27). The reasons for focusing on exon 18 in this study is that exon 18 polymorphisms are important on backfat thickness, feed yield and reproduction traits (28). When investigated the transcripts of the sequences, we found that the first SNP1 (T490C) caused with changing of phenylalanine to leucine (Phe-Leu) by occurring missense mutation. However, the other SNPs were synonyms and there were no changes on the transcripts in current study. El Moujahid et al. (29) found a significant association between 4 previously reported non-synonymous SNPs and the growth performance traits in meat-type chickens. In this study, the HW, SMW, BW, CW and FI values for the TT genotype were obviously higher than that of the CC and CT at SNP1 locus. When the effect of SNP2 was examined, it was not difficult to observe that the CC genotype has positive impact, and the highest phenotypic values were obtained for the CC. According to SNP3 the AA genotype has obviously positive impact while the GG genotype has negative affect on the phenotypic traits. The CC genotype at the SNP4 loci has positive relation on the HW, SMW, BW, CW and FI, while the TT has negative. Also, Hap1 (TTGT) and Hap5 (TCAC) have positive affect while the Hap2 (CTGT) and the Hap3 (TCGT) have negative affect on interested traits (HW, BW, SMW, CW, and FI).

De Vuyst et al. (30) found that both crossbred CT and TT beef cows wean significantly heavier beef calves than CC cows. There were several SNPs found in the porcine *LEP* and *LEPR* genes, suggesting that the SNPs lead to increased growth and fat (31). Buchanan et al. (32) revealed a SNP in the leptin gene of dairy cattle. This polymorphism, in which the first nucleotide is a thymine instead of cytosine in the 25th codon, changed arginine to a cysteine. However, homozygous animals carrying the T allele show no difference compared to animals carrying the C allele in terms of milk

fat and milk protein production and a daily milk yield of more than 1.5 kg. In addition, homozygous T allele-bearing animals developed higher fatty carcasses than those with the C allele (33). Also, El Moujahid et al. (29) reported relation of *LEPR* gene polymorphisms with growth and feed efficiency in meat-type chickens. Wang et al. (34) found a single nucleotide polymorphism (C/A) and three SNP genotypes in *LEPR* associated with fatness traits in chickens. Abbasi et al. (35) screened exons 9–11 of chicken *LEPR* but did not find any SNPs. Tarabany et al. (19) To the best of our knowledge, this is the first study to report the presence of two adjacent novel SNPs (A277G and A304G) in intron 8 of the *LEPR* gene of Japanese quail. GG/GG quails had significantly lower egg production and feed intake. Four SNPs loci (T490C, C528T, G537A, T571C) and five haplotypes (TTGT, CTGT, TCGT, TCAT, TCAC) were determined and the distributions of the SNP alleles and the haplotypes in quail lines were not the same in this study. This suggests that long-term selection for high body weight and low body weight in quail's results in different changes in the same locus of the *LEPR* gene.

Feed consumption, growth, development, energy metabolism and immune system functioning have a high economic importance in livestock. In this regard, there is a need for additional studies on genes that affect animal feed intake, the regulation of energy metabolism, yield and health. Leptin plays an important role in all of these mechanisms of economic importance in livestock. Therefore, studies of leptin will significantly contribute to animal nutrition, breeding and health. Yet, effects of the single nucleotide polymorphisms of leptin and *LEPR* genes in poultry animals are not adequately explained.

Therefore, it is possible to conclude that the effects of different genotypes, haplotypes, and SNPs on HW, SMW, BW, CW and FI are considerable. Since expectation of high correlation between the studied traits, that it is not a surprise to get these results of the long term bi-direction selection. These changes can be altered the function of the *LEPR*. However, to achieve a more accurate understanding of the role of leptin and its receptor, the DNA sequence of all of the SNP changes that benefit individuals and alter protein structure should be identified. However, conclusively demonstration of this effect requires the identification of all of the SNPs in the entire sequence of the *LEPR* in quails. Although it is very hard to interpret this data into livestock weight, surely these polymorphisms are worth a further investigation.

Acknowledgement

Selection studies were supported by the Scientific Research Projects Coordination Unit of Akdeniz University (Project ID: 2003.03.121.004). Molecular studies were supported by the Scientific Research Projects Coordination Unit of Akdeniz University (Project ID: 2012.01.0104.002) and the Scientific and Technological Research Council of Turkey (Project ID:114O047).

The authors certify that there is no conflict of interest with any financial organization regarding the material discussed in the manuscript.

References

1. Macajova M, Lamosova D, Zeman M. Role of leptin in Japanese quail development. Acta Vet Brno 2002; 71: 473–9.

2. Macajova M, Lamosova D, Zeman M. Role of leptin in farm animals: a review. J Vet Med A 2004; 51: 157–66.

3. Taouis M, Chen JW, Daviaud C, Dupont J, Derouet M, Simon J. Cloning the chicken leptin gene. Gene 1998; 208: 239–42.

4. Ashwell CM, Czerwinski SM, Brocht D, McMurtry M. Hormonal regulation of leptine expression in broiler chickens. Am J Physiol 1999; 276: 226–32.

5. Horev G, Einat P, Aharoni T, Eshdat Y, Friedman- Einat M. Molecular cloning and properties of the chicken leptin-receptor (CLEPR) gene. Mol Cell Endocrinol 2000;162(1/2): 95–106.

6. Denbow DM, Meade S, Robertson A, Mc Murtry JP, Richards M, Ashwell C. Leptin-induced decrease in feed intake in chickens. Physiol Behav 2000; 69: 359–62.

7. Kuo AY, Cline MA, Werner E, Siegel PB, Denbow DM. Leptin effects on feed and water intake in lines of chickens selected for high or low body weight. Physiol Behav 2005; 84: 459–64.

8. Lamosova D, Macajova M, Zeman M, Mozes S, Jezova D. Effect of in ovo leptin administration on the development of Japanese quail. Physiol Res 2003; 52: 201–9.

9. Doyon C, Drouin G, Trudeau VL, Moon TW. Molecular evolution of leptin. Gen Comp Endocrinol 2001; 124: 188–98.

10. Wang D, Jiang R, Wang T, Xu C, Kang X, Liu X. 2014. http://www.ncbi.nlm.nih.gov/nuccore/KJ639903

11. Wang D, Xu C, Wang T, et al. Discovery and functional characterization of leptin and its receptors in Japanese quail (*Coturnix japonica*). Gen Comp Endocrinol 2016; 225: 1–12.

12. Lagonigro R, Wiener P, Pilla F, Woolliams JA, Williams JL. A new mutation in the coding region of the bovine leptin gene associated with feed intake. Anim Genet 2003; 34: 371–4.

13. Huang JX, Lu L, Xi L, Luo XG, Liu B. Effects of age and strain on the expression of leptin receptor, neuropeptide Y and pro-opiomelanocortin in the hypothalamus of young chickens. Br Poult Sci 2011; 51: 696–702.

14. Liefers SC, Te-Pas MF, Veerkamp RF, Van-Der-Lende T. Associations between leptin gene polymorphisms and production, live weight, energy balance, feed intake, and fertility in Holstein heifers. J Dairy Sci 2002; 85: 1633–8.

15. Banos G, Woolliams JA, Woodward BW, Forbes AB, Coffey MP. Impact of single nucleotide polymorphisms in leptin, leptin receptor, growth hormone receptor, and diacylglycerol acyltransferase (DGAT1) gene loci on milk production, feed, and body energy traits of UK dairy cows. J Dairy Sci 2008; 91: 3190–200.

16. Chebel RC, Santos JEP. Association between leptin single nucleotide polymorphism and reproductive performance of lactating Holstein cows. Anim Reprod Sci 2011; 127: 126–34.

17. Lusk JL. Association of single nucleotide polymorphisms in the leptin gene with body weight and back fat growth curve parameters for beef cattle. J Anim Sci 2007; 85: 1865–72.

18. Boucher D, Palin MF, Castonguay F, Gariépy C, Pothier F. Detection of polymorphisms in the ovine leptin (lep) gene: association of a single nucleotide polymorphism with muscle growth and meat quality traits. Can J Anim Sci 2006; 86(1): 31–5.

19. El Tarabany MS, Saleh AA, El Araby IE, El Magd MA. Association of *LEPR* polymorphisms with egg production and growth performance in female Japanese quails.

20. Lei MM, Wu SQ, Li XW, Wang CL, Chen Z, Shi ZD. Leptin receptor signaling inhibits ovarian follicle development and egg laying in chicken hens. Reprod Biol Endocrinol 2014; 12: e25. doi: 10.1186/1477-7827-12-25

21. Nelson PR, Wludyka PS, Copeland KA. The analysis of means: a graphical method for comparing means, rates, and proportions. Philadelphia : Society for Industrial and Applied Mathematics, 2005.

22. Mendeş M, Yiğit S. Comparison of ANOVA-F and ANOM tests with regard to type I error rate and test power. J Stat Comput Simul 2013; 83(11): 2093–104.

23. Mendeş M, Yiğit S. An alternative approach for multiple comparison problems when there are a large number of groups: ANOM technique. J Anim Plant Sci 2018; 28: 1074–9.

24. Yalçin S, Oğuz I, Otles S. Carcass characteristics of quail (*Coturnix coturnix japonica*) slaughtered at different ages. Br Poult Sci 1995; 36: 393–9.

25. Göksoy EO, Mc Kinstry LJ, Wilkins LJ, et al. Broiler stunning and meat quality. Poult Sci 1999; 78: 1796–800.

26. Dridi S, Buyse J, Decuypere E, Taouis M. Potential role of leptin in increase of fatty acid synthase gene expression in chicken liver. Domest Anim Endocrinol 2005; 29: 646–60.

27. Jiang ZH, Gibson JP. Genetic polymorphisms in the leptin gene and their association with fatness in four pig breeds. Mamm Genome 1999; 10: 191–3.

28. Chen CC, Chang T, Su HY. Characterization of porcine leptin receptor polymorphisms and their association with reproduction and production traits. Anim Biotechnol 2007; 15: 89–102.

29. El Moujahid EM, Chen S, Jian S, Lu Y, Zhang D, Congliang, J, Yang N. Association of leptin receptor gene polymorphisms with growth and feed efficiency in meat-type chickens. Poult Sci 2014; 93: 1910–5.

30. De Vuyst EA, Bauer ML, Cheng FC, Mitchell J, Larson D. The impact of a leptin gene SNP on beef calf weaning weights. Anim Genet 2008; 39: 284–6.

31. Perez-Montarelo D, Fernandez A, Folch JM, et al. Joint effects of porcine leptin and leptin receptor polymorphisms on productivity and quality traits. Anim Genet 2012; 43: 805–9.

32. Buchanan FC, Van Kessel AG, Waldner C, Christensen DA, Laarveld B, Schmutz SM. Hot topic: an association between a leptin single nucleotide polymorphism and milk and protein yield. J Dairy Sci 2003; 86:3164–6.

33. Buchanan FC, Fitzsimmons CJ, Van Kessel AG, Thue TD, Winkelman-Sim DC, Schmutz SM. Association of a missense mutation in the bovine leptin gene with carcass fat content and leptin mRNA levels. Genet Sel Evol 2002; 34: 105–16.

34. Wang Y, Li H, Zhang YD, Gu ZL, Li ZH, Wang QG. Analysis on Association of a SNP in the chicken OBR gene with growth and body composition traits. Asian-Aust J Anim Sci 2006; 19(12): 1706–10.

35. Abbasi HA, Gharahveysi S, Abdullahpour R. Study of polymorphism of leptin gene receptor in Mazandaran fowls. Afr J Biotechnol. 2011; 10: 4024–8.

UČINKI SELEKCIJE LINIJ JAPONSKIH PREPELIC NA GEN ZA LEPTINSKI RECEPTOR, POVEZAN S PRIREJO MESA

K. Karabağ, S. Alkan, T. Karslı, C. İkten, İ. Şahin, M. Mendeş

Izvleček: Ta študija je bila izvedena z namenom raziskati učinke selekcije na polimorfizme posameznih nukleotidov (Angl., Single Nucleotide Polymorphism, SNP) v kodnem zaporedju gena za leptinski receptor (*LEPR*) in možne povezave med SNP in nekaterimi značilnostmi prireje mesa pri japonskih prepelicah. V študiji je bilo poleg kontrole uporabljenih petnajst generacij različno izbranih dveh linij (HBW in LBW) s telesno maso pri starosti petih tednov. 348-bp del kodne regije LEPR (18. ekson) je bil sekvenciran pri skupno 113 posameznikih iz treh linij prepelic, fragmenti pa so vsebovali štiri lokuse SNP (T490C, C528T, G537A, T571C) in pet haplotipov (TTGT, CTGT, TCGT, TCAT, TCAC). Zamenjava T490C je povzročila drugačnosmiselno mutacijo fenilalanina v levcin (Phe > Leu), vendar so bili drugi SNP-ji sinonimni in v transkriptih ni bilo sprememb. Ugotovljeno je bilo, da so prepelice z višjimi fenotipskimi vrednostmi imele genotip TT na lokusu T390C. Statistične analize so z vidika fenotipskih lastnosti pokazale značilne razlike med linijami prepelic, aleli SNP in haplotipi ($P < 0,05$), med linijami prepelic pa je bila različna tudi porazdelitev SNP in haplotipov ($P < 0,001$). Na podlagi vrednotenja vseh rezultatov smo ugotovili, da je selekcija petnajstih generacij linij japonskih prepelic (*C. coturnix japonica*) povzročila ključne spremembe v genu *LEPR*, povezanim z gospodarsko pomembnimi lastnostmi prepelic.

Ključne besede: SNP; haplotip; leptinski receptor; selekcija; japosnka prepelica

Slov Vet Res 2022; 59 (2): 99–111
DOI 10.26873/SVR-1347-2022
UDC 602.68:543.384:618.36

Original Research Article

IMMUNOLOCALIZATION OF HOXA11 AND HLX PROTEINS IN COW PLACENTA DURING PREGNANCY

Uğur Topaloğlu[1]*, Muzaffer Aydın Ketani[1], Mehmet Erdem Akbalık[1], Hakan Sağsöz[1], Berna Güney Saruhan[1], Bayram Bayram[2]

[1]Department of Histology and Embryology, Faculty of Veterinary Medicine Dicle University, Diyarbakır 21280, [2]Laboratory and Veterinary Health, Idil Vocational College Sırnak University, Şırnak 73200, Turkey

*Corresponding author, E-mail: ugur.topaloglu@dicle.edu.tr

Abstract: In addition to its many functions, the placenta is characterized by the intensity and characteristics of its hormonal functions. It has been reported that the development and vascularization of the placenta with normal fetal growth is regulated by many factors such as growth factors, transcriptional factors, and homeobox proteins. This study was conducted to determine the distribution and possible physiological roles of HOXA11 and HLX in the uterus and placenta of pregnant cows. In this study, 27 pregnant Holstein breed cow uteri and placentas obtained from private slaughterhouses were used as tissue samples. In order to determine the pregnancy period of the obtained uterus, the ages of the fetuses were calculated according to the age calculation formula. The uteri were classified under three different groups as the first (69-89 days), second (99-178 days), and third (190-269 days) periods of pregnancy so that there are 9 animals in each group. Tissue samples were then subjected to immunohistochemistry to demonstrate the presence of HOXA11 and HLX proteins. It was observed that HOXA11 and HLX immunoreactions occurred prominently in the luminal and glandular epithelial cells of the uterus during pregnancy, and the maternal epithelial cells of the placenta and UTC and TGC/BNCs. In addition, it was determined that HOXA11 immunoreaction also formed in some vascular endothelial cells. Immunohistochemical findings in this study suggested that Hoxa11 and HLX may affect the proliferation and differentiation of cells in the uterus and placenta during pregnancy in cows, and that these proteins may contribute to normal uterine physiology, placentation formation, and continuity of pregnancy in cows.

Key words: Hox proteins; HOXA11; HLX; placenta

Introduction

Prenatal growth and development affect lifetime well-being and general health, status, which is directly proportional to the placental transport of nutrients and oxygen. As the placenta is subjected to significant morphological and histological changes during pregnancy to provide for fetal growth, gaining insight into the developmental changes which may affect the growth and development of fetus/embryo is crucial (1). Besides its many functions, the placenta is characterized by its hormonal functions. Hormones secreted by trophoblasts and syncytiotrophoblast cells along the placental villi have important roles in the birth mechanism as well as in the formation and maintenance of pregnancy and the growth and development of the fetus (2).

The formation of the placenta in Eutherian mammals requires interaction between the chorioallantoic membrane and the maternal uterine tissue. Different degrees of invasiveness are observed among different types of placenta. The human hemochorial placenta has invasive trophoblasts that come into direct contact with maternal blood. On the other hand, in the epitheliochorial placenta observed in ruminants, the uterine epithelium and maternal blood vessels remain intact throughout pregnancy. On the contrary, the cow placenta is a more complex

Received: 26 May 2021
Accepted for publication: 23 May 2022

placental type in which different sites may have different levels of fetal-maternal interaction (3). The cotyledonary placenta in cattle is classified as synepitheliochorial based upon the presence of fetal-maternal syncytium and the number of cell layers between maternal and fetal circulations (4). Synepitheliochorial placenta is the most critical region of fetal-maternal communication for the continuation of pregnancy and embryonic development in ruminants (5). Normal fetal growth and development are regulated by maternal, fetal, placental, and environmental factors in accordance with the genetically predetermined growth potential. However, it was reported that the development and vascularization of the placenta in fetal growth are governed by many elements such as growth factors, transcriptional factors, and homeobox proteins (6).

Homeobox proteins have many subunits such as HOX/Hox, DLX/Dlx, MSX/Msx, HMX/Hmx, and PBC in the genomes of vertebrates such as humans and mice (7), HOX proteins are essential regulators of the fate and identity of the cell in the embryonic process, and their roles in embryonic stem cell differentiation and morphogenesis are important. Hox proteins have been reported to have many important roles according to their order and location on the chromosome, and their activation feature (6, 8). These roles appear in implantation, cell and tissue differentiation of developing embryos, the angiogenesis and neovasculogenesis of adult processes of the embryo, and the development of the vertebrate nervous and reproductive systems. However, it has been pointed out that they play important roles in adults, such as controlling the extraembryonic development of the placenta, sexual cycle regulating and the physiology of the uterus during pregnancy (6, 8, 9).

In mammals, along the paramesonephric ducts (Müllerian ducts), it has been shown that HOXA11, which is related to the abdominal-B (Abd-B), plays a role in the composition of the female genital anatomy and is expressed in the cervix (10). HOXA11, which is very important for the development and function of the female reproductive system, and has been reported to be necessary for the successful implantation of the blastocyst in mammals (11, 12). HOXA11 is responsible for significant changes in the reproductive biology of mammals (especially changes in the cellular functions of the female reproductive organs such as the uterus, cervix, vagina, and placenta). However, it has been reported that HOXA11 has plays critical roles in the proliferation and differentiation of endometrium cells in response to varying estrogen and progesterone concentrations, in the acceptance of the offspring by the uterus, and in the maintenance of pregnancy. Mutation defects occur in the organs of the female genital system, especially the uterus, and as a result infertility occurs (12, 13, 14 ,15).

Similar to the Drosophila's homeobox gene H2.0, Hlx is a distinct homeobox protein whose expression and protein sequence are highly conserved among mammals, birds, and fish. Hlx, which was initially detected in hematopoietic cells of myelomonocytic and B lymphocyte lineages in mice, is 86.5% identical to its homolog HLX (HB24/HLX1) in humans, and its homeodomain areas are completely preserved (16, 17). It has been pointed out that the HLX/hlx protein generally has important roles such as visceral organogenesis and enteric nervous system development, and is detected at the highest level in the normal liver, gall bladder, and mesenchyme of the developing mouse embryo. In recent studies, it has been reported that it was isolated in human and mouse placenta and is a potential regulator of placental development (16, 17, 18, 19, 20). It has also been reported that HLX has an active role in the proliferation, differentiation, and migration of extra villous trophoblast (EVT) cells in the human placenta (19, 21). Thus, it was reported that HLX is important for normal placental formation and, its deficiency causes fetal growth retardation and pregnancy impairment together with abnormal trophoblasts (22).

The present study was carried out to reveal the expressions of HOXA11 and HLX proteins, subunits of homeobox proteins, in cow uterus and placenta, which are critical for reproductive fertility and maintenance of animal husbandry, and also to identify their possible physiological roles in these tissues.

Material and methods

Obtaining the Tissue Samples

In the study, 27 pregnant Holstein breed cow uteri and placentas obtained from cows slaughtered in Diyarbakir and Hatay, Turkey were used as tissue samples (23). In order to determine the pregnancy period of the obtained uteri, the ages of the fetuses

were calculated. For this purpose, Crown-Rump Length (CRL) was measured. Then, the age of the fetus was calculated using the equation „y: 54.6 cm + 2.46 (x) cm", which was obtained as a result of the linear relationship between CRL and fetal age. In the calculation applied, y is a constant representing the fetal age, and x denotes CRL (24). According to this age calculation formula, the uteri were classified under three different groups as: the first (69-89 days), second (99-178 days), and third (190-269 days) periods of pregnancy.

Routine Histological Tissue Tracking and Staining

We obtained tissue samples in the placentomal and interplacentomal region of the pregnant cornu uteri in three different groups were subjected to fixation in 10% neutral formalin solution for 24 hours. The tissues were then washed with running tap water for 24 hours. Following the washing process, the tissues were treated with through graded alcohols, methyl benzoate, and benzol series. Subsequently, the tissues were kept in an incubator at 58 °C in paraffin dissolved in benzol for 30 minutes in order to acclimate the tissues to paraffin. Then, the tissues were kept in clean, melted paraffin in an oven at 58 °C for 4 hours. After these procedures, the tissues were enclosed in liquid paraffin and transformed into blocks. Serial sections of 5-µm thickness were taken from the prepared paraffin blocks in a rotary microtome (Leica RM-2125, Germany). The sections were put on Poly-L-Lysine (PLL) coated slides and subjected to immunohistochemistry staining.

Immunohistochemical Staining

Indirect streptavidin-biotin complex method of immunohistochemistry was performed on the serial sections taken on Poly-L-Lysine (PLL) slides to determine the presence of HOXA11 and HLX proteins (23). Serial sections were taken in distilled water after passing through xylol for deparaffinization and through alcohol series for dehydration. Then, the sections were kept in 3% H_2O_2 prepared with methanol for 20 minutes to prevent endogenous peroxidase activity in the tissues. In the next step, the preparations were washed three times in 0.01M phosphate buffer saline (PBS) for 5 minutes each. Following washing, citrate buffer (0.01M, pH 6) was prepared for antigen retrieval. The sections were boiled at 95 °C for 20 minutes and then left for cooling. After cooling, the sections were washed in 0.01M PBS. Afterwards, they were incubated with protein blocking solution (Ultra V Block, Thermo Fisher Scientific, Lab Vision Corporation) at room temperature for 15 minutes in order to prevent non-specific staining in the tissues. Afterwards, normal sections were incubated with HOXA11 (Rabbit polyclonal, catalog no: PA5-57341, Invitrogen) and HLX (Rabbit polyclonal, catalog no: PA5-44857, Invitrogen) primary antibodies diluted to 1/100, and negative control preparations were incubated with PBS overnight at 4 °C in order to determine the accuracy of staining. After incubation, the preparations were washed three times in 0.01M PBS for 5 minutes. Subsequently, they were subjected to biotinylated secondary antibody (Histostain Plus Bulk Kit, Zymed) for 20 min at room temperature and binding to the primary antibody was obtained. After this treatment, the preparations were washed three times again in 0.01M PBS for 5 minutes. Subsequently, the sections were reacted in streptavidin peroxidase solution (Histostain Plus Bulk Kit, Zymed) for 20 minutes. They were then washed three times for 5 minutes in 0.01M PBS. After these processes, the preparations were incubated with 3'3-diaminobenzidine hydrochloride (DAB) for 5-15 minutes to observe the antigen-antibody reaction. After the immunoreaction is visualized, the preparations were subjected to Mayer's hematoxylin for 2-3 minutes for background staining. Immediately after this, they were washed in running water. Then, the preparations washed in distilled water were passed through the alcohol and xylol series and closed with Entellan. The images of the preparations were taken with a Nikon Eclipse E400 (Nikon, Tokyo, Japan) research microscope attached to a Nikon Coolpix 4500 digital camera.

Semi-quantitative Evaluation

Immunohistochemical staining was assessed semi-quantitatively based on the intensity score. Intensity score was determined according to the positive staining intensity in the cells. Accordingly, the scoring process in the immunohistochemical staining results was carried out following this rubric: (0: Negative) no staining observed in cells at 40 and 100X magnification, (1:Weak) staining observed in cells only at 40 and 100X magnification, (2: Medium) staining easily observed in cells at 20X magnifications, (3: Strong) staining observed in cells at 4X and 10X magnifications (25).

Table 1: In cow placenta and uterus in the first period of pregnancy (69-89 days, n:9), in the second period (99-178 days, n:9), in the third period (190-269 days, n:9) distribution of HOXA11 and HLX proteins according to immunostaining intensity score (IS)

		Endometrium			Placenta maternalis			Placenta fetalis		
		Luminal Epithelium	Glanduler Epithelium	Stroma	Smooth Muscle	Maternal Epithelium	Maternal Stroma	UTC	TGC/BNC	Fötal Stroma
		IS	IS	IS	IS	IS	IS	IS	IS	IS
HOXA11	I. Period	2,11±0,6^{a}	2,00±0,7^{a}	1,67±0,7^{b}	0,67±0,7^{a}	0,67±0,5^{a}	0,22±0,44^{a}	1,44±0,73^{b}	0,89±0,6^{ab}	0,33±0,5^{a}
	II. Period	1,89±0,6^{a}	1,78±0,67^{a}	0,33±0,5^{a}	0,44±0,53^{a}	1,67±0,5^{b}	0,56±0,53^{ab}	2,11±0,6^{b}	1,33±0,5^{b}	0,67±0,71^{a}
	III. Period	2,00±0,5^{a}	1,44±0,53^{a}	0,56±0,73^{a}	0,67±0,5^{a}	1,11±0,6^{ab}	1,11±0,6^{b}	0,67±0,5^{a}	0,44±0,53^{a}	0,78±0,67^{a}
HLX	I. Period	1,67±0,5^{a}	1,56±0,53^{a}	0,89±0,33^{a}	0,78±0,44^{a}	1,33±0,5^{a}	0,78±0,44^{a}	1,22±0,44^{a}	1,22±0,44^{a}	0,33±0,5^{a}
	II. Period	1,78±0,44^{a}	1,78±0,44^{a}	0,67±0,50^{a}	0,33±0,50^{a}	1,44±0,53^{a}	0,78±0,44^{a}	1,33±0,5^{a}	1,44±0,53^{a}	0,67±0,5^{a}
	III.Period	1,56±0,53^{a}	1,33±0,50^{a}	0,78±0,44^{a}	0,56±0,53^{a}	1,11±0,6^{a}	0,33±0,5^{a}	0,89±0,33^{a}	1,00±0,00^{a}	0,22±0,44^{a}

Different superscripts in the same row show the statistical difference ($^{a\text{-}b\text{-}c}p<0,05$) in expression levels depending on the periods, and common superscripts show the similarity between the periods

The sections were examined independently by three expert histologists (U.T, M.A.K, M.E.A) and calculations were made by taking the mean of the scores reported by the observers. Positive immunoreaction parts of HOXA11 and HLX in the placentomal and interplacentomal regions were scanned at 40x, 100x, 200x, and 400x magnifications, and thereby, high expression areas were detected. Four sections randomly selected for each section were evaluated. The mean of the obtained scores was taken as a single score. The followings were assessed as placentomal and interplacentomal parts: maternal epithelial and stromal cells, fetal trophoblast and stromal cells, luminal and glandular epithelial cells of the uterus, and stromal and smooth muscle cells. We did not assess blood vessels in detail; however, the general appearance of the vessels was observed. In this study, 1090 observations were recorded for each tissue and 2320 observations were recorded for each uteri.

Statistical analysis

The data obtained as a result of immunohistochemical density scoring were evaluated statistically with *SPSS* Statistics for Windows, Version 25.0 (Armonk, NY, USA). The normality test was applied to the scores before the statistical analysis. In the normality test, the Shapiro-Wilk test was used depending on the number of samples in each parameter. The datasets for which normality tests were shown to be $p<0.05$ were analyzed using Kruskal-Wallis test (a non-parametric test). Tamhane›s T2 test was used for multiple comparisons between groups. The data obtained as a result are shown as mean ± standard deviation (SD). Any p value less than 0.05 was considered statistically significant (Table 1).

Results

HOXA11

In the fetal part of the placenta, it was found that HOXA11 immunoreaction in the chorion epithelium was localized in the nucleus and cytoplasm of some uninucleate trophoblast cells (UTC) and binucleated trophoblast giant cells (TGC/BNC) in the first and second periods, and only in the cytoplasm of the third period cells. Furthermore, HOXA11 was observed to induce a weak immunoreaction in fetal mesenchyme along with some fetal stromal cells (Figure 1A, B, C). It was observed that the moderate intensity immunoreaction observed in UTCs in the first and second periods significantly reduced in the third period cells ($p<0.05$). However, it was confirmed that the intensity of immunoreactions in TGC/BNCs decreased significantly in the third period compared to the second period ($p<0.05$), and took a value between of the first and that of the second period. It was observed that HOXA11 immunoreaction was very weak to weak in the epithelial and stromal cells of the maternal placenta. However, it was determined that the reaction intensity in maternal epithelial cells in the second period was slightly stronger compared to the other periods (Figure 1D, E, F). It was found that the observed immunoreaction increased significantly in the second period for maternal epithelium and in the third period for maternal stromal cells ($p<0.05$).

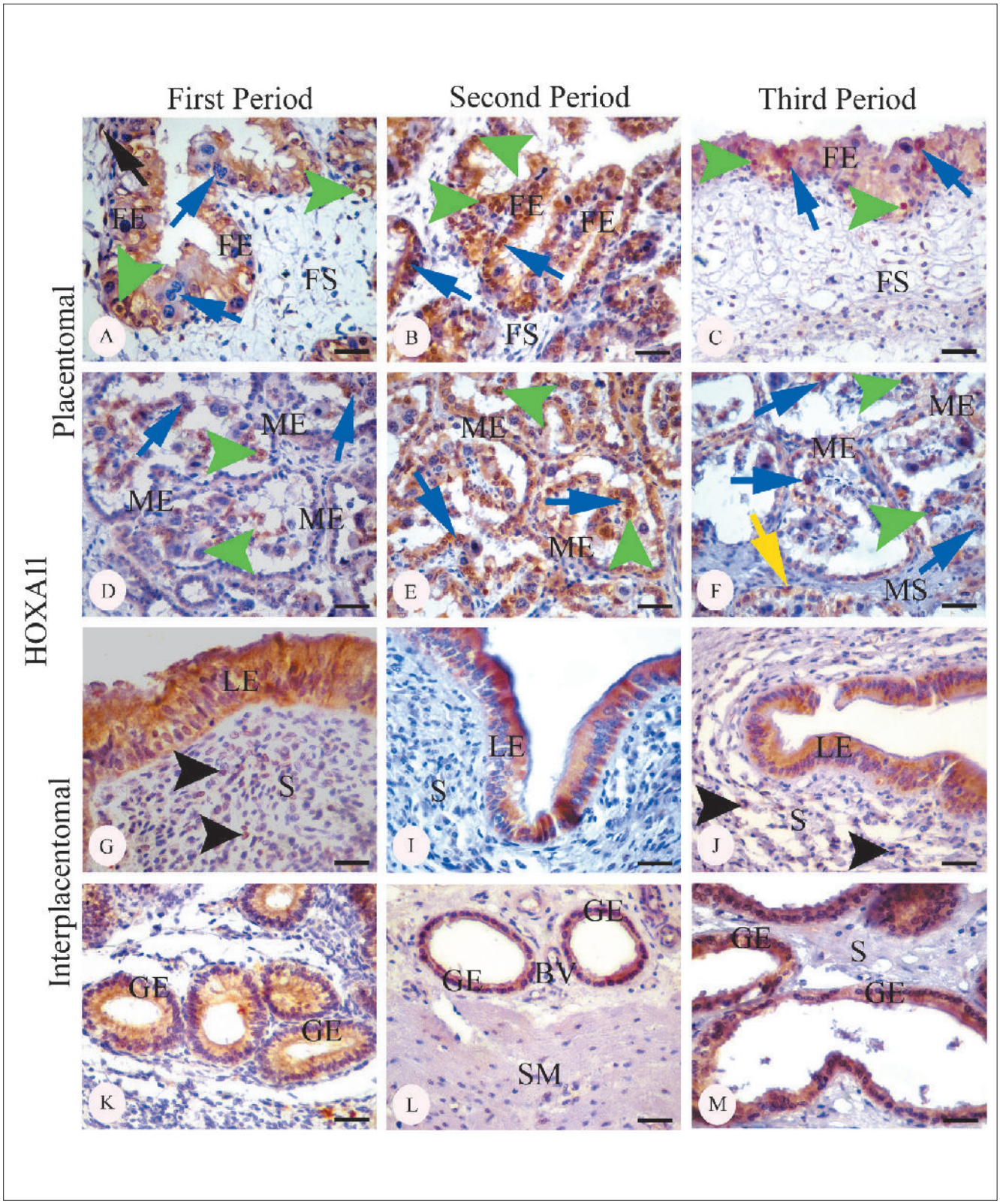


Figure 1: Immunolocalisation of HOXA11 in bovine placentomal and interplacentomal part, in the first period of pregnancy (79 days; A, D, G, K), second period (106 days; B, E, I, L), third period (249 days; C, F, J, M). FE: Fetal Epithelial, FS: Fetal Stroma, Green Arrowhead: Uninucleated trophoblast cell, Blue arrow: Binucleated trophoblast cell, Black arrow: Fetal stroma cells, ME: Maternal Epithelium, MS: Maternal Stroma, Yellow arrow: Maternal stroma cell, LE: Luminal Epithelium, S: Stroma, Black arrowhead: Stroma cell, GE: Gland Epithelium, SM: Smooth Muscle layer, BV: Blood Vessel. Scale Bar: 25 μm

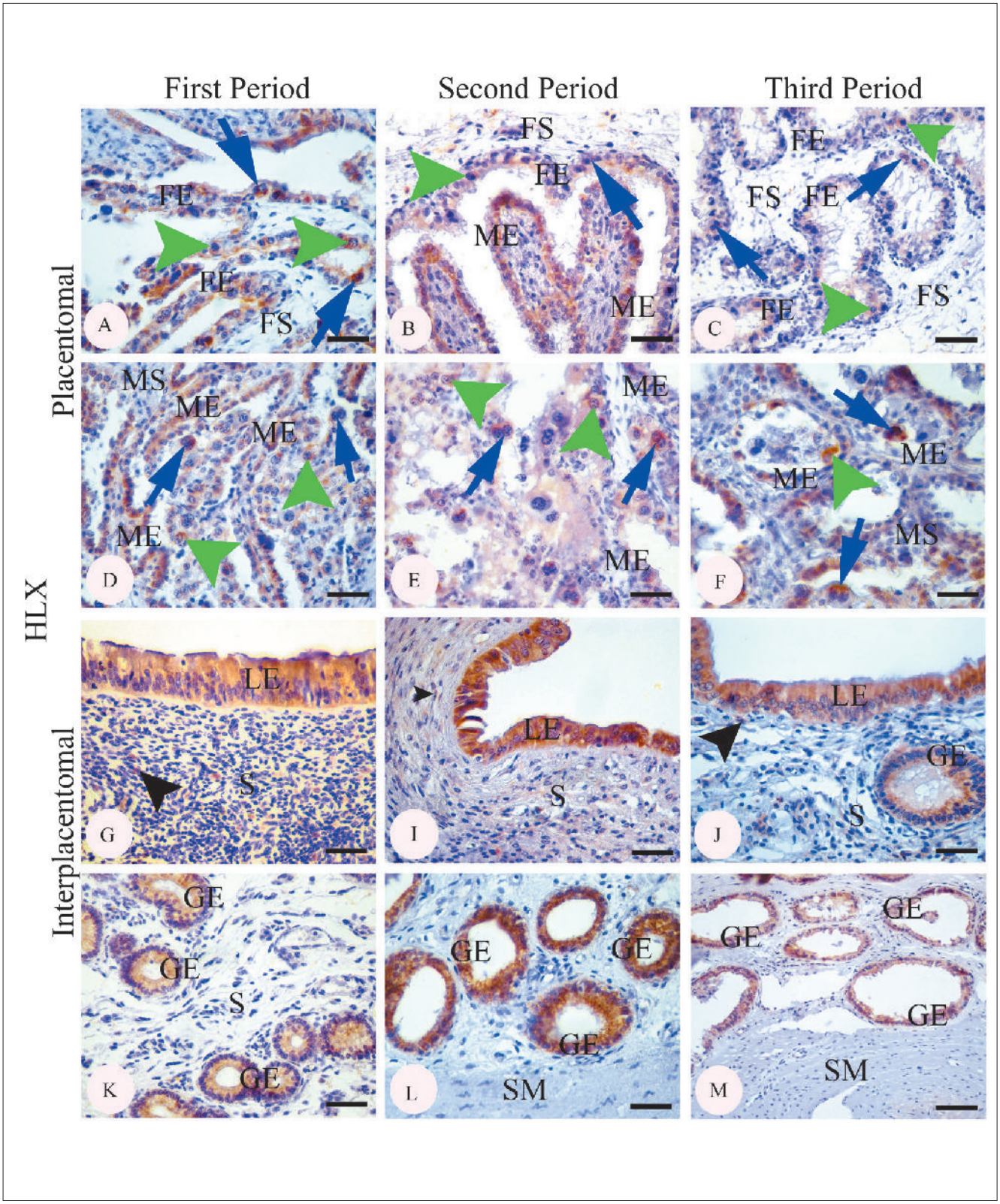


Figure 2: Immunolocalisation of HLX in bovine placentomal and interplacentomal part, in the first period of pregnancy (75 days; A, D, G, K), second period (123 days; B, E, I, L), third period (202 days; C, F, J, M). FE: Fetal Epithelial, FS: Fetal Stroma, Green Arrowhead: Uninucleated trophoblast cell, Blue arrow: Binucleated trophoblast cell, ME: Maternal Epithelium, MS: Maternal Stroma, LE: Luminal Epithelium, S: Stroma, Black arrowhead: Stroma cell, GE: Gland Epithelium, SM: Smooth Muscle layer cell. Scale Bar: (A, B, C, D, E, F, G, I, J, L): 25 µm, (K, M) 50 µm

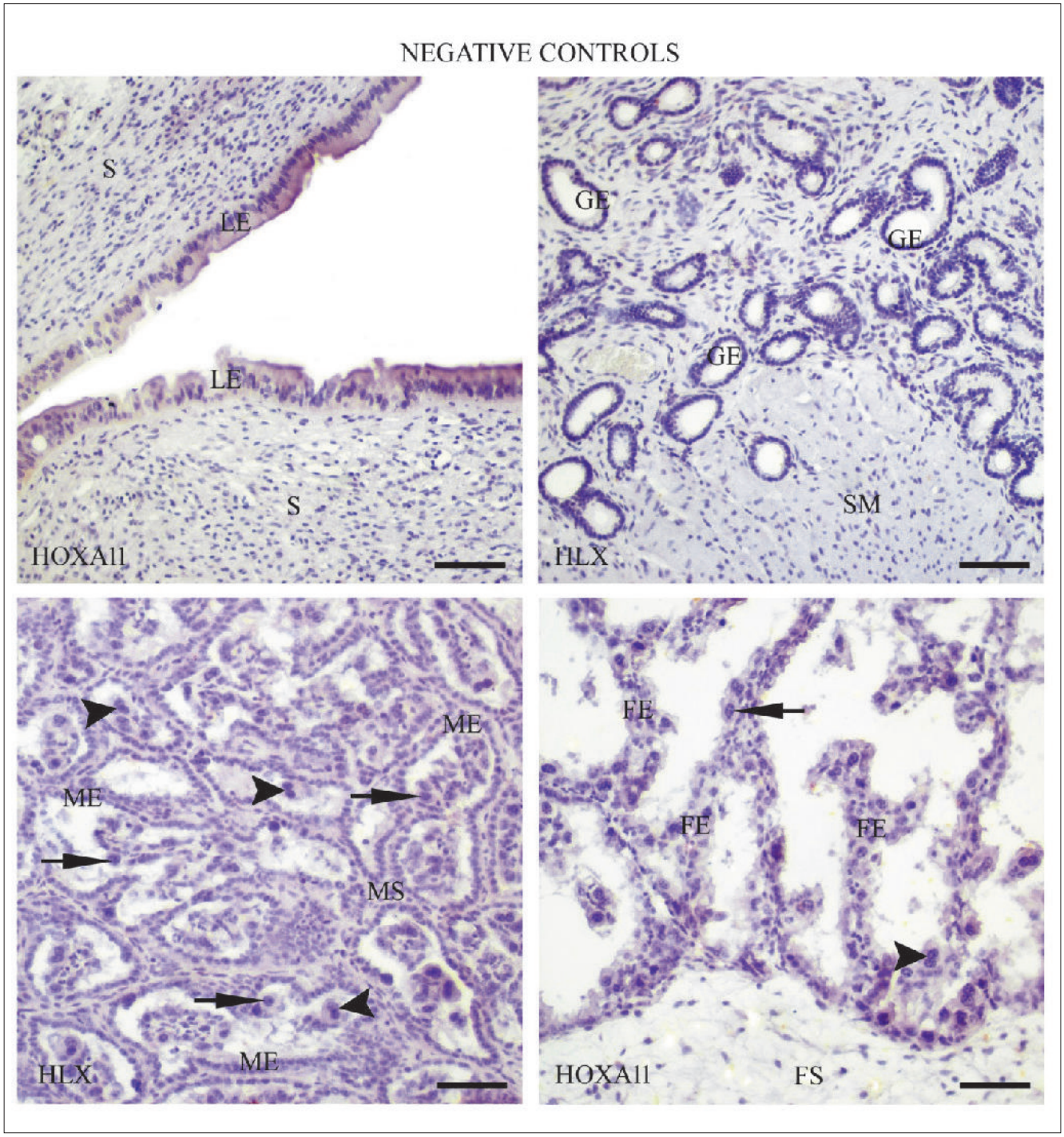


Figure 3: Negative control immunoreactivity for HOXA11 and HLX proteins. LE: Luminal Epithelium, S: Stroma, GE: Gland Epithelium, SM: Smooth Muscle layer, FE: Fetal Epithelial, FS: Fetal Stroma, ME: Maternal Epithelium, MS: Maternal Stroma, Arrow: Uninucleated trophoblast cell, Arrowhead: Binucleated trophoblast cell. Scale Bar: 50 μm

It was observed that a moderate positive immunoreaction occurred in the luminal and glandular epithelial cells of the endometrium in all three periods of pregnancy. It was found that especially the immunoreaction was localized in the apical cytoplasm of the luminal and gland epithelial cells (Figure 1G, I, J, K, L, M). It was observed that immunoreactivity was statistically similar in endometrial epithelium, gland, and muscle layer during the entire pregnancy ($p>0.05$). Moreover, it was determined that HOXA11 produced a positive immunoreaction in some stromal cells

of the uterus in the first and third periods, as well as in some vascular endothelial cells in the second period (Figure 1G, J, L). It was observed that immunoreaction in stromal cells decreased significantly in the second period compared to the first period ($p < 0.05$).

HLX

It was observed that the immunoreaction in UTC and TGC/BNC in the chorion epithelium belonging to the fetal part of the placenta was found to be in weak density in the cytoplasm in all three periods of pregnancy. On the other hand, a very weak immunoreaction was observed in the fetal stroma and mesenchyme. The HLX immunoreaction observed in the maternal part of the placenta was determined to be weak and weak-to-middle for the epithelium in all three periods of pregnancy, and it was found to be in very weak intensity for the stroma (Figure 2A, B, C, D, E, F). As a result of the statistical evaluation of the scores obtained from the placental parts, it was observed that there was no significant difference in the immunoreaction intensity of HLX in each period of pregnancy ($p > 0.05$).

HLX immunoreaction observed positively in pregnant cow uteri was found to occur in the cytoplasm of luminal and glandular epithelial cells from weak-to-medium density in all three periods of pregnancy. Furthermore, it was found that a very weak immunoreaction occurred in some stromal and smooth muscle cells of the uterus (Figure 2G, I, J, K, L, M). The statistical evaluation of the scores showed that the HLX immunoreaction was at similar levels in the uterus in all periods of pregnancy ($p > 0.05$).

It was observed that no immunoreaction occurred in negative control sections where PBS was applied instead of primary antibody, which verified the accuracy of the immunoreactivity in the interplacentomal and placentomal regions (Figure 3).

Discussion

Homeobox proteins encode transcription factors controlling embryonic development, and regulate the proliferation and differentiation of cells throughout the reproductive cycle. It has also been stated that they play critical roles in the growth and development of the endometrium together with estrogen and progesterone hormones that regulate endometrial physiology (26).

Homeobox proteins have been reported as one of the many transcription factors that regulate and control the proliferation and differentiation of placental cells. Furthermore, it has also been reported that they can contribute to the normal and pathological development of the human placenta, as in embryogenesis and organogenesis (27). Moreover, studies have indicated that developmental, morphological, and angiogenesis defects occur in the placenta caused by the absence of homeobox protein (28).

It has been reported that HOXA11, which is necessary for female fertility, is expressed in the cervix together with the lower segment of the uterus during the development of the female genital tract (29, 30). Studies have reported that HOXA11/Hoxa11 protein is expressed in adult human and mouse endometrium, and this expression reaches the highest level in the secretory phase of the sexual cycle in humans in relation with estrogen and progesterone. It was reported that the hox protein in mice (Hoxa11) and humans (HOXA11) plays a critical role in embryo implantation and it is expressed in the endometrial glands and stroma during the estrus cycle (31). In an experimental study in wild-type mice, it was reported that the Hoxa11 gene was localized in stromal cells in the endometrium; however, it was shown to be absent in the glandular epithelium. It was pointed out that infertility occurs as a result of uterine anomalies caused by the mutation of this gene (15). In another study, it was reported that HOXA11/Hoxa11 expression, which was observed at high levels during the secretory phase of the normal endometrium in humans and in the normal endometrium of mice, did not occur in uterus with endometriosis. In addition, the impairment of HOXA11 expression was reported to promote abnormal epithelial cell differentiation leading to epithelial ovarian neoplasia and decreased glandular and stromal tissue development in mice (26). In another study conducted in humans, it was stated that HOXA11 was strongly localized in the stromal, glandular, and luminal epithelial cell nuclei of the endometrium (32). In a study conducted on pigs, it was reported that HOXA11 was expressed in the uterus and it was shown that this expression, which was high at the beginning of pregnancy, decreased towards the end of pregnancy (33).

In the presented study, it was observed that HOXA11 was expressed in the cow endometrium as well as in that of humans, mice, and pigs. Hsieh-li et al. (1995) reported that, unlike other studies, no reaction occurred in the glandular epithelium of the wild-type mouse endometrium. However, parallel to the findings of Xu et al. (2014) in humans, in our study, it was found that a similar level of expression occurred in all three periods of pregnancy in luminal, glandular, and some stromal cells. However, in our study, a significant decrease was found in the expression of stromal cells in the second and third periods of pregnancy ($p<0.05$). These finding suggest that HOXA11 can contribute to endometrial functions during pregnancy in cows, to the proliferation and differentiation of epithelial and glandular cells, and to pregnancy physiology. Moreover, it was suggested that the reduced immunoreaction in stromal cells in the second period of gestation may be due to the stagnancy in the differentiation of stromal cells. Furthermore, the observation of HOXA11 immunoreactivity in some vascular endothelium suggested that this protein may play a role in the proliferation of some endothelial cells.

Although it is known that homeobox proteins are localized in placental cells, information about their roles is sparse. In a study performed in human placenta, it was observed that HOXA11 reacted positively in the cells of the placental villi, cytotrophoblast, intermediate trophoblast, and syncytiotrophoblast cells in the last period of pregnancy. This demonstrated that the HOXA11 gene expression has important roles in the formation and development of the human placenta, as well as in the regulation of differentiation and proliferation of the trophoblast cells. Furthermore, the fact that this protein is expressed in placental cells with choriocarcinoma shows that it plays a role in the development and pathogenesis of tumor diseases (34). In another study, it was observed that HOXA11 induces a strong immunoreaction in cytotrophoblast nuclei. However, it was reported that this immunoreaction is reduced in syncytiotrophoblasts (35).

Studies on the expression of HOXA11 in the placenta are scarce and its function has not been fully revealed. In parallel with the studies in the human placenta, in our study, HOXA11 was found to be expressed in cow placenta. It was found that the expression increased significantly in the stroma of the maternal placenta in the third period ($p<0.05$), and decreased significantly in the UTC and TGC/BNC in the fetal placenta in the same period ($p <0.05$). Thus, it was thought that HOXA11 could perform autocrine and/or paracrine functions in stromal cells in cow placenta as well as in human placenta. Furthermore, placental development and continuity may have critical roles in the proliferation and differentiation of trophoblasts. In addition, it is thought that the immunoreaction increasing in the second period of the maternal placenta occurs due to the metabolic activities of the differentiated epithelial cells.

H2.0-like homeobox (HLX, HB24) is one of the molecules that can control tissue growth caused by epithelial-mesenchymal cell interactions during human endometrial morphogenesis (36). In a study conducted in human endometrium, it was reported that the HB24 (HLX) protein was detected in the glandular and luminal epithelial cells and in the stromal cells of the endometrium (37). The effects of the Hlx gene, the homologue of HLX in rats, on endometrial or placental development are unknown. However, the Hlx gene has been reported to be concentrated in the lung, heart, skeletal muscle, and hematopoietic tissues and cells in adult mice; moderately intensely expressed in the liver, uterus, and ovaries; and it is expressed at a little intensity in the brain, kidney, and testis (36, 38). As in humans and mice, it was observed in our study that HLX induces a positive immunoreaction in the cow uterus. It was determined that immunoreaction occurred in the cytoplasm of the cells from weak-to-medium density in luminal and glandular epithelial cells in all three periods of pregnancy. It was also observed that a very weak reaction occurred in some stromal and smooth muscle cells. Thus, it was thought that HLX may have functional roles in the normal physiology of the cow uterus, in the preparation of the uterus for pregnancy, and in maintaining the pregnancy, as in humans.

Evidence has been presented that HLX (previously HLX1 and HB24), a member of the Homeobox gene family, may play an important role in normal placentation and development, especially trophoblast proliferation and differentiation (19, 22, 39). It was reported that HLX, which has an important regulatory role for the human placenta, is expressed in the villi and extravillous cytotrophoblast cells as well as in endothelial cells and fetal microcapillaries (22). In another study, it was stated that HLX expression seen in trophoblasts and stromal

cells in human placenta in early gestation (8-12 weeks) is dense in the nucleus and weak in the cytoplasm. In addition, expression was observed in villus cytotrophoblasts, endovascular, and multinuclear (giant cells) cytotrophoblasts, but not in syncytiotrophoblasts or syncytiums (39, 40, 41). In studies performed in human placentas with fetal growth retardation, it has been reported that HLX expression is decreased in trophoblasts, but does not occur in any stromal cells (39, 42).

H2.0-like homeobox (HLX), which is known as Hlx in mice, has been reported to be expressed in the labyrinth layer of the placenta, secondary giant cells, and the spongiotrophoblast layer in mice. It was also stated that the spongiotrophoblast layer described here is necessary for the structural support of the placenta. Moreover, although the presence of Hlx in the mouse placenta has not been fully revealed, morphological deterioration in the spongiotrophoblast layer in the placental tissues of mutant mice has been reported; however, it was demonstrated that the layer with giant cells on top was not affected (43). In another study conducted on Hlx mutant mice, it was reported that there were defects in placental development, which caused fetal growth retardation (44, 45).

It has been observed that HLX induces an immunoreaction in cow placenta as well as in human (22) and mouse (43) placentas. The observed immunoreactivity was found to be at a similar moderate intensity in the epithelial and stromal cells of the maternal placenta, and in uninucleate and binucleate trophoblast cells in all three periods of pregnancy. Thus, in cow placenta as well as in human and mouse placentas, HLX plays very important roles in the proliferation and differentiation of trophoblasts; in the growth and development of the placenta; and in the continuity of pregnancy. In addition, it has been reported that HLX, which has a role in normal placental development and differentiation of placental cells, should be at a certain level and order. Otherwise, it has been stated that, together with the dysregulation of HLX, cytotrophoblasts proliferate and differentiate abnormally, resulting in pathological conditions such as placental choriocarcinoma (19). The findings in our study support this information and it was observed that HLX has similar densities in all parts of the placenta.

As a result, it was observed that HOXA11 and HLX proteins produced a positive immunoreaction at certain concentrations in cow uterus and placenta during pregnancy. The immunohistochemical outcomes observed in the present study suggested that HOXA11 and HLX may affect the proliferation and differentiation of cells in the uterus and placenta during pregnancy in cows, and they may contribute to the normal uterine physiology, placentation formation, and the continuity of pregnancy in cows. In addition, we think that this study will shed light on new studies to be conducted on the mammalian uterus and pregnancy physiology, and that it can contribute to the emergence of new methods for the prevention and treatment of hereditary and pathological problems of the placenta, uterus and fetus.

Acknowledgement

This study is based on a project numbered as ‚VETERINER.19.004“, which is supported by the scientific research projects commission of Dicle University.

References

1. Estrella CAS, Kind KL, Derks A, et. al. Remodelling of the bovine placenta: comprehensive morphological and histomorphological characterization at the late embryonic and early accelerated fetal growth stages. Placenta 2017; 55: 37–46.

2. Roberts RM, Cross JC, Leaman DW. Interferons as hormones of pregnancy. Endocr Rev 1992; 3: 432–52.

3. Pereira FTV, Oliveira LJ, Barreto Rda S, Mess A, et al. Fetal-Maternal interactions in the synepitheliochorial placenta using the eGFP cloned cattle model. PLoS One 2013; 8(5): e64399. doi: 10.1371/journal.pone.0064399

4. Wooding FBP. The synepitheliochorial placenta of ruminants: binucleate cell fusions and hormone production. Placenta 1992; 13(2): 101—3.

5. Bridger SP, Haupt S, Leiser R, et. al. Integrin activation in bovine placentomes and in caruncular epithelial cells isolated from pregnant cows. Biol Reprod 2008; 79: 274–82.

6. Murthi P, Rajaraman G, Brennecke SP, Kalionis B. The role of placental homeobox genes in human fetal growth restriction. J Pregnancy 2011; 2011: e548171. doi: 10.1155/2011/548171

7. He B, Ni Z, Kong SB, Lu JH, Wang HB. Homeobox genes for embryo implantation: from mouse to human. Anim Model Exp Med 2018; 1: 14–22.

8. Deb P. Epigenetic mechanism of regulation of hox genes and neurotransmitters via hormones and lncrna. Arlington : University of Texas, 2017. Doctoral Thesis

9. Taylor HS. The role of hox genes in the development and function of the female reproductive tract. Semin Reprod Med 2000; 18(1): 81–9.

10. Zanatta A, Rocha AM, Carvalho FM, et. al. The role of the Hoxa10/HOXA10 gene in the etiology of endometriosis and ıts related infertility: a review. J Assist Reprod Genet 2010; 27(12): 701–10.

11. Kim JJ, Taylor HS, Lu Z, et. al. Altered expression of HOXA10 in endometriosis: potential role in decidualization. Mol Hum Reprod 2007; 13: 323–32.

12. Lynch VJ, Roth JJ, Takahashi K, et. al. Adaptive evolution of HoxA-11 and HoxA-13 at the origin of the uterus in mammals. Proc Biol Soc 2004; 271(1554): 2201–7.

13. Zhu Y , Cheng Z, Wang J, et. al. A novel mutation of HOXA11 in a patient with septate uterus. Orphanet J Rare Dis 2017; 12(1): e178. doi: 10.1186/s13023-017-0727-9

14. Wang LF, Luo HZ, Zhu ZM, Wang JD. Expression of HOXA11 gene in human endometrium. Am J Obstet Gynecol 2004; 191(1): 767–72.

15. Hsieh-Li HM, Witte DP, Weinstein M, et. al. Hoxa 11 Structure, extensive antisense transcription, and function in male and female fertility. Development 1995; 121(5): 1373–85.

16. Bates MD, Schatzman LC, Lints T, Hamlin PE, Harvey RP, Potter SS. Structural and functional characterization of the mouse Hlx homeobox gene. Mamm Genome 2000; 11(10): 836–42.

17. Hentsch B, Lyons I, Li R, et al. Hlx Homeo box gene is essential for an inductive tissue interaction that drives expansion of embryonic liver and gut. Genes Dev 1996; 10(1): 70–9.

18. Murthi P, Doherty V, Said J, Donath S, Brennecke SP, Kalionis B. Homeobox gene HLX1 expression is decreased in idiopathic human fetal growth restriction. Am J Clin Pathol 2006; 168(2): 511–8.

19. Rajaraman G, Murthi P, Quinn L, Brennecke SP, Kalionis B. Homeodomain protein HLX is expressed primarily in cytotrophoblast cell types in the early pregnancy human placenta. Reprod Fertil Dev 2008; 20(3): 357–67.

20. Lints TJ, Hartley L, Parsons LM, Harvey RP. Mesoderm-specific expression of the divergent homeobox gene Hlx during murine embryogenesis. Dev Dyn 1996; 205(4): 457–70.

21. Irving JA, Lala PK. Functional role of cell surface integrins on human trophoblast cell migration: regulation by TGF-beta, IGF-II, and IGFBP-1. Exp Cell Res 1995; 217(2): 419–27.

22. Rajaraman G, Murthi P, Brennecke SP, Kalionis B. Homeobox gene HLX is a regulator of HGF/c-met-mediated migration of human trophoblast-derived cell lines. Biol Reprod 2010; 83(4): 676–83.

23. Topaloğlu U, Ketani MA. The distribution of some homeobox proteins in the bovine placenta during gestation. Theriogenology 2021; 166: 71–82.

24. Harris RM, Fly DE, Synder BG, et al. The relationship of bovine crown rump measurement to fetal age. Agri-Practice 1983; 4(1): 16–22.

25. Detre S, Saccani JG, Dowset M. A ‘’Quik score’’ method for immunohistochemical semiquantitation: validation for oestrogen receptor in breast carcinoms. J Clin Pathol 1995; 48(9): 876–8.

26. Nova-Lampeti E, Aguilera V, Oporto K, et al. Hox genes in adult tissues and their role in endothelial cell differentiation and angiogenesis. In: Lenasi H, ed. Endothelial dysfunction: old conseptus and new challenges, [online] London : IntechOpen, 2018. doi: 10.5772/intechopen.76745

27. Novakovic B, Fournier T, Harris LK, James J, Roberts CT, Yong HEC. Increased methylation and decreased expression of homeobox genes TLX1, HOXA10 and DLX5 in human placenta are associated with trophoblast differentiation. Sci Rep 2017; 7(1): e4523. doi: 10.1038/s41598-017-04776-5.

28. Chui A, Pathirage NA, Johnson B, et.al. Homeobox gene distal-less 3 is expressed in proliferating and differentiating cells of the human placenta. Placenta 2010; 31(8): 691–7.

29. Taylor J, Ramos JG, Bosquiazzo VL, Toro MM, Luque EH. Developmental exposure to bisphenol an impairs the uterine response to ovarian steroids in the adult. Endocrinology 2008; 149 (11): 5848–60.

30. Taylor HS, Vanden-Heuvel GB, Igarashi P. A conserved Hox axis in the mouse and human female reproductive system: late establishment and persistent adult expression of the Hoxa cluster genes. Biol Reprod 1997; 57(6): 1338–45.

31. Du H, Taylor HS. The Role of Hox genes in female reproductive tract development, adult function, and fertility. Cold Spring Harb Perspect Med 2016; 6(1): art023002. doi: 10.1101/cshperspect.a023002

32. Xu B, Geerts D, Bu Z, et. al. Regulation of endometrial receptivity by the highly expressed HOXA9, HOXA11 and HOXD10 HOX-class homeobox genes. Hum Reprod 2014; 29(4): 781–90.

33. Lin R, Jia H, Chen S, Zhao S. Expression analysis of the porcine homeobox A11 gene and its association with litter size in large white pigs. Czech J Anim Sci 2016; 61(5): 243–9.

34. Amesse LS, Moulton R, Zhang YM, Pfaff-Amesse T. Expression of hox gene products in normal and abnormal trophoblastic tissue. Gynecol Oncol 2002; 90(3): 512–8.

35. Zhang YM, Xu B, Rote N, Peterson L, Amesse LS. Expression of homeobox gene transcripts in trophoblastic cells. Am J Obstet Gynecol 2002; 187(1): 24–32.

36. Quinn LM, Latham SE, Kalionis B. Homeobox gene HB24, a regulator of hematopoiesis, is a candidate for regulating differentiationof the extra-embryonic trophoblast cell lineage. Reprod Fertil Dev 1997; 9(6): 617–23.

37. Quinn LM, Kilpatrick LM, Latham SE, Kalionis B. Homeobox genes DLX4 and HB24 are expressed in regions of epithelial-mesenchymal cell interaction in the adult human endometrium. Mol Hum Reprod 1998; 4(5): 497–501.

38. Bates MD, Wells JM, Venkatesh B. Comparative genomics of the Hlx homeobox gene and protein: conservation of structure and expression from fish to mammals. Gene 2005; 352: 45–56.

39. Rajaraman G, Murthi P, Pathirage N, Brennecke SP, Kalionis B. Downstream targets of homeobox gene HLX show altered expression in human idiopathic fetal growth restriction. Am J Pathol 2010; 176(1): 278–87.

40. Liu H, Murthi P, Qin S, et. al. A novel combination of homeobox genes is expressed in mesenchymal chorionic stem / stromal cells in first trimester and term pregnancies. Reprod Sci 2014; 21(11): 1382–94.

41. Gabrielsen M, Nerhus M. Placentation and expression of homeobox genes in extravillous trophoblasts in first trimester pregnancies. Oslo : University, Faculty of Medicine, 2006. Student Thesis

42. Yuen N, Brennecke SP, Umstad MP, et. al. Expression of homeobox gene HLX and its downstream target genes are altered in placentae from discordant twin pregnancies. Twin Res Hum Genet 2018; 21(1): 42–50.

43. Murthi P, Bates MD, Sankaran G, Brennecke SP, Kalionis B. Role of homeobox gene Hlx expression in normal placental developmemt. Reprod Fertil Dev 2004; 16(suppl, 9): 271. doi: 10.1071/SRB04Abs271

44. Li Y, Behringer RR. Esx1 is an X-Chromosome imprinted regulator of placental development and fetal growth. Nat Genet 1998; 20(3): 309–11.

45. Sapin V, Blanchon L, Serre AF, Lemery D, Dastugue B, Ward SJ. Use of transgenic mice model for understanding the placentation: towards clinical applications in human obstetrical pathologies. Transgenic Res 2001; 10(5): 377–98.

IMUNOLOKALIZACIJA PROTEINOV HOXA11 IN HLX V POSTELJICI KRAVE MED BREJOSTJO

U. Topaloğlu, M. A. Ketani, M. E. Akbalık, H. Sağsöz, B. G. Saruhan, B. Bayram

Izvleček: Za posteljico so poleg številnih funkcij značilne intenzivnost in karakteristike njenih hormonskih funkcij. Poročali so, da razvoj in ožiljenost posteljice pri normalni rasti ploda uravnavajo številni dejavniki, kot so rastni dejavniki, transkripcijski dejavniki in homeoboks proteini. Ta študija je bila izvedena z namenom določiti porazdelitev in morebitno fiziološko vlogo proteinov HOXA11 in HLX v maternici in posteljici brejih krav. Za vzorce tkiv smo uporabili maternice in posteljice 27 krav pasem Holstein, pridobljenih v zasebnih klavnicah. Obdobje brejosti smo izračunali na podlagi starosti plodov po formuli. Maternice smo glede na obdobje brejosti uvrstili v tri skupine po 9 vzorcev: 1 (69-89 dni), 2 (99-178 dni) in 3 (190-269 dni). Prisotnost proteinov HOXA11 in HLX v vzorcih tkiv smo preverili z imunohistokemijo. Proteini HOXA11 in HLX so bili prisotni predvsem v luminalnih in žleznih epitelijskih celicah maternice ter v epitelijskih celicah maternalne posteljice, UTC in TGC/BNC. Prisotnost HOXA11 je bila zaznavna tudi v nekaterih žilnih endotelijskih celicah. Rezultati imunohistokemije v tej študiji kažejo na morebiten vpliv proteinov HOXA11 in HLX na proliferacijo in diferenciacijo celic v maternici in posteljici krav med brejostjo in na morebitno vlogo teh proteinov pri normalni fiziologiji maternice, nastanku posteljice in vztrajanju brejosti pri kravah.

Ključne besede: proteini Hox; HOXA11; HLX; posteljica

Slov Vet Res 2022; 59 (2): 113–23
DOI 10.26873/SVR-1317-2022
UDC 611.019:611.714:599.744.211:572.08

Case Report

MORPHOMETRICAL FEATURES OF THE CAVE BEAR AND BROWN BEAR HEAD SKELETON: A COMPARATIVE STUDY

Matjaž Uršič

Veterinary Faculty, University of Ljubljana, Gerbičeva 60, 1000, Ljubljana, Slovenia

E-mail: matjaz.ursic@vf.uni-lj.si

Abstract: The extinct cave bear (CB) is often depicted as a large heavy animal with a prominent, massive skull and severely shortened pelvic limbs. The size and robustness of the CB are comparable to those of the largest living ursids. Great similarities between CB and the brown bear (BB) species prompted our morphometric comparison. The goal of this study was to elucidate the potential differences in the morphometric skull characteristics of CB and BB species. Craniometric measurements were performed on the skulls of both bear species and were compared to identify craniometric features indicative of possible adaptations in both bear species. The results revealed a marked difference in skull size; however, the shape of the CB cranium is generally known to be similar to that of the BB. In CB, the total length of the skull was approximately 1.5 times longer, and the external bony nasal aperture was larger due to the relatively shorter nasal bones. The nose length and median palate length were relatively longer in the CB, and the infraorbital foramen was located more caudally and closer to the zygomatic process. The infraorbital channel of the CB was located over to the second superior molar roots whereas in BB the latter extends beyond the roots of the first to the second superior molar roots. A marked difference was the non-existence of the three most anterior premolars in the maxilla and mandible of the CB, Higher body of the mandible, which, together with the extensive biting surfaces of the cheeks teeth, indicates a predominantly plant-based diet of the CB. The braincase length was considerably shorter in the CB, resulting in a relatively small neurocranium volume. The pronounced frontal fossa in the CB skull continues caudally into a strongly developed frontal area, which gives the CB skull a prominent steep profile. In summary, comparative craniometry showed that CB had a smaller neurocranial volume and had herbivore-adapted jaws and teeth. These metric features of the head skeleton may be related to a lower adaptability to extreme climatic conditions to which they were exposed to during the last Pleistocene glacial period, which may have contributed to their extinction.

Key words: head skeleton; skull; mandible; craniometrical features; bear

Introduction

Bears are members of the *Ursidae* family. It is believed that bears evolved from the canid line during the late Oligocene and early Miocene (1). The family consists of eight extant species which are classified into three subfamilies: *Ailuropodinae* including the giant panda *(Ailuropoda melanoleuca), Tremarctinae* including the Andean bear *(Tremarctos ornatus)*, and *Ursinae* which includes six species: brown bear *(Ursus arctos)*, polar bear *(Ursus maritimus)*, American black bear *(Ursus americanus)*, Asiatic black bear *(Ursus thibetanus)*, sloth bear *(Melursus ursinus)*, and sun bear *(Helarctos malayanus)*. The six species in the *Ursinae* diverged in the last five million years (2). The cave bear (CB) *(Ursus spelaeus)* is an extinct species in the *Ursinae* subfamily that was continuously present in the middle and late Pleistocene. CBs were the most common subspecies among the Pleistocene animals (1, 3,). The Etruscan bear *(Ursus etruscus)* spread into Eurasia during the early Pleistocene, it gave rise to CBs and was an ancestral to BBs. It was

Received: 18 February 2021
Accepted for publication: 19 April 2022

initially smaller, but continued to trend toward a larger body size (4, 5).

Studies involving mitochondrial and genomic DNA extracted from CB remains showed that it was very closely related to BBs and polar bears but had split from the brown bear lineage before the BBs split from the polar bears (2). The name CB is used because the fossils of this species are usually found in caves that provide a favourable fossilisation environment. There is innumerable and unequivocal evidence of habitation by a number of different species in Eurasian cave sediments. CBs often used caves for hibernation, and for giving birth to cubs. Modern bears can create artificial shelters and hibernation dens; however, they readily hibernate in natural caves when appropriate conditions exist (4). This suggests that CBs may have spent more time in caves than BBs, which use caves occasionally, primarily for hibernation. The preponderance of bear remains found in Pleistocene cave sediments does not necessarily mean that CBs lived exclusively in caves. It may be that the caves provided more favourable conditions for the preservation of remains over a long period of time.

Extensive research and excavations of bone remains have been carried out in almost all important archaeological deposits (6, 7, 8, 9, 10). The CB bone remains are the most numerous of the large mammal species. Bones, teeth, and genetic research have shown that at least four different CB lineages existed before their extinction in the middle Pleistocene (11).

CBs had a wide geographical distribution throughout Europe. Fossil remains have been reported from Spain in the west to Great Britain in the north, in the northern parts of Greece, and as far east as the Urals (12). CBs were also frequent inhabitants of the Alps. In Slovenia, many archaeological sites with CB remains are known, including Potočka zijalka, Mokriška jama, Križna jama, and Divje babe caves, where large quantities of CB bones have been found (13).

The Eurasian brown bear (*Ursus arctos arctos*) is one of the most common subspecies of BB. The BB in the Balkans is a member of this subspecies belonging to the European population (14). Today, Slovenian bears are members of the Alpine-Dinaric-Pindos population, which occupies the area from the Eastern Alps in Austria and north-eastern Italy, to the Pindos Mountains in Greece (15) and extends over the territory of Austria, Italy, Slovenia, Croatia, Bosnia and Herzegovina, North Macedonia, Montenegro, Serbia, Kosovo, Albania, and Greece. The Dinaric-Pindos BB population number is assessed at 2100–2800 bears (16, 17). Slovenia is located on the north-western edge of the most densely populated area of the Dinaric bear population and is thus the westernmost part of the BB population in Central Europe (18).

During the Pleistocene ice age, the climate was too cold for BBs to survive in Europe, except in some places in Russia, Spain, and the Balkans, while the CB became extinct. The southern European BB is a relic from the late Pleistocene in comparison to the north-east European bear population, and spread throughout Europe only at the beginning of the Holocene (19).

In recent decades, many metric studies have been conducted on bears (20, 21, 22, 23, 24). Generally, the *Ursidae* represent the largest terrestrial carnivores, with a robust body, short tail, and short but powerful limbs. They are quadrupedal plantigrades. The head is large and robust with erect rounded ears, small eyes, grasping lips, and a particularly long tongue. The extinct CB is often depicted as a large heavy animal with a distinctive bulky skull and strongly shortened pelvic limbs in comparison to the thoracic ones. The size and robustness of the CB body are comparable to those of the largest extant ursids. The CB is thought to resemble a very large and cumbersome BB that fed mostly on plants (25). Therefore, we assumed that the CB and the modern BB shared similar morphological characteristics, which prompted our comparative craniometric study.

Craniometric characteristics have typically been used to describe the morphological characteristics of individuals to compare groups and to evaluate skull dimensions. Skulls differ in size and shape among the mammalian species. Considering that craniometry is becoming increasingly important in the characterisation of certain species, breeds and crosses, we compared the main metric features of the head skeleton of an extinct CB with that of a modern BB.

Material and methods

Two fossil skulls and three fossil mandibles of adult CBs, found at the archaeological site of Križna jama cave in Slovenia and owned by the palaeontological collection of the Natural History Museum of Slovenia were studied. The data

were compared to those of two adult BB skulls, including the mandibles, owned by the Anatomical Museum of the Veterinary Faculty University of Ljubljana. Craniometry was performed on the skulls and mandibles (Figures 1–5).

Several craniometric points and landmarks on the skull were determined for linear measurements. Firstly, the most important craniometrics points, shown in Figure 1, were marked: *akrokranion* (A), the most caudal point of the cranium vertex in the median plane; *prosthion* (P), the most rostral point of the interincisive suture; *basion* (B), the point in the middle of the ventral margin of the foramen magnum; *synsphenion* (S), the midpoint in the intersphenoid suture; *nasion* (N), the median point of the naso-frontal suture; *frontal midpoint* (F), the median point of the line joining both the most lateral points of the frontal bone on the occipital side of the orbit *ectorbitale* (Ect); *rhinion* (Rh), the median point of the line joining the most rostral points of the nasals; *staphylion* (St), the most caudal point of the palatine bone in the median plane; *palatinoorale* (Po), the median point of the palatine maxillary suture; *otion* (Ot), the most lateral point of the mastoid region; *zygion* (Zy), the most lateral point of the zygomatic arch; *entorbitale* (Ent), the naso-medial indentation of the orbit that corresponds with the inner angle of the living animal; *euryon* (Eu), the most lateral points of braincase; *coronion* (Cr), the highest point of the coronoid process; *infradentale* (Id), the most prominent median point at the rostral border of the alveoli of the mandible incisors.

The craniometric measurements of each skull were taken according to the scheme provided by von den Driesch (26) and are given in millimetres. The measurements were taken with a curved calliper in millimetres and a sliding calliper with an accuracy of 0.1 mm.

The following craniometric measures were taken for each skull (Table 1, Figure 1a, -b, -c): *the total length* (L1) from A to P; *the condylobasal length* (L2) from the caudal border of the occipital condyles to P; *the basal length* (L3) from B to P; *the basicranial axis* (L4) from B to S; the *basifacial axis* (L5) from S to P; *the neurocranium length* (L6) from B to N; *the upper neurocranium length* (L7) from A to F; *the viscerocranium length* (L8) from N to P; *the facial length* (L9) from F to P; *the greatest length of the nasal bone* (L10) from N to Rh; length of braincase; *the inner length of the cranial cavity* (L11) from B to cribriform plate; *the nose length* (L12) from medial orbital border to P; *the median palatal length* (L13) from St to P; *the length of the horizontal part of the palatine bone* (L14) from St to Po; *the length of the cheektooth row* (L15); *the alveolus M^2 to alveolus C length* (L18) of the caudal border of alveolus of M^2 to rostral border of alveolus of C; *the length of the molar row* (L16); *the length of the premolar row* (L17); *P^4 to M^2 length* (L19); *the greatest diameter of the auditory bulla* (D) from the most caudal point of the bulla on the suture with the jugular process up to the external carotid foramen; *the greatest mastoid breath* (W23) the distance between left and right Ot; *the breath dorsal to the external auditory meatus* (W24); *the greatest breadth of the occipital condyles* (W25); *the greatest breadth of the bases of the paracondylar process* (W26), *the greatest foramen magnum breadth* (W27); *the foramen magnum height* (H28); *the greatest neurocranium breadth* (W29) between left and right Eu; *the zygomatic breadth* (W30) Zy-Zy; *least postorbital breadth of skull* at the postorbital constriction (W31); *the frontal breadth,* Ect-Ect (W32); *the least breadth between the orbits* Ent-Ent (W33); *the greatest palatal breadth* (W34); *the least palatal breadth behind the canines* (W35); *the breadth at the canine alveoli* (W36); *the greatest inner height of the orbit* (H37); *the skull height* (H38); *the skull height without the sagittal crest* (H39); *the height of the occipital triangle* (H40); *the neurocranium capacity* (C42).

The neurocranium capacity is based on the cranial cavity volume. The cranial cavity openings were closed with cellulose wadding, and the neurocranium was subsequently filled with fine seeds of rice through the foramen magnum and repeatedly shaken to remove eventual air pockets. Finally, the seeds were tipped into a measuring cylinder and the content volume was read.

The measurements for each mandible were taken as follows (Table 2, Figure 1d): *the total length* from condylar process to Id (L1*); *the length from the angular process to Id* (L2*); the *length from the notch between the condylar process and angular process to Id* (L3*); *the length from the condylar process to the caudal border of the canine alveolus* (L4*); *the length from the notch between the condylar process and angular process to the canine alveolus* (L5*); *the length from the angular process to the caudal border of the canine alveolus* (L6*); *the length of the caudal border of the M_3*

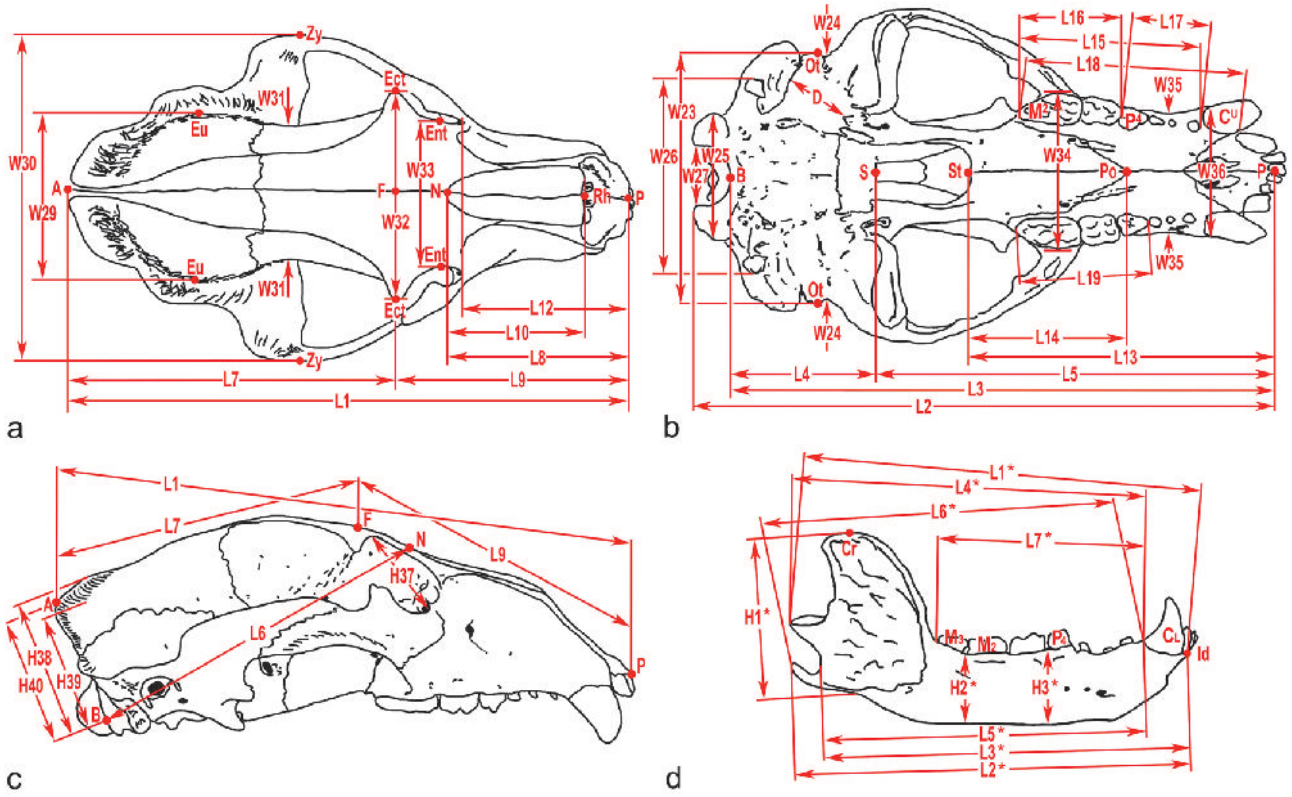


Figure 1: Craniometric measurements of the *Ursus* cranium, (a) dorsal, (b) ventral and (c) right side view and the *Ursus* mandible, (d) right side view

A akrokranion; B basion; Ect ectorbitale; Ent entorbitale; Eu euryon; F frontal midpoint; P prosthion; N nasion; O otion; S synsphenion; Zy zygion; L length, D diameter; W breadth; H height; Id infradentale; Cr coronion; C^U upper-, C_L lover- canine tooth; M^2 second upper-, M_1 first lower-, M_2 second lower-, M_3 third lover- molar tooth; P_4 fourth lover premolar tooth total L1; condylobasal L2; basal L3; basicranial axis L4; basifacial axis L5, neurocranium L6; uper neurocranium L7; viscerocranium L8; facial L9; greatest nasals L10; braincase L11; nose L12; greatest median palatal L13; horizontal part of the palatine L14; cheektooth row L15; molar row L16; premolar row L17; L18 from caudal border of alveolus of M^2 to rostral border of alveolus of C^U; L19 from P^4 to M^2; auditory bulla D; mastoid W23; dorsal to the external auditory meatus W24; occipital condyles W25; the bases of the paracondylar process W26; foramen magnum greatest W27; zygomatic W29; least skull W31; frontal W32; between the orbits least W33; greatest palatal W34; least palatal W35; at the canine alveoli W36; greatest inner H37 of the orbit; skull H38; skull H39 without the sagittal crest; occipital triangle H40; total L1*; L2* from the angular process to infradentale; L3* from the notch between the condyle process and angular process to infradentale; L4* from the condyle process to caudal border of the canine alveolus; from the notch between the condyle process and angular process L5* to caudal border to the C_L alveolus; L6* from the angular process to caudal border of the canine alveolus; L7* from the caudal border of the alveolus of M_3 to caudal border of the C_L alveolus; mandible ramus H1*; H2* of the mandible behind M_2; H3* of the mandible between P_4 and M_1

alveolus of to the caudal border of the canine alveolus (L7*); *the height of the vertical ramus from the basal point of the angular process to Cr* (H1**); the mandible height behind* M_2, measured on the buccal side (H2**); the mandible height between* P_4 *and* M_1 (H3*).

Owing to large differences in craniometric values between the two bear species, we normalised the CB skull measurements to the average of the total length of the BB skull. The average values of the BB measurements were used as a standard for comparison (100% value). The relative deviations of the CB normalised values were considered an expression of species dimorphism.

Similarly, the mandible measurements of the CBs were normalised to the average measurements of the BB mandibles.

Results and discussion

The bear species measurements are summarised in Table 1. The relative deviations for each craniometric parameter of the CB compared with the BB are graphically presented.

Several relative skull length parameters, such as the condylobasal length (L2), basal length (L3), upper neurocranium length (L7), facial length (L9), length of the cheektooth row

(L15), length of the caudal border of M^2 alveolus to the rostral border of the C alveolus (L18), the molar row length (L16), and the distance of the P^4 to M^2 (L19) showed less than 5% size-based deviations between the BB and CB skulls. The relative values of some width parameters, such as (W30) zygomatic breadth, (W34) greatest palatal breadth, and (W36), the breadth at the canine alveoli deviated even less.

The relative neurocranium length (L6) of CB was 6% shorter than that in BB, while the relative basicranial axis (L4) was 8% shorter.

The relative length of basifacial axis (L5), and certain relative values of the skull facial part, such as nose length (L12) and median palatal length (L13), were about 8%-10% longer in the CB than that in BB. Additionally, the relative length of the horizontal part of the palatine bone (L14) was 12% longer in the CB.

Some dimensions, such as the greatest mastoid breadth (W23), the greatest breadth of the occipital condyles (W25), zygomatic breadth (W30), the greatest palatal breadth (W34), the breadth of the canine alveoli (W36), skull height (H38), and height of the occipital triangle (H40), showed less than 5% relative deviations in the CB skull.

Some measured parameters, mainly of the cranial part of the head skeleton, such as the greatest breadth (W27), height of the foramen magnum (H28), the greatest breadth of the neurocranium (W29), and frontal breadth (W32), showed 13%-15% lower values in the CB. Additionally, some individual values of the skull facial part, such as the least breadth between the orbits (W33), the least palatal breadth (W35), and the greatest inner height of the orbit (H37), were 15-20% shorter in CB, and the greatest length of the nasal bones (L10), and the length of the braincase (L11) were also shorter by more than 15%.

The largest deviation was observed in the greatest diameter values of the auditory bulla (D), which was approximately 21% shorter, and of the least breadth of the skull (W31), which was 24% narrower in CB. Accordingly, the measured length of the braincase was 16% shorter. In the CB, this is reflected in the relatively smaller volume of the neurocranium capacity (C42). This is important because the brain fits into the cranial cavity. Therefore, it is obvious that a larger neurocranium cavity volume can accommodate a larger brain. Therefore, a larger brain in relation to body size could be considered an advantage for the individual with respect to behavioural adaptability (27), which is crucially important when an individual is exposed to new or changing environmental conditions. Furthermore, in a new environment, the survival rate of mammals with larger brains to their body weight is supposed to be higher than that of mammals with smaller brains. Because of the relatively small CB brain, this conjecture did not support the survival of CB species under the extreme conditions of the last Pleistocene glacial period. We can speculate that a smaller brain may be one of the crucial factors leading to reduced adaptability to extreme conditions, and thus one of the reasons for the CB extinction.

The dorsal surface of the skull facial part is formed by the dorsal surfaces of the nasal, incisive, and maxillary bones and the nasal processes of the frontal bones. In CB, the prominent feature is the unpaired and more extensive external bony nasal aperture. This is mainly due to the relatively shorter length of the nasal bones, which form the caudodorsal edge of the bony opening and relatively longer terminal line passing along the nasal processes of the incisive bones. According to the nose length (L12) or the median palatal length (L13), which were relatively longer in the CB, the external nasal aperture has an oblique caudodorsal position in the CB. In BB, the nasal opening is smaller and set more vertically.

The nasal process of the incisive bone is connected medially to the nasal bone, laterally to the maxilla, and in both bear species, to the frontal bone at the base level of the frontal process of the maxilla. The maxilla has a roughly pyramidal shape with its apex directed cranially and its wide base on the caudal side of the bone. There is a prominent elliptical infraorbital foramen for the passage of the infraorbital nerve and the vessel on the facial surface. In BB, this opening is located more rostrally and lies at a level above the anterior root of the M^1, whereas in the CB, the infraorbital foramen is located more caudally and closer to the zygomatic process, located nearly in front of the zygomatic arch and at a level above the interalveolar septum between M^1 and M^2 teeth. In BB, the alveolar process of the maxilla contains alveoli dentales for the roots of the canine tooth and six cheek teeth, four premolars, and two molars. Hence, the dental formula in the upper jaw of the BB is 3, 1, 4, 2. In contrast, in the CB

Table 1: The size-based dimorphism between the brown bear and the cave bear skulls, the deviations (%) of the CBn are graphically displayed

	BBa	CBa	CBn	DEVIATIONS of the CBn from BBa (%)
L1	306.5	445.0	306.5	0.0
L2	284.5	426.0	293.4	3.1
L3	266.0	398.0	274.1	3.0
L4	75.5	101.0	69.6	-7.9
L5	193.0	301.5	207.7	7.6
L6	174.5	237.0	163.2	-6.5
L7	167.0	241.0	166.0	-0.6
L8	138.0	220.0	151.5	9.8
L9	163.0	240.0	165.3	1.4
L10	82.0	101.0	69.6	-15.2
L11	128.0	157.0	108.1	-15.5
L12	118.0	185.0	127.4	8.0
L13	149.0	236.5	162.9	9.3
L14	73.5	119.0	82.0	11.5
L15	91.0	135.8	93.5	2.8
L18	111.8	164.0	113.0	1.1
L16	53.0	76.0	52.3	-1.2
L17	38.3	59.5	41.0	7.1
L19	66.5	94.1	64.8	-2.6
D	28.0	32.0	22.0	-21.3
W23	128.0	187.0	128.8	0.6
W24	124.0	191.0	131.6	6.1
W25	60.5	84.0	57.9	-4.4
W26	91.5	143.0	98.5	7.6
W27	30.0	37.0	25.5	-15.1
H28	20.5	26.0	17.9	-12.6
W29	97.5	122.0	84.0	-13.8
W30	173.0	248.0	170.8	-1.3
W31	68.5	76.0	52.3	-23.6
W32	91.0	120.0	82.7	-9.2
W33	66.5	88.0	60.6	-8.9
W34	78.0	110.0	75.8	-2.9
W35	51.0	65.0	44.8	-12.2
W36	67.0	98.0	67.5	0.7
H37	44.5	55.0	37.9	-14.9
H38	79.0	120.0	82.7	4.6
H39	67.0	105.0	72.3	7.9
H40	74.0	113.0	77.8	5.2
C42	332.5	375.0	258.3	-22.3

BBa - the average values of the brown bear skulls craniometric features used as the standard of comparison;
CBa - the cave bear skull average data;
CBn - the cave bear skull normalized data

maxilla, the three anterior premolars are absent and only the fourth molar, P^4, is present, giving the upper jaw dental formula: 3, 1, 1, 2. There is a distinct alveolar-free dorsal border between the canine and P^4, called the interalveolar margin, *margo interalveolaris*, or diastema.

In BB, the first three premolars usually appear in a rudimentary form with no real function. Often, they do not grow or may fall out later. Small interdental spaces were found between each premolar in the BB. In the CB, the alveolar juga of both canine alveoli were the most prominent above the diastema on the facial surface of the maxilla.

Table 2: The size-based dimorphism between the brown bear and the cave bear mandibles, the deviations (%) of the CBn are graphically displayed

	BBa	CBa	CBn	DEVIATIONS of the CBn from BBa (%)
L1*	206.0	329.7	206.0	0.00
L2*	210.3	329.7	206.0	-2.02
L3*	196.5	313.7	196.0	-0.25
L4*	178.0	287.7	179.8	0.99
L5*	168.0	270.3	168.9	0.55
L6*	181.3	287.0	179.3	-1.05
L7*	106.8	163.7	102.3	-4.20
H1*	83.3	148.7	92.9	11.48
H2*	46.3	86.7	54.2	17.09
H3*	40.3	76.0	47.5	17.99

BBa - the average data of the BB mandibles measurements used as the standard of comparison,
CBa - the cave bear mandible average data,
CBn - the cave bear mandible normalized data

The absence of the three anterior premolars in both the upper and lower jaws, a rather long diastema, and the blunt morphology of the molar cusps with broad occlusal surfaces indicate their function in grinding plant food.

Compared to the BB, the upper margin of the nasal dorsum is relatively horizontal in the CB. In the BB, it runs caudodorsally and continues without a sharp boundary into the frontal area. In the CB, there is a distinct unpaired midsagittal depression at the transition from the nasal to the frontal region. The frontal fossa (*fossa frontalis*) extends caudally on the frontal bones from the nasal bones to a strongly developed frontal area. This feature gives it a distinctive and characteristic steep profile in the lateral view, which is not present in the BB (Figure 2).

Figure 2: Cave bear (a) and brown bear (b) cranium, right side view

Note that the characteristic steep profile of the cave bear skull and strongly developed frontal area (arrow) is not present in the brown bear

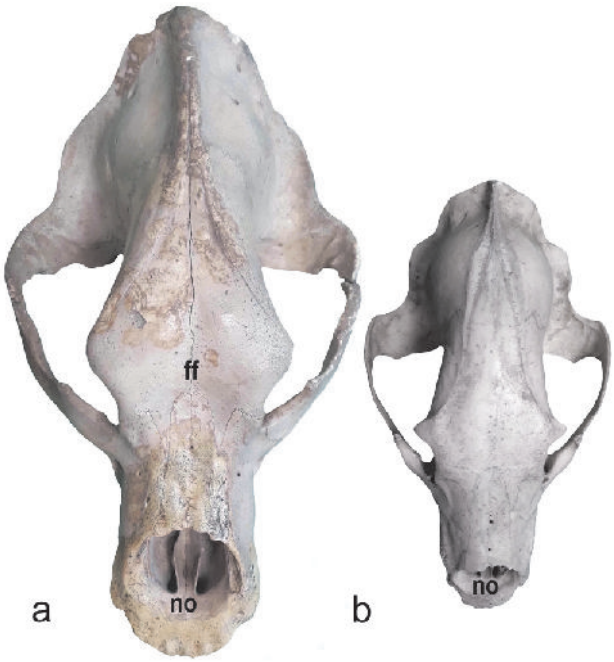


Figure 3: Cave bear (a) and brown bear (b) cranium, dorsal view

An extensive bony nasal opening (no) and the frontal fossa (ff) extending from the nasal bones to the frontal bones are characteristic of the cave bear skull

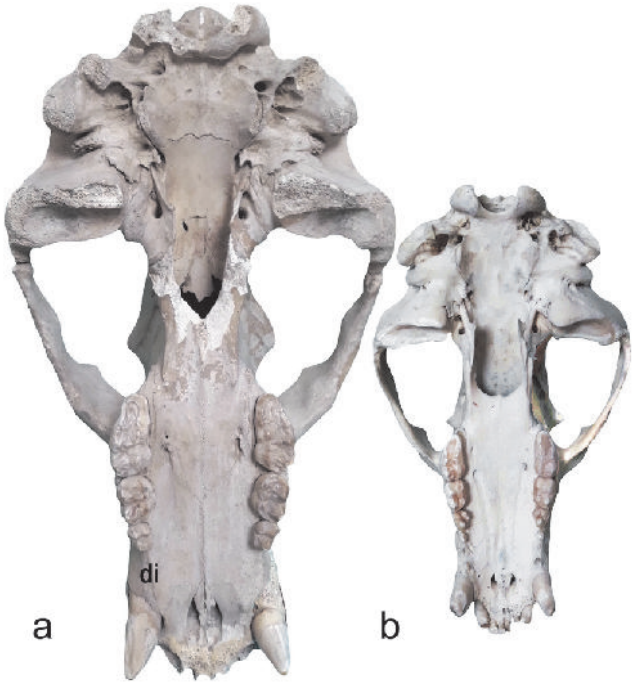


Figure 4: Cave bear (a) and brown bear (b) cranium, basal view

Note the absence of the first three upper premolars and the presence of the maxillary diastema (di) in the cave bear skull

The prominent dorsal part development of the CB skull, especially in the frontal area, indicates a relatively high extent of the frontal sinus between the ecto- and endo-calvar lamine, whereas in BB, the frontal sinus is narrower. According to Torres Pérez-Hidalgo, the CB frontal bones are extremely thick; possibly because of their large size and protecting function, they are pneumatized to reduce their weight (28) and may have functioned as shocks absorbers to protect the brain. The CB had a larger olfactory tract than BB (4, 28). Thus, the enlargement of the frontal sinuses could have been an pleiotropic effect of the turbinates development into the nasal cavity (29). The anterior part of the powerful masticatory temporal muscle was attached to an enlarged frontal region. The sinuses also serve as a reservoir for gases such as nitric oxide and hydrogen sulphide, which play an important role in hibernation control by lowering the heart rate and body temperature. The extensive sinuses may have contributed to a longer hibernation duration in the CB, which would have been beneficial during the long winters of the late Pleistocene. It is possible that death from starvation owing to prolonged hibernation could be one of the factors contributing to their extinction (30).

On the lateral surface of the cranial part of the skull are the orbit and the prominent zygomatic arch. The latter is a laterodorsally convex bridge formed by a laterally compressed zygomatic bone and a dorsoventrally compressed zygomatic process of the temporal bone.

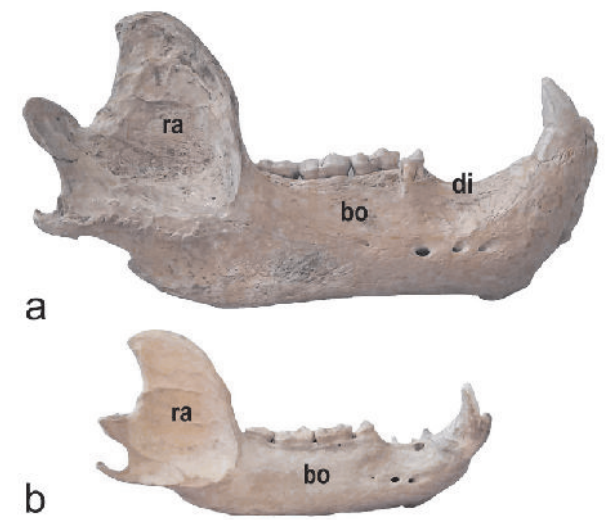


Figure 5: Cave bear (a) and brown bear (b) mandible, right side view

Note a more pronounced and higher ramus (ra) and body (bo) and the absence of the first three premolars with a pronounced diastema (di) in the cave bear mandible

The zygomatic breadth (W30) of these two species did not differ. In both species, it represented 56% of the total skull length. The ratio of the zygomatic breadth to the total length of the skull determines the shape of the skull, which is very similar in both species. This is consistent with the data of Grandal-d' Andlage & López-González 2005 (29), who reported that the cranium shape of *U. spelaeus* was generally extremely similar to that of *U. arctos*.

Owing to the relatively deep frontal fossa and the steep transition of the nasal area into the frontal region, the upper part of the face is positioned more caudally in the CB than in the BB. According to the total length of the skull, this is reflected in the approximately 10% longer viscerocranium length (L8) and 8% longer nose length (L12) in CB. The width of the greatest palatal breadth (W34) measured across the outer borders of the alveoli and the breadth of the canine tooth alveoli (W36) did not show any discernible differences between the two species. The least palatal breadth (W35), measured behind the canine teeth, was 12% narrower in the CB. This is visually reflected in the slight narrowing of this part of the nose.

Both palatal lengths, the median palatal length (L13) and length of the horizontal part of the palatine bone (L14), were 9%–12% longer in CB, while the length of the cheektooth row (L15) did not differ considerably. The premolar row length (the length of P^4, including diastema) seemed to be longer in the CB, because most of its anterior part is a toothless diastema. Therefore, all cheek teeth, P^4, M^1 and M^2, were located behind the lip opening, *rima oris*. The more caudal placement of the cheek teeth in the oral cavity indicates their primary chewing function, presumably biting plant food, as clearly shown by their wide occlusal surfaces.

In the CB, the bony opening of the intrapharyngeal ostium above the caudal border of the hard palate was circular in cross-section, while in BB, it was transversely oval. In both species, the unclosed orbital margin was slightly irregular in shape, while the orbits in the CB were closer together. The distance of the least breadth between the orbits (W33) was 9% narrower in CB, and so was the distance between the zygomatic process of the frontal bone (W32). The greatest inner height of the orbit (H37) was approximately 15% lower in the CB than in the BB, suggesting that the eyeballs may have been smaller in the CB.

The orbital opening faces more rostrally in the CB skull. The shorter distance between the orbits (W33) may indicate that the CB had a larger binocular visual field. A dorsally convex ventral orbital crest (*crista orbitalis ventralis*) of the frontal bone, which is more prominent in the CB, indicates a well-developed pterygoid muscle. The crest demarcates the medial boundary between the orbital and the ventrally positioned pterygopalatine fossa. The rostral end of the fossa funnels down to the infraorbital channel, which is located dorsal to the roots of the second superior molar, while in BB, the position of the entire infraorbital channel is more rostral over almost the entire length of M^1. M^2 was the only upper cheek tooth in CB, which was according to the total length of the skull 7% longer than than that in BB. All other maxillary cheek teeth were shorter, according to the total skull length in the CB.

There is a prominent external sagittal crest on the dorsal surface of the neurocranium, which is longer in the CB. It is located along the entire length of the interparietal suture from the level of the least breadth of the skull (W31) and runs caudally, where it is limited by the transverse nuchal crest, which marks the transition between the dorsal and caudal surfaces of the skull. The BB braincase is almost hemispherical. The relatively short external sagittal crest in the BB is limited only at the position of the interparietal bone and the parietal part of the occipital bone. The width of the least breadth of the skull (W31) at the neurocranium is approximately 24% lower in the CB. Additionally, the greatest neurocranium breadth (W29) between the Eur points is 13% lower in the CB. The right and left temporal lines diverge from the sagittal crest and continue rostrally to the zygomatic process.

The temporal fossa is the area on both sides of the sagittal crest and ventrolateral from the temporal lines. In BB, this is a relatively convex surface on each side of the dorsal part of the skull, whereas in CB, it is rather flat because of the narrower neurocranium. The skull is narrow and high in the CB. These features may have accounted for a more powerful masticatory musculature, especially the temporal and masseter muscles in the CB. As pointed out by Mazza et al. (31), anterior parts of the temporal

muscles are inserted into the high, broad, and robust frontal bones. Consistent with the masticatory muscles arrangement in the CB, the masticatory force must have been stronger in the posterior part of the jaw, where the strongest molars are located. The upper second molar acted against the lower second and third molars. The amount of food that could be processed between the CB teeth was probably small, as the gap size was more limited than that in carnivores that crushed food with the premolars.

Considering the size of the CB mandible (Table 2), all mandibular length data were not considerably different from the comparative norm, while the mandibular ramus was approximately 11% higher than in BB. The mandibular body was also 17%–18% higher in the CB. These findings of expanded mandibular ramus and body further support the hypothesis that CB had better developed masticatory muscles than BB, especially the masseter and pterygoid muscles. Therefore, the tongue must have been larger as well, which is also implied by the relatively deep intermandibular space. All these facts further confirm the assumption that CB was primarily a herbivorous species.

In summary, the comparative craniometry of the CB and BB skulls revealed that CB had a smaller neurocranial cavity volume and thus a smaller brain size, which may reflect a lower adaptability to extreme climatic changes to which they were exposed to during the last Pleistocene glacial period. Additionally, herbivore-adapted jaws and teeth were not an evolutionary advantage in the vegetation-poor era. All these facts might have led to the extinction of the CB.

References

1. Kurten B. Pleistocene bears of North America I, Genus *Tremarctos*, spectacled bears. Acta Zool Fenn 1966; 115: 1–120.

2. Kumar V, Lammers F, Bidon T, et al. The evolutionary history of bears is characterized by gene flow across species. Sci Rep 2017; 7: e46487. doi: 10.1038/srep46487

3. Kurten B. Pleistocene Mammals of Europe. London : Weidenfeld and Nicholson, 1968.

4. Stiner MC. Cave bear ecology and interactions with Pleistocene humans. Ursus 1999; 11: 41–58.

5. De Torres PH, Trinidad J. The European descendants of *Ursus etruscus* C. Cuvier (Mammalia, Carnivora, Ursidae)". Bol Inst Geol Miner Esp 1992; 103(4): 632–42.

6. Hofreiter M, Capelli C, Krings M, et al. Ancient DNA analyses reveal high mitochondrial DNA sequence diversity and parallel morphological evolution of Late Pleistocene cave bears. Mol Biol Evol 1992; 19: 1244–50.

7. Orlando L, Bonjean D, Bocherens H, et al. Ancient DNA and the population genetics of cave bears through space and time. Mol Biol Evol 2002; 19: 1920–33.

8. Rabeder G, Hofreiter M, Nagel D, Withalm G. New taxa of Alpine cave bears (Ursidae, Carnivora). Cah Sci Mus Lyon 2, 2004; 2: 49–67.

9. Hofreiter M. Evolutionsgeschichte alpiner Höhlenbären aus molekulargenetischer Sicht. Mitt Komm Quartärforsch Österr Akad Wiss 2005; 14: 67–72.

10. Rabeder G, Debeljak I, Hofreiter M, Withalm G. Morphological responses of cave bears (Ursus spelaeus group) to high-alpine habitats. Höhle 2008; 59: 59–72.

11. Stuart AJ. Vertebrate faunas from the early Middle Pleistocene of East Anglia. In: Turner C, ed. The Early Middle Pleistocene in Europe. Rotterdam : Balkema, 1996: 9–24.

12. Rabeder G, Nagel D, Pacher M. Der Höhlenbär. Stuttgart: Jan Thorbecke Verlag, 2000.

13. Rabeder G, Hofreiter M, Withalm G. The systematic position of the cave bear from Potočka zijalka (Slovenia). Mitt Komm Quartärforsch Österr Akad Wiss, 2004; 13: 197–200.

14. Ruskov M, Markov G. Der Braunbar (*Ursus arctos* L.) in Bulgarien. Z Säugetierkd, 1984; 39: 358–68.

15. Swenson JE, Dahle B, Gerstl N, Zedrosser A. Action plan for the conservation of the brown bear in Europe.Convention on the Conservation of European Wildlife and Natural Habitats (Bern Convention), Nature and environment, N0. 114. Strasbourgh, France : Council of Europe Publishing, 2000.

16. Zedrosser A, Dahle B, Swenson JE, Gerstl N. Status and management of the brown bear in Europe. Ursus, 2001; 12: 9–20. https://www.jstor.org/stable/3873224

17. Linnell J, Salvatori V, Boitani L, 2007. Guidelines for population level management plans for large carnivores in Europe. A Large

Carnivore Initiative for Europe, Report Prepared for the European Commission. http://www.lcie.org/.

18. Strategija upravljanja z rjavim medvedom (*Ursus arctos*)v Sloveniji, 2002 (Brown bear (*Ursus arctos*) management strategy in Slovenia, 2002). Ministry of the Environment and Spatial Planning and Ministry of Agriculture, Forestry and Food, Ljubljana, Slovenia, 2002: 1–31. https://www.gov.si/assets/ministrstva/MOP/Dokumenti/Narava/Velike-zveri/1b50c822a1/strategija_rjavi_medved_2002.pdf

19. Spassov N. Evidence for a late Pleistocene isolation and a separate taxonomic status of the Mediterranean brown bear and the conservation value of the Balkan bear population. Hist Nat Bulg 1997; 7: 109–13.

20. Gunchev R. Study on stocks, biology and ecology of brown bear (*Ursus arctos* L.) in the Balkan mountains. Sofia : Higher Institute of Forestry, 1989. Dissertation PhD

21. Glenn L. Morphometric characteristics of Brown Bears on the central Alaska Peninsula. In: Bears: their biology and management. 4th International Conference on Bear Research and Management. Kalispell, Montana, 1980: 313–9.

22. Ohdachi S, Aoi T, Mano T, Tsubota TS, Growth sexual dimorphism and geographical variation of skull dimensions of the Brown bear *Ursus arctos* in Hokkaido. J Mamm Soc Jap 1992; 17 (1): 27–47.

23. Meijaard E. Craniometric differences among Malayan sun bears (*Ursus malayanus*). Evolutionary and taxonomic implications. Raffles Bull Zool 2004; 52 (2): 665–72.

24. Mihaylov R, Dimitrov R, Raichev E,et al. Morphometrical features of the head skeleton in brown bear (*Ursus arctos*) in Bulgaria. Bulg J Agric Sci 2013; 19 (2): 331–37.

25. Christiansen P. What size were Arctodus simus and Ursus spelaeus (Carnivora: Ursidae)? Ann Zool Fennici 1999; 36: 93–102.-

26. Von den Driesch A. A guide to the measurement of animal bone from archaeological sites. Peabody Museum Bulletin 1. Harvard : Peabody Museum of Archaeology and Ethnology, 1976: 1–138.

27. Sol D, Bacher S, Reader S, Lefebvre L. Brain size predicts the success of mammal species introduced into novel environments. Am Nat 2008; 172 (1): 63–71.

28. Torres Pérez-Hidalgo T. Osos (Mammalia, Carnivora, Ursidae) del Pleistoceno de la Península Ibérica. Publicaciones Especiales del Boletín Geológico y Minero, Madrid, 1988: 1–316.

29. Grandal-d'Anglade A, Pérez-Rama M, García-Vázquez A, González-Fortes GM. The cave bear's hibernation: reconstructing the physiology and behaviour of an extinct animal. Hist Biol 2019; 31, 429–41.

30. Grandal-d'Anglade A, López-González F. Sexual dimorphism and ontogenetic variation in the skull of the cave bear (Ursus spelaeus Rosenmüller) of the European Upper Pleistocene. Geobios 2005; 38: 325–37.

31. Mazza P, Rustioni M, Boscagli G. Evolution of ursid dentition, with inferences on the functional morphology of the masticatory apparatus in the genus Ursus. In: Moggi-Cecchi J, ed. Aspects of dental biology: Palaeontology, anthropology and evolution. Florence : International Institute for the Study of Man, 1995: 147–57.

MORFOMETRIČNE ZNAČILNOSTI SKELETA GLAVE JAMSKEGA IN RJAVEGA MEDVEDA: PRIMERJALNA ŠTUDIJA

M. Uršič

Izvleček: Izumrli jamski medved je pogosto upodobljen kot robustna, težka žival z masivno lobanjo in izrazito skrajšanima medeničnima okončinama. Po velikosti ga lahko primerjamo z največjimi vrstami današnjih medvedov. Domnevne podobnosti med jamskim in rjavim medvedom so spodbudile našo primerjalno študijo, s katero želimo pojasniti morebitne razlike v morfometričnih značilnostih lobanj, iz česar je mogoče sklepati na možne prilagoditve pri obeh vrstah. Oblika lobanje je pri jamskem medvedu precej podobna obliki pri rjavem medvedu, ugotovili pa smo izrazito razliko v velikosti. Pri jamskem medvedu je celotna dolžina lobanje približno 1,5-krat daljša, zunanja koščena odprtina vhoda v nosno votlino pa je zaradi razmeroma krajših nosnih kosti obsežnejša. Dolžina nosu in mediana nebna dolžina sta pri jamskem medvedu daljši. Podočnična odprtina leži nekoliko kavdalneje in bližje ličnemu loku. Posledično se podočnični kanal pri jamskem medvedu nahaja le nad koreninami drugega molarja, medtem ko slednji pri rjavem medvedu sega še nad korenine prvega molarja. Razlika je bila opazna tudi v neizraslih prvih treh premolarjih tako v zgornji kot spodnji čeljusti jamskega medveda ter izrazitejšem telesu spodnje čeljustnice. Vse to, vključno z obsežnimi griznimi ploskvami molarjev, nakazuje pretežno rastlinsko prehrano. Dolžina možganske votline je pri jamskem medvedu opazno krajša, kar se odraža v sorazmerno majhni prostornini. Globoka čelna jama se kavdalno strmo nadaljuje v močno razvito čelno področje. Manjša prostornina možganske votline, rastlinski hrani prilagojene čeljusti in površine zob ter nekatere druge metrične značilnosti okostja glave bi lahko bile vzročno povezane z nižjo prilagodljivostjo ekstremnim podnebnim razmeram, katerim je v zadnjem pleistocenskem ledeniškem obdobju jamski medved neuspešno kljuboval in kar bi lahko znatno prispevalo k njegovemu izumrtju.

Ključne besede: okostje glave; lobanja; spodnja čeljustnica; kraniometrične lastnosti; medved

SLOVENIAN VETERINARY RESEARCH
SLOVENSKI VETERINARSKI ZBORNIK

Slov Vet Res 2022; 59 (2)