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A REVIEW OF INTRODUCED CERVIDS IN CHILE

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ABSTRACT

We review the extent of exotic deer distributions in Chile, which are encountered in all provinces, including Tierra del Fuego, except for possibly Region III: many deer are contained in >100 enclosures. Red deer (*Cervus elaphus*) by far have the largest feral population of exotic cervids in southern South America, providing source animals that can easily cross the Andes between Chile and Argentina. Red deer were introduced from Europe to the central valley of Chile in 1928. Since the 1940s, feral populations have expanded from Argentina into Chile, by way of easily accessible, low-elevation mountain passes of the Andes, accompanied by further direct shipments from Argentina. The area occupied by 1990 was about 3400 km², but this increased to 7700 km² by 2002. The overall area invaded by 2002 was between 37°42'S-54°55'S and 73°36'W-69°50'W (Argentina and Chile combined, though not contiguous). Negative ecological impact from red deer in Chile have been described since 1981, and red deer figure in the Chilean Pest Manual. A conservative rate for the red deer invasion is 1 km/year, but likely is more rapid where habitat modifications facilitate movement. The pre-Columbian northern limit of the native cervid huemul (*Hippocamelus bisulcus*) was 30°S, and as red deer have occupied all habitat types currently used by huemul, they could thus spread >750 km further north. To the south, all areas are suitable for red deer. Invasion patterns will depend on additional intentional introduction and enclosures on both side of the Andes due to the omnipresent risk of escapes. Fallow, axis and roe deer (*Dama dama*, *Axis axis*, *Capreolus capreolus*, respectively) also have been introduced to Chile and occur in many enclosures. Fallow deer recently escaped on Chiloé Island, became established and raise concerns due to the potential negative impacts on several endemic species on the island. The striking lack of information on feral deer may relate to the policy and laws about firearms and restricted access to hunting areas, resulting in the apparent absence of popular hunting, which could be a potential tool should the invasion continue and lead to future deer overabundance.

Additional keywords: exotic deer, Chile, distribution, *Cervus elaphus*, *Dama dama*, *Axis axis*, *Capreolus capreolus*.

INTRODUCTION

Phoenicians and Romans likely perceived only positive outcomes when translocating fallow deer (*Dama dama*), and for the same reasons Acclimatization Societies everywhere were going strong until the early 20th century. Releasing ungulates allowed alternative production including hunting and thus presented economic benefits. However, releases were also done without economic motives, sometimes to add to depauperate local fauna and improve sport hunting opportunities. Such motives resulted in releases of several ungulate species in southern Latin America, intensifying in the early 1900s (Flueck and Smith-Flueck 1993). Interest in impacts from such releases began already 7-8 decades ago in New Zealand, with government-supported control efforts since 1923 (Caughley 1983). Thereafter, as the economic liability became apparent, global research efforts and publications on invasive species increased exponentially (Kolar and Lodge 2001). Today awareness about impacts from invasive species is quite elevated, and exotic species have been recognized as a leading global threat to native biodiversity and ecosystem function (Pimentel *et al.* 2000; Sala *et al.* 2000; Olson 2006). Exotic deer may have undesirable impacts even at low density, but overt problems are certain when they become

overabundant. For a recent workshop on overabundant deer, held during the 7th International Deer Biology Congress in Chile, we reviewed information on exotic deer present in Chile.

RESULTS

We reviewed all sources of information available to us to provide an update on exotic cervids in Chile. Exotic cervids are encountered in all provinces, including Tierra del Fuego, except for possibly Region III (Fig. 1); from limited information, many of the deer in these provinces live in captive herds, which total over 100.

Red deer (*Cervus elaphus*) were introduced to central Argentina in 1906 and then taken to the Andean foothills in Argentina in the early 1920s (Flueck and Smith-Flueck 1993). In 1928, a shipment of red deer from Europe arrived to the central valley of Chile (Wollenhaupt 1983). Since the 1940s, red deer have expanded from Argentina into Chile, by way of easily accessible, low-elevation mountain passes of the Andean range, accompanied by further direct shipments from Argentina. The area occupied by 1990 in Chile was estimated at 3400 km², but this increased to 7700 km² by 2002 (Flueck *et al.* 2003). The overall area invaded by 2002 was between 37°42'S-54°55'S and 73°36'W-69°50'W (Fig. 2, Argentina and Chile combined, though not contiguous). Based on linear rates of expansion reported for Chile (Wollenhaupt 1983), a conservative rate of 1 km/year for the north-south and east-west dispersals can be assumed in Chile, but likely is frequently more rapid, particularly due to substantial presence of settlers with livestock, which have opened up forests through cattle use and intentional fires, thus allowing red deer to advance more efficiently. The pre-Columbian northern limit of the native cervid, the huemul (*Hippocamelus bisulcus*), was 30°S, and as the red deer has invaded all other known habitat types used by huemul, the potential northern limit for red deer could be >750 km further north of the present distribution. To the south, all areas are suitable for red deer. Overall invasion patterns in Chile will depend on additional local introduction on both side of the Andes and human population density. Already there are several major focal populations from which the invasion is radiating in various directions. Intentional introduction may still occur, but of more concern are new approved deer enclosures and the omnipresent risk of escapes. Captive enclosures represent high risks due to cases of escapees, which would provide source animals for feral populations. Enclosures in Argentina present the same risks for Chile as deer can easily cross the Andes. Red deer by far have the largest feral population of exotic cervids in southern South America and in Chile they are registered from the areas of Bullileo (Parral, region VII) about 35°S and then in all regions southward including Tierra del Fuego (Jaksic *et al.* 2002; Iriarte 2008; Urrutia and Ojeda 2008).

Fallow deer first came to Chile in 1887 and were released to several sites (Lever 1985), currently occur in regions IX, X, XI and more recently appeared in region V by the coast where they are numbering 6-800. Recently, fallow deer had also been taken to an enclosure on Chiloé island, from where they escaped and have established themselves in the surrounding area. Total numbers in Chile were estimated at more than 8000 deer (Iriarte 2008). Axis deer (*Axis axis*) are found in semi-captive enclosures in Region VII and XI where hunting opportunities are provided (Iriarte 2008); we are not aware of any feral populations. Roe deer (*Capreolus capreolus*) were brought to a semi-captive hunting ranch in 1990 in the region X (Jaksic *et al.* 2002; Iriarte 2008) and also appear to be confined.

DISCUSSION

Fallow deer escaping and establishing populations recently on Chiloé Island might be of more immediate concern, considering that this island has several endemic species including medium sized mammals. Otherwise, fallow, axis and roe deer have apparently not been reported as problems, in part because most may be under confined conditions.

Red deer placed recently in enclosures on Tierra del Fuego present environmental risks in case of escapees from the current enclosures. Due to the lack of physical barriers between Chile and Argentina, Jaksic *et al.* (2002) suggested the two countries should coordinate such policy moves in order to

prevent the entry of unwelcome invaders. The list of worrisome species provided by Jaksic *et al.* (2002) now also includes Himalayan tahr (*Hemitragus jemlahicus*), which was introduced to Argentina in 2000 and to Andean foothills a few years later (Flueck 2010).

The feral state of red deer on the other hand is much further advanced both geographically and numerically. Although there is likely substantial local information, there is a striking absence of well-founded published information in Chile. Even presence/absence data are very rudimentary, especially for more remote areas, and we are not aware of any population or individual based studies on free-ranging red deer, although ecological impacts from these deer in Chile have been described since 1981 and red deer figure in the Chilean Pest Manual (Urrutia and Ojeda 2008). The lack of information about feral deer population may also relate to the strict policy and laws about firearms and limited access to hunting areas. One result is the apparent absence of a popular large game hunting segment which, if active, would generate various lines of information, including presence/absence, age structure, diseases, and progress of the invasion. Thus, large game like exotic deer and boar (*Sus scrofa*) can only be hunted on ones own land or with the permission of another private landowner, and hunting on public lands is non-existing (SAG 2004). Consequently, deer hunting in Chile is currently aimed mainly at clients using hunting ranches. When private land suffers from overabundant deer, frequently the owners will find a way to correct the situation. In contrast, overabundance problems on public land depend on government intervention, and a popular hunting segment often is a primary tool to implement management. The continuation of the invasion by exotic deer will likely be a future challenge for Chile.

ACKNOWLEDGMENTS

We like to thank two anonymous reviewers for their valuable suggestions.

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FIGURES

Figure 1. Geographical location of Chile. Left: the internal division into Regions. Center: bulk of red deer distribution and habitats. Right: the Southern Cone.

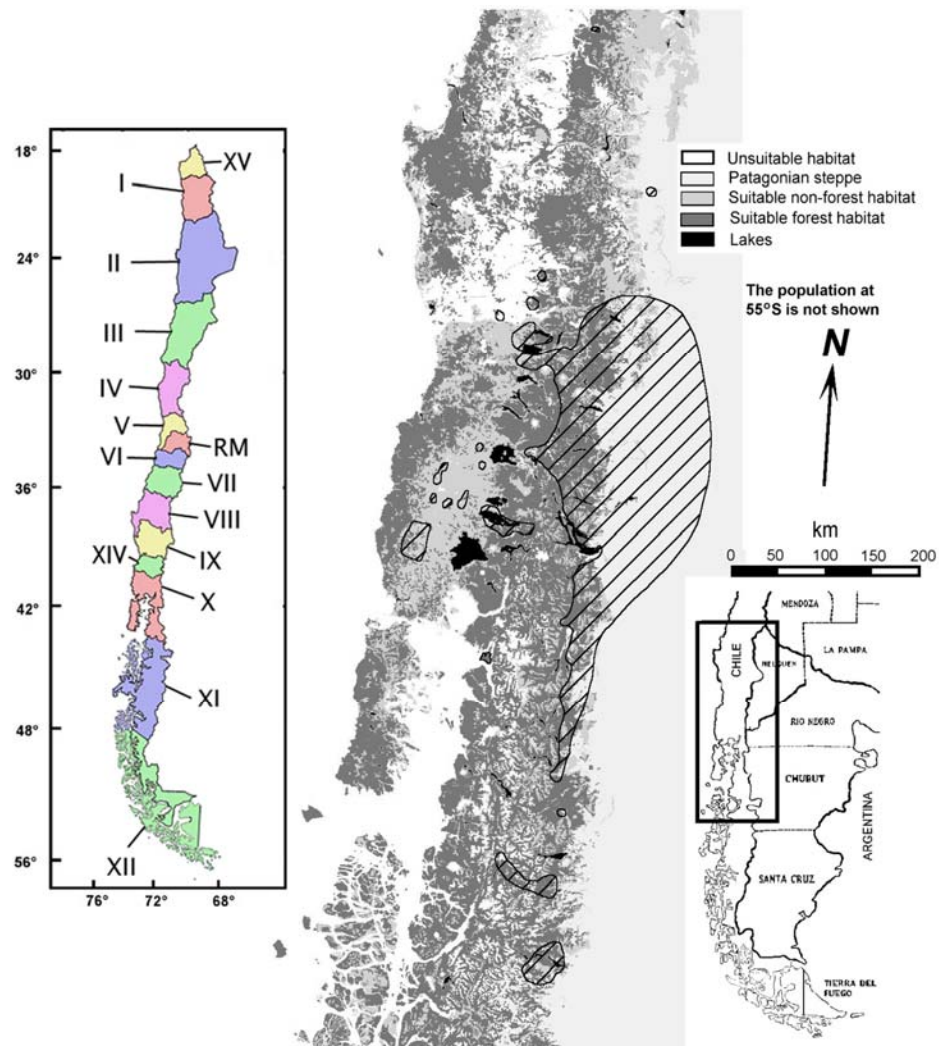
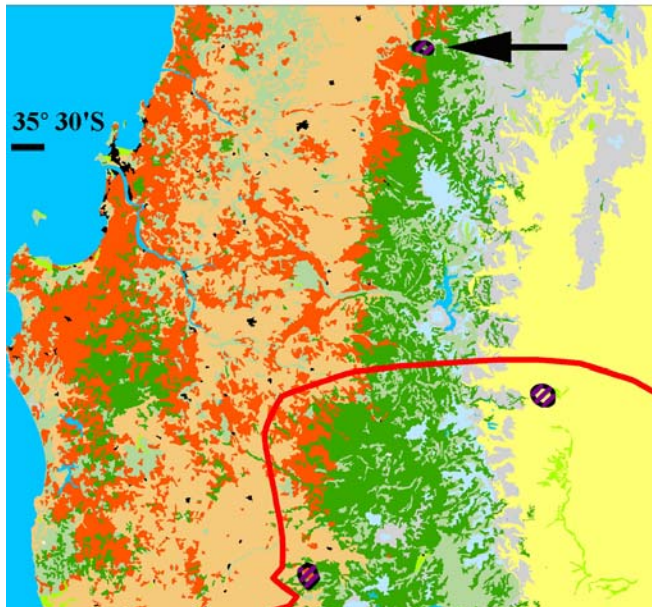
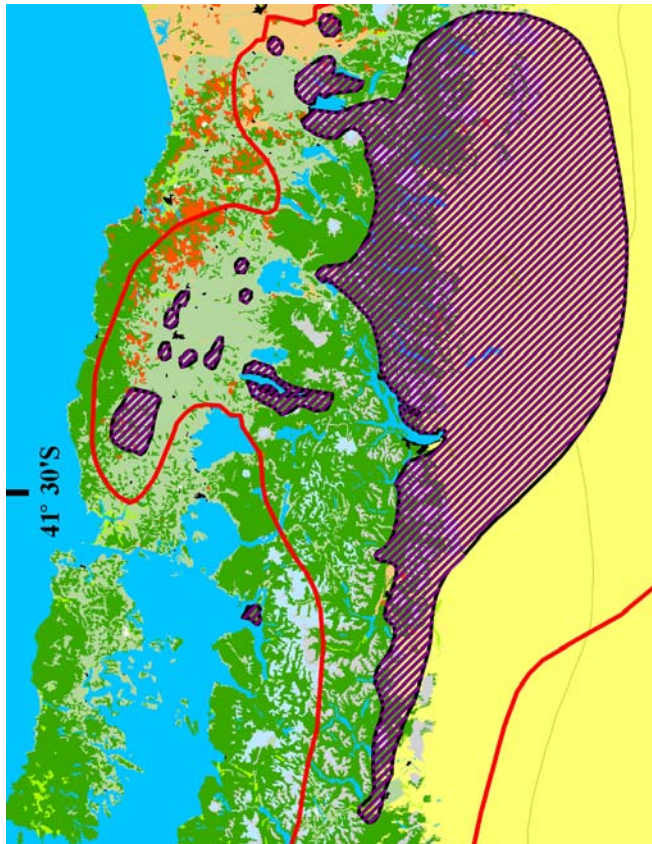


Figure 2. Distribution of feral red deer in Chile and Argentina, approximately year 2000: (a) northern section, (b) central section, and (c) southern section (without showing populations on Staten Island, nor recent enclosures in region XI and in Tierra del Fuego).

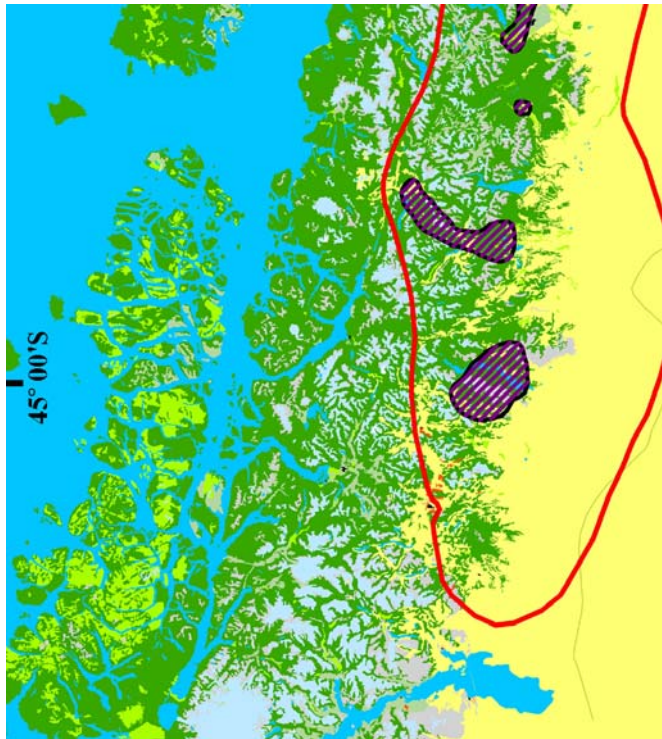
(a)



(b)



(c)



INFLUENCE OF RANCH INFRASTRUCTURE ON THE SPATIAL DISTRIBUTION OF WHITE-TAILED DEER ON SOUTH TEXAS RANGELAND

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ABSTRACT

In Texas, white-tailed deer (*Odocoileus virginianus*) have replaced cattle as the primary source of income from rangeland. Changes in ranch infrastructure required for production of high quality deer for the hunting industry may influence the distribution of animals over the landscape. To determine the effects of high fencing, annual kernel home ranges were determined for 6 white-tailed deer (3 bucks, 3 does wearing GPS collars) inside, and 6 deer outside, a 200 ha high fenced enclosure. On a second ranch, in 4 seasonal trials each lasting 12 days, 6 different deer and 9 cows with GPS collars were tracked more intensively. Interactions between deer and cattle were calculated from synchronized 5 minute locations using Spatial Analyst in ArcView9. Data were then thinned to hourly locations to avoid problems of spatial autocorrelation. Habitat preference was calculated by chi-squared test of proportional use and availability of ecological sites. Response to anthropogenic features was assessed by comparing the distribution of animal locations with an equal number of random points on a distance surface grid. There was no evidence that high fencing constrained home range size. The idea that cattle limit access of deer to preferred ecological sites and water was not supported. Both species showed extensive overlap in spatial distribution but were separated in time. Both deer and cattle were located closer to ranch roads than random distribution. Ranch roads offer easy travel corridors for animals in shrub dominated landscapes. Animal distributions can be manipulated to some extent by changes in ranch infrastructure and the placement of supplemental feed.

Additional keywords: cattle, GPS, habitat selection, home range *Odocoileus virginianus*.

INTRODUCTION

In Texas, white-tailed deer (*Odocoileus virginianus*) have replaced cattle as the primary source of income from ranchland (Adams *et al.* 2000; IAFWA 2002; Connor *et al.* 2010). Many ranches still run both cattle and hunting enterprises but the emphasis on hunting has lead to changes on the landscape due to the different infrastructure needed for producing and hunting trophy deer. Ranches are frequently surrounded by “deer-proof” high fencing, dirt roads are cleared to service the numerous hunting blinds and additional water sources and supplemental feeding locations are developed for the deer. Habitat use by animals is affected by the distribution of resources on the landscape (Roath and Krueger 1982; Owens *et al.* 1991). Thus changes in the locations of water and feed and altered ease of access via roads cut through shrubby vegetation may influence the distribution of animals over the landscape. How animals use the landscape and their spatial response to anthropogenic features is of importance to land managers. Land use patterns of herbivores can have long-lasting effects on plant community structure and the sustainability of forage resources (Bailey *et al.* 1996; Hobbs 1996; Russell *et al.* 2001). White-tailed deer cannot be managed and herded like cattle, however, knowledge of animal movements relative to natural and anthropogenic features can be used to modify animals’ use of space by changing the placement of critical resources and thus alter the impact of animal foraging behaviour on the landscape.

This paper is a synthesis of two studies on large private ranches in the South Texas Plains ecoregion. It is aimed at deciphering how ranch infrastructure and management practices may affect local scale distribution of white-tailed deer. We hypothesized that home range size of individuals may be compacted when deer are confined within a high fence due to potential overcrowding. We also predicted that deer will stay closer than is predicted from random distribution to resources such as

water, supplemental feeding sites and tracks providing ease of travel in brush dominated rangeland. A more detailed analysis of deer use of rangeland and their interaction with cattle can be found in Cooper et al (2008).

MATERIALS AND METHODS

Two large private ranches in South Texas Plains Ecoregion of Texas, USA participated in this study. This semi-arid region has an annual average temperature of 23°F and is characterized by mild winters and hot summers. Annual precipitation is variable but averages 620 mm/year with most rain falling in spring and again in September. The study years of 2005-6 and 2009 fell within dry periods receiving only half (308 and 363 mm respectively) the average annual rainfall, nevertheless soil moisture was adequate to maintain the deep rooted shrubs that characterize this shrub dominated savanna. Habitat quality remained adequate to keep the deer in satisfactory condition. The study of the effect of high fencing was conducted on the Hillman ranch. The soils of the high fenced pasture and surrounding areas were predominantly sandy loams and clay loams which supported a mixed brush community with scattered thickets of blackbrush (*Acacia rigidula*). More open areas formerly created by mechanical brush clearance were characterized by small trees of honey mesquite (*Prosopis glandulosa*). Vegetation and habitat for the deer within the 200 ha high-fenced pasture and in the surrounding area was similar, thus home ranges sizes of deer on either side of the fence were directly comparable. The study of deer distribution relative to ranch roads, water, supplemental feeding sites and the presence of cattle was conducted in a 1211 ha low fenced pasture on the Harris ranch. Soil types graded from clay loams (58%) on the alluvial area of the dry gravel riverbed into less productive shallow ridge sites (42%) with thin calcareous soils. Clay loam soils supported scattered small trees of honey mesquite and live oak (*Quercus virginiana*) with an understory of mixed shrubs, native grasses and seasonal forbs. The narrow riparian fringe supported taller plants of the same species and was surrounded by a low fence to keep cattle out. Shallower soils on calcareous ridges and rocky areas supported a variety of spiny shrubs including acacias (*A. berlandieri*, *A. rigidula* and *A. greggi*) and prickly pear cactus (*Opuntia lindheimeri*) with sparse herbaceous vegetation. Within this area small trees of live oak and Texas persimmon (*Diospyros texana*) grew in the drainage lines (Meek 2007).

To assess the effect of high fencing on kernel home range size GPS (Global Positioning System) collars (Lotek GPS 3300 S and LR, Lotek Wireless, Ontario, Canada) were placed on 6 white-tailed deer (3 bucks, 3 does) inside, and 6 deer (3 bucks, 3 does) outside, a 200 ha high fenced enclosure on the Hillman ranch. Collars collected 1 GPS location every 3 hours for 1 year from February 2009 until March 2010. Annual kernel home range sizes were calculated in Home Range Extension in ArcView 3.2 (ESRI, Redlands, CA, USA). Kernel home ranges values for the 95% and 50% utilization areas were reported with standard deviations and extents compared by *t*-test. Significant difference was set at $P < 0.05$.

On the Harris ranch, animal locations were monitored over 1 year from July 2005 through July 2006 during 4 trials each lasting 12 days. In each trial 6 white tailed deer and 9 cows were fitted with GPS collars programmed to take a position location every 5 minutes. Different individuals were used in each trial. Inter-specific interactions between deer and cattle were calculated from synchronized 5 min locations using Spatial Analyst in ArcView9. To assess animal distributions relative to habitat and ranch infrastructure data was thinned to hourly locations to avoid statistical problems of spatial autocorrelation (Swihart and Slade 1985). Habitat preference was calculated by chi-squared test of proportional use and availability of ecological sites. Effects of anthropogenic features were assessed using analysis of variance to compare animal hourly locations and an equal number of random points on a distance surface grid. Animal locations were also examined during the 2 hours before and after dusk and before and after dawn when animals are most actively foraging (Montgomery 1963), this was confirmed by activity sensors on the GPS collars.

Helicopter drop-netting and collaring of deer and use of cattle was approved by Texas A&M Institutional Animal Care and Use Committee Animal Use Protocols # 2004-49 and # 2009-003.

RESULTS

On the Hillman ranch, the high fence failed to contain the deer, even though the fence was well maintained 4 of the deer moved through the fence utilizing holes created by feral pigs. Three does moved in and out of the high fenced enclosure throughout the year but the buck which left the enclosure during the rut in December was killed by poachers before he could return. A full year of data was obtained for 2 bucks and 2 does inside and 2 bucks and 3 does outside the high-fenced pasture.

Being contained within the high fence had no effect on the 95% home range size of bucks (inside 119.4 ± 0.8 ha, outside 130.0 ± 0.0 ha), or their 50% core use areas (inside 69.74 ± 1.8 ha, outside 67.5 ± 0.0 ha). Home range sizes of does appeared to be slightly smaller within the high fence (95% KHR inside 75.5 ± 11.5 ha, outside 116.0 ± 20.3 ha, 50% KHR inside 42.1 ± 8.9 ha, outside 73.8 ± 11.9 ha), but because home range sizes were very variable this difference was not significant. Since there was little difference in home range size of bucks and does the genders were combined to compare home range size inside and outside the high fence; no difference in home range size was found (95% KHR: $t = 1.001$; 50% KHR: $t = 1.211$, $P > 0.05$, $n=9$).

On the Harris ranch, deer exhibited seasonal selectivity in habitat use ($\chi^2_2 = 25.84 - 66.75$, $P < 0.05$). Bucks favored the more productive clay loam flats. Does used the clay loam sites in similar proportion to their availability but favored sites with denser tree canopy including riparian areas and drainage areas within the rocky areas. Cattle showed strong selectivity for clay loam flats and avoided rocky terrain ($\chi^2_{7.9} = 43.24 - 94.95$, $P < 0.001$). Although both deer and cattle favored the clay loam areas extensively overlap was limited by temporal separation. GPS collared deer and cattle rarely came within 2 km of each other. Close inter-specific interactions were rare (0.16% of deer relocations were within 100 m of cattle), yet when deer and cattle did come close individual deer did not show avoidance of cattle until inter-specific distances were < 50 m.

Deer and cattle shared use of the 3 watering locations. Cattle stayed closer to water sources than the deer and were found within 100 m of water for 5 - 9% of relocations depending on the season. Deer were distributed farther from water than the cattle and were only recorded within 100 m of water on $1 \pm 0.6\%$ of relocations. Deer usually came to drink at dusk after the cattle had dispersed to graze ($P < 0.001$).

Providing supplemental feed in the form of protein pellets is a common deer management tool in Texas. Cattle were fenced out of the deer feeders. Deer were found within 100 m of the 6 feeders dispensing protein pellets on $1 \pm 1\%$ of relocations. Bucks were consistently distributed closer to the feeders than random ($P < 0.001$) but the distribution of does was random relative to the feeders.

Both deer and cattle were located closer to ranch roads than random distribution ($P < 0.001$). Two thirds, ($64 \pm 3\%$) of cattle relocations were within 100 m of a road and $17 \pm 3\%$ were on the road. Similarly, half ($53 \pm 8\%$) of deer relocations were within 100 m of a road and $5 \pm 2\%$ were on a road. Deer use of roads was greatest in November when shelled corn was sprinkled on the road to improve visibility of wildlife for pre-hunt population surveys.

DISCUSSION

Concern that the current trend towards enclosing deer ranches within high fencing will limit the movement of deer and lead to inbreeding are ameliorated by the discovery of the relative permeability of 'deer proof' fences to deer. The 200 ha enclosure used in this study was large enough that the home range size of the enclosed deer was not compressed by fencing or competition with conspecifics.

Also the idea, commonly expressed by deer hunters, that cattle limit access of deer to preferred ecological sites and water was not supported, instead there was short term temporal separation of species distributions when using shared resources including both favored habitat types and localized resources like water. White-tailed deer maintained a voluntary distance from cattle, a tolerance distance of about 50 m from cattle has also been recorded for mule deer (*O. hemionus*) (Loft *et al.* 1993). Deer had almost exclusive use of rocky terrain which was avoided by cattle (Stewart *et al.* 2002; Cooper *et al.* 2008). This included the more fertile pockets of deep soil in depressions and along creeks running through the rocky areas, these areas were particularly favored by does which typically select denser cover than bucks (DePerno *et al.* 2003). Thus rougher terrain can be managed primarily for wildlife.

Ranch roads and the provision of supplemental feed had the most noticeable effect on the distribution of deer. Cattle are known to use ranch roads as pathways of least effort (Roath and Kroeger 1982; Ganskopp *et al.* 2000), and it is logical that deer too will use cleared roads to traverse spiny, shrubby country (Cooper *et al.* 2008). Land managers can cut additional tracks through shrub dominated pastures to disperse livestock grazing patterns and possibly to open areas for deer browsing. Provision of supplemental food is thought not to change the home range size of deer (Cooper *et al.* 2006) but may alter the distribution of animals within their home range. Cattle use of habitat can be modified by strategic placement of supplemental feed (Bailey and Welling 1999) and it may be possible to do the

same for deer. Both bucks and does used the supplemental feed, although feeder placement only influenced the distribution of bucks. This was partly because more feeders were located in the clay loam areas preferred by bucks and partly because bucks tend to dominate does at the feeders.

CONCLUSION

Changes in ranch infrastructure implemented as land use transitions from cattle only operations to mixed cattle and deer hunting or hunting only ranches, can influence the distribution of animals over the landscape. Ranching both deer and cattle achieves optimal use of the forage base because the dietary overlap of white-tailed deer and cattle is small; the deer are browsers whereas cattle are grazers. In addition these species separate temporally when utilizing shared prime habitats. Judicious placement of feed and water and planned placement of roads can be used to obtain optimum dispersal of deer and cattle over the landscape.

ACKNOWLEDGEMENTS

This research was supported by funds provided by the US Department of Agriculture, Rocky Mountain Research Station, Forest Service, Joint Venture Agreement #03-JV-11221-602-281, Joe Skeen Institute for Rangeland Restoration and Hispanic Serving Institutions grant 2007-38422-18038. We thank Mike Harris and Betty Hillman for use of their property, and Andrea Wappel, Shane Sieckenius, Rose Cooper and students of South West Texas Junior College Wildlife Management Program for technical help.

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THE TIMING OF ANTLER CASTING OF REEVES' MUNTJAC (*MUNTIACUS REEVESI*)

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ABSTRACT

For 22 Reeves' muntjac (*Muntiacus reevesi*) in captivity in England the exact casting dates of left and right antlers were recorded for a total of 106 antler cycles. On only 15.1 per cent of occasions were both antlers cast on the same day but more than half were cast within 3 days. All except 3 castings occurred in May and June with 26 May as the median date for both left and right antlers.

Additional keywords synchrony, asynchrony, deer, cervid.

INTRODUCTION

Various authors have stated that both antlers are often cast on the same day (e.g. Goss, 1983) but the species of deer and the number of antler cycles over which observations were made rarely have been reported.

A colony of captive Reeves' muntjac provided the opportunity to test whether this generalisation applied to this primitive cervid, native to China and Taiwan but introduced to England where now it is widely distributed and abundant.

METHODS

From 1973, until the present time, muntjac have been observed in England (at latitude 52°20') within three enclosures (each at least 1000 m², with trees, shrubs, grass and other vegetation) situated adjacent to the house of the author. The deer were fed on herbage, root crops and fruits. The dates of casting of both antlers have been recorded for 22 bucks, totalling 106 antler cycles. The maturity of the bucks ranged from first head of antlers to 15th head. Casting was defined as synchronous when both antlers were cast on the same day; an interval of one or more days was asynchronous.

RESULTS

Only 15.1% of the 106 antler cycles were synchronous. In 40.6% of cases the left antler was cast one or more days before the right one, whereas in 44.3% of cases the reverse was true. Regarding the difference in days between casting the first and second antler, for 52.7% of cases the difference was up to 3 days, for 38.5% of the cases the delay was 4-8 days and in 8.8% the difference was 9-13 days.

The greatest number of paired casting dates for an individual buck was 13 ($n = 2$). Of these two bucks, one cast synchronously on 3 occasions, the other only once. Two bucks for which there are data for 10 and 11 cycles respectively never cast synchronously but another buck for which 8 sets of dates are known, cast synchronously in 4 successive years. Only 10 of the 22 bucks ever cast synchronously.

Over the years individual bucks showed a range of intervals between casting left and right antlers e.g 0 to 10 days (over 10 antler cycles); 2 to 13 days (over 4 cycles).

An individual buck seldom cast on the same date in different years. The smallest difference between an individual's earliest and latest casting dates was 9 days (over only 2 cycles) but 9 bucks gave examples of more than 3 weeks, even up to 52 days.

All casting dates were between 1 May and 17 July but only 3 were in July. For first heads ($n = 16$) the range (9 May to 26 June) was almost as wide as for older bucks. No correlation was found between the age of a buck and the time of casting.

The median date for all castings of left and right antlers was 26 May. An earlier report on a small subset of the present data had reported 27 May for the left and 26 May for the right (Chapman and Chapman 1982).

DISCUSSION

Although the incidence of synchronous casting was only 15.1%, more than half the pairs of antlers were cast within 3 days. For comparison with other species, the most comprehensive data are those for a park herd of red deer (*Cervus elaphus*) where 146 casting dates for 25 stags showed that synchrony occurred in 60.3% (Bartoš and Perner 1987). The range of intervals between casting of left and right antlers was not given.

For sambar (*Cervus unicolor*) data has been published for 67 antler cycles of an unspecified number of stags in a park in India (Acharjyo 1982). Synchronous casting occurred on 44.8% of occasions and 86.6% of castings of both antlers occurred within 3 days. The maximum interval between casting left and right antlers was 6 days. For hog deer (*Axis porcinus*) a delay of several days was sometimes observed in a park in Australia (Mayze and Moore 1990) and for roe deer (*Capreolus capreolus*) up to 2 days has been reported (Raesfeld 1970).

These few examples show that asynchronous castings are not unusual and were frequent in the population of Reeves' muntjac that was studied. Within the many other species of deer there are likely to be variations in synchrony/asynchrony.

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STAGES IN THE REINTRODUCTION OF CHINESE WATER DEER TO SHANGHAI, CHINA

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ABSTRACT

Chinese water deer (*Hydropotes inermis inermis*) is endangered in China. To aid the conservation of this species, we have conducted series introduction efforts based on historical literature researches of the deer populations, and investigation of public attitude and survival requirements of the deer. We have reintroduced Chinese water deer from Zhoushan Archipelago to Huaxia Park to breed a releasing stock in March 2007, then acclimatized the descendents in Punan woodland and Binjiang forest Park. We released deer in Nanhui East Shoal in early 2010. The project aimed to re-establish the deer population in suburban area of Shanghai gradually.

Additional keywords: Chinese water deer, reintroduction, Shanghai.

INTRODUCTION

Chinese water deer (*Hydropotes inermis inermis*) is an endangered subspecies, native to China which is ranked as VU in IUCN Red Data book of 2009. The deer like living in swampy, open grasslands. The distribution in China used to extend to Liaodong Peninsula, North China plain and the shores of the lower reaches of the Yangtze River and adjacent lake areas, within the region extending from latitude 28° to 42°N and the eastern limit at longitude 110°E (Sheng 1992). In the 19th century, the Chinese water deer was introduced to Britain and France and established several local populations in England (Cooke and Farrell 1998). However, the population of the deer in China has declined rapidly and its distribution is now fragmented and limited to the coastal areas of Jiangsu, Zhoushan Archipelago of Zhejiang, the east of Anhui and Poyang Lake areas in Jiangxi (Sheng *et al.* 1999; Chen *et al.* 2009).

We started the project reintroducing Chinese water deer to Shanghai in 2006. The goal of our project is to recover the Chinese water deer population through establishing a viable, free ranging population in the suburbs and wetlands of Shanghai, thereby enhancing the long-term survival of these deer; and re-establishing a key species in the ecosystem and biodiversity of the Yangtze River Estuary.

THE PROJECT OF CHINESE WATER DEER REINTRODUCTION

Pre-study for reintroduction

The feasibility study and background research included an assessment of the taxonomic status of individuals to be reintroduced, the historical information about the loss and fate of the deer from the re-introduction area and the views of local people. These studies led us to choose the stock and the site for reintroduction.

Chinese water deer, which was native to Shanghai region since the Neolithic Age, but extirpated in the wild at the beginning of the twentieth century, was said to be moderately widespread in the 1880s in this area, such as Qingpu and Fengxian (Cao 1996; Allen 1940). Therefore, according to the IUCN guidelines for re-introductions, Shanghai region, as the species' former natural habitat and distribution range, is a suitable site for the re-introduction of Chinese water deer. The potential risks of ecological invasion that might be caused by the introduction of Chinese water deer will be minimal and the species will not play significant negative effects on local greening or daily life of citizens. It is a species of high fertility, capable of establishing a stable population in a comparatively short period. Chinese water deer could be the keystone species for the ecological reconstruction of Shanghai's region.

Re-introductions are generally long-term projects that require the commitment of long-term financial and political support, and also need support from local people. An assessment of attitudes of local people to the proposed project is necessary to ensure long term protection of the re-introduced population; the project should be fully understood, accepted and supported by local communities. We

did a social survey in 2005, in which 2000 people were interviewed. As a result, 97.1% of the sample favoured the reintroduction project (Su *et al.* 2008).

In order to decide from where to obtain the deer, a study was conducted on genetic variation within different populations of Chinese water deer. The *cytb*, D-loop and 12SrRNA of mtDNA were sequenced from the samples collected non-invasively from Zhoushan of Zhejiang, Yancheng of Jiangsu and Jishan of Jiangxi. The results showed that Chinese water deer exhibit high genetic diversity and the population in Yancheng has the highest (Chen 2006). But, the last censuses show that the population of the deer in Yancheng coastal areas was around 372 in the spring of 2007 (Chen *et al.* 2009). It would be difficult to capture enough individuals from Yancheng. The mtDNA study also showed that no obvious phylogenetic structure among haplotypes was found in the samples from Zhoushan and Yancheng and no clear divergence between the populations from Zhoushan and Yancheng (Chen 2006). It implied that if we can introduce Chinese water deer from Zhoushan without worrying on gene flow among them if the reintroduction program is successful.

IMPLEMENTED STEPS OF INTRODUCTION PROJECT

The first step ---- breed a releasing stock in Shanghai

The project selected a plot in Shanghai first to establish a breeding population for future releasing. At the end of 2006, a 5400m² area was prepared for the deer in Huaxia Park (N31°11'37", E121°38'35"), a Park within the city Green belt of Pudong. In March 2007, 21 Chinese water deer were released into Huaxia Park. The stock population has been increasing year by year. We can find the population number and the growth rate in Table 1. The population growth reached a peak at 96.3% in the second year, 2008. Then the growth rate declined in the following two years. The behaviour has also shown that the deer have adapted to the climatic conditions and we have the availability of suitable release stock, according to the IUCN guidelines for re-introductions, pre-project activities (Ma, 2009). So we conducted the acclimatization step at 2009.

Table 1. The stock population growth

Time	Population size	Growth rate
March 2007	21	
December 2007	27	28.6%
December 2008	53	96.3%
December 2009	77	45.3%
December 2010	96	24.7%

The second step ---- acclimatization

The acclimatization of release stock was designed and finished before release stage. We have selected two typical habitats suitable for the liberation of individually marked deer to more open areas around the city. One place is the Punan woodland; the other is Shanghai Binjiang Forest Park, a Suburban Park in the estuary.

The Punan woodland(30°57'03" ~30°58'06"N , 121°17'58"~121°18'36"E) is in Yexie County, Songjiang District which is in the southeast of Shanghai City, which abuts Huangpu River in the north. The principal part, Yexie Section of Huangpu River Conservation Forest, is characterized by wetland, where the dominant species are water-tolerant plants just like the natural wetland forests. 20 deer from stock were transferred here for acclimatization in October, 2009. And in November, 2009, another 14 deer were acclimatized in the other location, Shanghai Binjiang Forest Park, which is located in the most northern part of Pudong New Area, where Huangpu River, Yangtze River, and East China Sea converge. The Park presents the unique "Convergence of Three Rivers" geographic landscape in Shanghai, and is the gateway landscape on entering Shanghai from the waterway.

All the acclimatized individuals were tagged, and specific individuals were fitted with telemetry collars, with which 24-hour continuous statistics have been collected to analyze their dispersion model, home range formation, circadian activity rhythm, etc, with the aim of monitoring their long-term adaptation. In addition, studies on the foraging behavior, energy metabolism and vigilance behavior toward humans of the released stock are undertaken. The effect of re-introduced species will have on the local ecosystem and habitat is also studied. Biotic and abiotic habitat requirements and interactions

with the deer have been quantified in terms of plant changes, biomass, and water quality and soil composition.

The third step ----releasing

The first release site, where a feasibility study was made, is Nanhui East Shoal. Nanhui East Shoal (30°50'54.13" ~31°00'02.17"N, 121°53'03.42"~121°58'25.55"E) is the existing wetlands located on the estuary of Yangtze River and plays an important role in ecological protection and restoration for Shanghai. A wildlife sanctuary covering 122.5km² was founded in this area in July 2007. It is a typical wetland and the main vegetation in this area is *Phragmites australis* community, the *Scirpus mariqueter* community, and the *Scirpus triqueter* community, etc. There are more than 100 macrobenthic fauna and fish species discovered and 249 bird species were recorded during 2006-2008 (Nanhui wildlife protection station, pers. comm. 2010). This area is a key habitat for the migrating birds because it is on the route of East Asian–Australasian. It may also meet the habitat requirements of Chinese water deer. Comparing the study on diet of Chinese water deer in Zhejiang Zhoushan Archipelago and Songjiang Punan Conservation Forest, at least 24 species of plants in spring could be the potential food resources for the deer, and the heights of reed could reach 134.7 cm with cover-80% to 90% in spring (Chen 2010), which could provide the deer with ideal shelter. These factors indicate that Nanhui East Shoal is a suitable area to release Chinese water deer in Shanghai, though typhoon, birds of prey, domestic dogs and passing-by motor vehicles could potentially threaten the lives of the deer. Early in 2010, we started to liberate some deer there with GPS collars. We will track these deer and hope that the deer can reproduce and gradually re-establish a natural population in Nanhui East Shoal.

DISCUSSION

Wildlife can offer people a great deal of satisfaction, but now the opportunities for seeing deer in the field are less than ever as their distributions continue to shrink. We hope that the conservation project will attract the attention of more and more people in China, as elsewhere. However, as a protected species, the two top threats are habitat loss and hunting the unweaned fawns, for the sake of the milk from their stomachs, because of a superstitious belief that this helps digestion in people (Su *et al.* 2004). The challenge is how to ensure that natural populations can be maintained in the face of growing human demands on the environment.

All of our studies need to be monitored continuously to evaluate the strategy although we have some good examples to follow, especially the milu (Père David's deer, *Elaphurus davidianus*), a deer which lived in warm swampy habitats but disappeared from China many years ago. Milu was reintroduced successfully to a coastal area of Yancheng and adjacent areas of Dongting Lake. But, the key difference is that a nature reserve was created for the milu reintroduction, but there is no reserve for Chinese water deer. It will be hard for long-term conservation and reintroduction. All of our work seeks to establish a self-sustainable wild Chinese water deer population as well as to promote the ecological restoration, in the hope of improving the overall ecosystem and biodiversity in this area. Sustained support from local government and people will be required and it will be a long scientific and social project.

ACKNOWLEDGEMENTS

This project was partially funded by Shanghai Agriculture, Shanghai Pudong Science and Technology Commission, Shanghai Wildlife Conservation and Management Bureau Shanghai WWF Shanghai Office, and got local support from Shanghai Amenities and Greenization Bureau, Pudong Greenbelt Construction and Management Bureau, Songjiang Wildlife Conservation and Management Bureau, Nanhui Wildlife Conservation and Management station, Binjiang Forest Park. We also thank Ma Feiyan, Tian Xinxin, Yin Xiaojun, Yang Jinyan, Zhu Yujie, Zhangchao, Cai Jiahang.

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Figure 1. Sites of the reintroduction project: (i) Huaxia Park, breed a releasing stock; (ii) Punan Woodland and Binjiang forest park, acclimatize the deer; (iii) Nanhui east shoal, release the deer.

STUDIES ON MOLECULAR GENETIC DIVERSITIES OF CHINESE SIKA DEER AND WAPITI

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ABSTRACT

Sika deer (*Cervus nippon*) and wapiti (*Cervus elaphus*) are the two most important species in China in that they are the only deer farmed for the production of precious traditional Chinese medicine, the velvet antler. These two deer species are widely distributed in China with distinguishable morphological differences within each species in the different geographical location. These differences have justified the existence of subspecies/breeds. However, thus far the molecular genetic relationship between these subspecies/breeds is not clear. In the present study, we used 20 relevant microsatellite markers to analyse them. We found that Chinese wapiti breeds/subspecies could be classified into four clusters, and Chinese sika deer classified into two clusters. The microsatellite genetic markers that are related to velvet production were identified through establishment of a general linear model in the study. We believe our results can provide useful information to Chinese deer farm industry for molecular breeding toward further increasing production of velvet antlers.

Additional keywords: Wapiti; Sika deer; microsatellite marker, genetic diversity.

INTRODUCTION

China is one of the few countries in the world where most deer species reside (Sheng and Ohtaishi 1993). Amongst these species, sika deer (*Cervus nippon*) and wapiti (*Cervus elaphus*) stand out as they are most abundant in China and successfully farmed for production of the velvet antler (Zhao and Shen 1998). Only the velvet antlers from these two species are formally listed in Chinese Pharmacopeia as traditional medicine (Zhao and Shen 1998). Currently, there are a number of subspecies/breeds of each species in China, and we think it is very important to understand the molecular genetic diversities of each deer species, sika deer and wapiti, from the velvet production and pharmacology points of view.

MATERIAL AND METHODS

In this study, we analyzed the genetic diversities of Chinese Northeast (60 ♂), Gansu (74 ♂), Zuoqia (67 ♂), Tianshan (58♂, 73 ♀), Altai (26♂, 31♀) and Tarim (66♂, 57♀) wapiti; and Dongfeng (50♂, 10♀), Xingkaihu (29♂, 33♀) and Zuoqia (65♂) sika deer using 20 microsatellite markers (Table 1). Blood sampling from each experimental deer was carried out under general anaesthesia; 10 mL blood was collected into a tube containing ACD anticoagulant from deer jugular vein, and stored at -20°C. The software packages used for the calculations in the study were as follows: POPGEN32 for allele frequencies, number of effective alleles and heterozygosity; SAS for PIC; DISPAN for genetic distance; PHYLIP3.5 and TreeView for clustering graph; GLM procedure of SAS8.1 for the least square of variance between velvet production traits and the 16 microsatellite markers. In evolution genetics, population differentiation time was calculated based on the formula of Nei's standard genetic distance: $D=2\alpha T$. Where α =average microsatellite mutation rate per generation (estimated at 4.5×10^{-5} and 4.8×10^{-5} in Chinese wapiti and sika deer respectively), and T = differentiation time/generation interval (the deer generation interval is 6-7 years).

Economic traits, yield (weight) of green velvet antler, were split and assigned to the genotypic effects of multiple microsatellite loci. A multi-factored linear model was established to estimate the genotypic effects on the value of the economic traits:

$$Y_{\text{abcdefghijklnm}} = \mu + \text{BL42}_a + \text{BM203}_b + \text{BM4107}_c + \text{MGTG7}_d + \text{TGLA226}_e + \text{TGLA127}_f + \text{BM2320}_g + \text{JAB1}_h + \text{ET H11}_i + \text{BM1225}_j + \text{BM3628}_k + \text{BMC1009}_l + \text{IDVGA-71}_m + \text{BM6506}_n + e_{\text{abcdefghijklnm}} \quad \text{Where}$$

$Y_{\text{abcdefghijklnm}}$: yield of velvet antler (5th cut), μ : mean value of weight, BL42_a : Genotypic effects of BL42 locus ($j=1, 2, 3$), BM203_b : Genotypic effects of BM203 locus ($i=1, 2$), BM4107_c : Genotypic

effects of BM4107 locus ($j=1, 2, 3$), MGTG7_d : Genotypic effects of MGTG7 locus($j=1, 2, 3$), TGLA226_e : Genotypic effects of TGLA226 locus($j=1, 2, 3$), TGLA127_f : Genotypic effects of TGLA127 locus ($j=1, 2, 3$), BM2320_g : Genotypic effects of BM2320 locus ($j=1, 2, 3$), JAB1_h : Genotypic effects of JAB1 locus ($j=1, 2, 3$), ETH11_i : Genotypic effects of ETH11 locus ($j=1, 2, 3$), BM1225_j : Genotypic effects of BM1225 locus ($j=1, 2, 3$), BM3628_k : Genotypic effects of BM3628 locus ($j=1, 2, 3$), BMC1009_l : Genotypic effects of BMC1009 locus ($j=1, 2, 3$), IDVGA-71_m : Genotypic effects of IDVGA-71locus ($j=1, 2, 3$), BM6506_n : Genotypic effects of BM6506 locus ($j=1, 2, 3$), $e_{\text{abcdefghijklmn}}$: random error.

Table 1. Primer sequences used in the study

BL42 ^A	CAAGGTCAAGTCCAAATGCC	GCATTTTTGTGTTAATTTTCATGC
BM203	GGGTGTGACATTTTGTTC	CTGCTCGCCACTAGTCCTTC
MGTG7 ^A	TTCATTGCAGCACTATTTACAATAG	TAAGTTCCCTGTATCATTTTTTGAA
TGLA226	AGTGAATCCAGATAAGATGTATCA	ATGAAAAGAAGCAATATCGTAAC
TGLA127	CAATTGTGTGGTAGTTTGGACATTC	CACTATTGCAAAGGACCTCCAATT
BM2320	GGTCCCAGCAGCAGTAGAG	CCCATGTCTCCCGTTACTTC
TGLA10	CTAAATTTATCCCACTGTGGCTCTT	CAATCTGCAGTAGCATACATCCTTG
BM757	TGGAAACAATGTAAACCTGGG	TTGAGCCACCAAGGAACC
JAB1	CAGCCATTAAGGGCTGGGATTCC	GATTTCTGGAGGAGGCTCACAGCAG
ETH11	GGCGGACAAGAGTTCTCAGAGAC	CTTGACACATCATTGAGCACACATGC
BM5004	TCTGGAGTGAATGTTTCTGAGG	TTGTGATGAGCACCTGAAGG
BM3628	CTGAGATGGACTCAGGGAGG	GTTGGATTGGAAAGGTTAGGC
BM1225	TTTCTCAACAGAGGTGTCCAC	ACCCCTATCACCATGCTCTG
IDVGA-29	CCCACAAGGTTATCTATCTCCAG	CCAAGAAGGTCCAAAGCATCCAC
IDVGA-71	GCTAGGCATCTGGCAAATAG	CCTCAGAGAAGCCTGGTCAT
BMC1009 ^A	GCACCAGCAGAGAGGACATT	ACCGGCTATTGTCCATCTTG
BM4107 ^A	AGCCCCTGCTATTGTGTGAG	ATAGGCTTTGCATTGTTTCAGG
BM6506 ^A	GCACGTGGTAAAGAGATGGC	AGCAACTTGAGCATGGCAC
BOVIRBP ^A	GTATGATCACCTTCTATGCTTCC	CCCTAAATACTACCATCTAGAAG
BM848	ATGCAAATCTGCTACATCACCTAT	CCCAACTCTCACATCCAGAT

^AFrom cattle or sheep.

RESULTS AND DISCUSSION

Among the 20 microsatellite markers, 14 were derived from the red deer (Bonnet 2002) and the remaining from cattle and sheep (Wang *et al* 1998). These markers consist of 4 monomorphic loci (TGLA10, BM757, BM5004 and IDVGA-29) and 16 polymorphic loci (BL42, BM203, MGTG7, TGLA127, BM2320, JAB1, ETH11, BM1225, BM3628, BMC1009, IDVGA-71, BM4107, BM6506, BOVIRBP, TGLA226 and BM848) (Fukui *et al* 2001; Fyllensten *et al* 1983; Slate *et al* 2002; Mahmut, *et al* 2001; Coulson *et al* 1998). In total, 27 alleles were detected in both wapiti and sika deer.

Allele numbers and allele number in each locus of each subspecies of Chinese wapiti and sika deer were tabulated (Tables 2 and 3).

The mean number of effective alleles was 1.43. Polymorphism information content of these loci was between 0.1187-0.9134. Mean heterozygosity value of these subspecies/breeds was between 0.0014-0.9180 (Table 4). Zuoja wapiti and sika deer had higher heterozygosity value than the other tested

subspecies/breeds being 0.3342 and 0.2803 respectively; indicating extra genes were introduced into these two breeds during breeding.

The results of systemic hierarchical clustering were consistent with the geographical distribution of these deer subspecies/breeds (Fig. 1).

Based on our results, Chinese wapiti breeds/subspecies could be classified into four clusters. The first cluster includes Altai Wapiti and Tianshan Wapiti; the second cluster Northeast Wapiti and Zuoja Wapiti; the third cluster Gansu Wapiti; and the fourth cluster Tarim Wapiti. Chinese sika deer could be classified into two clusters. The first cluster consisted of Dongfeng sika deer and Zuoja sika deer; and the second cluster Xingkaihu sika deer. The time span of genetic divergence of Chinese wapiti and sika deer population was calculated 86-2086 years. In sika deer, the time span of this divergence of Dongfeng and Zuoja Sika deer was the shortest: 110 years.

Table 2. Allele numbers and allele number in each locus of each subspecies of Chinese wapiti

Locus	Northeast		Altai		Tianshan		Gansu		Tarim		Zuojia	
	Allele number	No of effective allele	Allele numbers	No of effective allele	Allele numbers	No of effective allele	Allele numbers	No of effective allele	Allele numbers	No of effective allele	Allele numbers	No of effective allele
BL42	2	1.5571	2	1.9781	2	1.6476	2	1.8689	2	1.7546	2	1.4139
BM203	2	1.4274	2	1.7610	2	1.3502	2	1.3318	2	1.8732	2	1.9190
MGTG7	2	2.0000	2	1.9847	2	1.9826	2	1.6528	2	1.9779	2	1.5736
TGLA226	2	1.9731	2	1.5888	2	1.8089	2	1.9459	2	1.6868	3	2.1151
TGLA127	2	1.9651	2	1.7610	2	1.2344	3	1.3250	3	1.7297	1	1.0000
BM2320	1	1.0000	2	1.2962	2	1.1540	1	1.0000	2	1.0415	1	1.0000
TGLA10	1	1.0000	1	1.0000	1	1.0000	1	1.0000	1	1.0000	1	1.0000
BM757	1	1.0000	1	1.0000	1	1.0000	1	1.0000	1	1.0000	1	1.0000
JAB1	1	1.0000	1	1.0000	1	1.0000	1	1.0000	1	1.0000	1	1.0000
ETH11	3	2.0619	3	2.0351	3	2.0230	3	1.7089	3	2.0658	3	2.2803
BM5004	1	1.0000	1	1.0000	1	1.0000	1	1.0000	1	1.0000	1	1.0000
BM1225	2	1.9651	2	1.9403	2	1.4868	2	1.7041	2	1.8422	2	1.7749
BM3628	2	1.8000	2	1.8366	2	1.5759	2	1.7208	2	1.4896	2	1.9556
IDVGA-29	1	1.0000	1	1.0000	1	1.0000	1	1.0000	1	1.0000	1	1.0000
BMC1009	1	1.0000	1	1.0000	1	1.0000	2	1.0868	1	1.0000	2	2.0000
IDVGA-71	2	1.6000	2	1.9781	2	1.9560	2	2.0000	2	2.0000	2	2.0000
BM4107	2	1.6000	2	1.9975	2	1.9629	2	1.1643	2	1.6052	2	1.9632
BM6506	2	1.3006	2	1.5662	2	1.6397	2	1.9369	2	1.9841	2	1.1463
BOVIRBP	1	1.0000	1	1.0000	1	1.0000	1	1.0000	1	1.0000	1	1.0000
BM848	1	1.0000	1	1.0000	1	1.0000	1	1.0000	2	1.0934	1	1.0000
Mean	1.6000	1.4125	1.6500	1.4862	1.6500	1.3911	1.7000	1.3724	1.7500	1.4572	1.6500	1.4571
s.d.	0.5982	0.4278	0.5871	0.4424	0.5871	0.4004	0.6569	0.3941	0.6387	0.4318	0.6708	0.4873

Table 3. Allele numbers and allele number in each locus of each subspecies of Chinese sika deer

Locus	Xingkaihu		Dongfeng		Zuojia		Total	
	Allele numbers	No of effective allele	Allele numbers	No of effective allele	Allele numbers	No of effective allele	Allele numbers	No of effective allele
BL42	2	1.2952	1	1.0000	1	1.0000	2	1.9099
BM203	1	1.0000	1	1.0000	1	1.0000	2	1.9903
MGTG7	2	1.9370	2	1.9731	2	1.9959	2	1.9433
TGLA226	3	1.5047	3	1.8927	3	1.8135	3	2.1105
TGLA127	3	2.2898	3	2.0737	3	2.5157	3	1.7178
BM2320	1	1.0000	1	1.0000	1	1.0000	2	1.6875
TGLA10	1	1.0000	1	1.0000	1	1.0000	1	1.0000
BM757	1	1.0000	1	1.0000	1	1.0000	1	1.0000
JAB1	3	2.8723	3	2.9652	3	2.6273	3	1.3532
ETH11	2	2.0000	2	2.0000	2	2.0000	3	2.0547
BM5004	1	1.0000	1	1.0000	1	1.0000	1	1.0000
BM1225	2	1.3361	2	1.3213	2	1.6575	2	1.6791
BM3628	1	1.0000	1	1.0000	1	1.0000	2	1.9921
IDVGA-29	1	1.0000	1	1.0000	1	1.0000	1	1.0000
BMC1009	2	1.1772	2	1.6423	1	1.0000	2	1.3481
IDVGA-71	2	2.0000	2	2.0000	2	2.0000	2	1.9867
BM4107	2	1.0853	2	1.6632	2	1.5806	2	1.8895
BM6506	2	1.1032	2	1.4922	2	1.0786	2	1.6087
BOVIRBP	2	1.2750	1	1.0000	1	1.0000	2	1.0207
BM848	1	1.0000	1	1.0000	1	1.0000	2	1.0151
Mean	1.7500	1.3938	1.6500	1.4512	1.6000	1.4135	2.0000	1.5654
s.d.	0.7164	0.5393	0.7452	0.5566	0.7539	0.5599	0.6489	0.4271

Table 4. The mean gene frequency of each allele in each locus of each subspecies or breed

Subspecies or breed	Allele A	Allele B	Allele C
Northeast Wapiti	0.6662	0.2663	0.0175
Altai Wapiti	0.6447	0.2807	0.0246
Tianshan Wapiti	0.6370	0.2903	0.0227
Gansu Wapiti	0.6351	0.2958	0.0191
Tarim Wapiti	0.6494	0.2750	0.0256
Zuojia wapiti	0.6579	0.2668	0.0253
Xingkaihu sika deer	0.6516	0.2570	0.0414
Dongfeng sika deer	0.6600	0.2567	0.0333
Zuojia sika deer	0.6845	0.2216	0.0439

In wapiti, the time span of this divergence of Tarim and Northeast Wapiti was the longest: 793 years; whereas, Altai and Tianshan Wapiti were the shortest: 86 years. Consequently, the time spans of

genetic divergence of those sub-species/breeds that distribute in the same geographic region were relatively short and vice versa.

The microsatellite genetic markers that are related to velvet production were identified through establishment of a general linear model. There was a significant difference between the AA type and the AB type at the BM4107 locus in Zuoja sika deer ($P<0.05$), and velvet antler yield of the AA type was significantly higher than that of the AB type ($P<0.05$) (Table 5). Likewise, a significant difference was detected between the AB type and the BB type at the TGLA226 locus in Xingkaihu sika deer ($P<0.05$), and velvet antler yield of the AB type was significantly higher than that of the BB type ($P<0.05$) (Table 6).

Table 5. Least square mean and multiple comparisons of microsatellite genetic markers and velvet antler yield in Zuoja sika deer

Classification variable	Level	Least square mean	Standard error	t-value/probability
BM4107	AA	3401.32	1499.33	762904/0.110474 0.0108*/0.9130
	AB	-102.58	1551.52	-2.762904/-2.55702 0.0108*/0.1173
	BB	3296.78	1719.31	-0.110474/2.55702 0.9130/0.1173

Note: level of significance set at 0.05.

Table 6. Least square mean and multiple comparisons of microsatellite genetic markers and velvet antler yield in Xingkaihu sika deer

Classification variable	Level	Least square mean	Standard error	t-value/probability
TGLA226	AB	5406.33	543.22	2.582211/1.599684 0.0416*/0.1608
	BB	4067.56	299.08	-2.58221/-1.08282 0.0416*/0.3205
	BC	4567.71	556.72	-1.59968/1.082817 0.1608/0.3205

Note: level of significance set at 0.05.

Overall, our results could provide useful information for an in depth understanding of molecular genetic diversities of Chinese sika deer and wapiti, and for molecular breeding of these deer breeds toward further increasing in velvet antler production.

ACKNOWLEDGEMENTS

The authors wish to thank all the Biotechnology Unit staff from Institute of Wild Economic Animals and Plants for the help of collecting blood samples, and Mr Zhao Haiping for helping with the partial data analysis.

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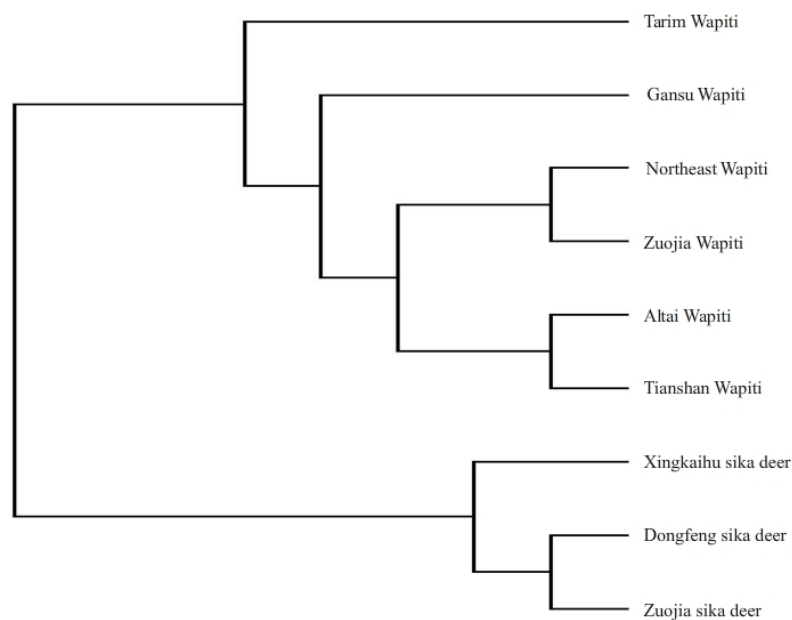


Figure 1. Dendrogram of subspecies/breeds of Wapiti and sika deer using UPGMA/Da method.

EFFECTS OF DIETARY PROTEIN LEVELS ON PRODUCTION PERFORMANCE AND SERUM IGF-1 LEVELS IN SIKA DEER

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ABSTRACT

The purpose of this study was to investigate the influence of dietary protein levels on performance of sika deer. Twenty four male sika deer were randomly assigned into 3 treatments; the deer in each treatment were fed by one of the diets containing 12%, 16% and 20% of crude protein (CP), respectively. The results showed that during antler growth period the average body weight gain in 12%, 16% and 20% CP levels was 17.3kg, 19.9kg and 14.4 kg respectively; and the antler yield was 2224g, 2518g and 2246g for each treatment. A significant positive relationship ($P<0.01$) was found between antler yield (AY) and serum IGF-I concentration , and the regression equation was: $\text{IGF-1}(\text{ng/mL})=3.55\text{AY}(\text{g})-1659.4$ ($R^2=0.77$, $n=8$). It was concluded that 16% dietary protein was optimal for body weight gain and antler yield during the antler growth period in the sika deer.

Additional keywords: Sika deer, protein, body weight, velvet antler, IGF-I.

INTRODUCTION

Apart from other domestic animals, the purpose of deer farming in China is to obtain high value medicinal material, velvet antlers. The major deer species for velvet antler production is sika deer. During the long history of sika deer domestication, strategies have been developed by Chinese deer farmers to maximize velvet antler yield, one of which is nutritional enhancement. Gao and Yang (1993) reported that proper dietary energy and protein level can improve velvet antler production of sika deer. Gao *et al.* (1997) showed that body weight gains of the yearling sika deer were significantly influenced by the interaction between dietary energy and protein. However, further research on nutrients and hormone regulating mechanisms for velvet antler growth are needed, especially how do the dietary energy, protein, and other nutrient levels regulate the velvet growth and development.

Velvet antler, tissue of bone and cartilage, was reported to be regulated by insulin-like growth factor 1 (IGF-1) (Li *et al.* 1999). Insulin-like growth factor (IGF-1), a constituent of the IGF axis, is a peptide structurally related to insulin that is secreted by many tissues (Sadighi *et al.*, 1994). Since IGF-1 has been demonstrated to influence cartilage growth, it was supposed to be an important candidate as antler stimulating hormone (Suttie *et al.* 1989, 1992). Furthermore, IGF-1 level in animals is highly correlated with nutrition (Breier *et al.* 1986). In the present study, we investigated whether dietary protein level would have effects on growth of deer bodyweight and velvet yield by regulating serum IGF-1 level.

MATERIALS AND METHODS

Experimental diets and animals

Twenty four 4-year-old sika deer stags (around 90 kg bodyweight) were selected during antler growth period (from 15 April to 7 August). All deer were randomly assigned to 3 groups with 8 deer in each. Each deer was reared in individual pens and fed 3 times every day (early 0700, noon 1100, night 1700 hours) with 1.6 kg concentrate and 4.2 kg corn silage as-fed.

The deer in each group was fed with one of the formulated diets containing 12%, 16% and 20% crude protein, respectively. These diets were made up of corn silage, corn meal, soybean meal, wheat bran, fish meal *etc.* (Table 1).

Sampling, analyses and measurements

Each animal was weighed before feeding at the beginning and the end of the trial. Velvet antler weight of each deer was accurately recorded at velveting.

Table 1. Experimental diets and nutritional levels (dry matter basis)

Ingredient	Ration 1	Ration 2	Ration 3
Corn meal (%)	46.0	33.4	20.6
Soybean meal (%)	11.5	21.0	30.6
Wheat bran (%)	3.0	5.0	7.0
Fish meal (%)	3.0	4.5	6.0
Corn silage (%)	35.0	35.0	35.0
Dicalcium phosphate (%)	0.85	0.43	-
Calcium carbonate (%)	0.15	0.25	0.40
Salt (%)	0.45	0.42	0.40
Nutritional level			
DM (%)	87.07	87.26	88.22
CP (%)	12.02	16.00	20.02
ME (MJ/kg) ^A	11.63	11.67	11.71
Ca (%)	0.66	0.65	0.66
P (%)	0.43	0.43	0.43

^A ME values were obtained by measurement.

Table 2. DM intake, CP intake, body weight gain, velvet yield and serum IGF –I levels of sika deer

Within rows, values followed by different letters are significantly different

	Dietary treatment		
	12% CP	16% CP	20% CP
Bodyweight gain (kg)	17.27 ± 5.87	19.90 ± 6.11	14.43 ± 2.79
Velvet yield (g)	2224 ± 379.4	2518 ± 337.2	2246 ± 246.9
DM intake(g/day)	2460 ± 17.6	2470 ± 21.6	2472 ± 27.2
CP intake(g/day)	295 ± 0.9a	393 ± 1.1b	492 ± 1.3c
Serum IGF-I (ng/mL)			
Pre-hard button casting	479 ± 31.1	491 ± 36.7	480 ± 26.2
Velveting point	1018 ± 73.3a	1194 ± 84.9b	1027 ± 71.1a
Post –velveting	1048 ± 66.2a	1211 ± 85.7b	1005 ± 68.4a

The blood was collected via jugular vein puncture before feeding just prior to hard button casting, velveting and last day of the trial period. After centrifuging for 10 min at 5000 rev/min after blood coagulation, serum was collected and frozen at -20 °C for later analysis. Serum IGF-1 level of each animal was measured using immunoradiometric assay kits obtained from Diagnostic Systems Laboratories (Shanghai, China). Dry matter (DM) and crude protein were determined using conventional methods. DM intake and crude protein (CP: Kjeldahl-N×6.25) intake were analyzed according to AOAC (2003) procedures.

Statistical analysis

Data were given as mean ± s.e.m. Results were analyzed by one-way ANOVA followed by Turkey's test. Differences were considered significant when $P < 0.05$. Multivariate linear regression model was used for analyses of relations between serum IGF-1 levels and body weight and antler yield.

RESULTS AND DISCUSSION

Effects of dietary protein levels on dry matter intake and crude protein intake

The results indicated that intake of crude protein per day was significantly influenced by dietary protein level ($P < 0.01$). The CP intakes were higher for the groups receiving 20% CP than for those receiving 16% CP or 12% CP, the CP intakes were higher for the groups receiving 16% CP than for those receiving 12% CP ($P < 0.01$) (Table 2). The results were similar to those of Gao *et al.* (1993). There was no significant difference between the different groups in DM intake.

Effects of dietary protein levels on bodyweight gain and velvet antler yield

During the antler growth period the average bodyweight gain of the three groups of deer fed 12%, 16% and 20% dietary protein was 17.27 kg, 19.90 kg and 14.43 kg respectively (Table 2). The bodyweight

gains of the group fed 16% CP were numerically higher than those fed 12% CP and 20% CP, but there were no statistically significant differences among different dietary protein level groups ($P>0.05$). The deer showed best body weight gain in 16%CP group.

When deer were fed 12%, 16%, and 20%CP diet, average velvet antler yield was 2224 g, 2518 g and 2246 g respectively (Table 2). Velvet antler yield of 16% dietary protein was 13% and 12% higher than the groups of 12% and 20% of dietary protein, respectively. However, dietary protein level had no statistically significant effect on velvet antler yield ($P>0.05$).

Effects of dietary protein levels on serum insulin growth factor 1 (IGF-1)

During the whole antler growth period, serum IGF-1 levels of deer fed 12%, 16%, and 20% dietary protein were determined at three different growth intervals: pre-hard button casting, velvetting point, and post-velvetting (Table 2).

Dietary protein had no effect on serum IGF-1 level at pre-hard button casting ($P>0.05$), moreover, the serum IGF-1 content in 16% CP group showed higher serum IGF-1 level than those in 12% and 20% groups at velvetting point, and post-velvetting ($P<0.05$).

IGF-1 levels increased when velvet antler was growing and reached the highest when velvet antler was harvested. IGF-1 level in the post-velvetting period was much higher than that in pre-hard button casting period. These results were similar to those reported on other deer species (Suttie *et al.* 1985, 1989; Schams *et al.* 1992; Suttie *et al.* 1992), suggesting that IGF-1 is also the antler growth regulator of sika deer.

Further data regression analysis showed that there was high correlation between serum IGF-1 and bodyweight (BW) ($P<0.01$) and antler yield (AY) ($P<0.01$), and the regression equations were $IGF-1(ng/mL) = 0.06BW(kg)-52.14(R^2 = 0.69, n = 8)$ and $IGF-1(ng/mL)=3.55AY(g)-1659.4 (R^2 = 0.77, n = 8)$, respectively.

In addition, deer serum IGF-1 level at velvetting was very close to that of post-velvetting, meaning that even after the first set of velvet was harvested, circulating serum still retained high level of IGF-1. It was not clear how serum IGF-1 level changed after the first velvetting till regeneration of the second set of velvet. Further study is needed to investigate the regulatory mechanism of IGF-1 during velvet growth cycle.

CONCLUSIONS

The present study showed that the intake of crude protein per day was significantly influenced by the dietary protein level. The dietary protein level had no significant effect on bodyweight and velvet antler yield when sika deer were fed the routine levels of crude protein. When diet contained 16% crude protein, there is a trend to show that sika deer had best bodyweight gain and antler yield, although not statistically significant. Serum IGF-I was highly related to bodyweight gain and velvet antler yield during velvet antler growth period for sika deer.

ACKNOWLEDGEMENTS

The authors are grateful for the financial support from the Ministry of Science and Technology, the assistance for the helping on the preparation of this manuscript from Dr Li Chunyi and Dr Yan Haijie.

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FACTORS AFFECTING THE NUMBER OF STRO-1⁺ STEM CELLS DERIVED FROM REGENERATING ANTLER AND PEDICLE CELLS OF RED AND FALLOW DEER.

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ABSTRACT

Mesenchymal stem cells positive to surface antigen STRO-1 were isolated from regenerating antlers of red deer (*Cervus elaphus*) and fallow deer (*Dama dama*) using a magnetic cell separation method. In this study we analysed factors potentially affecting the number of STRO-1⁺ cells in the cell cultures. With regard to the STRO-1 antigen, we evaluated data from 188 MACS[®] separation procedures of cell cultures cultivated in DMEM and 10% foetal calf serum of four fallow deer males (130 procedures) and four red deer males (58 procedures). The analysed factors were the sampling site of the antler or the pedicle, cell passage and type of the cell culture (mixed or STRO-1 negative cell cultures). The percentage of obtained STRO-1⁺ cells varied greatly from 0.4% to 38.9% for fallow deer and from 1.8% to 16.5% for red deer. We have not found any significant influence of the sampling site. The passage and the type of culture were significant factors for both fallow and red deer cells. The highest numbers of STRO-1⁺ cells were obtained from the second passage from both fallow and red deer cell cultures (24.6% +/- 14.37, 5.5% +/- 3.03 respectively). Our experiments revealed that we can maximize the number of STRO-1⁺ cells in the cultures by manipulating the cultivation factors.

Additional keywords: magnetic cell separation, cell culture passage, culture conditions, STRO-1⁺ stem cells.

INTRODUCTION

Stem cell based origin of deer antlers has been discussed over the last decade and has been indirectly confirmed by various transplantation and deletion experiments of the antlerogenic and pedicle periosteum (Li and Suttie 2001, 2006, Mount *et al.* 2006, Li *et al.* 2007, Kierdorf *et al.* 2007, 2009). Rolf *et al.* (2006, 2008) described the localization, isolated and characterized stem cells from pedicles and regenerating antlers of fallow deer (*Dama dama*) using the STRO-1 mesenchymal stem cell surface marker (Dennis *et al.* 2002). A creation of a new stem cell line from antlerogenic cells and successful xenotransplantation of these cells followed (Cegielski *et al.* 2008, 2010).

Each year, the pedicle periosteum gives rise to the antlers (Li *et al.* 2007). As shown by various authors, antlerogenic and pedicle periosteum cells express markers of undifferentiated multipotential cells (e.g. Oct4, Nanog) and mixed cultures of these cells could differentiate into osteocytes, chondrocytes and adipocytes under appropriate culture conditions (Li and Suttie 2006, Mount *et al.* 2006, Berg *et al.* 2007). We assume, that the STRO-1 positive (STRO-1⁺) cells, as a part of the antlerogenic tissue, could be one of the most important stem cell populations which give rise to chondroprogenitors and osteoprogenitors. STRO-1⁺ cell cultures differentiate *in vitro* along osteogenic, chondrogenic and with antler re-growth unrelated adipogenic lineages. Identification of particular cell types participating at the amazingly rapid antler re-growth might help to elucidate mechanisms behind this unique mammalian phenomenon frequently discussed as a suitable biomedical model for bone regeneration. Furthermore, such cells could provide an appropriate culture model e.g. to study hormonal influences during antler regeneration.

A common problem of mesenchymal stem cells is the isolation of sufficient quantities of cells and their subsequent expansion. The culture conditions and cell passaging are crucial for maintaining their undifferentiated potential. Another problem with STRO-1⁺ cells is their rather wide range of incidence in the cell cultures (Stewart *et al.* 1999). Stewart *et al.* (1999) isolated 10-50% from murine bone marrow-derived cell line and 2-80% from adult human bone marrow stromal cells. In their opinion, the

exact proportion of STRO-1⁺ cells was remarkably donor-dependent for the human cell cultures. Moreover they reported that STRO-1 negative (STRO-1⁻) fractions can give a rise to STRO-1⁺ cells.

In the present study we isolated mesenchymal stem cells positive to superficial antigen STRO-1 from regenerating red deer antlers by the same method as applied previously in fallow deer (Rolf *et al.* 2008). These cells should have served us as a model to examine the influence of hormones, particularly steroids and insulin like growth factor 1 (IGF-1), whose function is controversial in antler research (Bartoš *et al.* 2009). Additionally they could also support the prediction of inter-individual differences of antler growth, as indicated by our previous study on mixed antler cell cultures (Kužmová *et al.* 2011). For both fallow and red deer, the number of obtained STRO-1⁺ cells in the antler and pedicle cell cultures varied greatly. Due to initially planned hormonal experiments we wanted to avoid STRO-1⁺ cell cultures expansions by supplementation with growth factors. Besides, by expansion in standard cultivation conditions (DMEM and 10% FCS) or even in a special serum free expansion medium (Miltenyi Biotec, Germany) the STRO-1⁺ cells lost their positivity rapidly (unpublished observation). For it was difficult to expand the STRO-1⁺ cells after separation, it became essential to determine the factors influencing the abundance of STRO-1⁺ cells in the cell cultures prior to the MACS[®] separation. In this study we present a detailed analysis of the factors sampling site, cell passage and type of cell culture, which possibly affected the percentage of STRO-1⁺ cells in the fallow deer and red deer cell cultures.

MATERIALS AND METHODS

The tissue was collected from growing antlers 15 to 90 days after antler casting from four anesthetized red deer males and four fallow deer males by bioptic punch 1 and 2 cm below the growing antler tip corresponding to the antler growth and cartilaginous zone (Matich *et al.* 2003). To obtain additional samples from antler bone, antler periosteum and pedicle periosteum the four adult fallow deer were slaughtered and sampled according to Rolf *et al.* (2008). The age of the deer males was between two and six years. The obtained samples were mechanically minced into pieces (approx. 0.5 – 1 mm³). The tissue pieces of red deer were incubated for four hours at 37°C in Dulbecco's Minimal Eagle Medium (DMEM) supplemented with antibiotics and 200 U/mL Type II Collagenase (Gibco/Invitrogen, Czech Republic) and vortexed every 20 min. (Kužmová *et al.* 2011). The cells of fallow deer were let grown out of cultured tissue pieces. All cells and tissue pieces were cultured in DMEM supplemented with 10% FCS under standardized conditions (37°C and 5% CO₂). Because the cultivation density was not specified in some of the primary tissue cultures, it could not be analyzed as one of the factors.

The mixed cell cultures were passaged after reaching subconfluence up to 2nd passage for red deer and up to 7th passage for fallow deer. Mixed cell cultures were labeled with a primary antibody, surface antigen STRO-1 (R&D Systems, Germany), coupled with secondary antibody IgG-MicroBeads (Miltenyi Biotec, Germany) and separated with MACS[®] (Miltenyi Biotec, Germany) according to the manufacturer's protocols. The cells were counted before and after MACS[®] separation using a CASY cell counter. After the MACS[®] separation the STRO-1⁻ fraction was further cultivated until the cells reached subconfluence and then separated to examine the number of STRO-1⁺ cells (these cultures will be referred to as STRO-1⁻ cultures and their passage numeration continues from the primary cultures). To ensure that no STRO-1⁺ cells remained in the negative fractions, the procedure was repeated immediately after the first separation in few cases. Two of the STRO-1⁻ fractions have been further cultivated in DMEM and 10% FCS until they reached subconfluence and separated again up to six cultivation and separation procedures (always cultivating only the negative fraction). These multiple-times cultivated and separated STRO-1⁻ cell cultures were not statistically evaluated and are discussed separately in this study. All MACS[®] separations were performed in the same laboratory as part of a long term study on antler stem cells. The data from fallow and red deer were statistically analysed separately. The procedures of 188 MACS[®] separation were analysed, 130 for fallow deer and 58 for red deer. For the statistical analysis we used the General Linear Mixed Model (GLMM) using the PROC MIXED procedure (SAS V9.0) with least-square-means (LSMEANS) and the Tukey-Kramer adjustment for multiple comparisons. To account for the repeated measures on the same individuals, all analyses were performed with individual males as a random factor. The dependent variable was the percentage of obtained STRO-1⁺ cells (calculated from the total number of cells after separation) and was analysed in relation to the following factors: (a) the sampling site (antler growth zone and antler cartilaginous zone for fallow and red deer; antler bone, antler periosteum and pedicle periosteum for fallow deer), (b) cell passage and (c) type of cell culture (mixed cell culture and STRO-1⁻ cell culture).

RESULTS

We examined the influence of particular sampling site and cultivation factors on the percentage of STRO-1⁺ cells in the cell cultures. The percentage of obtained STRO-1⁺ cells varied between 0.4% and 38.9% for fallow deer and between 1.8% and 16.5% for red deer.

We did not detect any significant influence of the cell sampling site of the antler or pedicle on the percentage of obtained STRO-1⁺ cells.

For both red and fallow deer cells the passage was a highly significant factor (red deer: $F_{2,51} = 8.41$, $P < 0.001$, fallow deer: $F_{6,122} = 33.6$, $P < 0.0001$). In general, the highest percentage of STRO-1⁺ cells was obtained from the second passage, both in fallow deer (24.6% $\pm$ 14.37) and red deer (5.5% $\pm$ 3.03) as shown in Figure 1.

The type of culture was also a significant factor (red deer: $F_{1,51} = 4.44$, $P < 0.05$, fallow deer: $F_{1,122} = 18.75$, $P < 0.0001$), but the results differed between fallow and red deer. While the percentage of obtained STRO-1⁺ cells from a mixed culture was significantly higher compared to the STRO-1⁻ culture of fallow deer, it was the opposite for red deer cells (Fig. 2).

By immediate repetition of the MACS[®] separation procedure on the STRO-1⁻ fraction, we hardly detected any STRO-1⁺ cells remaining in the STRO-1⁻ fractions. However, after subsequent cultivation we isolated STRO-1⁺ cells from multiple-times cultivated and sorted STRO-1⁻ cell cultures.

DISCUSSION

This study focused on factors which could influence the yields of STRO-1⁺ cells in cell cultures derived from regenerating antlers and pedicle of fallow deer and red deer. The amount of obtained STRO-1⁺ cells was highly variable as it was also described in Stewart *et al.* (1999). We determined that the number of passages and type of cell culture were both significant factors. On the other hand, we found no significant influence of the sampling site of antlers or pedicles. It is hence probable that the cultivation procedure affected the yields of STRO-1⁺ positive cells up to the level that overrides the anticipated effect of sampling site, particularly the pedicle periosteum, which is supposed to be the initiation tissue for antler re-growth (Li *et al.* 2007).

In our experiments, we have isolated STRO-1⁺ cells not only from subsequently cultivated STRO-1⁻ fractions as previously reported by Stewart *et al.* (1999), but also from multiple-times cultivated and sorted STRO-1⁻ cell cultures. As STRO-1⁺ cells were not detected in STRO-1⁻ cell fractions right after the separation, the STRO-1⁺ cells isolated from subsequently cultivated negative cultures had to arise from the STRO-1⁻ cells. At this stage we can only speculate about the explanation. First, negative cultures may contain earlier precursor cells which after some time of cultivation become STRO-1⁺. Second, as a result of the *in vitro* cultivation, 10% of FCS and passaging, differentiated cells might de-differentiate and become STRO-1⁺. Such de-differentiation is typical especially for chondrocytes cultivated as monolayers (Barbero *et al.* 2003). Third, the used method was not sensitive enough to separate cells which possess only small number of STRO-1 surface proteins, and subsequently, their number increased during further cultivation.

The second passage of the cell cultures cultivated in DMEM and 10% FCS seemed to be the most suitable for the isolation of the greatest numbers of STRO-1⁺ cells. This could be explained by the de-differentiation of particular cells in the cultures as well as by the presence of cells in various stages of expression of STRO-1 marker, their continuously changing multilineage potential and ongoing differentiation (Yu *et al.* 2010, Stewart *et al.* 1999, Barbero *et al.* 2003). Moreover, Simmons and Torok-Storb (1991) found a comparable pattern of a significant increase in the proportion of STRO-1⁺ cells after two weeks of cultivation, followed by a progressive decline. They concluded that it could be a culture epiphenomenon unrelated to normal *in vivo* conditions, or it occurred due to the maturation of stromal precursors into more differentiated stromal cell types as discussed above.

ACKNOWLEDGEMENTS

All experiments were performed with the approval of the Central Commission for Animal Welfare, Czech Republic and Animal Care Committee of Germany. This study was supported by the Czech Science Foundation (grant number 523/08/0808); the Ministry of Agriculture of the Czech Republic (grant number MZe 0002701404), and the German Research Society (DFG, grant numbers RO 2520/1-1 and RO 2520/1-3). The internship was made possible by bilateral agreement between Charles

University in Prague, Czech Republic and The George August University in Göttingen, Germany.

We would like to thank to all who participated in collecting of the tissue samples and took care of the animals.

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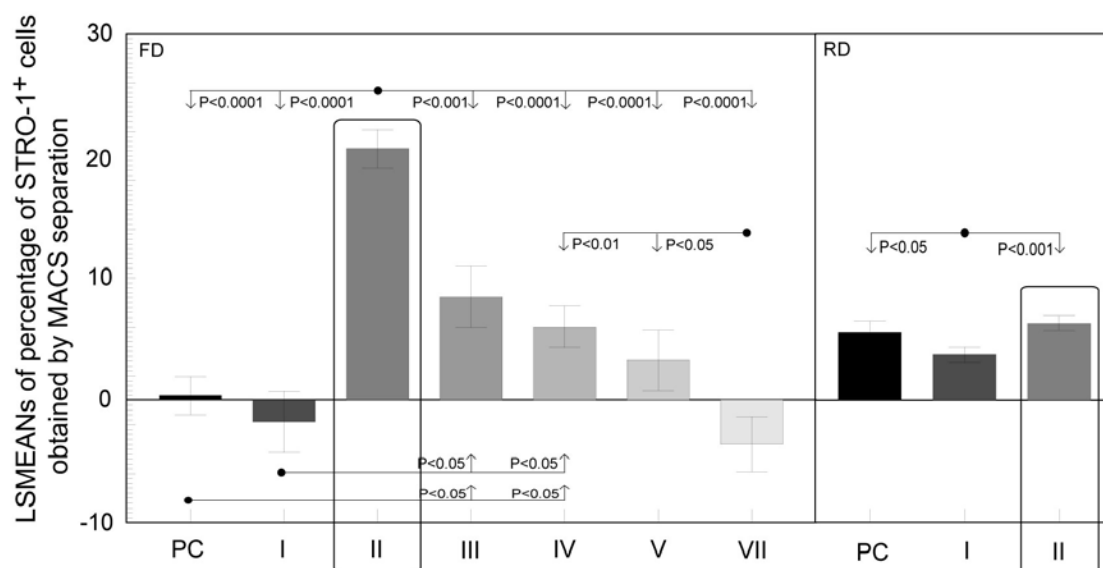


Figure 1. Effect of the passage (primary culture PC, passage I. – VII.) on the STRO-1⁺ quantities by fallow (FD) and red deer (RD). The 6th passage of the fallow deer cell cultures was not separated.

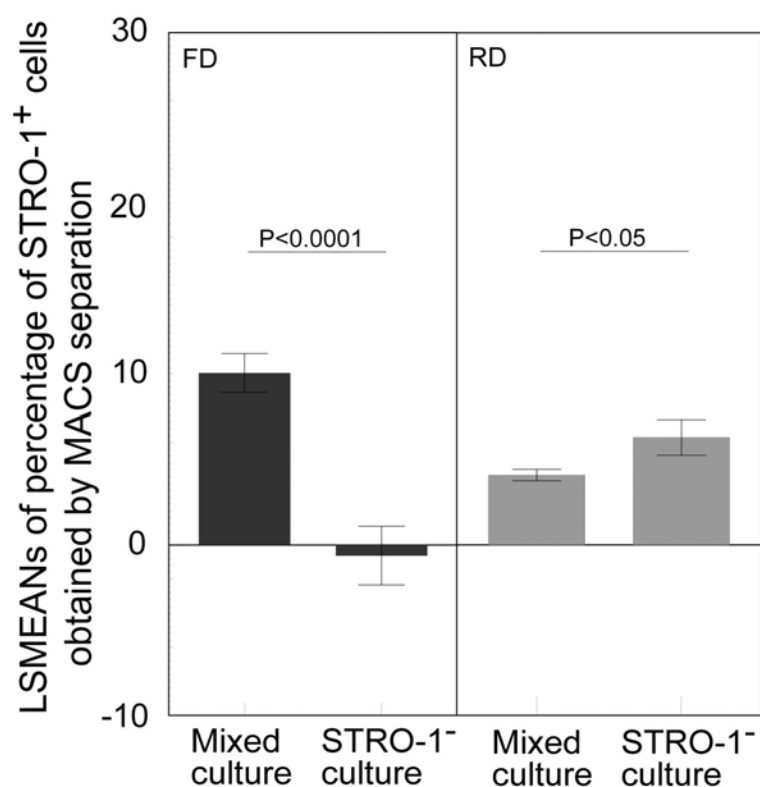


Figure 2. Effect of the type of culture on the STRO-1⁺ quantities by fallow (FD) and red deer (RD).

PAMPAS DEER (*OZOTOCEROS BEZOARTICUS*) PARASITOLOGICAL ASSESSMENT IN URUGUAYAN POPULATIONS

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ABSTRACT

The pampas deer (*Ozotoceros bezoarticus*) is one of the most endangered neotropical mammal in Uruguay. Although it has a wide geographic distribution in southeastern South America (from 5° to 41° S), the habitat required by this species has been greatly reduced by agriculture and urbanisation. The objective of this study was to determine the parasitological composition fauna and estimate the parasite load in the two Uruguayan pampas deer populations. We surveyed the copro-parasite load analyzing the effect of seasonality along the year, the micro ecosystem environment and the topography and also the livestock of the paddock. The taxonomic assessment was based of adult individuals obtained from the *post mortem* necropsy examination in individuals found dead in the field. The genera of the endoparasite recorded through the morphological eggs, larvae and or adults were *Trichuris*, *Capillaria*, *Strongyloides*, *Fasciola*, *Paramphistomum*, *Moniezia*, *Haemonchus*, *Ostertagia*, *Trichostrongylus*, *Oesophagostomum* and *Coccidias* ooqueste. Furthermore, once we have diagnosed the same genera of helminthes in domestic ruminants, is required to take into account and balance the carrying capacities of the field paddock, and in particular perform periodic surveys of the livestock parasite load that will be helpful to maintain under control with lower values.

Additional keywords: *Ozotoceros bezoarticus*, parasites, Uruguay.

INTRODUCTION

The pampas deer (*Ozotoceros bezoarticus*) is one of the Cervidae of the Uruguayan local fauna, and formerly occupied a range of open habitats such as grassland, pampas, savanna and cerrado (Brazil). Before cattle introduction in Uruguay, the pampas deer was reported by several European naturalists to be one of the most widespread and common species of the Uruguayan's grasslands. Although it has a wide geographic distribution in southeastern South America (from 5° to 41° S), the habitat required by this species has been greatly reduced by agriculture and urbanisation.

However at the beginning of the last century a clearly pampas deer eradication campaign was initiated, similar situation as bison in Americas. This species was persecuted believing that the grassland needs to be use in exclusivity by the introduced livestock. However, some isolated and small populations have survived in close contact with cattle (Cosse *et al.* 2009).

The process of pampas deer population decline is clearly visible in Uruguay, which is home to two endemic subspecies: *O. b. arerunguaensis*, represented by a population with around 1000 individuals in Arerunguá (Salto north of the country), and *O. b. uruguayensis*, with less than 400 individuals, located at Los Ajos in Rocha southeastern Uruguay (González 2010).

However, the area encompassed by these habitats has been dramatically reduced to less than 1% of that present in 1900 (González *et al.* 1998). The two Uruguayan remaining populations are located in private ranches in "Arerunguá" region (31° 65' S, 56° 43' W, Salto Department) and in "Sierra de los Ajos" (33° 45' S, 54° 02' W, Rocha Department, González *et al.* 2002). Currently, the pampas deer has a limited population growth related to the carrying capacity of the land (= ranches) they occupy. Even though pampas deer have been recognized by the Uruguayan government as threatened, declaring the species a living Uruguayan Natural Monument (Ministerial Decree 12/985), no management guidelines have yet been issued, nor any action taken to its effective conservation. The effective protection of the species relies on secure habitat and the participation and commitment of landowners. In Uruguay, where protected areas cover less than 1% of the surface area, it is crucial to design strategies that integrate private land into the conservation system. For almost two decades we are studying and analyzing the demographic trends, genetic variability, ecology and medicine issues (Hernández *et al.*

1994; González *et al.* 1998; González *et al.* 2002; Hernández 2002; Cosse *et al.* 2007; Cosse *et al.* 2009).

In order to analyze pampas deer health conditions and to better understand the effect of habitat and niche overlapping with livestock, we conducted a parasitological study based on samples surveyed on the Uruguayan wild populations. These data represent the first parasitological systematic study of pampas deer, conducted over two decades. Our main results clearly showed the pampas deer shared the parasite fauna with livestock, and we proposed management guidelines to improve the health conditions of the agro-ecosystems in Uruguay.

MATERIAL AND METHODS

Study areas

The Pampas deer parasite surveys were conducted seasonally from 1992 to 2009 in both pampas deer populations located in Arerunguá and Sierra de los Ajos localities.

The pampas deer inhabit in both regions in private ranches with livestock. The pampas deer is free ranging in this complex scenario of discrete patches with different kinds and amounts of livestock and crops. The pampas deer distribution occurred mainly in four ranches of approximately 10,000 ha (33°50'01''S; 54°01'34''W) within the “Bañados del Este” Biosphere Reserve, Rocha Department in the southeast of the country.

The main activities of these ranches are livestock (cattle and sheep ranching) and crops as rice for human consumption and ryegrass (*Lolium* sp., *Poaceae*) for pasture. The landscape is low, rolling hills; the parent material consists of Quaternary unconsolidated sediments (clays, argillaceous muds and sands and, locally at rocky points, igneous or metamorphic rocks). The dominant soils are deep, poorly drained and fine textured (gleysols). Altitudes ranging from –5 to 100 masl, the annual average rainfall is 1,000 mm and the average annual temperature is 16°C (Ramsar Sites Database, 2002).

The other pampas deer population is mainly located in ten ranches in an area of 30,000 ha. (between 31° 48' 55'' S; 56° 33' 30'' W to 31° 44' 65''S; 56° 45' 20'' W.), close to the “El Tapado” stream and about 12 km southwest of the Arerunguá River, Salto Department. The main activities of these ranches are livestock (cattle and sheep ranching). The area is a rolling, hilly area dominated by natural temperate grasslands; it is treeless, dissected by streams and small valleys and interrupted by rocky outcrops and ridges. The soil is mainly superficial composed by basaltic Brunosols and Vertisols. The altitudes ranging from –5 to 300 masl, and the annual average rainfall is 1500 mm and the average annual temperature is 18.5°C. This area integrates a Conservation Priority Area in Uruguay (González and Sans, 2009).

Sample collection

Fresh pampas deer feces were collected in plastic bags and stored refrigerated and send to the Parasitological Laboratory at Facultad de Veterinaria Regional Norte. In each sample was identified and labeled with the information regarding the individual, age category and gender, the date, geographic coordinates, the ranch and paddock name.

Laboratory analysis

The coproparasite analysis was performed in the laboratory comprising the macro and microscopic examination to visualize possible forms of dissemination. We analyzed in total 829 samples from Arerunguá and 233 from Los Ajos populations. In both populations an annual seasonally study was performed using 216 samples from Arerunguá during 1995 and in Los Ajos in 33 samples during 2003-2004 in feces from tagged animals.

Techniques were used to perform qualitative enrichment by flotation concentration, method of Willis–Mollay as described by Ueno and Gonçalves (1998) and sedimentation method Hapich and Boray (1969). To determine nematode indistinguishable from eggs we culture larvae by the method Roberts and O'Sullivan (1950). For the estimation of the parasite load we used nematode eggs counts (EPG) by the Mc Master method as described by Whitlock (1948) and Thienpont *et al.* (1986). The recognition of the adult forms of endoparasites was made from *post mortem* examination by necropsy in one animal recently dead at Arerunguá. The samples were stored in formol at 10% (Soulsby 1987).

The collection of ectoparasites were carried out by inspection and examining the skin of dead animals (4) in both studied populations, and in Los Ajos population in twelve (6 females and 6 males) alive adults animals captured and eartagged (González and Duarte 2003).

Statistical analyses

We calculated in each annual study the EPG average and dispersal values for each population. The EPG values were transformed to the logarithmic scale to compare the main variables: EPG related with the spatial location (the paddock), and season collected, to conduct an Analysis of Variance (ANOVA) considering a significant value of 0.05 using StatSoft, Inc. (1999).

RESULTS

Pampas deer gastrointestinal parasites diversity

We found in the feces, in both Pampas deer populations, 10 genera of Helminth belonging to: *Trichuris*, *Capillaria*, *Strongyloides*, *Fasciola*, *Paramphistomum*, *Moniezia*, *Haemonchus*, *Ostertagia*, *Trichostrongylus* and *Oesophagostomum*. Additionally we recorded Coccidias ooquiste. The *Moniezia* genus was diagnosed by visualization of the proglottids at juvenile category and the morphology of the eggs.

In the coprocultures we found the larvae stages of gastrointestinal nematodes of short, medium and long tail, corresponding to the genera *Ostertagia*, *Trichostrongylus*, *Haemonchus* and *Oesophagostomum*.

In the field we have the opportunity to find an animal recently dead from Arerunguá population. We performed a necropsy and we diagnosed according to microscopic morphological characteristics in the abomasum males and females of *Haemonchus* spp., males of *Ostertagia* spp. and immature stages. In the small intestine we found *Trichostrongylus* spp. In the abomasal mucosa we observed nodular lesions compatible with those produced by immature forms of nematodes. Additionally the histopathological study showed a granulomatous gastritis, the presence of parasitic granuloma and we also observed cross sections of larvae in the submucosa.

Parasites load in Arerunguá population

In total we analyzed 829 fecal samples. The gastrointestinal eggs nematode parasite load values varied from minimum to 0 to 2000 EPG. From these range 74% of the sample represent less to 100, 11% from 100 to 200, 6% from 200 of 300 and the remaining 9% among 300 to 2000.

We conducted an annual study during 1995 in Arerunguá population analyzing 216 samples. We compared the EPG values obtained in feces that were collected seasonally during the months of January, May, August and December, in different paddocks ("Tala", "Bañado" and "Perado") of a ranch (Fig. 1). The geometric mean general elimination of gastrointestinal nematode eggs was 115 EPG.

The ANOVA showed that the parasites load is highly significantly correlated with the season of the year ($F = 7.854$; $P = 0.0001$), and also with the spatial location in the ranch (paddock) ($F = 5.780$; $P = 0.0036$) and the interactions of both variables month-paddock ($F = 2.953$; $P = 0.0087$). The significant EPG values were recorder in the month of August (winter in the Southern Hemisphere). In Uruguay winter is severe cold and humid ranging from 4 to 15 Celsius degree and 260 mL, (Dirección Nacional de Meteorología, 2010) promoting the environmental conditions to develop and increase the parasite load (Fig. 1).

Parasites load Los Ajos population

In total we analyzed 233 fecal samples. The gastrointestinal eggs nematode parasite load values varied from minimum to 0 to 3600 EPG. We conducted an annual study in Los Ajos population, using 33 samples during 2003-2004. The samples were obtained from 5 captured animals and ear tagged that were survey along a year. We compared the EPG values obtained from feces counting collected seasonally during the months of October, January, May and June, in a large paddock (800 ha; Fig. 2). In spite of the small sample size some trends were possible to be analyzed, surveying the tagged animal around a year. The EPG values ranges were from 50 to 400 EPG, being the average 84.84. The ANOVA results showed a significant value of the variable season with the EPG value ($F = 3.347694$; $P = 0.032560$). The maximum EPG values obtained in October (Spring) the season with high humidity and precipitation values in Los Ajos location.

Pampas deer ectoparasites

The ectoparasite *Lipoptena* sp. (Diptera: Hippoboscidae) was found in recently dead animals and in all the alive animals examined (12). This ectoparasite was previously reported in pampas deer individuals from Uruguay (Jackson and Langguth 1992).

DISCUSSION

Interaction of pampas deer parasite fauna and livestock

The genera of helminthes identified in the pampas deer being shared in the domestic ruminants' species and none were exclusively for *Cervidae* species. Similar findings were reported for pampas deer individuals analyzed from Brazilian population from Pantanal (Nascimento *et al* 2000) and also observed in Hawaii in axis (*Cervus axis*) in which the helminth communities found seem derived from cattle (Mc Kenzie and Davidson 1989). Additionally, McGhee and Nettles (1981) studies also suggested that cross-transmission of *Haemonchus contortus* occurs between deer species and domestic animals.

Moreover in the whitetail deer the gastrointestinal parasites and ectoparasites recorded were the same species as in the cattle (Prestwood *et al.* 1975; Pursglove *et al.* 1976; Cook *et al.* 1979; Davidson *et al.* 1980; Richardson and Demarais 1992; Romero Castañón *et al.* 2008). However in a survey performed by Prestwood *et al.* (1976) on whitetail deer that shared the pastures with sheep, of the 11 parasites found, only 5 species (45.4%) also were found in sheep. Those findings strongly suggested that the parasite faunas of deer and sheep are quite distinct, and sharing only 45% of the genera parasitic would unlikely to be a reservoir of common parasites in sheep in the southern United States (Prestwood *et al.* 1976).

However in our analyzed pampas deer sample the parasite genera found are usually reported in the Uruguayan livestock. Furthermore, the main genera of nematodes in domestic species in Uruguay are in cattle: *Cooperia*, *Ostertagia*, *Haemonchus* and *Trichostrongylus*, and in sheep: *Haemonchus*, *Trichostrongylus* and *Ostertagia* (Nari and Fiel 1994).

Moreover in Uruguay the domestic ruminants as sheep and cattle, are the main hosts of the parasites species *F. hepatica* and *Paramphistomum* (Nari and Fiel 1994). In the case of the wild species can be infected because share the paddock with livestock and may act as a reservoir for the disease. Tietz *et al.* (2007) recorded in other neotropical deer species as, the gray brocket deer (*Mazama gouazoubira*) the genera *Paramphistomum* in Brazil. The finding of eggs of *F. hepatica* and *Paramphistomum* in Arerunguá and Los Ajos populations respectively, were correlated with the environmental and topographical features that enable the development of the intermediate host, and where it meets the requirements for the parasite habitat.

We also confirmed the presence of infective larvae of *Haemonchus*, *Ostertagia* and *Trichostrongylus* by finding parasitic adults stages at the necropsy. According to the study performed by Nascimento *et al.* (2000) regarding the natural infection in neotropical deer species in the states of Mato Grosso do Sul and Sao Paulo, they found in pampas deer the following nematodes species *Haemonchus*, *Trichostrongylus* and *Cooperia*, with a wide range of variation in the intensity of infection and with high percentages of immature forms.

Besides the finding that 9% of all samples analyzed from Arerunguá were the high EPG values with a range between 400 and 2000, is consistent with that reported also in domestic ruminants where 5 to 10% carry the majority of the population of nematodes in Uruguay (Nari and Fiel 1994).

The infections of helminthes in pampas deer samples were almost always expressed in low abundance, mean intensity and also are characterized by several species. According to these observations, Nascimento *et al.* (2000) reported that all the deer were infected with one or several species of *Trichostrongyloidea*, and most of the analyzed cases exhibited low parasite load values. Similar finding were also reported by Ditchkoff, *et al* (2005) for the white-tailed deer, correlating the allelic composition of the MHC-DRB to resistance to one class of parasite, either abomasal nematodes or ectoparasitic ticks. Based on previous report the existence of several species and low EPG values which may be interpret to subclinical and or immunizing infections. The minor and progressive infections in wild animals would stimulate the immune process and thus would make them more resistant to parasites than domestic animals.

Conservation and management implications

The pampas deer currently is an endangered species in Uruguay and agro-ecosystems (ranches) play an important role in conservation management. On these ranches where pampas deer co-inhabit with livestock it is essential to regulate the stocking and carrying capacity of the wild species, and avoid overcrowding conditions that will promote the parasites infections and affect the livestock and pampas deer health.

Moreover, once we have diagnosed the same genera of helminthes in domestic ruminants, are required to take into account and balance the carrying capacities of the field paddock, and in particular perform periodic surveys of the livestock fecal parasite load that will be helpful to maintain under control with treatment (Kelly *et al.* 2010; Woodgate and Besier, 2010).

Specifically we recommend reducing stock densities on ranches in Arerunguá that have large sheep numbers, and leaving some unstocked paddocks to separate the pampas deer from the domestic stock. Chemical control of parasite numbers and regulating livestock stocking rates will help to reduce the exchange of parasites between the livestock and the pampas deer.

Finally we conclude that the structure of parasite communities in the pampas deer is correlated to the environment and the niche shared with the livestock. In winter, for example, management practices should be designed to avoid stress conditions that favor the increase of the parasite load and cross infections in the agro-ecosystem.

ACKNOWLEDGEMENTS

Our study was funded by: CSIC-UdelaR; PEDECIBA, MGAP from Uruguay, Wildlife Trust Alliance, Whitley Award Fund For Nature. Special thanks to landowners families Arrarte, Castro, and Constantín, for authorizing access to field sites. The authors are grateful to Prof. Luis Lavarello and Luis Salvarrey for statically assistance. We are also very grateful to the critical comments of two anonymous Referees and the Editor that greatly improves de quality of the manuscript.

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Figure 1. The geometric mean EPG values of gastrointestinal nematodes per paddock (▼Tala, ◆ Bañado, ▲ Perado) and season in Arerunguá population.

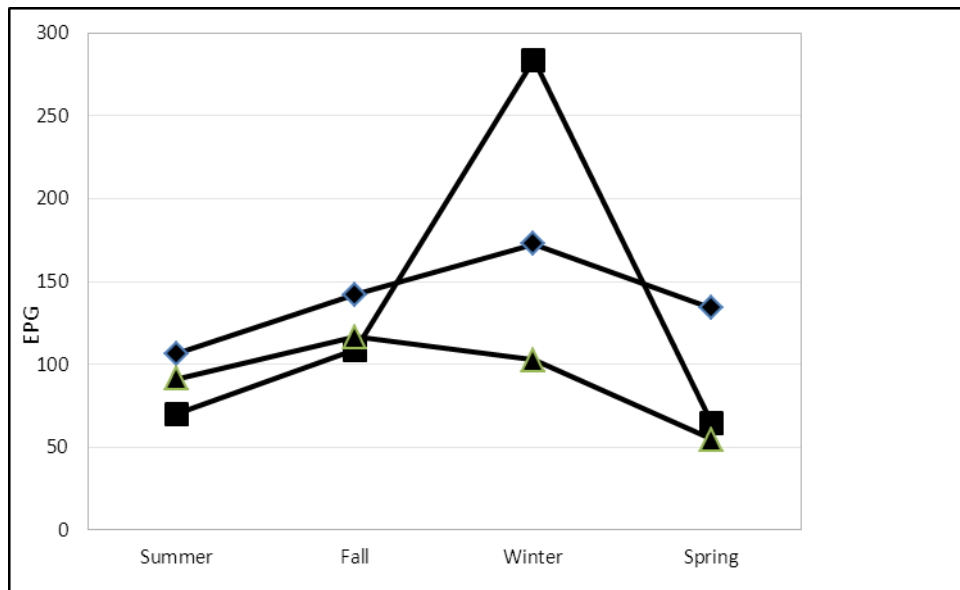
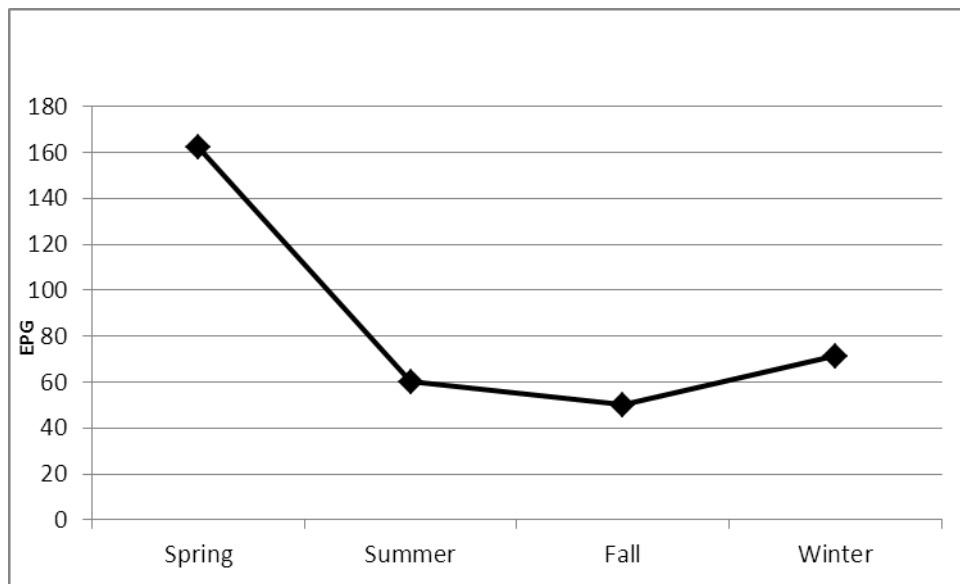


Figure 2. The geometric mean EPG values of gastrointestinal nematodes per season in Los Ajos population.



HYPODERMOSIS IN PANNONIAN RED AND FALLOW DEER POPULATIONS

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ABSTRACT

Hypodermosis in European Cervidae is of high economic importance in regard of the losses for leather processing. The hides of deer shot during autumn-winter is full of the warble-holes, while the summer hides have reduced value because of the cicatrized patches. Authors examined the hypodermosis of red deer (*Cervus elaphus* L.) and fallow deer (*Dama dama* L.) in Pannonia (Western Hungary) during two consequent hunting seasons (October-February). The overall prevalence (68.6%), as well as the mean intensity (85.5 larvae/host), were relatively high, and hypodermosis has a general distribution. The prevalence in stags was 100% and in yearling hinds 97.4%, respectively. In the majority of the infected adult hinds a large number of dead larvae were present, what should be the result of acquired immunity induced by the developing larvae in previous season(s). The lower prevalence in calves is related to the time of birth (early May), thus about half of them or more is missing the time of oviposition. Hypodermosis is less common in fallow deer that seems to be an unsuitable host for *Hypoderma* larvae. There was no infected fawn, as they were born in early June only, when the oviposition season is over. For the purpose of the industrial quality deer leather only hides of calves/fawns are useful after careful inspection. Peroral chemotherapy against hypodermosis is contraindicated in terms of venison-contamination, ecological-nature conservational, and other problems.

Additional keywords: red deer *Cervus elaphus* L., fallow deer *Dama dama* L., hypodermosis, prevalence, intensity, Western-Hungary

INTRODUCTION

Larvae of skin bot flies or grubs (Hypodermatidae) are causing subcutaneous myiasis with serious economic losses in many domestic (Bovidae) and wild (Cervidae) Eurasian ruminants. In European cervids three *Hypoderma* species occur. *H. actaeon* (Brauer 1858) is known to be a stenoxen parasite of red deer (*Cervus elaphus* L.); in contrast *H. diana* (Brauer 1858) may occur in all cervid species, however its main host is the roe deer (*Capreolus capreolus* L.), meanwhile *Hypoderma* (*Oedemagena*) *tarandi* is coexisting with reindeer (*Rangifer rangifer* L.) and only exceptionally developing in other deer species according to Grunin (1965) and Zumpt (1965).

Before detailing our study it is necessary to overview the biology of the warble flies briefly (see Grunin 1965). Adult females produce about 500 eggs, what they put on the deer trunk and leg hairs in strings usually. The swarming period is known to happen in April for *H. diana* and May for *H. actaeon*. Due to this difference in the chronobiology there is about one month delay in all phases of *H. actaeon*'s development, as compared to *H. diana*. After the 2-3 days long incubation period the 0.6-0.8 mm long first stage larvae (L1) penetrate the skin and start their migration lasting for about six months. In October-November they will settle in the subcutaneous connective tissue of the dorso-lateral parts of the back including the shoulder and hindquarters. Settling down there each specimen makes a breathing aperture (2-3 mm in diameter) on the skin needing access to the open air after the first moult. Settled larva lying in the "nest" stays here for about 4 ½-5 months starting as second stage larvae (L2). At the half they moult again turning to third stage (L3). The latter ones are yellowish then turning to be darker continually from light grey to completely black in colour. Forming similar to 'a glove of garlic' their final size is around 22-25 mm x 8-10 mm. In the period between late February and late April mature black larvae leave the host through the breathing aperture, fell to the ground and crawl to a suitable place to pupate. After the 2-4 weeks long pupa period hatched imagos meet for mating then females search for host animals. They are ephemers with a life span of 7-10 days flying even several hundreds of kilometres.

According to the chronobiology of warble development in relation to the deer hunting seasons in Hungary – red deer hinds and calves from 1 September, fallow does and fawns from 1 October to 28-29 February – the pathological/economic consequences are the followings. Most of the hind hides are

useless for leather production because of the high number of holes or reduced in quality due to the blotches or tears resulted by the cicatrized reparation of the warble breathing apertures.

Here we report the findings of examining *Hypoderma* larvae occurrence in Pannonian (W.-Hungarian) red deer and fallow deer (*Dama dama* L.) populations. Extensive studies on deer hypodermosis have been conducted in central Europe (Brauer 1863; Sugár 1976) and more recently in Spain (Martinez *et al.* 1990; Perez *et al.* 1995; San Miguel *et al.* 2001).

MATERIALS AND METHODS

Pannonia (Western Hungary) is dominated by hill-country between 150-700 m a.s.l., with two major flatlands, one in the NW corner and another in the central part on the East. However, roe deer is more numerous and generally dispersed everywhere, red deer also shows an overall distribution in the area, but it usually concentrated in and around the large forest blocks. Fallow deer is common in two regions of the South, but there are some populations in the North as well. Moufflon (*Ovis ammon musimon*) has some island-like populations in the higher mountains, meanwhile wild boar (*Sus scrofa ferus* L.) is common everywhere.

Carcasses were examined in Öreglak Venison Processing Plant Ltd. during two consequent hunting seasons, September-February 2007-08 and 2008-09, because most of the deer shot in Pannonia is processed there.

During carcass inspection it was possible to classify the host specimens like calf/fawn, yearling adult hind/doe and stag/buck according to the body size and shape, and the situation of the radius-ulna and tibia-fibula epiphysis ossification.

After skinning, the surface of the carcasses, as well as the inner surface of the hides, were examined. All living *Hypoderma* larvae were collected and taken into polyethylene-bags for laboratory investigation.

The localization of larvae in relation to body parts as well as the number and status (stage, estimated time of mortality) of the dead larvae were registered, in some cases documented by digital images.

In the laboratory larvae collected from the individual carcasses/hides were classified and separated according to the larval stages such as L₁, L₂, L₃. and mature L₃. The species of the larva specimens were identified using the characteristics described and illustrated by Grunin (1965), Zumpt (1965), Sugár (1976), and Papp & Szappanos (1992). Larva specimens were put into 40 % ethanol solution for the more precise morphologic examination and for documentation.

Data were taken into Microsoft Excel tables, then the prevalence (percentage of infected hosts) and mean intensity (number of larvae/infected hosts) values were calculated separately for the two hunting seasons, deer age and sex, and in total, respectively according to the definitions by Margolis *et al.* (1982). To calculate intensity live larvae were considered only. Altogether data of 159 red deer and 183 fallow deer inspected in the two hunting seasons were analysed.

RESULTS

Red deer

Characteristic data of red deer investigations are presented in Table 1. The overall prevalence is 68.6%. There is a little difference between the two season's prevalence values, except calves, what is mainly resulted by the different ratios of infected adult hinds and calves in the two seasons. Age or sex groups did not differ significantly. Combined data of the two seasons show that two-third of the adult hinds and less than half of the calves had *Hypoderma* larvae. The prevalence in yearling hinds and stags is extremely high, and there was only one yearling free of larvae among 39 individuals. It is important to notice that in 9/36 (25%) positive adult hinds exclusively dead larvae were present, 20 (55.6%) had dead larva specimens beside alive ones and 7 (19.4%) animals harboured alive larvae only.

Concerning mean intensity there is an important difference between the two seasons. Looking at the different age and sex groups the intensity in adult hinds seems to be constant. In contrast, the main intensity values of the two seasons differ strongly influenced by the yearling hinds' as well as calves' infection rate and intensity values. The probable explanation of the increased intensities in calves and yearling hinds as well as the increased prevalence in calves in the 2nd season is that higher ratios of the

Table 1. Prevalence (P%) and intensity (In) of red deer hypodermosis

P, number of positive animals; N, number of examined animals

Season	All animals		Hinds		Stags		1.5-year-old hinds		Calves	
	P/N	P% (In)	P/N	P% (In)	P/N	P% (In)	P/N	P% (In)	P/N	P% (In)
2007-08	37/60	61.7 (39.2)	13/24	54.2 (27.8)	-	-	21/22	95.5 (49.6)	3/14	21.4 (16.0)
2008-09	72/99	72.7 (120.5)	23/30	76.7 (27.1)	13/13	100 (256.8)	17/17	100 (165.5)	19/39	48.7 (57.5)
Total	109/159	68.6 (85.5)	36/54	66.7 (27.4)	13/13	100 (256.8)	38/39	97.4 (101.5)	22/53	41.5 (51.9)

Table 2. Prevalence (P%) and intensity (In) of fallow deer hypodermosis

P, number of positive animals; N, number of examined animals

Season	All animals			≥1.5-year-old (2)			Fawns		
	P/N	P%	In	P/N	P%	In	P/N	P%	In
2007-08	4/84	4.8	20.2	4/51	7.8	20.2	0/33	0	0
2008-09	25/99	25.3	7.9	25/77	32.5	7.9	0/22	0	0
Total	29/183	15.8	9.6	29/128	22.7	9.6	0/55	0	0

animals were examined later in the season (December – February), when (a) higher proportion of larvae settled under the skin, and (b) *H. actaeon* larvae arrived here, too (delayed phenology by one month, see above). The highest value (256.8 larvae/host) was found in stags followed by yearling hinds (101.5).

Fallow deer

Data of fallow-deer examinations are shown in Table 2. The combined prevalence is low (15.8%) and there is a strong difference between the two seasons. It is obviously resulted by the difference values found in deer older than 1.5 year, because all fawns were free of larvae.

Mean intensity is also low (9.6 larvae/host). However the single season values are much higher in 2007-2008 in contrast to the prevalence values of the two seasons. It is worth to comment that in an important proportion (20/29) of the infected fallow deer only dead larvae, seemingly young L2 specimens, were found. Although the identification of the larvae collected is not yet completed, in red deer both *H. actaeon* and *H. diana* seems to be quite prevalent according to the strong morphological characteristics in the posterior peritremes of L3 larvae. In contrary, in fallow deer L3 larva was not present, and the differentiation of L1 and L2 larvae is more difficult morphologically, if possible at all, like in the case of *H. bovis* and *H. lineatum* (see Balkaya *et al.* 2010).

DISCUSSION AND CONCLUSIONS

The results of this investigation confirm the previous observations of Sugár (1976) and Sugár *et al.* (2001) for Hungary in regard of the high prevalence of both *H. actaeon* and *H. diana* in Pannonian red deer. Surprisingly, Perez *et al.* (1995) and San Miguel *et al.* (2001) in Southern Spain found only *H. actaeon* in contrast to the previous detection of *H. diana* exclusively (Martinez *et al.* 1990) in similar southern areas of Spain. The prevalence as well as intensity values were high in stags and yearling hinds. The lower values in adult hinds are indicating acquired immunity, what is well documented in

cattle (Baron and Colwell 1991; Otranto 2001). On the other hand, the lower prevalence in calves is related to their birth time (early May), therefore about half of them will miss the oviposition by adult flies.

Hypodermosis is less common in fallow deer, this species seems to be an unsuitable host for *Hypoderma* larvae. There was no infected fawn, as they born in early June only (one month later than the red deer calves), when the oviposition season is over.

On the basis of the findings the occurrence of *H. actaeon* and *H. diana* in Pannonia seems to be quite frequent and general. For purpose of the industrial quality deer leather only hides of calves/fawns are useful after careful inspection. Peroral chemotherapy against hypodermosis is contraindicated in terms of venison-contamination, ecological - nature conservational (Ridsdill-Smith 1988), and other problems.

ACKNOWLEDGEMENTS

Authors would like to acknowledge the help of the team in Öreglak Venison Processing Plant Ltd., especially for Géza Vörös, Ferenc Takács, János Berta, György Nyers, Roland Tóth and our vet colleagues, dr. János Geszti and dr. Zoltán Tóth.

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SURGICAL AMPUTATION OF FORELEG IN SOUTH ANDEAN DEER (HUEMUL, *HIPPOCAMELUS BISULCUS*) IN COYHAIQUE, CHILE

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ABSTRACT

Huemul (*Hippocamelus bisulcus*) is the southernmost neotropical deer. In Chile, two isolated populations are found: one in Nevados de Chillan (Bio-Bio Region), and the other southernmost population in Aysen and Magallanes Regions. Different, past and recent factors, like habitat loss and fragmentation, poaching, cattle diseases, and domestic and feral dogs attacks have driven huemul into the category of an endangered species.

Frequently, a government institution, the Agriculture and Livestock Service in Aysen Region, offers veterinarian attention to huemul individuals found with a variety of injuries of different origins. The first clinic case of trauma surgery on a juvenile male, found in the wild with an exposed fracture of the distal radius growth plate (Salter-Harris type I fracture) with concurrent fracture of the distal epiphysis of ulna, is presented in this work.

Anesthesia, reduction of articular loosening and surgical technique protocols used in two surgeries are described. With these actions it was attempted, firstly, to reduce the fracture and afterwards saving the individual's life by amputating the foreleg. The excellent recovery state of the individual in semi captivity conditions are reported, as well as the possibility of its function as a reproductive male.

This surgery represents a landmark amongst other medical procedures conducted on this species. Hence the importance of this work is presented to the scientific community, veterinarians and the institutions and people devoted to the species' conservation.

INTRODUCTION

Hippocamelus bisulcus (Patagonian huemul or simply huemul) is a neotropical deer with the southernmost distribution in the Southern Cone of South America, in Argentina and Chile. In both countries the species is considered endangered throughout its distribution range. In Chile, two isolated populations are found: one in Nevados de Chillan (Bio-Bio Region), and the other southernmost population in Aysen and Magallanes Regions. Several factors have determined this conservation state, such as: illegal poaching, fragmentation and habitat loss, disease and parasite transmission from domestic cattle (González *et al.* 2009), wild dogs predation and attacks, and highway accidents.

The Agricultural and Livestock Service (SAG for its acronym in Spanish), in the Aysen Region of Chile frequently handles calls regarding huemul found in poor conditions. The reported cases are mainly offspring and juveniles that have lost their mothers, also specimens that enter enclosed areas without being able to find their way out, and individuals harassed and bitten by dogs.

Here we report on the rescue of a juvenile huemul male found in Coyhaique, Aysen Region, which was conducted in a joint effort between SAG, a private veterinarian, and the Fauna Andina Los Canelos Foundation; on its trauma treatment with an ultimately surgical amputation of left forelimb; and on its recovery and its current state.

CASE STUDY

On 16 February 2009, SAG received notice from the National Forestry Service (CONAF) of the finding of a huemul suffering a fracture, on an estate near Monreal Lake in Coyhaique Province, Aysen Region. Staff from SAG alongside CONAF personnel organized the search of the specimen and its relocation to Coyhaique on 17 February 2009.

Diagnosis

On 17 February, diagnosis and intervention were conducted. An X-Ray exam, under anesthesia (2.5 mg/kg ketamine, 3 mg/kg xylazine intravenously), revealed an open fracture injury of the distal radius growth plate (Salter-Harris type I fracture) (Figure 1), and a concomitant fracture of the distal epiphysis of the ulna bone. An immediate intervention was decided by proceeding with the reduction and fixation of the fracture through two Kirchner nails 3 mm in diameter.

Treatment

The main goal was to reduce the probability of losing the member, despite the fact that a change in direction of the bone axis took place some time ago, probably due to a previous fracture and deterioration suffered by an exposed broken bone. As a postsurgical treatment, an Amoxicillin-Clavulamic acid dose of 11mg/kg every 12 h was used. To reduce the pain, the analgesic Ketoprofen in a dose of 1 mg/kg every 24 h was also administered. Three days after the trauma treatment, the bones came out of place again, which may have been caused by: (a) the change in the normal alignment, provoked by a previous fracture that produced new forces which the fracture reduction could not resist; and (b) the direction of forces acting when the animal changed from a resting to a standing position. After the failure of the fracture reduction, a difficult decision had to be made, either losing the specimen or deciding to amputate a bone structure with structural failure and periosteal damage, with the obvious possibility of osteomyelitis caused by the time since the fracture was exposed to the environment conditions in the field.

Expectancy

If the treatment was to be successful, it was expected that the individual could survive in conditions of semi-confinement provided with adequate environmental conditions and proper care.

Amputation

An amputation through a scapulo humeral disarticulation was chosen as a sound alternative. This area was selected to avoid unnecessary weight of a longer and heavier stump. The skin was incised all around the limb in the upper third of the humerus. Then muscle, vascular and nerve structures were cut, and using a periosteal elevator, tendons were released to reach the joint and the joint capsule to remove the humerus. The surgery was finished performing a tobacco-pouch suture around the muscles to reduce dead space, then subcutaneous tissue and skin were sutured routinely.

Relocation to a safer place

To improve the chances of a good recovery, the individual was transferred to a location with better conditions of topography, climate and protection from predator attack. On 9 March 2009, 21 days since capture, aerial transportation to the recovery location was conducted with the support of Fauna Andina Los Canelos Foundation. The animal was sedated and transported in a wooden crate.

Post-operation evolution and rehabilitation

One year and five months after the surgery, the following features of the huemul buck are highlighted: an improvement in the quality of fur and weight gain were noted; the individual showed a very satisfactory general condition and continued to adapt to its new environment, walking and grazing without major problem. Because of these results, the possibility of the individual being used for breeding is considered viable by the involved institutions (Fig. 2).

DISCUSSION

Despite several surgeries reported in different species of *Artiodactyla*, this is a pioneer intervention on *H. bisulcus* which opens important opportunities for the treatment of severely damaged huemul specimens.

This case report demonstrates that, provided timely rescue action, proper diagnosis and adequate surgical procedures are conducted, a huemul damaged in the wild can be recovered in a rehabilitation centre offering the opportunity of extending the animal's life span after a serious accident, trauma or injury, which otherwise could have resulted in the death of the individual. Our decision to amputate is consistent with other reported surgeries in cervids, camelids and bovids (Butt *et al.* 2001; Kreycik *et al.* 2005; Newman and Anderson 2009; Quessada 1993) who chose amputation as a humane alternative to euthanasia. The use of a xylazine/ketamine protocol to anesthetize *H. bisulcus* seems to be as effective as in *Cervus elaphus* (Quessada 1993).

In contrast, the huemul buck performed well during 20 days of hospitalization. Moreover, it shows that huemul can tolerate prolonged periods of recumbency during recovery after surgery and are able to ambulate on three legs. As noted for South American camelids (Newman and Anderson 2009), this huemul buck made an excellent orthopaedic patient.

CONCLUSION

The Patagonian huemul can be treated surgically in major operations, adopting stringent antiseptic measures, stress control, monitoring of respiratory and circulatory functions and proper monitoring of the rumination during anesthesia and recovery. Anesthesia can be accomplished with wide safety margins. Recovered animals, well adapted to their new conditions of life, despite having just three limbs can be an important asset for *ex situ* conservation and even reproduction efforts of this endangered species.

ACKNOWLEDGEMENTS

The authors thank Chilean Agriculture and Livestock Service, Patagonia Veterinarian Clinic and Patagonia Andina Los Canelos Foundation for their financial support. The authors also would like to acknowledge staff from SAG and CONAF park rangers for their field work support, and also the invaluable help of Sergio Herrera-Encina offering comments on previous versions of this paper.

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Figure 1. Exposed fracture of left forelimb in huemul.



Figure 2. Huemul individual recovered after left forelimb amputation.



AN OPTIMUM HABITAT MODEL FOR THE WHITE-TAILED DEER (*Odocoileus virginianus*) IN CENTRAL VERACRUZ, MEXICO

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ABSTRACT

The modeling of wildlife habitat availability for animal species has important implications for explaining the distribution of the organisms in the wild, effective management and conservation. In this study we evaluated an Optimum Habitat Model based on estimation of the Habitat Suitability Index (HSI), for the white-tailed deer (*Odocoileus virginianus*) in Central Veracruz, Mexico. We generated a GIS-based model from a combination of the main habitat requirements that influence the presence of white-tailed deer. Six variables were used for modeling the optimum habitat. With these data layers we calculated the HSI for deer in the study area. Two strip transects (500 x 2 m) were established in 18 localities, where all tracks and signs from white-tailed deer were recorded. The HSI estimated for the study area ranging from -0.667 to 0.905, where the lowest values were associated with poor habitat quality. Eighty six percent of total surface included intermediate and low habitat quality. We found 51.16% fecal groups in intermediate habitat quality. White-tailed deer frequently used oak forest, tropical deciduous forest and pastures. A Classification Tree Analysis indicated that temperature and aspect were the main habitat features influencing the white-tailed deer presence in the study area. Although there is high anthropogenic pressure in the zone, forest patches help to maintain some suitable habitat for small populations of this species. Conservation and restoration of the vegetation cover is necessary to promote deer populations recovery in Central Veracruz, Mexico.

Additional keywords: deer signs, habitat quality, oak forest, tropical deciduous forest, anthropogenic pressure.

INTRODUCTION

To improve habitat management effectiveness, evaluation of habitat is necessary to understand how environmental factors affect distribution, density and dynamics of wildlife populations. Actually there are many techniques and tools to analyze wildlife–habitat relationships (e.g. habitat suitability models, species-use analysis and pattern recognition); these tools may use information about existing vegetation conditions or land-cover databases (Felix *et al.* 2004). Also with these methods it is possible to predict the effects of environmental changes, by natural or anthropogenic causes, in the maintenance of the species (Delfín-Alfonso *et al.* 2009; Mandujano 1994; Vaughan 1994).

The white-tailed deer (*Odocoileus virginianus*) have gained substantial management attention throughout North America due to their overabundance (McShea *et al.* 1997), emerging diseases (O'Brien *et al.* 2002; Williams *et al.* 2002), and their importance as a natural and economic resource (Halls *et al.* 1984). In Mexico since prehispanic cultures to modern times the white-tailed deer has a high value as food, hunting, ornamental, curative and in ceremonial activities (Mandujano and Rico-Gray 1991). These actions have reduced their populations and in some places this specie is locally extinct (Villarreal 1999).

Several studies have proposed some abiotic and biotic habitat attributes as essentials for the maintenance of deer populations. Some of these attributes are: vegetation cover for escape, vegetation cover for weather protection, temperature, slope, aspect, water availability, patch size and other (Gallina 1994; Mandujano 1994; Rothley 2001).

The aim of this study was to evaluate an Optimum Habitat Model based on estimation of the Habitat Suitability Index ((U.S. Fish & Wildlife Service, 1991) to identify favorable areas for the white-tailed deer (*Odocoileus virginianus*) in Central Veracruz, Mexico.

METHODS

This work was carried out in central Veracruz, Mexico (19°15'–20°00'N, 96°15'–97°30'W), within an area of 5139 km². This area is characterized by altitudinal gradient ranging from sea level to 4200 m above sea level, allowing the presence of a great variety of vegetation types (Fig. 1).

We generated a GIS-based model from a combination of the main habitat requirements that influence the presence of white-tailed deer in the area, five environmental variables were used for modeling the optimum habitat. We used the following data layers: Mean annual temperature (<http://www.worldclim.org>), vegetation cover (National Forest Inventory, Series II, 2002) 1:250,000 scale, Digital Elevation Model (DEM) 1:50,000 scale (INEGI, 1999), from which we obtained the slope and aspect for the study area using ArcView GIS 3.2. (ESRI 1996). Finally, the information on rivers, roads and villages was obtained from the 1:250,000 scale vectorial map (INEGI 1994).

With these data we calculated the Attribute Importance Index (*AII*), its value ranging from 0 (low quality) to 1 (high quality). This value is obtained with the following formula (Delfin-Alfonso *et al.* 2009): $AII = I_v/n$ where I_v is the importance value of each category for an attribute and n is the number of categories in which an attribute was divided. The importance value was assigned considering the category relevance for the white-tailed deer, using bibliographic information (Table 1).

With the *AII* we calculated the habitat quality with the Habitat Suitability Index (HSI) for deer in the study area with the following formula (Delfin-Alfonso *et al.* 2009):

$$HSI = \left[\frac{IIA_1 + IIA_2 + IIA_3 + 2 IIA_4 + 2 IIA_5}{\sum IIA_n} \right] - pv$$

where IIA_1 = aspect, IIA_2 = slope, IIA_3 = water availability, IIA_4 = temperature, IIA_5 = vegetation type and pv = pressure value.

Table 1. Habitat attributes evaluated with their respective categories and values

Habitat attributes	Category	I_v	AII	Quality
Aspect	N, NO.NE	3	1	High
	Flat	2	0.67	Intermediate
	S, SE, SO, E, O	1	0.33	Low
Slope (°)	0-9°	3	1	High
	10-13°	2	0.67	Intermediate
	>14°	1	0.33	Low
Water sources	Perennial	3	1	High
	Intermittent	2	0.67	Intermediate
	Without	1	0.33	Low
Temperature (°C)	5 a 21 °C	3	1	High
	21 a 29 °C	2	0.67	Intermediate
	>29°C	1	0.33	Low
Vegetation	Tropical deciduous forest, tropical subdeciduous forest, desert shrubland.	5	1	High
	Pine forest, Fir forest, Oak forest, Juniper forest.	4	0.80	High
	Tropical rainforest, water bodies.	3	0.60	Intermediate
	Cloud forest	2	0.40	Low
	Agriculture, pastureland, high prairie.	1	0.20	Low
	Urban areas, mangroves, dunes, without vegetation.	0	0	Not applicable

The *pressure value* was calculated using information about roads and towns. The assessment on the effect of roads and human settlements in the distribution and habitat use of white-tailed deer and other cervids, have reported that the number of fecal groups increases with the distance from them, especially during the hunting season or places where hunting is common (Rost and Bailey, 1972; Vogel, 1989; Kilgo *et al.*, 1998; Manor and Saltz, 2005). This study established a gradient of impact where the risk for deer is a function of distance from its location to roads and human settlements, with the assumption that if the deer is very close to any of them, it is more likely to be detected by a human and therefore the level of risk will be higher. Since the differences in type of roads and human density represent a

different risk for the white-tailed deer, these were divided in categories according to their potential impact on the deer. For each category three risk buffers were established: high, medium and low, depending on its proximity to the roads or settlements (Table 2). Once the maps were generated for three levels of risk, each map was assigned a *pressure value* (*pv*), where the categories high, medium and low were replaced by values 1, 0.6 and 0.3 respectively. The areas outside the risk buffers and whose level of impact was null, a value of 0 was assigned. We overlap both maps (roads and human settlements) and for the new polygons produced we assigned the highest pressure value from the base maps.

Table 2. Risk buffers for the white-tailed deer related with roads and human population

Roads	High risk buffer (m)	Intermediate risk buffer (m)	Low risk buffer (m)
Trail, dirt road	100	200	300
Paved road, railroad	200	400	600
State highway, Federal highway.	300	600	900
Population (No. of inhabitants)			
< 15	300	600	900
16 - 150	500	1000	1500
151 - 1500	1000	2000	3000
1501 - 10000	2000	4000	6000
10001 - 100000	2500	5000	7500
100001 - 1000000	3000	6000	9000

The data of white-tailed deer presence in the study area was obtained from field surveys in two dry seasons (February and May 2008; February-March de 2009) and one wet season (August-September 2008). Two strip transects (500 x 2 m) were established in 18 localities, where all tracks and signs from white-tailed deer were recorded. These transects were located in the tropical deciduous forest, oak forest and rangelands.

We used a Tree Analysis to explain variation of a single response variable (white-tailed deer presence) by one or more explanatory variables (environmental variables and anthropogenic pressure). Due the response variable is categorical we apply a Classification Tree Analysis to identify the main environmental variables related with the white-tailed deer presence in the study area (Andersen *et al.* 2000). The tree is constructed by repeatedly splitting the data, defined by a simple rule based on a single explanatory variable. At each split the data is partitioned into two mutually exclusive groups, each of which is as homogeneous as possible. The splitting procedure is then applied to each group separately. The objective is to partition the response into homogeneous groups, but also to keep the tree reasonably small. The size of a tree equals the number of final groups (De'Ath and Fabricius 2000). Also we apply a Chi-squared test to know if there were significant differences in the deer presence between sites with different habitat quality and vegetation type. The Bonferroni Confidence Interval was used when we found these differences (Neu *et al.* 1974).

RESULTS

The HSI estimated for the study area was from -0.667 to 0.905, where the lowest values were associated with poor habitat quality. Eighty six percent of total surface (5167.71 km²) included intermediate and low quality habitat (4455.9 km²) and only 13.52% was high quality habitat (711.81 km²) (Fig. 2).

We recorded 203 faecal groups at 18 sites sampled, furthermore another 30 tracks were found (rubblings, bedsites and fingerprints). We found two times more tracks in the intermediate quality habitat than in high and low quality habitats. The Chi-squared test found significant differences in the use of habitat taking into account the quality ($\chi^2 = 9.82$, d.f. = 2, $\alpha = 0.05$). Considering the available surface of each type of habitat quality and applying the Bonferroni Confidence Interval, the low quality habitats were avoided by white-tailed deer. Intermediate and high quality habitats were used by the deer as the intervals predicted.

White-tailed deer frequently used oak forest (58.6% of the tracks), rangelands (23.6%) and tropical deciduous forest (17.7%). We found significant differences in the use of the vegetation types by the

deer ($\chi^2 = 8.97$, $\alpha = 0.05$). The oak forest was preferred, whereas the tropical deciduous forest and the rangelands were avoided, considering the results of the Bonferroni Confidence Interval.

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A Classification Tree Analysis indicated that temperature and aspect were the main habitat features influencing the white-tailed deer presence in the study area. When the temperature was higher than 28.15 °C and the aspect was W, NW or N the analysis predicted the deer presence. Other important habitat attributes were anthropogenic pressure and slope; we found that in localities with high anthropogenic pressure on the deer, refuge in sites with pronounced slope.

DISCUSSION

The analysis of the environmental variables that influence the presence of white-tailed deer in the study area, considered the temperature as the most important in explaining the occurrence of this species. Although temperatures between 20-25°C are usually considered as thermoneutral for most medium-large animals (Moen 1968), for white-tailed deer thermoneutral limit is 30°C. Above this temperature the deer extensive loss of water from evaporate cooling, could threatens their survival (Moen, 1973). Although the model predicts that with temperatures below to 28.15°C deer will be absent, this boundary is because most of our records were restricted to warm thermal zone in the study area, where temperatures range from the 22°C to $\geq 26^\circ\text{C}$. The absence of tracks for the temperate zone in the study area is because these animals were removed from the region due to excessive hunting and wood cutting in the middle of last century (*com. pers.* Gallina). So the model will have more accurate predictions in places where exist a better representation of the all environmental variables that satisfies the species requirements. Although we try to obtain more representative records of deer presence in the zone, most of them were located outside of the study area.

The aspect was also an important variable to predict the deer presence, mainly West, North West and North are preferred (Sánchez-Rojas *et al.* 1997; Mandujano *et al.* 2004), the latter two types of orientation are used preferentially by deer because having less sunshine have more favorable microclimatic conditions (Mandujano and Gallina, 1994) and retain more moisture due to the presence of trees with dense foliage (Mandujano *et al.* 2004).

The oak forest was the most used habitat by the white-tailed deer, the presence in this type of vegetation of *Quercus* sp. and *Leucaena leucocephala* as dominant species promotes it use due both species form a major part of the deer diet (Gallina *et al.* 1981). This type of vegetation has been associated with greater abundance of deer, especially when presented in rough terrain and has a good percentage of vertical vegetation cover, which allows protection from natural predators and humans (Ortiz-Martínez *et al.* 2005). In this study the oak forest was located in areas with a lower degree of anthropogenic pressure and pronounced slopes.

Although tropical deciduous forests have higher plant species richness, a high production of biomass (Arceo 1999) and species with high nutritional value (Silva-Villalobos *et al.* 1999) this type of vegetation was not used by the white-tailed deer as we expected. One factor that may have influenced the reduced use of this type of vegetation is the high anthropogenic pressure in the zone. A study carried out in a tropical deciduous forest in Puebla, Mexico showed a higher density of deer in areas with lower levels of human disturbance (López-Téllez *et al.* 2007), so possibly this could explain the low number of tracks found in this type of vegetation in this study.

Conservation and restoration of the vegetation cover is necessary to promote deer populations recovery in Central Veracruz, Mexico.

ACKNOWLEDGEMENTS

Support for this study was obtained from CONACYT (Project number 52925 “Situación actual de la distribución del venado cola blanca en el Estado de Veracruz” to Sonia Gallina and fellowship number 212500 to Beatriz Bolívar) and Veracruz State Government for support the project “Estudio poblacional del venado cola blanca en la Sierra Madre Oriental del Estado de Veracruz” (SEDESMA/UA/PDIA/008/06). We want thank Norma Corona for her help with the GIS and Octavio Rojas for his comments. We are grateful for the field help to: Antonio Vázquez, Luis Arturo Escobedo, Arturo González and Rolando González Trápaga.

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Figure 1. Study Area of Central Veracruz, Mexico, with classification of vegetation types.

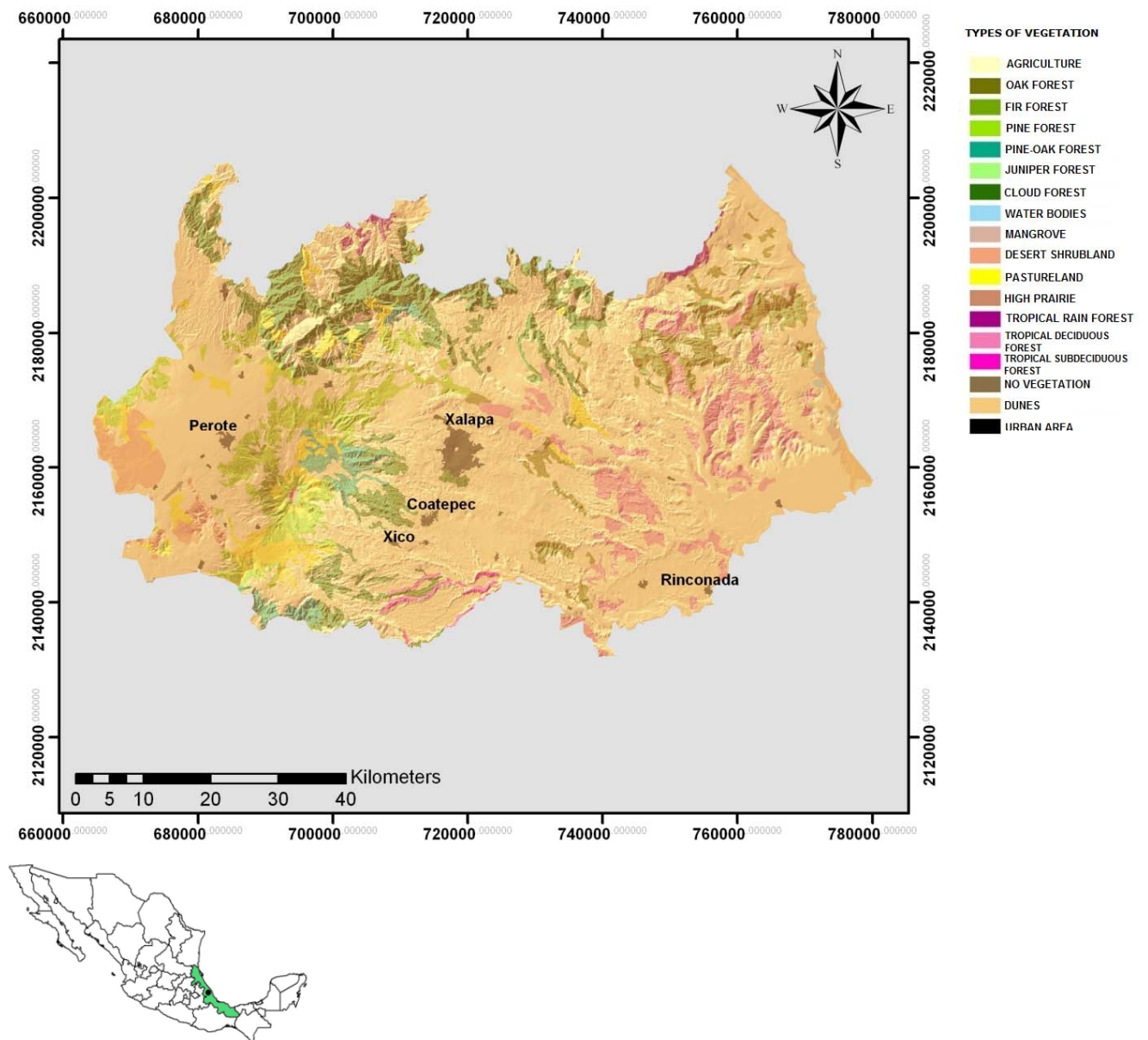
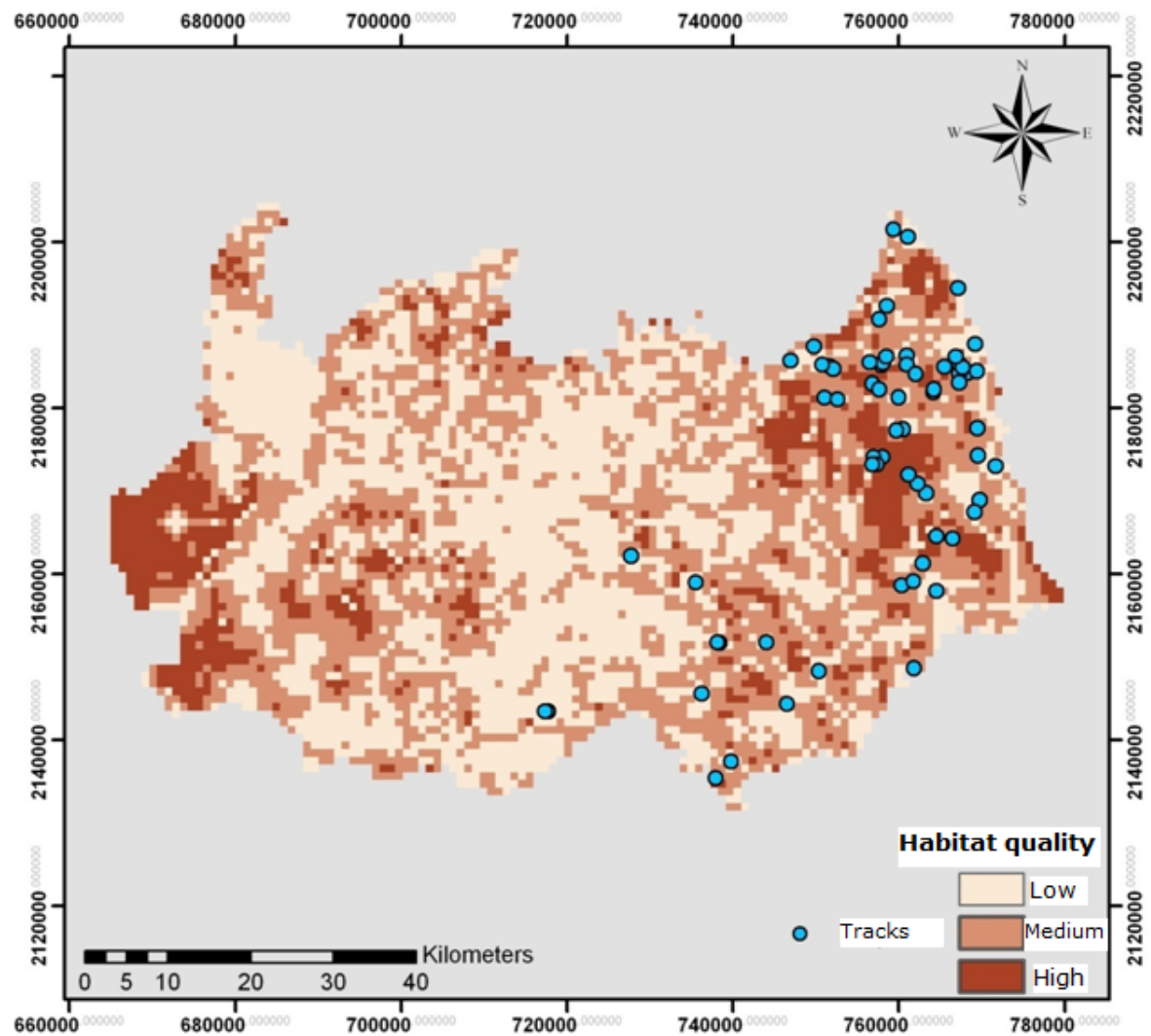


Figure 2. Deer Habitat Quality Categories in Central Veracruz, Mexico, according the Optimum Habitat Model. The blue points are the presence of deer.



PERIODONTAL DISEASE AND FLUORIDE BONE LEVELS IN TWO SEPARATE IBERIAN RED DEER POPULATIONS

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ABSTRACT

The mandibles of 209 red deer (*Cervus elaphus hispanicus*) from 6 months to 15 years old, shot between 2001-2002 in the Sierra de Andújar Natural Park ($n = 173$) and National Park of Doñana ($n = 36$) in southern Spain, were studied in order to assess spatial variations in the occurrence of periodontal disease and bone fluoride levels in two separate populations. Similarities were found in periodontal disease prevalence between the study areas (20.74% in Sierra de Andújar Natural Park and 33.3% in National Park of Doñana), and enamel defects or abnormal abrasion (7-10 %, respectively). There were also variations in fluoride levels according to the area and the occurrence of periodontal disease, irrespective of age. Fluoride content was significantly lower in animals from Sierra de Andújar (250.5 ± 158.9 mg F/kg ash) than in deer from Doñana (752.4 ± 451.0 mg F/kg ash), and the higher levels of bone fluoride occurred in mandibles showing periodontal disease in both areas (358.6 ± 201.7 mg F/kg and $1,224.9 \pm 422.34$ mg F/kg, respectively). The fluoride levels were lower than expected, typical of non-polluted areas, and there was no significant influence of the occurrence of enamel defects on the bone fluoride concentration. In these areas pathologically increased attrition, enamel discolouration or enamel surface lesions seem not to be caused by fluorosis, so that special care must also be taken in using macroscopic lesions instead of fluoride determination as an indicator for monitoring the magnitude of environmental contamination. Research into the global mineral content and possible deficiencies in certain minerals such as P and Ca interacting with fluoride is necessary in order to understand the high prevalence of periodontal disease in younger animals and the higher concentrations of fluoride in mandibles with periodontal disease.

Additional Keywords: *Cervus elaphus hispanicus*, Doñana, enamel defects, fluoride, monitoring pollution, periodontal disease, red deer, Andújar Natural Park.

INTRODUCTION

Periodontal disease is common in many wild, domestic and feral mammals and has been reported several times in free-living ungulates like Suidae (e.g. Samuel and Woodall 1988; Woodall 1989) or Cervidae (e.g. Geiger *et al.* 1992; Flueck and Smith-Flueck 2008). This disease is important because it may have consequences for the welfare, condition and longevity of animals, and is therefore a good indicator for monitoring herd welfare. Periodontal breakdown, periodontitis and the occurrence of marked periodontal disease have been reported in severely fluorotic wild red deer (*Cervus elaphus*) as a consequence of enamel defects and excessive tooth wear resulting from the toxic effect of fluoride (Kierdorf *et al.* 1996; Schultz *et al.* 1998). In the animals' environment fluorides can occur both naturally (in rock weathering, volcanic emissions, sedimentary formations that contain fluoride-bearing minerals derived from the parent rock, fluoride-rich clays, or fluorapatite) and because of human activities (e.g., phosphate rock mining and use, aluminium manufacturing, chemical fertilizers, drinking water fluoridation) (Ozsvath 2009). Elevated fluoride contents in the bodies of wild ruminants are mostly the consequence of feeding on contaminated plant material or the ingestion of soil and water that contain high concentrations of fluorides. Because of this, monitoring fluorine bioaccumulated in bone has been studied as an indicator of fluorine contamination in the natural environment (Machoy *et al.* 1991; Kierdorf *et al.* 1996; Kierdorf *et al.* 1997; Kierdorf and Kierdorf 2000b; Jelenko and Pokorny 2010).

In southern Spain there are several possible environmental sources of fluoride such as ceramic manufacturing and brick production, mining or the use of phosphate fertilizers. Previous studies have found high levels of tooth wear (Azorit *et al.* 2003) and severe dental disfigurement as well as the occurrence of enamel defects compatible with lesions described by Kierdorf *et al.* (1996) and Schultz *et al.* (1998) in fluorotic deer. However the occurrence of periodontal disease in red deer has still not been well documented. The purposes of this study were: (i) to describe for the first time the occurrence of periodontal disease in two separate deer populations of southern Spain, (ii) to determine bone fluoride levels in order to monitor possible environmental pollution, and (iii) to test variations of fluoride concentration in different areas and in different animals. We expect high levels of bone fluoride to coincide with the occurrence of enamel defects or unequal tooth wear, and consequently with periodontal disease.

MATERIALS AND METHODS

Animals

The mandibles of 209 red deer (*Cervus elaphus hispanicus*) shot between 2001-2002 in the Sierra de Andújar Natural Park ($n = 173$) and National Park of Doñana ($n = 36$) were studied. The ages were determined by a combination of tooth eruption and replacement patterns (Azorit *et al.* 2002), and using the stand counting of incremental lines described by Azorit *et al.* (2004). Ages ranged from 6 months to 15 years. Table 1 shows the origins and ages of the animals, classified in seven age classes.

Table 1. Age and origin of red deer (*Cervus elaphus hispanicus*) studied

Age class	Age in years	Study areas		Total
		1 (<i>Sierra de Andujar</i>)	2 (<i>Doñana</i>)	
1	< 1	7	2	9
2	1-2	15	3	18
3	2-3	14	5	19
4	3-5	35	9	44
5	5-8	45	8	53
6	8-12	27	6	33
7	> 12	30	3	33
Total		173	36	209

Periodontal disease characterization and enamel defects

The primary symptoms of periodontitis are tooth-supporting tissues loss ("attachment loss") and the formation of true gingival and/or bony pockets following mucogingival deformities and bad conditions around the teeth. But as all mandibles were boiled to clean off soft tissue before their storage and examined afterwards, we did not attempt to differentiate gingival or soft tissue lesions. We classified disease-positive specimens as those with chronic periodontal disease. Many changes in the periodontal disease classification system have been simply semantic, allowing for a more continuous organization

of conditions rather than a separation of periodontitis from other diseases or disorders also affecting the periodontium (Wiebe and Putnins 2000). In this study we follow a reduction of the Samuel and Woodall (1988) methods and descriptions of Wiebe *et al.* (2001).

Specimens having enamel defects in at least two cheek teeth other than M₁ showing altered appearance of the enamel were recorded as positive. The lesions ranged from demarcated to hypoplastic according to the DDE Index (FDI 1982) or showed pronounced abrasion of the enamel ridges on their occlusal surfaces, or pathologically increased wear leading to abnormal tooth shape, lesions scored as 3-5 in the DL Index (Kierdorf and Kierdorf 1999).

We did not grade the periodontal or dental lesions, just the presence or non-presence of affection.

Fluoride determination

Bone samples were obtained by drilling holes into the ventral side of the cleaned and dried mandibles and collecting the bone powder. This region of the jaw consists solely of compact cortical bone. Between 100 and 150 mg of bone powder per sample were dry-ashed over 5 h in a muffle furnace at 560°C. Precisely weighed samples (50 ± 0.01 mg) of bone ash were dissolved in 25 mL of 0.1 M perchloric acid, using closed plastic tubes and constant shaking, for about 24 hours. Then 5 mL of dissolved bone ash were neutralised with 5 mL of KOH 0.1 mol/L and finally buffered to 5.5 PH with a total ionic strength adjustment buffer (10 mL of TISAB II, Orion Research Beverly, MA, USA). Fluoride determination was performed with a fluoride ion-specific combination electrode (Orion, model 96-09) connected to an Orion 920A meter. The standard used for electrode calibration was prepared from a certified Orion standard containing NaF. Fluoride values given and used for calculation are arithmetic means of the results of two parallel analyses per bone ash sample. Concentration is expressed as milligrams per kilogram of bone ash (= ppm). The control of the analytical procedure was performed analysing a 0.6 ppm F patron solution, prepared with a certificated standard. This patron was analysed periodically and the incertitude calculated. The precision shows the random error and was calculated using the standard deviation of the patron result. In the same way, the exactitude shows the systematic error. The total incertitude of method using both parameters was about 5.29%.

Statistics

The statistical analysis was performed using SPSS Statistics 17.0. We considered levels of fluoride as a dependent variable. By applying the statistical method of multifactorial analysis of variance we assessed differences in the concentration of fluoride according to the factors of area, occurrence of enamel defects and occurrence of periodontal disease, using the factor age as a covariate. A previous data logarithmic transformation was realized in order to satisfy the normality (Shapiro-Wilk W test) and homoscedasticity (Levene's test) assumptions of the analysis of variance. All statistical tests were therefore performed on the transformed data. Once the statistically significant factors had been detected we performed a post hoc comparison analysis by using the Least Significant Difference (l.s.d.) method.

RESULTS AND DISCUSSION

Periodontal disease and enamel defects

Mandibles from both areas exhibited dental defects (Fig. 1) and periodontal disease characterized by lesions from alveolar bone loss and recession of alveolar crests to more severe changes with distortion or destruction of alveoli and exposure of tooth roots (Fig. 2). But severe osteomyelitis, actinomycosis like-lesions or "lumpy jaw" were not present in this survey. About 7% of the mandibles from Sierra de Andújar Natural Park and 10 % from Doñana exhibited permanent cheek teeth with enamel defects. There was also a slight difference of periodontal disease prevalence between study areas with 20.7% and 30.3% respectively, but not statistically significant. In both study areas deer older than 9 years had the highest occurrence of disease of more than 50%, but the youngest animals were also greatly affected. Prevalence values of 33.3% and 38.5% were recorded in deer of 5-8 years, and even 8% of deer 3-4 years old showed this disease.

It appears that the occurrence of periodontal disease was higher than expected and there was a high prevalence at young ages, especially when compared to similar studies such as Geiger *et al.* (1992), where no lesions were found in jaws of red deer 8 years or younger.

In both study areas there was widespread starvation due to extreme drought in 1995, coinciding with a very high population density and subsequent nutritional deficiencies, especially for the younger animals. A high prevalence of disease in deer born in this period (deer 5-6 years old in our study) could be explained with reference to the poor nutritional ecology they experienced as calves, which would predispose poor health as adults. On the other hand, all animals used in our study were selectively culled, meaning that their body condition would be worse than the rest of the population (the animals in the poorest condition are normally those culled), and they would also have a higher predisposition to disease.

The periodontal disease pathophysiology may vary between different species due to infectious, genetic, and/or age-related factors (Miles and Grigson 1990), and studies have provided evidence that hormones, heredity, and other host factors influence periodontal disease incidence and severity (Wactawski-Wende 2001). Soft and high-sucrose diets (Wiebe *et al.* 2001) and bacterial plaque appear to be the primary etiological factors that cause predisposition for the disease in primates and carnivores (Fagan 1980; Gual-Sill and Suárez de Gual 1996), whereas in ungulates the etiological factors reported for this disease have been molar progression, abnormal tooth wear, and abnormal separation between molars resulting in impaction of coarse food between teeth and gingival and root irritation (Alexander 1983; Gual-Sill and Suárez de Gual 1996). Systemic loss of bone density in osteoporosis, including that of the oral cavity, may provide a host system that is increasingly susceptible to infectious destruction of periodontal tissue, so that bone porosity may give an indication of susceptibility to periodontitis (Glickman 1979; Whalen and Krook 1996; Wactawski-Wende 2001). Poor environmental conditions during the animals' birth and growth are important factors of disease because of malnutrition, deficiency of selenium, vitamin C and specially Ca or P. Similarly to osteoporosis (Palmer 1993), periodontal disease might also be nutritional in origin due to nutrient imbalances like food scarcity, mineral and trace element deficiencies, and excessive resorption of alveolar bone.

Table 2. Analysis of variance for LOGFluoride - Type III Sums of Squares

<i>Source</i>	<i>Sum of squares</i>	<i>d.f.</i>	<i>Mean square</i>	<i>F-ratio</i>	<i>P-value</i>
Covariates					
Age in years	10.1498	1	10.1498	38.69	0.0000
Main effects					
A: Area	25.1722	1	25.1722	95.6	0.0000
B: Periodontal disease	3.81005	1	3.81005	14.52	0.0002
C: Enamel defects	0.056327	1	0.056327	0.21	0.6436
Residual	53.5158	204	0.262332		
Total (corrected)	108.709	208			

All *F*-ratios are based on the residual mean square error.

Bone fluoride concentration and its variations

Fluoride concentration was influenced by all the factors studied except for enamel defects (see Table 2 and Figure 3). There was a significant influence of the area and occurrence of periodontal disease on the bone fluoride concentration, independently of age. Fluoride content was significantly lower in animals from Sierra de Andújar (250.5 ± 158.9 mg F/kg ash) than in deer from Doñana (752.4 ± 451.0 mg F/kg ash) and the higher levels of bone fluoride occurred in mandibles showing periodontal disease in both areas, recording 358.6 ± 201.7 mg F/kg ash and $1,224.9 \pm 422.34$ mg F/kg ash, respectively (*F*-ratio 42.20, *P*-value 0.0000 and *F*-ratio 13.88, *P*-value 0.0014, post hoc comparison analysis by l.s.d. method).

Variations in the water composition between Doñana and Andujar Natural Park could be the reason for differences in fluoride levels because fluoride levels are usually controlled by the solubility of fluorite (CaF_2), and high concentrations of fluoride are often associated with soft, alkaline and calcium-deficient water (Ozsvath 2009). But higher fluoride levels were also found in animals with periodontal disease irrespective of age or area.

The deposition of fluoride in rachitic animals has been reported in two studies, both of which referred to elevated levels of fluoride (in Zinpkín *et al.* 1958). Differences in deposition of fluoride were found to be due to the rachitogenic diet (extremely limited in phosphorus) rather than to inanition. These animals had higher concentrations of fluoride but lower levels of phosphorus. Phosphorus deficit causes secondary nutritional hyperparathyroidism and subsequent jaw and maxillar damage, as well as predisposition to periodontal disease (Gual-Sill and Suárez de Gual 1996).

An elevated prevalence of periodontal disease could be related to a deficiency of phosphorous in deer that have grown in poor nutritional conditions or with a rachitogenic diet, and a greater deposition of fluoride in these animals may be due to an extreme limitation of phosphorus. But more studies on the effects of phosphorous deficiency are needed in order to verify this theory.

In relation to fluoride, the different levels found in our animals may be due to a C/P imbalance. It is similarly possible that differences in the composition of the bone mineral itself, the C/P ratio for instance, could play some part in skeletal fluoride deposition (Zipkin *et al.* 1958). Research into the global mineral content and possible deficiencies in certain minerals such as P and Ca interacting with fluoride is necessary to understand the highest concentrations of fluoride in mandibles with periodontal disease.

The fluoride levels were lower than expected, typical of non-polluted areas (Jelenko and Pokorny 2010) and healthy red deer (Kierdorf *et al.* 1995; Kierdorf *et al.* 1996; Richter *et al.* 2010). Also contrary to expectations there was no high fluoride level in specimens with presence of enamel defects. Between 1719 and 3411 mg F/kg ash, is the bone fluoride concentration in fluorotic deer (Richter *et al.* 2010), and only one specimen from Doñana was in this range (1960 mg F/kg ash).

Enamel defects, pathologically increased attrition, discolouration or occurrence of partial regression of the alveolar process similarly to that described by Kierdorf *et al.* (1996) and Schultz *et al.* (1998) in fluorotic deer, were found in our samples but are probably not solely related to fluoride levels, and these lesions could not be called fluorosis. Unequal tooth wear, with some teeth appearing young next to heavily worn M₁ and M₂ teeth, has been very frequent in our survey and maybe caused by mineral deficiencies of Ca or P rather than by high fluoride levels.

The diagnosis of fluorosis is a controversial one, and as enamel opacities can be caused by a variety of other factors (for instant rickets, caeliac disease, malnutrition, high altitude and premature birth) preventing the misclassification of defects is not easy even in human dentition (Browne *et al.* 2005). Therefore, special care must also be taken in using macroscopic lesions instead of fluoride determination as an indicator for monitoring the magnitude of environmental contamination or fluorosis.

ACKNOWLEDGMENTS

This study was supported by the P07-RNM-03087 (Consejería de Ciencia e Innovación de la Junta de Andalucía) and CGL-2008-00832 (Ministerio de Educación y Ciencia) projects and was approved by the Bioethical Committee of University of Jaén. We thank Muñoz-Cobo J, Cubillas E and F, Castilla M, Barato V, Reina P, Sánchez L, Gutierrez R, Salas J, Guerrero F, Novoa P, García R, Cuenca E, Muñoz LM, García G, and Portela C, for their helpful comments, collecting deer samples and field or laboratory assistance. Also to Araque G, Deutor B, Oya R and all CICT, University of Jaén, Spain.

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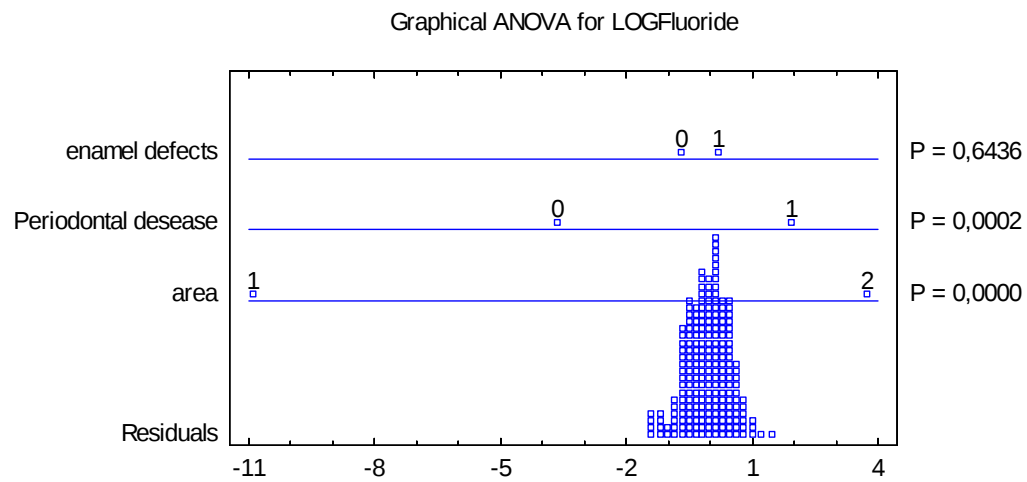


Figure 1. A: Incisor from red deer 1.5 years old with lightly enamel opacities and surface lesion. B: Disfigurement and enamel surface lesion on Pm₃ and Pm₄ (133 mg F/kg ash). C: Enamel surface lesion in M₃ and severe attrition with M₁ and M₂ appearing heavily worn in relation the rests ones (193 mg F/kg ash) D: Pathologically increased attrition of premolars and molars. The lack of ridges in M₂ indicate a reduced hardness of their enamel, likely due to hypomineralization (183 mg F/kg ash). E: Severe disfigurement and enamel surface lesion on M₃ . (967 mg F/kg ash).



Figure 2. All specimens show bone loss extending into the apical third of the root with severe molars and premolars attrition. Bone resorption exposing roots of teeth partially or completely to the apex together with osteoporosis condition can also be seen. Bone fluoride content: 601 mg F/kg, 923 mg F/kg and 403 mg F/kg for LN14, LN344 and R0936 respectively (specimens from Andújar) and 1,190 mg F/kg for VA173 (specimen from Doñana).

Figure 3. Variation of fluoride concentration by area (1, Sierra Andújar Natural Park, 2. Doñana) enamel defects and periodontal disease occurrence (1, with lesion or disease, 0, without)



ASSESSING RED DEER ANTLERS DENSITY WITH WATER DISPLACEMENT METHOD VERSUS A NEW PARAMETRIC VOLUME MODELLING TECHNIQUE USING CAD-3D

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ABSTRACT

Two methods of global volume measurement were compared in order to develop a simple and reliable method for estimating whole antlers density. We used ten cast antlers, previously dried and weighed, from ten different red deer. The volumes were determined by the traditional water displacement methods versus a new parametric volume modelling technique using CAD-3D (Computer Aided Design-3Dimensions) which is now being used in the biomedical industry in applications such as medical implant design, tissue engineering and in developing a better understanding of anatomical functionality and morphological analysis. The process paths to follow in the generation of CAD models from cast antlers were described. The whole antlers density was estimated from the weight and volumes measurement and a paired sample comparison procedure was performed to assess differences between volumes as well as densities. Cast antlers weights ranged from 219.93–1,857.9 g, and the volumes estimated in cm³ with the hydrostatic method were 732.45 ± 474.06 and 730.65 ± 492.59 by 3CAD method. The global antlers densities in g/cm³ of dry matter with the hydrostatic method (density A) were 1.112 ± 0.120 ranging from 0.915 to 1.345 (Shapiro-Wilks, *P*-value: 0.449), and with the 3CAD method (density B) 1.112 ± 0.158 ranging from 0.939 to 1.326 (Shapiro-Wilks, *P* value: 0.751). There no were differences of volumes (*t*-statistic: 0.95, *P*-value: 0.37) or densities (*t*-statistic: 0.54, *P*-value: 0.60) between methods and the correlation coefficient between density A and B was 0.968. Both methods had similar reliability, although the computing process with CAD 3D system calculates antler volume faster than the traditional hydrostatic weighing, also avoiding cast damage and the methodological problems with larger or smaller antlers or floatability due to low densities which occur when using the hydrostatic method.

Additional keywords: cast antlers density, hydrostatic weighing, modelling CAD-3D, red deer.

INTRODUCTION

Trophy hunting worldwide always seeks larger, higher quality antlers, but in Spain studies on the factors affecting antler size and quality have only increased in the last two decades (e.g. Alvarez *et al.* 1991; Soriguer *et al.* 1994; Azorit *et al.* 2002; Gómez *et al.* 2006; Landete-Castillejos *et al.* 2007; Torres-Porras *et al.* 2009; Gaspar-López *et al.* 2008, 2010; Landete-Castillejos *et al.* 2010). In this Mediterranean region where the limiting season is summer instead of winter, as in central and northern Europe, the antlers of free-living red deer (*Cervus elaphus hispanicus*) are especially influenced by climatology and herd management because drought affects body and antler size negatively, and the effects are more severe in populations of high density (Azorit *et al.* 2002; Torres-Porras *et al.* 2009). There is also an increasing interest in finding a specific index to help managers improve quality and monitor changes in this region. Antlers' size and their mineralization may play an interesting role as indicators of the nutritional situation before and during their growth. Specific gravity or global density includes both size and mineralization degree characters. The global density of the antlers is a variable depending on growth, development, spongy bone percentage, mineral composition and the stag's condition and age (Chen *et al.* 2009). Therefore a study of density may be useful as an index of quality for monitoring stags and/or their environmental condition. Local density may be determined directly, but techniques are so labour and economically expensive that studies are limited to only 15–30 antlers per study (Landete-Castillejos *et al.* 2007, 2010; Estevez *et al.* 2008). However methods based on knowledge of exact volume and weight of antlers could give information on the whole antler's density in larger sample sizes. Thus, a simple and reliable method to estimate the volume of an antler in order to compute its density seems necessary.

In order to obtain a simple and reliable method for estimating whole antler density we compared two methods of global volume measurement, the traditional water displacement or Archimedes method versus a new parametric volume modelling CAD-3D. This method has traditionally been used to assist in engineering design and modeling for representation, analysis and manufacturing, but advances in Information Technology have created new uses for CAD with many novel and important developments, particularly in biomedical applications in which the CAD based bio-tissue model provides critical information of tissue's biological, biophysical, and biochemical properties. This technique is now being used in the biomedical industry in applications such as customized medical implant design, tissue engineering and in increasing understanding of anatomical functionality and morphological analysis (Hollister *et al.* 200; Lal *et al.* 2004; Sun *et al.* 2004a, 2004b).

We use this new methodology to generate a 3D-CAD model of deer antlers from a set of homologous points to determine their volume, and we assess its usefulness in studying whole antler density in comparison to the traditional hydrostatic method. First we estimate the volumes with the two methods describing both Archimedes' method and the process paths to follow in the generation of CAD models and the reconstruction techniques. Next we study the densities and then we compare and discuss the results showing the strengths and weaknesses of each method.

MATERIALS AND METHODS

Animals

Ten cast antlers of ten different red deer (*Cervus elaphus hispanicus*) were collected from the Sierra Morena Mountains (Jaén, Spain) in the spring of different climatic years (1995–1998), so the casts belonged to stags of different ages, quality and condition. The cast antlers were dried at 105°C in an automatic pottery kiln with Siemens computer technology and weighed precisely until constant weight was achieved (an average of 48 h was necessary).

Volume Measurement Methods

Water displacement method. Volumes for reference were determined by water displacement following a modification of Miller's *et al.* (1985) method. Previously, the bases and all porous surfaces of antlers were varnished to exclude water from the internal spaces. Then the casts were submerged in an overflowing bath with a control tube and the water displaced by the antlers collected. The more frequent problems found with this method were the difficulty of measurement with large specimens, the damage of cast appearance because of the varnish, and the floatability of several casts of lower densities. We resolved this situation using an additional weight previously submerged, and with the possibility of holding the cast if necessary. All measurements were made always by the same researcher in order to minimize systematic error. In order to control the analytic procedure and to detect its precision we took four volume measurements per cast. The precision and random error was calculated by the standard deviation and coefficient of variation. Volume values given and used for calculation of subsequent density are arithmetic means of the results of the four measurements per cast.

Parametric volume modelling in CAD-3D. First we determined the homologous parameters of each cast, and then we performed a method of volume modelling using CAD-3D (Computer Aided Design-3Dimensions). This method is based on the branched-tree shape of deer antlers. A set of homologous points are located at the junction points of each branch along the central beam of the antler. These points are the centres of a set of rings. A swept volume is modelled through all the rings to obtain the central stock. Tips are modelled in the same way.

The central beam consists of an interpolation function solved by means of cubic polynomial curves named Bezier and B-spline. Mathematical discussion of these curves has been extensive from the first publication (Bézier 1972; Forrest 1972). Bezier and B-spline curves have been widely used in Computer Aided Design to model smooth curves and surfaces, and more recently to model body parts in medical and biological research (Viceconti *et al.* 1998; Sun *et al.* 2005; Kurazume *et al.* 2009).

This kind of curve has an interesting advantage because changes in homologous interpolation points lead only to local changes in the curve. Control points can be viewed on the graphic area and be moved by the user interactively to change the curve shape and obtain better curve adjustment. The curve shape is also controlled by tangent vectors located on interpolation points. In CAD systems it is possible to view simultaneously a photograph of the real piece to improve precision adjustment (see Figure 1a). A swept volume is modelled through all the rings to obtain the central stock. A perpendicular plane-to-spine curve containing each point is rendered in order to create the circle as a cross-section of the

central beam at this point. A Lofted volume is modelled to attach the set of circles along the centre line (Figure 1b). In a second stage tips are modelled in the same way, and only two more rings are necessary for each one (Figure 1c). A third process smoothes the surfaces and bevels the sharp edges (Figure 1d).

This method requires a measurement of XYZ coordinates, ring diameters at homologous points and edge radius by means of a simple tape measure and a calliper. This task can be performed outside the laboratory (Figure 1e). The parameters are then saved in an Excel template file previously linked to a parametric 3D model generated in a commercial CAD-3D system (SolidWorks) (Table 1).

Density estimations and statistical analysis

Density in g/cm³ dry matter of the whole antlers was estimated from the weight and volume measurements previously described. Using SPSS Statistics 17.0, an univariable analysis and paired sample t-tests were performed to assess differences between volumes as well as densities calculated using the two different methods. A normality test, the Shapiro-Wilk test, was performed to test for normality of the samples. A correlation coefficient was calculated in order to assess the relationship between the densities calculated with both methods.

RESULTS

Table 2 shows the weights, length, number of points and circumference at the burr as well as the volumes and the densities of each cast antler estimated using both methods.

The weights ranged between 219.93–1,857.9 g. The volumes estimated in cm³ using the CAD-3D method were 730.65 ± 492.59 ranging from 207.48 to 1,400.67. The volumes obtained with the hydrostatic method were 732.45 ± 474.06 ranging from 225 to 1,413 and the standard deviation and coefficient of variation among the four volume measurements taken per cast ranged between 0.82–7.07 and 0.06–3.07%, respectively. There were no significant differences of volumes between the two methods (*t*-statistic: 0.95, two-sided *P*-value: 0.37) when volume averages of these four hydrostatic measurements were used. The data are from a normally distributed population (Shapiro-Wilk, *P*-value: 0.88).

The global antler densities in g/cm³ of dry matter estimated with the Archimedes method (density A in Table 2) were 1.112 ± 0.1584 , ranged between 0.915–1.345 (with CV: 14.247%), while the densities estimated with the CAD-3D method (density B in Table 2) were 1.112 ± 0.120 , ranged between 0.939–1.326 (with CV: 11.5312%).

There were no significant differences of densities between the methods (*t*-statistic: 0.54, two-sided *P*-value: 0.60), and the data are from a normally distributed population (Shapiro-Wilk *P*-value: 0.25). The correlation coefficient between density A and B was 0.968.

DISCUSSION

Both hydrostatic and CAD-3D methods provide a similar value of whole cast antler volumes. Differences of densities among antlers (as well as weights and volumes) were likely because the casts belonged to stags of different years, ecological conditions during the antlers' growth, age, quality and condition. The densities found were lower than densities reported by Less (1982) (1.74 g/cm³), Currey (1979) (1.86 g/cm³) and Landete-Castillejos *et al.* (2010), who reported 1.750 ± 0.006 g/cm³ for standard seasons and 1.722 ± 0.010 g/cm³ for those with low quality antlers. However, our results were in the same range of the specific gravity of the whole antlers estimated from the difference in weight of the antlers in air and in water by Hyvärinen *et al.* (1977) (values from 0.96 to 1.44 g/cm³) using a hydrostatic balance. They also coincide with the results of Chen *et al.* (2009), who use the estimation of compact bone density (1.7 ± 0.04 g/cm³) and cancellous bone density (0.50 ± 0.05 g/cm³) to obtain total antler density (1.35 ± 0.10 g/cm³). This may be due to the fact that we determine the whole density in cast antlers rather than a local or partial density in different areas of the antlers.

Cast antlers' density may be confounded by many variables such as the year of cast harvest (with differing climatic conditions in our study area), the trophy size, the time they remain in the field before being harvested (Cyrille Barrette, per. comm.) and also by the methodology used in their determination. The overall density (specific gravity) is a potentially useful parameter that can provide important information, but all these variables should be controlled in ecological studies.

Our results show no differences between densities calculated from volume measurements determined by water displacement and parametric modelling using CAD-3D, so they have similar reliability.

Measurement by means of water displacement requires several hours, it must be performed very carefully, and measuring equipment must be in a laboratory and is certainly more complex and sometimes more difficult to attain. The more frequent problems found with the hydrostatic method were the difficulty of measurement in large specimens, the control of water measurement with smaller antlers and the floatability of several casts of low densities. Some larger antlers require the use of a huge tank, with the obvious infrastructure problems. Low density makes some pieces float and it is necessary to dip them, aided by a previously sunk weight. We were aware of these possible variations and it was necessary to repeat several times the water displacement measure in order to control the analytic procedure, to detect their standard variation and to obtain the most reliable values of volumes. This highlights a consistent weakness of this method, being at times difficult to control and generally less accurate than CAD-3D.

The new methodology based on parametric modelling using CAD-3D is a cheap technique that can be applied outside the laboratory without a hydrostatic balance and could give information on the whole antlers density in a larger sample sizes.

The main advantage of this method is time reduction and easy performance. Using this method, an antler measurement takes about twenty minutes. It can be carried out using a simple tape measure by non-experts, and is possible to perform far from the laboratory. Another reason for using this new method is a lesser manipulation of the antler, avoiding possible damage. CAD-3D is simpler and more robust than hydrostatic weighing because it avoids cast damage, variability between specimens and the methodological problems of antler size and floatability.

We found that the computing process with the CAD-3D system is simpler and faster than traditional hydrostatic weighing and calculates antler volume easily in an accurate way. By means of this new process it is possible to obtain a large amount of geometric data from a population of deer in a short time.

We have developed models taking into account all the usual antler configurations and tip numbers, but special considerations have to be taken into account in relation to defective antlers or those outside the typical morphology range. Certain types of defective or irregular antlers that do not match with the branched-tree scheme proposed are at the moment not possible to measure with this method. In this first stage, the CAD-3D model has only been developed to depict non-defective antlers. To solve these defective cases an extended 3D model should be developed so that defective antlers belonging to frail specimens can be studied.

Similar computer modelling techniques have been successfully applied in freeform surfaces to model bones and body organs, and later in manufacturing for rapid prototyping and mould processing (Kurazume et al 2009, Sun et al 2005). Modelling human body parts in a CAD based virtual environment, also referred to as Bio-CAD modeling, was a first step in the field of computer aided tissue engineering (Sun *et al.* 2004a, 2004b). The CAD-3D model can also be used in surgical assessment and planning of various bone pathologies (Minns *et al.* 2003). The latest development of this technology has been successfully used to quantify the microstructure function relationship of tissues and design tissue micro-architectures (Lin *et al.* 2003; Darling *et al.* 2004), and to (non-destructively) evaluate porosity (Müller *et al.* 1996). Our study demonstrates the ability and advantages of this method in the generation of antler morphology by creating a virtual model geometrically equivalent to real antlers (Figure 2). This method may be used easily with cast as well as skull trophies, but only for purposes of morphology studies because of the difficulty involved in measuring the weight of antlers in skull.

ACKNOWLEDGEMENT

This study was supported by the project P07-RNM-03087 (Consejería de Ciencia e Innovación de la Junta de Andalucía) and CGL-2008-00832 (Ministerio de Educación y Ciencia). We thank to pottery “Los Magníficos” from Bailén (Spain) for their helpful assistance.

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Figure 1. Picture describing the process paths to follow in the generation of CAD models. (a) Simultaneous-view photograph of real antler for better adjustment; (b) Volume modelled along the centreline; (c) Tip modelling; (d) Sharp edges smoothing; (e) Set of parameters in a 3D antler model, including **Table 1**. Parameters saved in an Excel template file linked to a CAD-3D model

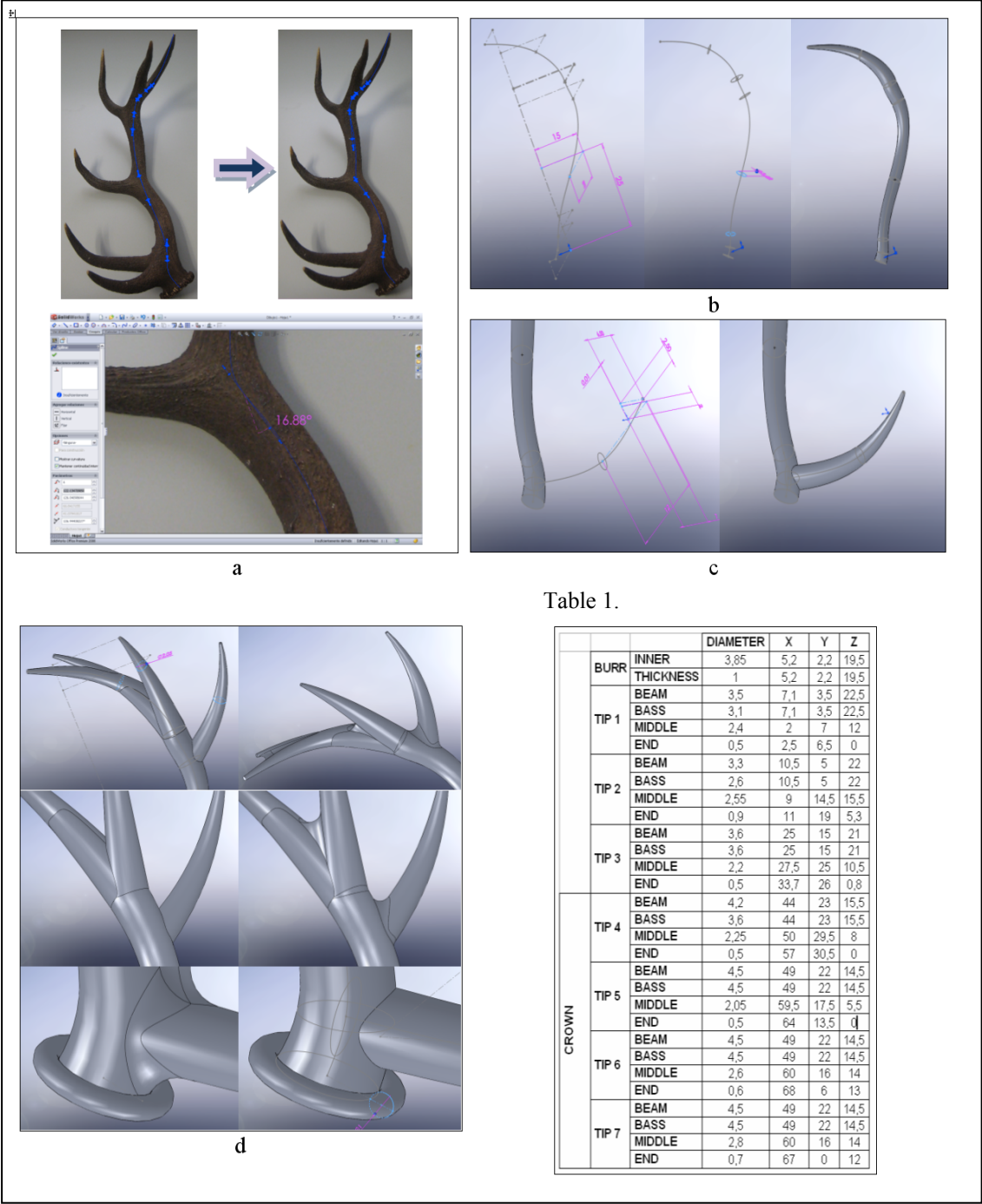


Figure 2. The simultaneous-view real antler photographs of the four first antlers studied

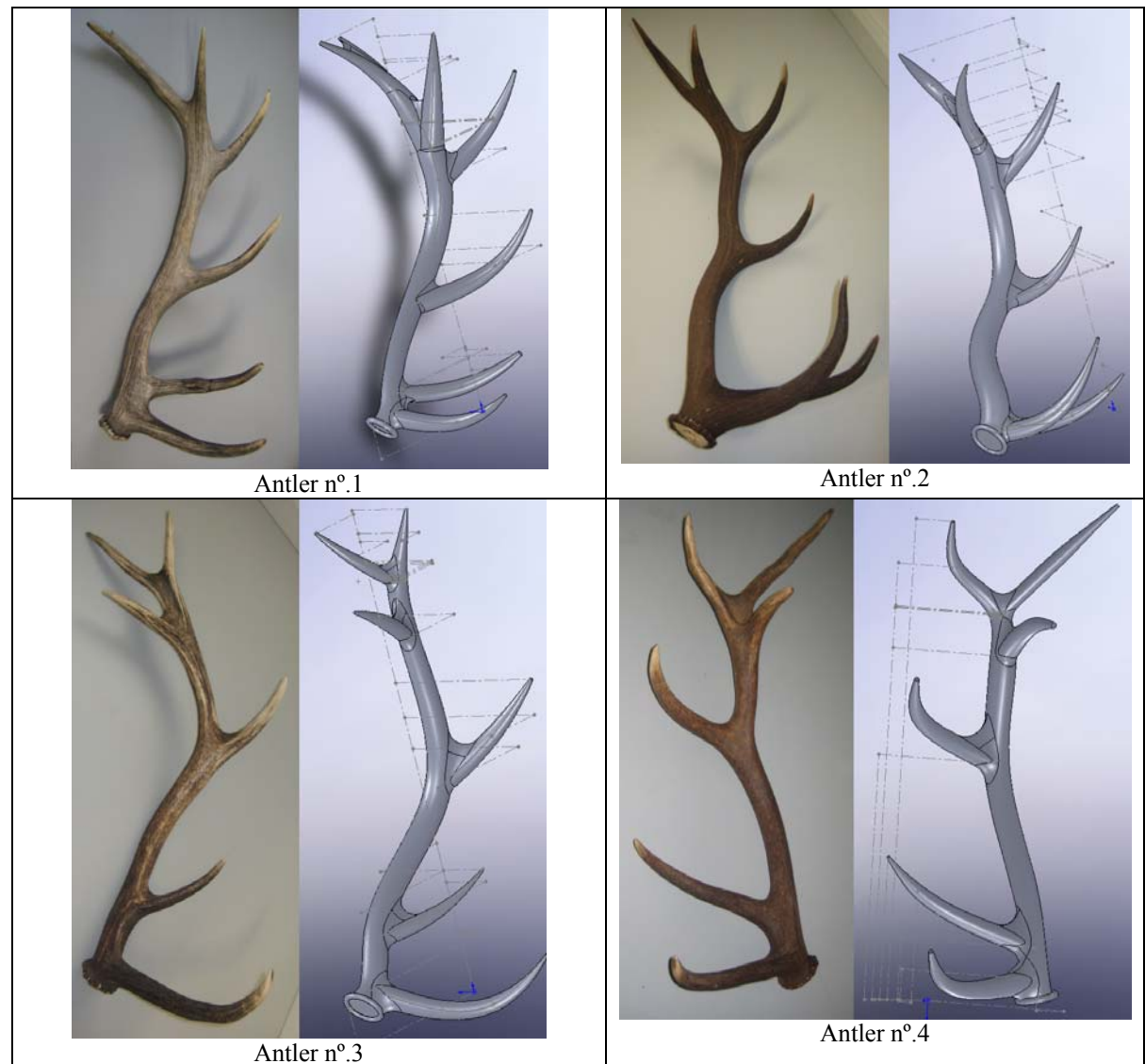


Table 2. Weights, volumes, densities and characteristic of cast antlers bellowed to 10 different red deer
Density A is calculated using volume A (Archimedes) and density B using volume CAD-3D

Cast antlers	Year	Weight (g)	No. of points	Length (cm)	Circumference at the burr	Volume A (cm ³)			Density A (g/cm ³)	Volume CAD-3D	Density B (g/cm ³)
						Range	Mean ± s.d.	CV			
1	1998	1343.97	7	81.9	38.5	1230-1241	1240 ± 4.97	(0.40%)	1.08384677	1253.52	1.07215681
2	1995	1022.86	6	79.8	48.0	1115-1124	1118 ± 3.74	(0.33%)	0.91490161	1089.11	0.93917052
3	1997	660.20	6	66.8	39.8	639-641	640 ± 0.82	(0.13%)	1.0315625	626.75	1.05337056
4	1997	658.28	6	58.8	34.5	518-522	520 ± 1.63	(0.31%)	1.26592308	529.92	1.24222524
5	1998	1857.89	6	78.4	55.0	1411-1413	1412 ± 1.63	(0.15%)	1.31578612	1400.67	1.32642949
6	1996	1587.37	7	90.8	45.0	1359-1361	1360 ± 0.82	(0.06%)	1.16718382	1360.84	1.16646336
7	1998	346.89	6	50.5	40.0	256-260	258 ± 1.63	(0.63%)	1.34453488	274.53	1.26357775
8	1996	262.20	5	53.0	31.0	278-284	282 ± 2.83	(1.00%)	0.92978723	274.21	0.95620145
9	1996	219.93	3	45.4	27.5	225-240	230 ± 7.07	(3.07%)	0.95621739	207.38	1.06051693
10	1996	324.33	5	45.6	29.5	288-298	292 ± 4.32	(1.48%)	1.11071918	289.56	1.12007874

SEASONAL AND SPECIFIC DIET VARIATIONS IN SYMPATRIC RED AND FALLOW DEER OF SOUTHERN SPAIN: A PRELIMINARY APPROACH TO FEEDING BEHAVIOUR

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ABSTRACT

The Sierra Morena Mountains have an important ecological value in southern Spain where plant-communities typical of the siliceous soil of Mediterranean ecosystems and savanna-like landscapes of the Iberian Peninsula known as “dehesas” are a dominant part of the agroforestry system. In this area red deer (*Cervus elaphus hispanicus*) are the most abundant and important game, but fallow deer (*Dama dama*) density has increased considerably since the 1950's, when they were introduced in some game reserves. When there is an overabundance of deer the subsequent selective foraging and over browsing have a negative ecological impact, making wise herd management necessary. In this context, studies of feeding behaviour and use of food resources by sympatric herbivores are very important for understanding differences in resource exploitation, their impact on vegetation and their ecology. Therefore, as part of a research project in a Mediterranean environment, we studied their diet composition, analysing a collection of rumen contents samples from 81 red deer and 69 fallow deer shot monthly by research staff during 2008–2009 in Sierra de Andújar Natural Park, Spain. The rumen contents were mixed and samples of about 250 g were stored frozen until analyzed. These were later thawed and washed through sieves with 1cm and 0.5 cm meshes. The rumen contents were classified into three groups: (1) Browse (woody, tree foliage and shrub parts, including forbs, broad green leaves, stems and shoots), (2) Grasses (graminoids, monocots, aquatic plants, and fungi and mushrooms which we included in this group because they were present, albeit as a small proportion), and (3) Fruits (acorns, olives and seeds). The material was sorted, oven-dried to constant weight and weighed. The mean percentage dry weight composition of each of the three groups with respect to the total weight of the sample was then compared using a multifactorial analysis of variance with a previous data transformation. We assessed differences of diet composition according to the factors sex, species, age and seasons (bi-monthly). Diet composition was found to vary throughout the year in both species and there was a statistically significant monthly and seasonal percentage difference of browses, grasses and fruits. Grasses were ingested more in spring, and browses were an important food resource in two periods; at the end of winter and at the end of summer. Both red and fallow deer behave as intermediate or mixed-feeders. However, red deer ingested a higher proportion of browse than fallow deer. Differences between the two sexes in red deer were also detected. Male red deer are more browsers than hinds at the beginning of spring, and they have a second peak of browsing in September-October. Nevertheless, in July–August hinds tend to be more browsers than both other sexes and other species. There were also differences in relation to fruits. Fallow deer, females especially, ingested a higher proportion of fruits, during a longer time period (September to February), than red deer. Acorns from *Quercus* sp. and olives were the most abundant fruits, being an interesting source of energy reserves for nutritional constraint periods.

Additional keywords: diet, *Cervus elaphus hispanicus*, *Dama dama*, feeding behaviour, Mediterranean constraint, monitoring variations.

INTRODUCTION

The Sierra Morena Mountains have an important ecological value in Southern Spain. Plant-communities typical of siliceous soil of Mediterranean ecosystems and savanna-like landscapes of the Iberian Peninsula known as “dehesas” are a dominant part of the agroforestry system, with *Quercus rotundifolia*, and *Quercus suber* as the most abundant trees. This area has a typical Mediterranean macroclimate characterised by irregular distribution of rainfall, reaching the highest rates in spring and autumn, and an almost total lack of rain together with high temperatures during summer. Wild ungulate hunting is the primary economic activity in this area, where red deer (*Cervus elaphus hispanicus*) is the

most abundant and important game and fallow deer (*Dama dama*) density has increased considerably since the 1950's, when they were introduced in some game reserves. Harvest rates are higher than in other European areas with an increase in the last two decades, and the influence of drought means that more animals are shot in dry years in order to adjust population densities (Azorit 2005).

When there is an overabundance of deer the subsequent selective foraging and over browsing have a negative ecological impact, making wise herd management necessary. In this context, studies of feeding behaviour and use of food resources by sympatric herbivores are very important for understanding differences in resource exploitation, their impact on vegetation and their ecology. Therefore, we studied diet composition in order to assess seasonal and specific diet variations in sympatric red and fallow deer. The main aims were to describe the different types of vegetation ingested by both species in this Mediterranean environment, evaluate the variations of diet composition through the years, and assess differences between species, sexes and age, giving preliminary information about feeding behaviour.

MATERIALS AND METHODS

We analysed a collection of rumen contents samples from 81 red deer and 69 fallow deer shot monthly during 2008–2009 by research staff of Lugar Nuevo (Sierra de Andújar Natural Park, Spain). In this official estate around 375 red and 450 fallow deer were harvested from 2002 to 2009 through intensive regular management culls intended to reduce population density, which decreased by 23.3 red and 4.0 fallow deer per km² during the study period.

Table 1 shows number of animals used in this study, their sex and age. The ages were determined by studying a combination of teeth eruption and replacement patterns (Brown and Chapman 1990; Azorit *et al.* 2002) and growth marks observed in cementum from decalcified and non-decalcified molars following a stand counting method described by Azorit *et al.* (2004). Finally the mandibles were assigned to five different age classes, animals younger than one year being excluded.

Table 1. Sex and age of red and fallow deer studied

Class of age	Red deer		Fallow deer		Totals
	Males	Females	Males	Females	
2	11	6	14	9	40
3	16	14	12	14	56
4	0	11	2	3	16
5	4	8	1	8	21
6	2	9	2	4	17
Totals	33	48	31	38	150
	81		69		

The rumen contents were mixed and samples of about 250 g were frozen and stored until analyzed. These were later thawed and washed through sieves with 1 cm and 0.5 cm meshes. The rumen contents were classified into three groups:

1. browse (woody, tree foliage and shrub parts, including forbs, broad green leaves, stems and shoots),
2. grasses (graminoids, monocots, aquatic plants, and also fungi and mushrooms) and
3. fruits (acorns, olives and seeds).

The material was sorted, oven-dried to constant weight and weighed. The mean percentage dry weight composition of each of the three groups with respect to the total weight of the sample was then compared.

Statistical analysis

The statistical analysis was performed using SPSS Statistics 17.0. We have considered 3 dependent variables: browse, grasses and fruits and by applying the statistical method of multifactorial analysis of variance we have assessed differences of diet composition according to the factors: sex, species, ages and seasons (bi-monthly). A previous data transformation was realized in order to satisfy the normality and homoscedasticity assumptions of the analysis of variance. Once the statistically significant factors had been detected we performed a post hoc comparison analysis by using the Least Significant Difference (l.s.d.) method.

RESULTS AND DISCUSSION

Diet composition was found to vary throughout the year in both species. As expected, significant differences in the diet (browse, grasses and fruits) between seasons were found for browses ($F = 9.095$, P -value = 0.000) for grasses ($F = 38.443$, P -value = 0.000) and for fruits ($F = 50.169$, P -value = 0.000). For grasses, the interaction among sex, species and seasons was not statistically significant ($F = 1.554$ and P -value = 0.096). The only differences observed in September-October were between males red deer and fallow deer. Coinciding with the study by Bugalho and Milne (2003) we observed that when grasses are widely available, deer were predominantly grazers, whereas they switched to a browser strategy when grass quality and availability fell.

In the case of browses, the diet composition depended on sex and species ($F = 10.879$, P -value = 0.000) in such a way that in January-February and September-October red deer were more browsers than fallow deer, and even in March-April male red deer ingested a higher proportion of browse than the rest. In July-August hinds were more browsers than the males (Figure 1).

With respect to fruits, the variations due to sex, species and seasons were also statistically significant ($F = 2.351$, P -value = 0.005). In January-February fallow deer females ingested the most and during September-October the fallow deer males ate the most fruits, while in November-December female red deer ate less fruit than the rest and the fallow deer ate more fruit than red deer. Fallow deer ingested a higher proportion of fruits than red deer and for a longer time period (September to February). Male fallow deer were the first to begin eating fruit while red deer were still browsing. This could lead us to conclude that fallow deer, as a more frugivorous animal, are a potential competitor of red deer.

Acorns from *Quercus* sp. and olives were the most abundant fruits, being an interesting source of energy reserves for nutritional constraint periods. In the Mediterranean ecosystem of southern Spain, because of the lack of rainfall and the high temperatures, the limiting season is the summer (especially at the end of the summer) instead of the winter of the northern ecosystem. But a second constraint period might occur at the end of winter if the scarcity of fruits coincides with lack of rainfall.

Both red and fallow deer, as indicated by the results of previous studies, behave as intermediate or mixed-feeders, adapting to seasonal changes in the quantity and quality of available food by feeding alternatively on grasses or on browse and forbs (Hofmann 1989). However, global results for red deer are: 19% browse, 57% grasses, 24% fruit; and, for fallow deer: 5% browse, 62% grasses, 33% fruit. Therefore, in constraint periods greater damage to the vegetation by browsing may be provoked by red deer, generally more browsers than fallow deer, especially when grass and fruit are scarce.

In our study area, both deer species are more grazers than browsers, grasses are ingested more in Spring, and browses (including forbs) are an important food resource in two periods: at the end of Winter and at the end of Summer. In other European areas an annual diet dominated by browse has been recorded, while the proportion of grasses eaten by red deer ranges from 5 to 95% of the diet varying between different localities and seasons (Clutton-Brock and Albon 1989). The red deer as well as fallow deer are mainly browsers from spring to autumn and grazers in winter (Putman 1986; Verheyden-Tixier et al. 2008), although it might be as well to make explicit the fact that these results relate to red deer in non-Mediterranean regions where the availability of grass in winter is reduced (in contrast to Spain). As a matter of interest, these results about the choice of grasses or browse in different seasons are similar to those reported for red deer in northern Australia (Dryden and Finch 2002), because of the similar climatic conditions. Gebert and Verheyden-Tixier (2001) show that diet variation of European red deer is associated with environmental factors like habitat and season. The availability of each food item seems to be the main source of variation, and the switch from a browser to a grazer diet was seen to be related to a change in the availability of the nutrients (Verheyden-Tixier et al. 2008).

We found a lack of significant differences in diet composition due to age but the differences between the two sexes in both species may be due to the fact that small ruminants are less able to exploit lower quality forage than larger ones because the metabolic requirement to gut capacity ratio increases with decreasing body size (Demment and Van Soest 1985). In spite of intensive management culls we have observed species and sexual segregation, with animals covering different habitats in different seasons. This would support several theories of sexual segregation. Clutton-Brock et al. (1982) showed a sexual difference in diet quality during winter, with hinds eating a better quality diet than stags.

Our observations seemed to confirm that the deer modified their feeding strategy in relation to grass availability and quality. However, the general affirmation that browse contains higher levels of soluble sugars and protein, compared to grasses (Gordon 2003) is contradicted in recent work (Verhey-Tixier *et al.* 2008). Research on the relationship between nutritional plant attributes and food selection according to species and sex would be an interesting contribution.

ACKNOWLEDGEMENTS

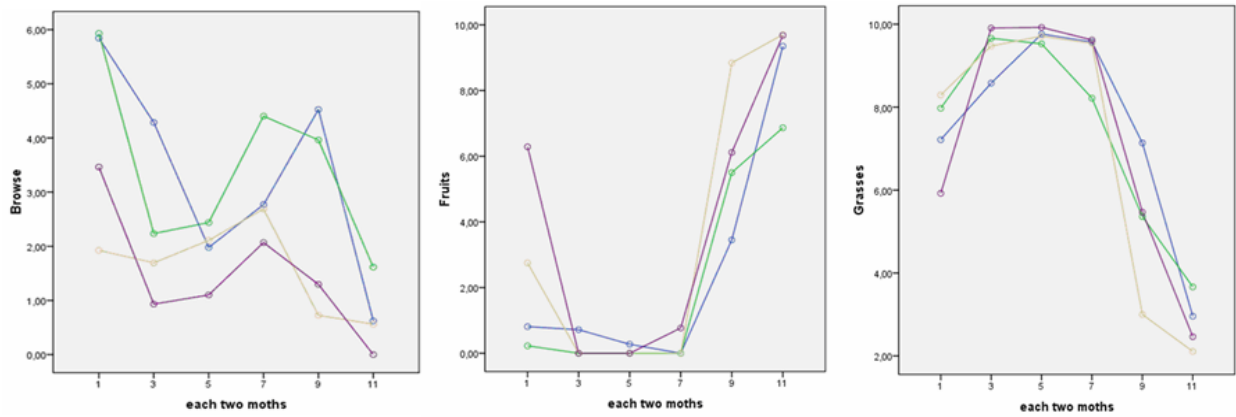
This study was supported by the projet *P07-RNM-03087* from the Consejería de Innovacion Ciencia y Empresa, Junta de Andalucía, and by the Organismo Autonomo de Parques Nacionales, Ministerio de Medio Ambiente, Medio Rural y Marino, Spain. We thank Muñoz-Cobo J, Cubillas E and F, Castilla M, Barato V, Reina P, Sánchez L, Gutierrez R, Salas J, Guerrero F, Novoa P, García R, Cuenca E, Muñoz LM, and García G, for their helpful comments, collecting deer samples and field or laboratory assistance.

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Figure 1. Variation of diet through the year (1: January–February, 2: March–April; 3: May–June; 4: July–August; 5: September–October; 6: November–December).

- **MALE RED DEER**
- **FEMALE RED DEER**
- **MALE FALLOW DEER**
- **FEMALE FALLOW DEER**



FAECAL 11-KETOETIOCHOLANOLONE MEASUREMENT IN IBERIAN RED DEER (*CERVUS ELAPHUS HISPANICUS*): VALIDATION OF METHODOLOGY USING HPLC-MS/MS

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ABSTRACT

11-Ketoetiocholanolone is a cortisol metabolite widely determined by immunoassay in monitoring stress in several vertebrates. However, these assays have certain limitations with respect to specificity. Also, differences in the excretion of FGM among species and even sexes make validation necessary in each case. Therefore, our aims were: first, to develop and validate a HPLC-MS/MS (High-pressure liquid chromatography- tandem mass spectrometry) methodology for monitoring 11-K in faeces of Iberian red deer (*Cervus elaphus hispanicus*); next to investigate the capability of our method to determine variations of this FGM in a longitudinal study. Finally we assessed the correspondence between faecal 11-K levels and plasma cortisol. An ACTH test was performed on six red deer stags translocated and kept in captivity for a week and faecal samples were collected twice a day. One single blood and faecal sample from another seven stags was also collected after two weeks in captivity. We used an Agilent 1100 HPLC system using a Waters ODS2 column (125 × 3 mm, 5 µm) and operating conditions were previously optimized by direct injection of the standard analyte into the ion source. The longitudinal study results show a first peak increase in 11-K 36 hours after the adrenocorticotropine test and handling and a second peak at 120 hours of being kept indoors. Maximum concentrations of 11-K ranged from 22.71–375.68 ng/g. In the second stag group, levels of 25.09 ± 20.53 ng/g had a correlation of $R^2 = 0.88$ to the plasma cortisol, 54.6 ± 55.1 ng/mL. This technique is capable of detecting changes in the levels of faecal 11-K. The values determined have a good correlation to the cortisol concentration in blood, and we also detected differences in different individuals' responses to the same stressors.

Additional keywords: red deer, *Cervus elaphus hispanicus*, monitoring, stress, faeces, glucocorticoid metabolites.

INTRODUCTION

Faecal glucocorticoid metabolites (FGM) measurement is a valuable non-invasive tool for studying the physiological response of free-living animals to a variety of stressors, and provides a useful monitoring technique in wildlife management and conservation. 11-Ketoetiocholanolone (11-K), also called 11-oxoetiocholanolone, is a cortisol metabolite widely determined by immunoassay in monitoring stress in several vertebrates (Keay *et al.* 2005). However, differences in the metabolism and excretion of FGM among species and even sexes and confusing factors in the interpretation of data make a validation necessary in each case (Möstl and Palme 2002; Millspaugh and Washburn 2004; Palme 2005). Furthermore, these immunoassays have certain limitations with respect to specificity because of cross-reactivity with related hormones like etiocholanolone, an important quantitative metabolite of testosterone (Ganswindt *et al.* 2003). Recently the use of high-performance liquid chromatography mass spectrometry (HPLC-MS) for steroid analysis has increased in order to avoid this problem (Shimada *et al.* 2001; Miksik *et al.* 2004; Hauser 2008; Rauh 2009).

Therefore, our aims were: first, to develop and validate a HPLC-MS/MS methodology (High-pressure liquid chromatography-tandem mass spectrometry) for monitoring 11-K in faeces of Iberian red deer (*Cervus elaphus hispanicus*); next to investigate the capability of our method in monitoring the trend of this FGM in a longitudinal study using deer under stressful stimuli like translocation, adrenocorticotrophic hormone (ACTH) injection and captivity. Finally we assessed the correspondence between faecal 11-K levels and circulating cortisol in plasma.

MATERIALS AND METHODS

Study area

The study was performed in *Lugar Nuevo*, an official game estate of the Sierra Andújar Natural Park (Jaén), located in the Sierra Morena Mountains in Southern Spain. This estate is characterized by a Mediterranean ecosystem and covers about 10,000 hectares. An adequate infrastructure for the capture, handling, manipulation, and management of wild red deer in a nature environment is available in this Game Reserve as well as other facilities and experienced staff (see Fig. 1).

Experimental deer

This study was performed in accordance with the Spanish policy for animal experimentation and was approved by the Bioethical Committee of University of Jaén.

Two experimental animal groups were studied: a first group (A) of stag red deer for the development and validation of the HPLC-MS/MS methodology and the longitudinal study; and a second group (B) of different stags kept in two weeks captivity in order to assess the correspondence between faecal 11-K levels and plasma cortisol. The age of deer in each experimental group was in the same range (from 5 to 7 years old). The experimental procedures were development at the same period of the year to avoid seasonal fluctuation, before the mating season (July-August) in the study area.

Wild deer (A) were captured in this same estate (Fig. 1b) and transported one at a time to a special facility for handling (Fig. 1c, d). Immediately, each experimental deer was moved down a raceway into a drop-floor mechanical restraint device. Adrenocorticotropin Nuvacthén Depot® (Lab. Pedró, S.A., Barcelona, Spain) was administrated intramuscularly to stimulate adrenal hormone production in a dose of four UI as recommended by Ingram *et al.* (1997). A blood sample was extracted from the jugular vein and a faecal sample directly from the rectum (Fig. 1e-h). Every manipulation lasted 2-5 min and the deer's eyes were covered with a mask during all manipulation procedures. The deer were kept in a special enclosure nearby for one hour and then a second blood sample was extracted after adrenocorticotrophic hormone (ACTH) stimulation (Fig. 1i). The deer were then housed separately in indoor-outdoor enclosures, exposed to natural photoperiod and had free access to water and alfalfa (23% DM, 16.1% CP, 43.3% NDF and 31.7% ADF), provided *ad libitum* (Fig. 1j).

In order to assess longitudinal 11-K excretion, subsequent faecal samples were collected twice a day at 0800 and 2000 hours for a week. The most recently-defecated faeces were always collected.

It was not possible to obtain blood samples at the end of the longitudinal study from this deer group (A), so a second group (B) of seven stags was studied in order to asses the correspondence between faecal 11-K levels and circulating cortisol in plasma. Faeces from the rectum and blood samples were taken from each individual all on the same day after two weeks in captivity. The deer were kept in a controlled fenced area of about two hectares on a mixed pasture and had fresh water and alfalfa provided *ad libitum* (enclosure shown in Fig. 1d). A lower level of stress was expected in relation to the A group.

All faeces and previously centrifuged blood samples were cooled instantly and stored after 45 minutes at -80°C until laboratory analysis. All plasma samples were measured by dissociation-enhanced lanthanide fluorescence immunoassay for cortisol (PerkinElmer Life and Analytical Science, Wallac Oy, Turku, Findland), according to the manufacturer's instructions. Intra-assay coefficient of variation: 5%–13.9%

inter-assay coefficient of variation: 5.7–15% (kit AXSIM DE ABBOT).

FGM extraction

FGM extraction procedures, separation, detection, identification and quantification procedures were optimized. Faeces were dried in a lyophilizer for 76 h. Once freeze-dried, glucocorticoid metabolites were extracted from faeces. Briefly, 0.5–0.7 g of dried faeces were placed in a glass tube with 4 mL of 100% methanol. Then samples were vortexed at high speed in a multitube vortexer for 8 h. We centrifuged samples at 4000 g for 30 min. The supernatant was introduced into a glass tube, and the pellets processed again as previously described. The second supernatant was mixed with the first and vacuum dried. The residue was then resuspended in 6 mL of water/methanol 9:1. This solution was loaded on a Solid Phase Extraction, SPE cartridge (Waters TM Sep-Pak vac 5cc reversed phase C18), previously conditioned with ultrapure water, MeOH and water, and eluted with water and methanol. The water eluate was discarded and the steroid enriched methanolic solution dried in vacuum and reconstituted with 40 μL of methanol. The recovery of steroids from the SPE cartridge was assessed by eluting a standard solution at 1 mg/L in the same way described above and comparing the HPLC/MS

profile with that of the original 1 mg/L solution. This experiment showed the recoveries from the cartridge to be quantitative.

Chromatography separation

Chromatographic separation from a set of steroids known to be present in faeces was achieved on an Agilent 1100 HPLC system (Agilent, Palo Alto, CA, USA) using a Waters ODS2 column (125 × 3 mm, 5 µm, Waters, Milford, Massachusetts, USA). Thus, 20 µL of the sample solution in methanol was injected, and elution was carried out by gradient between mobile phase A (methanol–formic acid 1000:1, v/v) and B (water–formic acid 1000:1, v/v). Gradient started from 50% A to 65% A in 20 min, then to 100% A in 5 min and 100% A for a further 5 min. The flow rate was set to 0.4 mL/min.

Mass spectrometry identification and quantification

The whole flow was directed into an ion trap mass spectrometer Esquire 6000 (Bruker Daltonics, Bremen, Germany); electrospray ionization in positive mode (ESI+) was used. MS/MS experiments were carried out by fragmenting precursor ion 287.0 ([M–OH]⁺), and the most abundant fragment ion 228.9 was used for quantification. Operating and fragmentation conditions were previously optimized by direct injection of a solution of the standard analyte into the ion source (Capillary voltage: 4 kV; Cap exit: 100 V; Skimmer 20 V; Trap Drive: 40; Fragmentation Amplitude: 0.45 V).

Quantification was performed by measuring the area under the peak for 11-K in the extracted ion chromatogram (EIC) for fragment 228.9. A lineal calibration curve was generated with 5 points over a range of 10–500 ng/mL. The limits of detection (LOD) and quantification (LOQ) were determined at a signal-to-noise ratio (S/N) of 3 and 10, respectively. To evaluate the influence of the complex matrixes of faeces over ionisation (matrix effect), a standard solution was spiked to the extracts and the signal obtained from this was compared to that coming from pure standard solution at the same concentration, showing that the area for the former was 40% of the standard. The 11-K (CAS number: 739-27-5) used as standard was purchased from Sigma-Adrich Quimica, SA (Madrid, Spain). The inter/intra assay coefficients of variation were from 7% to 15% (the minimum was inherent to the instrument).

Data analyses

The statistical analysis was performed using SPSS Statistics 17.0. Shapiro-Wilk W and Levene's test was used to verify the normality and homoscedasticity of the data. A simple regression was performed on data from stags B to describe the relationship between plasma cortisol and faecal 11-K. Similarly to the report by Young *et al.* (2004), several calculations for each deer of the longitudinal study were performed in order to summarize faecal 11-K values: (i) an overall mean of 11-K concentration (the mean value of all faecal samples analyzed for the collection period), (ii) a mean baseline (the mean excluding all values greater than the overall mean plus 1.5 standard deviation), and (iii) a mean peak (the mean including all values greater than the overall mean plus 1.5 standard deviation). Finally a post hoc grouping of the deer and a multiple sample comparison were used to detect differences among deer. For the group A stags the overall mean of 11-K concentration was used in this analysis.

RESULTS AND DISCUSSION

In relation to the HPLC procedure and methodology, the calibration curve showed good lineal regression ($r^2 > 0.9909$) and the limits of detection and quantification were between 1 and 10 ng/mL for the analyte with an injection of 20 µL samples. The overall extraction efficiency was not greater than 60%, and the values for within-day and between-day precision and accuracy were < 15%. In spite of a relatively low overall extraction efficiency, and the detection limits achieved being lower than for other validated methods (Hauser *et al.* 2008), this technique was capable of detecting the changes in the levels of faecal 11-K.

Figure 2 shows the results of the longitudinal study in deer group A. The first detectable level of 11-K as a consequence of the combined effects of transportation to an unfamiliar environment, adrenocorticotropine administration, handling and restraint during the treatment were recorded 12-24 hours later. In one individual (A3) a sole peak was detected at 24 h, but in five of the six deer a major increase in 11-K was detected at 36 h after treatment and a second peak was reached at 120 h of being kept indoors, before returning to the baseline.

The first increase peak in faecal 11-K within 24-36 h was similar to that found by Goymann *et al.* (1999) after handling and translocation of another species. However, in ruminants the first increase peak was recorded about 6-18.7 h (Palme *et al.* 1999), 10-26 h (Washburn and Millsbaugh, 2002) or

approximately 18-24 h after the injection of exogenous adrenocorticotrophic hormone (ACTH) (Huber *et al.* 2003b).

In response to several stimuli like those mentioned above the adrenal cortex rapidly synthesizes and secretes glucocorticoids. As a consequence plasma cortisol rises within 10-30 min in mammals (Broom and Johnson, 1993) or about 60 min in ruminants (Palme *et al.* 1999). However the appearance of elevated concentrations of glucocorticoid metabolites in faeces is much slower. The delay in detection in faeces is due to the time necessary for digesta to pass through the digestive tract (Palme *et al.* 1997). Probably for this reason no concentrations of 11-K were detected in the faecal samples collected at the same time (day 0) of treatment, whereas the plasma cortisol levels of A stags ranged from 10 to 86 ng/mL before the adrenocorticotrophic administration and reached levels of 51-88 ng/mL one hour after the treatment and handling (see Table 1). From the plasma cortisol analysis we can see that the deer of group A were stressed because of transportation and handling before the adrenocorticotrophic stimulation, but as we were not able to detect faecal 11-K we can assume that they were not affected by any stress before their capture.

The first peaks of 11-K detected at 36 h were probably due to stimulation during day 0 of the treatment, but the explanation for the great peak at 120 h is more difficult and a peak of concentration at 120 h is not frequently found (Washburn and Millspaugh 2002). The new circumstance of captivity and a variation of the food conditions of the deer may have caused this second peak found in the majority of deer. The food intake might have been reduced in captivity in spite of the availability for the deer of food *ad libitum*. The rise of glucocorticoids on day 5 might exhibit the catabolic functions in terms of mobilizing muscle protein and enzymatic reaction converting amino acids to glucose during the gluconeogenesis due to a new situation of poor nutrition or adaptation.

As we can see in Table 1 which shows the overall mean, the mean baseline the mean peak and the calculated increases, maximum concentrations of 11-K ranged from 22.71–375.68 ng/g, which reflected a 9 to 15-fold increase over the basal levels. These results are similar to those of Huber *et al.* (2003a), who recorded levels about 150–300 ng/g in August and September. Our increases are also similar to ones recorded in similar studies (Palme *et al.* 1999; Washburn and Millspaugh 2002; Huber *et al.* 2003b). Only stag A6 showed an increase (45-fold) higher than the general range (about 2.3-24 times).

Table 1. Faecal 11-Ketoetiocholanolone (ng/g) detected in faecal samples from the longitudinal study of stags A group and plasma cortisol of samples extracted on day 0 of the study

Stags	Faecal 11-Ketoetiocholanolone (ng/g)					Plasma cortisol ^A (ng/mL)
	Mean	Mean baseline (mean $\pm$ s.d.)	Mean peak	Increase	Maximum	
A1	5.64 $\pm$ 8.29	1.60 $\pm$ 2.37	15.80 $\pm$ 9.77	9.85	22.71	51–71
A2	13.28 $\pm$ 8.26	8.36 $\pm$ 4.97	20.98 $\pm$ 2.68	2.51	22.56	<10–53
A3	8.34 $\pm$ 12.32	4.54 $\pm$ 3.54	22.39 $\pm$ 17.54	4.93	34.80	<10–65
A4	7.15 $\pm$ 9.72	0.98 $\pm$ 1.97	15.38 $\pm$ 9.99	15.66	26.90	86–88
A5	81.86 $\pm$ 115.75	18.76 $\pm$ 26.09	230.22 $\pm$ 125.61	12.27	319.04	44–51
A6	88.86 $\pm$ 146.83	6.76 $\pm$ 13.14	290.74 $\pm$ 120.11	43.02	375.68	45–55

^A Range shows values before and after adrenocorticotrophic hormone, respectively.

Figure 3 shows the results of the punctual study of the correlation between plasma cortisol and 11-K determined in deer group B. Correlation was found between cortisol circulating and 11-K from faeces in the second stag group, and levels of 25.09 ± 20.53 ng/g (8.09-57.87) had a correlation of $R^2=0.88$ to the circulating cortisol, 54.6 ± 55.1 ng/mL (20-154) (P -value = 0.0016).

Inter-deer variability concerning plasma cortisol as well as faecal 11-K was observed when both mean levels and peaks were compared. This inter-individual variability was partially unexpected. We expected to find lower levels of stress in stags B because these were not submitted to adrenocorticotrophic stimulation. However there were high-responding and low-responding deer in both groups A and B so different individuals' responses to the same stressors were also detected. Table 2 shows faecal 11-Ketoetiocholanolone (ng/g) averages of the post-hoc grouped deer and differences from a multiple sample comparison. The stags of group A1-4 had similar stress levels to the deer of

B1-4 which had not received the adrenocorticotrophic hormone (ACTH) test injection. The B5-7 deer had intermediate levels and faecal 11-K concentrations from the A5 and A6 stags were significantly higher than all the others. These latter deer had higher levels of plasma cortisol on the first day of treatment, so we believe that the handling and restraint had a greater effect on the stimulation of stress response than the ACTH injection. We used 4 UI/100 kg, as recommended by Ingram *et al.* (1997) because this dose was not supraphysiological, although in most of our deer it might have been insufficient.

Table 2. Faecal 11-Ketoetiocholanolone (ng/g) averages of the post-hoc grouped deer and differences from a multiple sample comparison

Stags	N	Faecal 11-K (ng/g)			Homogeneous groups
		Average	Minimum	Maximum	
A1234	4	8.60 ± 3.31	5.64	13.28	X
B1234	4	9.77 ± 1.35	8.09	10.91	X
B567	3	45.51 ± 12.92	32.09	57.86	X
A67	2	85.36 ± 4.95	81.86	88.86	X
<i>F</i> -ratio 78.03, <i>P</i> -value:0.0000; Levene's 2.42558, <i>P</i> -value:0.132696					

Further work would need to investigate the independent effect of different food types, feeding rates, food intake or different conditions of captivity. The HPLC-MS/Ms methodology seems to be adequate since it was able to detect proportionally the increased adrenal cortisol secretion in faeces. The values found have a good correlation to the cortisol concentration in blood, and we also detected differences in different individuals' responses to the same stressors. Achieving an overall extraction efficiency greater than that obtained in this study (60%) would be useful for detecting very low levels of faecal glucocorticoid metabolites and so recommendable for future research.

ACKNOWLEDGEMENTS

This study was supported by the P07-RNM-03087 and CGL-2008-00832 projects and was approved by the Bioethical Committee of University of Jaén. We thank Muñoz-Cobo J, Cubillas E and F, Castilla M, Barato V, Reina P, Sánchez L, Gutierrez R, Salas J, Guerrero F, Novoa P, García R, Cuenca E, Muñoz LM, García G, Carrasco D, and Portela C, for their helpful comments, collecting deer samples and field or laboratory assistance. Also we wish to thank to two anonymous reviewers for helping to improve earlier version of the manuscript, to Araque G, Deutor B, Oya R and all CICT (Centro de Instrumentación Científico-Técnica), University of Jaén, Spain.

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Figure 1. Study area, installations and facilities for capture, handling, management and keeping of studied deer:

- (a) Map of Lugar Nuevo in the north of the Jaén Province.
- (b) Area and enclosure for capture using supplemental food as bait.
- (c) Procedure for the transport and translocation of deer.
- (d) White arrow shows the fenced area of about two hectares used for keeping deer.
- (e and f) Facilities for handling deer.
- (g and h). Drop-floor, mechanical restraint device.
- (i) Captive deer one hour after adrenocorticotrophic stimulation.
- (j) White arrow shows enclosure where deer were kept for a week for the longitudinal study.



Figure 2. Concentration of faecal 11-K (ng/g) determined every 12 h throughout a week in group A stags (a-f).

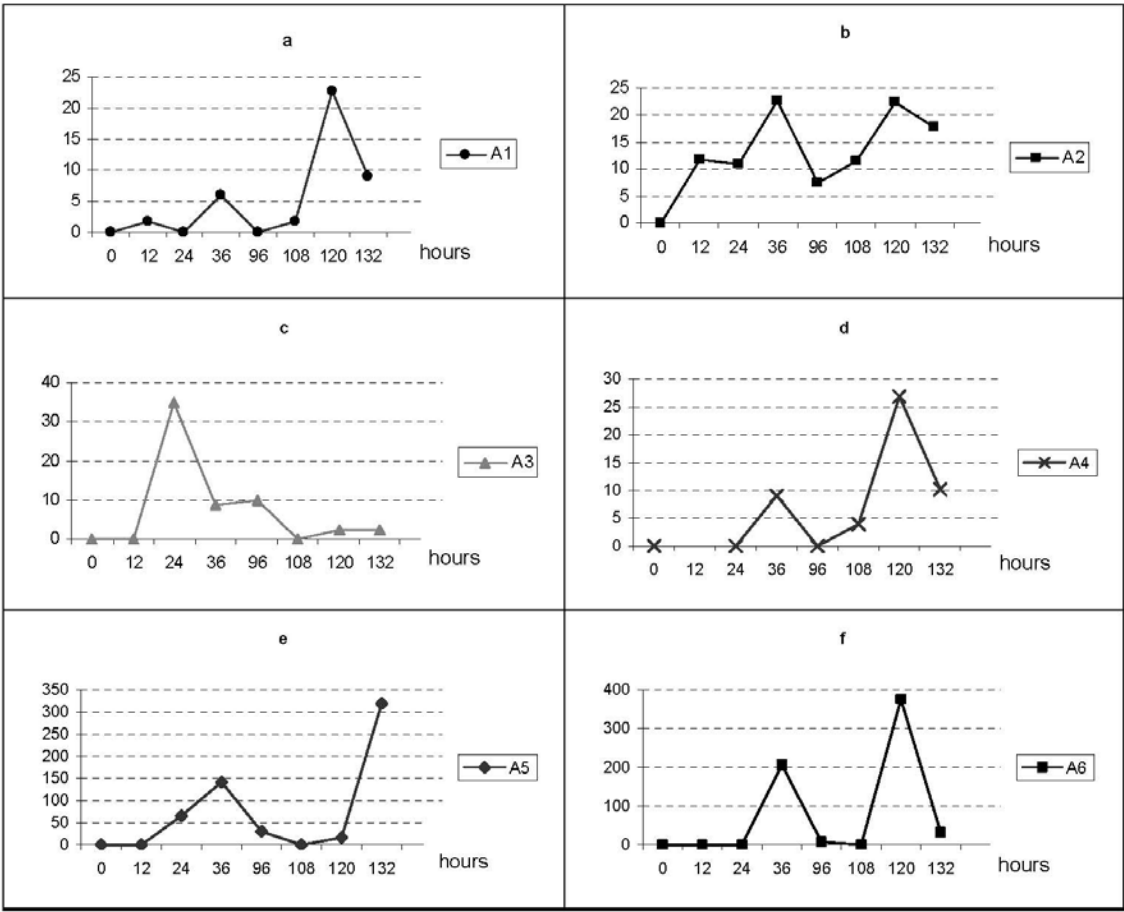
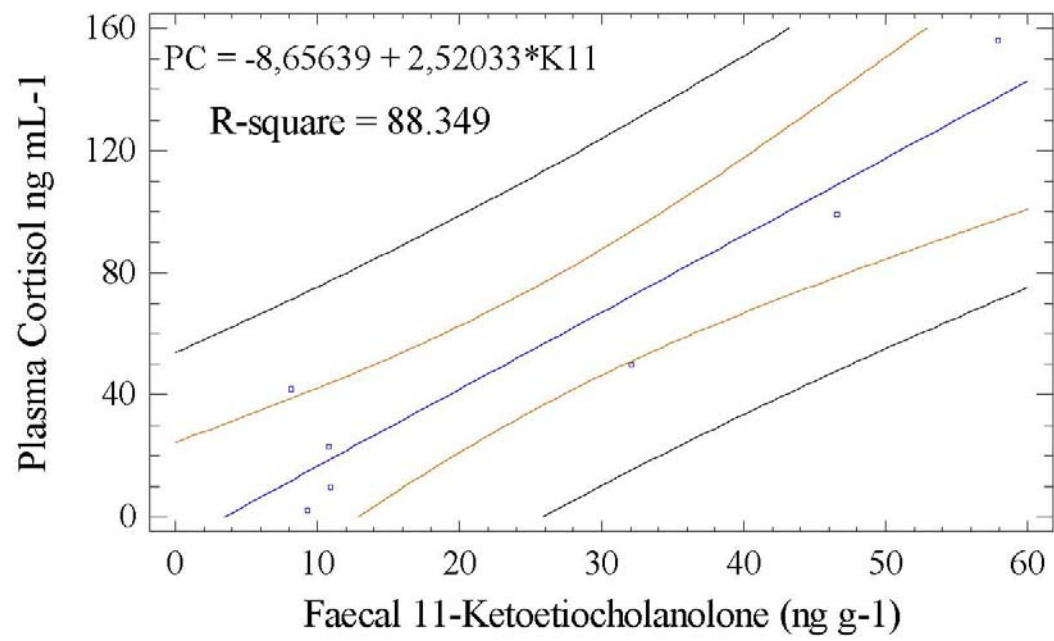


Figure 3. Relationship between circulating cortisol and 11-K from faeces in group B stags.



RENIN-ANGIOTENSIN SYSTEM-REGULATING AMINOPEPTIDASES: MONITORING SEASONAL VARIATIONS IN RED DEER AND FALLOW DEER FROM A MEDITERRANEAN ECOSYSTEM

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ABSTRACT

The circulating renin-angiotensin system (RAS) is well known for its systemic role in the regulation of blood pressure, renal hemodynamics and fluid homeostasis. However, in mammals several organs also contain a local RAS, including male and female reproductive tissues. In the present study we have analyzed serum, APA (regulating aminopeptidase A), ASAP (aspartyl aminopeptidase), APN (aminopeptidase N) and APB (aminopeptidase B) RAS-regulating aminopeptidases in a free-living population of red deer (*Cervus elaphus hispanicus*) and fallow deer (*Dama dama*) as part of a study of annual cycles of growth and condition. Our aim is to detect seasonal variations in these specific activities and their relationship to the reproductive behaviour of both species in a Mediterranean environment. In both males and females there was a maximum peak of specific activity in autumn, but a second peak was detected in spring for males while in females specific activity was also higher in summer. These changes may be related to a different endocrine status according to their seasonal cycle, the decreased photoperiod in autumn and the normal timing of the seasonal growth cycle. Thus, specific RAS-regulating aminopeptidase activities modification could reflect the functional role of angiotensins through the annual cycle of both species, also suggesting an important role of these peptide hormones in the regulation of these biological processes.

Additional keywords: APA, ASAP, APN, APB, RAS-regulating aminopeptidases, Mediterranean ecosystem, *Cervus*, *Dama*.

INTRODUCTION

It is well known that the renin-angiotensin system (RAS) plays an important role in mammals in general homeostasis including the regulation of systemic blood pressure and of fluid and electrolyte balance. As well as its classical roles, a local RAS has been found in both male and female reproductive systems in several mammals. Thus, in females, binding sites for angiotensin II (AngII) have been identified in rodent ovarian follicles (Speth *et al.* 1986; Husain *et al.* 1987) and corpora lutea (CL) (Pepperell *et al.* 1993, 2006). Renin- and prorenin-like activities have been shown in bovine follicles (Schultze *et al.* 1989), and angiotensin-converting enzyme (ACE) is also present in rodent ovarian follicles and CL (Speth and Husain 1988). Furthermore, evidence has been accumulating that AngII locally regulates ovulation, oocyte maturation (Kuo *et al.* 1991; Yoshimura *et al.* 1992), and steroidogenesis (Yoshimura *et al.* 1996). These findings suggest that the ovarian RAS may function independently or in conjunction with the systemic RAS, and regulate ovarian function through the paracrine/autocrine actions of AngII.

In males, several studies have also provided evidence for the presence of the RAS in reproductive tissues, such as the detection of immunoreactive renin in the Leydig cells of rat and human testes (Parmentier *et al.* 1983; Naruse *et al.* 1985). Similarly, other studies have shown the presence of renin, angiotensin I (AngI), AngII and angiotensin receptors in normal rat Leydig cells and a murine Leydig cell line (Pandey *et al.* 1984; Pandey and Inagamy 1986). Furthermore, angiotensin-converting enzyme (ACE) activity has been identified in testes, localized predominantly in the germinal cells. Only minor activity has been found in purified adult rodent Leydig and Sertoli cells (Velletri *et al.* 1985a, 1985b), where the existence of a new ACE homolog (ACE2) has been described (Douglas *et al.* 2004). A putative role has therefore been proposed for AngII in modulating the action of gonadotropin in Leydig cells, thus modulating steroidogenesis and testosterone production (Khanum and Dufau 1988).

A main role for AngII has therefore been proposed in both male and female steroidogenesis. However, although AngII has normally been considered the main bioactive peptide of the RAS, some studies

suggest that the major player is AngIII (Reaux *et al.* 2000, 2001; Reudelhuber 2005). In fact, AngIII has most of the properties of AngII and shares the same receptors (Szczepanska-Sadowska 1996; Barrett *et al.* 2004). AngIII arises through deletion of the N-terminal aspartic residue by glutamyl-aminopeptidase (GluAP: EC 3.4.11.7) and aspartyl-aminopeptidase (AspAP: EC 3.4.11.21). In combination, the enzymes are designated as aminopeptidase A (APA). Furthermore, AngIII is metabolized to angiotensin IV by arginyl-aminopeptidase (aminopeptidase B, APB: EC 3.4.11.6) and alanyl-aminopeptidase (aminopeptidase N, APN: EC 3.4.11.14) (Ward *et al.* 1990; Barrett *et al.* 2004).

Our aim is to analyze in free-living populations of red deer (*Cervus elaphus hispanicus*) and fallow deer (*Dama dama*) as part of a study of annual cycles of reproduction, growth and condition, serum APA, ASAP, APN and APB RAS-regulating specific aminopeptidase activities, to detect seasonal variations in these specific activities and their relationship to the reproductive behaviour of both species in a Mediterranean environment.

MATERIALS AND METHODS

Animals

Our study was based on animals shot during sport hunting, herd management culls and programs for population control in 2008 in the eastern area of Sierra Morena, southern Spain. A total of 306 blood samples were collected from 172 red deer and 134 fallow deer shot at different times of year covering the main features of the annual life cycle. Blood samples were centrifuged 10 min at 3000 ×g to obtain the serum. Samples were frozen and stored at -80°C until use.

RAS-Regulating Aminopeptidases Assay

AspAP was determined fluorometrically in triplicate, with aspartyl-β-naphthylamide (AspNNap) as the substrate, as previously described (García *et al.* 2003). Briefly, 10 μL of each supernatant was incubated with 100 μL of the substrate solution (100 μmol/L AspNNap, 1.5 mmol/L BSA, and 2 mmol/L MnCl₂ in 50 mmol/L HCl-Tris buffer pH 7.4.) for 30 min at 37°C.

GluAP was measured in the same way (García *et al.* 2003) using as substrate glutamyl-β-naphthylamide (GluNNap): 10 μL of supernatant was incubated for 30 min at 37°C with 100 μL of the substrate solution (100 μmol/L GluNNap, 1.5 mmol/L BSA, 0.65 mmol/L DTT, and 50 mmol/L CaCl₂ in 50 mmol/L HCl-Tris buffer pH 7.4).

Aminopeptidase N and aminopeptidase B were also measured fluorometrically using alanyl-β-naphthylamide (AlaNNap) or arginyl-β-naphthylamide (ArgNNap) as the substrate, as previously described (García *et al.* 2003). Ten μL of each supernatant were incubated for 30 min at 37°C with 100 μL of the substrate solution: 100 μmol/L AlaNNap or 100 μmol/L ArgNNap, 1.5 mmol/L bovine serum albumin (BSA), and 0.65 mmol/L dithiothreitol (DTT) in 50 mmol/L phosphate buffer, pH 7.4.

All reactions were stopped with the addition of 100 μL of 0.1 mol/L acetate buffer, pH 4.2. The amount of β-naphthylamine released as a result of the enzymatic activity was measured fluorometrically at a 412-nm emission wavelength with an excitation wavelength of 345 nm. Proteins were quantified in triplicate using the method of Bradford (Bradford 1976), with BSA as a standard. Specific soluble and membrane-bound AspAP, GluAP (APA), APN, and APB activities were expressed as picomoles of Asp- or Glu- or nanomoles of Ala- and Arg- β-naphthylamide, hydrolysed per minute per mg of protein, using a standard curve prepared with the latter compound under corresponding assay conditions. The fluorogenic assay was linear with respect to time of hydrolysis and protein content.

Statistics

After an exploratory study of the data to check for possible entry mistakes, missing values and sexual differences a multivariate analysis of variance with a previous square root data transformation using SPSS Statistics 17.0, was performed in order to assess differences according to species, age and seasons. More specifically, males and females were studied separately and a seasonal categorisation performed.

RESULTS AND DISCUSSION

Table 1 shows mean values in red deer and fallow deer and Figure 1 shows RAS-regulating specific aminopeptidase activities in both sexes at different seasons of annual cycle. In APA activity our results show no significant differences between sex and species, but ASAP, APN and APB activities were higher in male red deer than in male fallow deer ($P = 0.03, 0.04, 0.005$), although no differences were found in females of either species. Seasonal variations in APA and APN activity were not statistically

significant either in males or females of both species. However, ASAP and APB activities showed significant seasonal changes. In both males and females there was a maximum peak of concentration in autumn, but a second peak was detected in spring for males and in females there was a high concentration also in summer.

As expected from the involvement of RAS in both male and female steroidogenesis regulation, the aminopeptidase activities may be described concomitantly to the estrous cycle of females and coinciding with the highest plasma testosterone concentration in males. The maximum peak concentration in autumn coincides with the mating season and rut in both species. The red and fallow deer show a seasonal reproductive pattern shaped by photoperiod (Suttie *et al.* 1984; Rolf and Fischer 1996; Asher *et al.* 1999). These changes may be related to a different endocrine status according to their seasonal cycle and the decreased photoperiod in autumn. Mating takes place during short days, when the plasma levels of melatonin are high and those of prolactin are low. Perhaps differences in melatonin and prolactin levels between males and females throughout the year could explain the second peak of specific aminopeptidase activities found in spring for males and in summer for females. This is because we suppose that melatonin and prolactin are related to RAS-regulating specific aminopeptidase activities.

Melatonin is the hormone involved in the transduction of photoperiodic information to the endocrine system leading to precise timing of reproduction, and prolactin concentrations have been measured in several studies as an indicator of photoperiodic control (Rolf and Fischer 1996; Webster *et al.* 1999; Bartos *et al.* 2009).

However, in females steroid hormones also determine prolactin production, particularly estradiol, which promotes prolactin synthesis (Curlewis *et al.* 1992). Circulating oestrogens in particular increase in concentration during pregnancy and are also known to increase prolactin secretion, and lactating hinds show higher levels of prolactin. Plasma prolactin concentration increases during May (García *et al.* 2002) may be in relation to low levels of specific aminopeptidase activities in summer instead of spring, as occurs in males.

On the other hand, in males this photoperiod control generates a different seasonal cycle in relation to the antlers' growth process which starts in spring after the casting off of the previous set when plasma testosterone is low (Suttie *et al.* 1984). The physiological mechanisms controlling antler growth are not understood exactly, and the involvement of changes in secretion of growth hormones and insulin has been disputed (Bartos *et al.* 2009).

Thus, RAS-regulating aminopeptidase activities modification could reflect the functional role of angiotensins through the annual cycle of both species, also suggesting an important role of these peptide hormones in these biological processes. In order to explain the exact role of these specific activities and/or their endogenous substrates, further studies of seasonal, sexual and specific variations and their relationship to levels of reproductive steroids of both species in this Mediterranean environment is necessary. New research would test its possible role in antler growth regulation.

ACKNOWLEDGEMENTS

This study was supported by P07-RNM-03087 and CGL-2008-00832 projects and was approved by the Bioethical Committee of University of Jaén.

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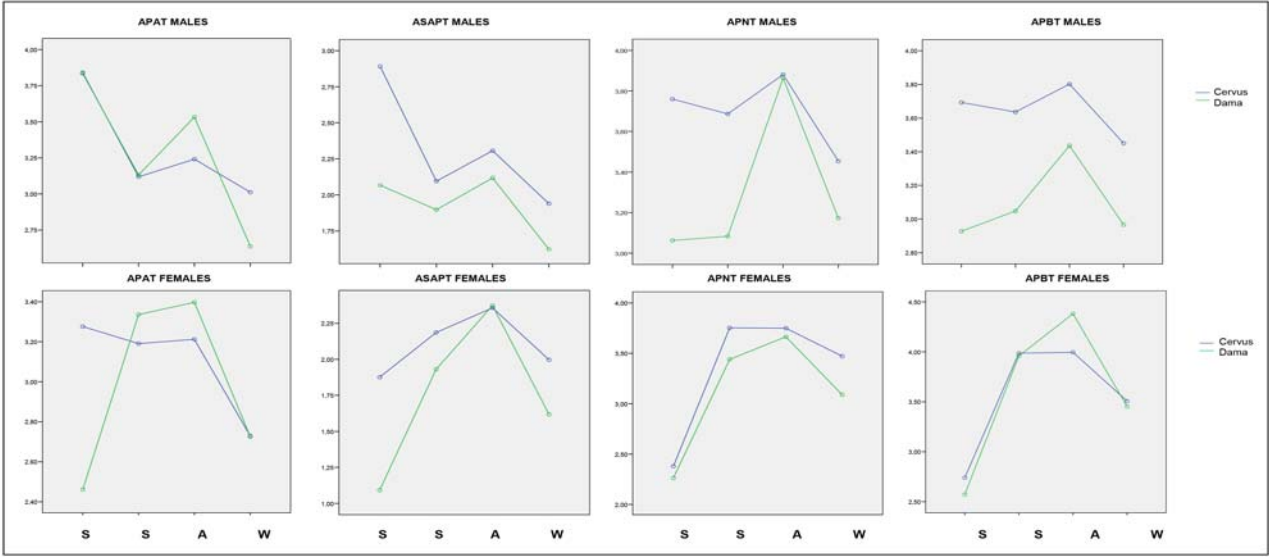
Table 1. Specific serum aminopeptidase activities in red and fallow deer

Note: the results are expressed in picomoles per minute per mg of protein

RAS-regulating aminopeptidase	Mean $\pm$ s.e.m.	
	Red deer (n = 172)	Fallow deer(n = 134)
APA	23.94 $\pm$ 22.06	30.15 $\pm$ 25.29
ASAP	9.20 $\pm$ 5.32	9.21 $\pm$ 6.72
APN	39.51 $\pm$ 23.67	35.35 $\pm$ 28.43
APB	45.06 $\pm$ 31.71	61.97 $\pm$ 75.36

Figure 1. RAS-regulating specific aminopeptidase activities in red and fallow deer in different seasons of their annual cycle.

Note: APAT (regulating aminopeptidase A₁), ASAPT (aspartyl aminopeptidase), APNT (aminopeptidase N) and APBT (aminopeptidase B) all with a square root transformation.



DOES CARCASS SUSPENSION TECHNIQUE INFLUENCE REINDEER (*RANGIFER TARANDUS TARANDUS*) MEAT QUALITY ATTRIBUTES?

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ABSTRACT

A total of 8 reindeer steers (ages 3–6 years old) were used in the study to evaluate the effects of carcass suspension technique on meat tenderness, colour and water-holding capacity (WHC). Carcasses were split along the spine and sides randomly allocated to pelvic suspension (hung using a butcher hook through the obturator foramen) or normal Achilles tendon suspension (control treatment). From all 16 carcass halves meat samples were collected from the loin (*M. longissimus*), inside (*M. semimembranosus*) and shoulder (*M. triceps brachii*) for sensory evaluation and measurements of shear force (tenderness). Loin samples were also evaluated for meat colour at 1 day post slaughter and for purge after vacuum-packaged chilled storage (+2°C) for 1, 2 and 3 weeks. No significant effects of carcass suspension technique were found for reindeer meat colour and WHC (purge). Shear force values for loin samples from pelvic suspended carcasses were significantly lower ($P = 0.001$) compared with Achilles tendon suspended carcasses, and there was a similar trend for inside samples though not significant ($P = 0.06$). There was no effect of carcass suspension technique for shear force values of the shoulder samples. The trained panel judged loin and inside samples from pelvic suspended carcasses to be more tender ($P \leq 0.001$) while no effect of carcass suspension technique on tenderness was found in the shoulder samples. Juiciness was not affected by carcass suspension. This study demonstrated that pelvic suspension improved tenderness in the most valuable cuts from the reindeer carcass (loin and inside).

Additional keywords: reindeer, meat, venison, carcass suspension, tenderness, sensory evaluation.

INTRODUCTION

Variation in meat tenderness and techniques developed to minimise this variation, have been investigated in several animal species over a long time period. For beef, inconsistent tenderness is considered to be the main reason for consumer dissatisfaction (Koohmaraie 1996). It is well known that the conditions during rigor development (e.g. muscle pH decline, temperature decline, temperature/pH relationship interactions and carcass treatments including suspension) are very important in controlling meat tenderisation (Dransfield 1994). Carcass suspension techniques have been studied for beef and shown to affect the tenderness of different muscles (Hostetler *et al.* 1970; Lundesjö Ahnström *et al.* 2003). In Alaska, reindeer is marketed as a premium red meat so assurance of a high quality product is important for the relationship between producer and consumer. The purpose of this study was to evaluate the effects of two different carcass suspension techniques on tenderness, colour and water-holding capacity in reindeer meat.

MATERIALS AND METHODS

Animals

A total of 8 reindeer steers (ages 3–6 years old) from the Reindeer Research Program herd (University of Alaska Fairbanks, Alaska, USA) were used in the study. The reindeer were transported to Delta Meat and Sausage (Delta Junction, Alaska) and slaughtered under USDA inspection. Carcasses were split along the spine and sides randomly allocated to pelvic suspension (hung using a butcher hook through the obturator foramen) or Achilles tendon suspension (control treatment). From all 16 carcass halves meat samples were collected at 1 day post slaughter from the loin (*M. longissimus*), inside (*M. semimembranosus*) and shoulder (*M. triceps brachii*) for sensory evaluation and measurements of shear force (tenderness). Samples were frozen and stored at -20°C until analysis. In addition, fresh loin samples were evaluated for meat colour at 1 day post slaughter and for purge after vacuum-packaged chilled storage (+2°C) for 1 week, 2 weeks and 3 weeks.

Tenderness, colour and purge measurements

For tenderness measurements, loin samples were cooked in bags submerged in boiling water until the internal temperature of the sample reached 75°C. Internal temperature in each loin was monitored with copper-constantan thermocouples (Type T, Omega Engineering, Stamford, CT, USA) and a Barnant scanning digital thermometer (Model 692-0000, Barnant Co., Barington, IL, USA). After cooking, the samples were immediately cooled in running tap water. Ten 1 cm x 1 cm cross-section slices (bites) were prepared from the cooked sample with the muscle fibres running longitudinally along the slice. Each sample was then sheared with the long axis of the fibres running perpendicular to the blade, using a TA.XT Plus texture analyzer (Texture Technologies, NY, USA) instrument equipped with a Warner-Bratzler blade attachment at a head speed of 3.5 mm/s. Tenderness values were registered as the maximum shear force (peak height).

A Minolta Chroma meter (CR-300, Osaka, Japan) was calibrated against a white tile ($L^* = 97.06$, $a^* = 0.41$ and $b^* = 1.72$). The aperture was 89 mm, illuminant D65 and 10° Standard observer were used. CIALab L^* (lightness) a^* (redness) and b^* (yellowness) were measured in triplicate on each sample on a freshly cut 2.5 cm thick steak after 2 h of blooming at +2 °C. For purge measurements, loins were removed from their packages, dabbed dry with a paper towel and then weighed. Purge loss was calculated as the difference in the weight of the loins before and after storage expressed as a percentage of the original weight of the loins.

Trained sensory panel

The work was performed at the Co-operative Extension Service, Food Product Development Kitchen (University of Alaska Fairbanks). A descriptive test, conventional profiling (ISO 6564, 1985), was carried out by a selected and trained sensory panel (ISO 8586-1, 1993) consisting of seven members. The sensory training was performed in accordance with ISO 6564 (1985). All assessments were carried out in a sensory laboratory with separate booths equipped with Compusense® *five*, an automated data collection system (Compusense Inc., 2004) and under normal white light (ISO 8589, 1988).

Upon thawing, the loin samples were placed in a refrigerator at 3 °C for 17 h. The meat was then cooked in a conventional oven at 150°C to a core temperature of 70°C. Internal temperature in each loin was monitored with copper-constantan thermocouples (Type T, Omega Engineering, Stamford, CT, USA) and a Barnant scanning digital thermometer (Model 692-0000, Barnant Co., Barington, IL, USA). Samples, 3mm in width were cut across the muscle grain (one slice of meat), placed in plastic cups coded with three-digit numbers and were served to the panel members in randomised order, at room temperature and in two replicates. The following attributes were evaluated by the panel; tenderness and juiciness. An unstructured continuous line scale from 0 (low intensity) to 10 (high intensity) was used.

Statistical analysis

The statistical analyses were carried out with the Statistical Analysis System (SAS Institute, 2003) using the GLM and MIXED procedures. The model for comparing shear force, meat colour and purge included the fixed effect of treatment group. Significance was defined as $P \leq 0.05$. For the trained panel work, the model included the random effects animal and panel member, as well as the fixed effect of treatment group.

RESULTS AND DISCUSSION

No significant effects of carcass suspension technique were found for reindeer meat colour and WHC (purge) in the present study (Table 1). Shear force values for loin (*M. longissimus*) samples from pelvic suspended carcasses were lower ($P = 0.001$) compared with Achilles tendon suspended carcasses. There was a similar trend towards lower shear force values for the inside (*M. semimembranosus*) samples of pelvic suspended carcasses, though not significant ($P = 0.06$). No effect of carcass suspension technique was demonstrated for shear force values of the shoulder (*M. triceps brachii*) samples (Table 1). In agreement with the mechanical shear force measurements, the trained panel judged loin and inside samples from pelvic suspended carcasses to be more tender ($P \leq 0.001$) while no effect of carcass suspension technique on tenderness was found in the shoulder samples. Juiciness was not affected by carcass suspension (Table 1).

Texture, flavour and tenderness are attributes valued by consumers in relation to the eating quality of meat. Different populations of consumers have different preferences for these quality attributes, something that affects the market for all types of meat. However, regardless of the consumer group, the consistency of meat quality is very important, and the product should be of the same quality every time

it is purchased. In the Australian beef grading system, Meat Standards Australia (MSA), these consumer important sensory quality attributes have been weighted in an overall score where tenderness represents 40%, flavour 20%, juiciness 10% and overall liking 30% (MSA 2001).

Sims *et al.* (2004) have reported that the tenderness of the following muscles was significantly improved ($P \leq 0.05$) in fallow deer (*Dama dama*) venison as a result of pelvic suspension; *Mm. longissimus*, *biceps femoris*, *semimebranosus*, *adductor femoris* and *vastus lateralis*. Their results agree with studies on beef, where the tenderness of *Mm. longissimus*, *semimembranosus* and *adductor femoris* was positively affected (more tender) by pelvic suspension (Hostetler *et al.* 1970; Bouton *et al.* 1973). Therefore, the positive effects of pelvic suspension on tenderness have been demonstrated for the most valuable cuts of a carcass; the loin (*M. longissimus*) and various cuts from the hindquarter. The same conclusion could be made about reindeer carcasses included in the present study. Smulders *et al.* (2006) reported a difference in tenderness between right and left side beef loins (*M. longissimus*), i.e. the loins were more tender on the side from which the carcass had been shackled during bleeding compared with the contralateral side. In the present study all reindeer carcasses were shackled in the left hind leg, but the random allocation of pelvic suspension to each carcass half would have counteracted a systematic effect of potential impact of shackling on tenderness.

Sensory scores for tenderness and juiciness in reindeer meat (*M. longissimus*, loin) have been reported to be high (Renecker *et al.* 2005; Rincker *et al.* 2006; Wiklund *et al.* 2003), and the results from the present study are in good agreement with those studies. Venison is more tender than beef, and, for some deer species like reindeer and fallow deer, ageing of the meat beyond 1-3 days post slaughter is not necessary (Barnier *et al.*, 1999; Sims *et al.* 2004). However, variation in venison tenderness between deer species has led to different processing protocols for carcasses from the different species.

In New Zealand, Red deer (*Cervus elaphus*) carcasses are electrically stimulated to accelerate the rate of tenderisation and thereby improve tenderness at short ageing periods (Wiklund *et al.* 2001). Electrical stimulation has been used for reindeer carcasses during field slaughter in Alaska where the same positive effect on meat tenderness was observed (Wiklund *et al.* 2008). However, Wiklund *et al.* (2008) reported that electrical stimulation resulted in variation of tenderness improvement between different meat cuts on the reindeer carcass. The authors concluded that it would therefore be necessary to balance the benefit of positive effects of electrical stimulation on tougher cuts such as shoulder meat with potential 'over-tenderising' of high value primal cuts. In this study, both shear values and sensory evaluation did not indicate that the loin and inside (*M. semimembranosus*) were too tender (i.e. meat with deteriorated texture). However, there may be a need to further investigate the potential for over-tenderisation of high value primal cuts, particularly since the tenderness improvement achieved by pelvic suspension is so clearly concentrated to those cuts.

Conclusions

This study demonstrated that pelvic suspension improved tenderness in the most valuable cuts from the reindeer carcass (loin and inside). Tenderness improvement was possible by changing the carcass suspension technique even in a meat type that is already very tender – compared with e.g. beef and red deer venison – without ageing beyond 1–3 days *post mortem* and without using electrical stimulation of the carcass. Pelvic suspension did not alter meat colour, WHC (purge) or sensory evaluated juiciness of the reindeer meat. Results from this study will help producers, processors, and retailers to deliver reindeer meat of a consistently high quality to the market.

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Table 1. Meat quality characteristics and sensory scores (least squares means $\pm$ s.e.) in reindeer meat from pelvic suspended and Achilles suspended carcasses

Within rows, means followed by different letters are significantly different ($P \leq 0.05$)

Trait	Pelvic suspension ($n = 8$)	Achilles tendon suspension (control, $n = 8$)
Shear force (kg)		
Inside (<i>M. semimebranosus</i>)	3.0 ± 0.16	3.5 ± 0.16
Loin (<i>M. longissimus</i>)	$4.3a \pm 0.27$	$6.4b \pm 0.27$
Shoulder (<i>M. triceps brachii</i>)	4.3 ± 0.37	4.9 ± 0.37
Minolta colour		
Loin		
L*	35.4 ± 0.53	34.7 ± 0.53
a*	14.0 ± 0.32	14.2 ± 0.32
b*	5.4 ± 0.20	5.4 ± 0.20
Purge (%)		
Loin		
1 week	5.5 ± 0.73	4.6 ± 0.73
2 weeks	7.5 ± 0.42	6.8 ± 0.42
3 weeks	10.2 ± 1.15	10.7 ± 1.15
Sensory evaluation scores		
Tenderness		
Inside	$6.0a \pm 0.18$	$7.2b \pm 0.19$
Loin	$4.2a \pm 0.19$	$6.4b \pm 0.18$
Shoulder	5.4 ± 0.18	5.2 ± 0.18
Juiciness		
Inside	6.0 ± 0.18	6.5 ± 0.19
Loin	5.6 ± 0.19	5.6 ± 0.18
Shoulder	6.0 ± 0.18	5.9 ± 0.18

DESCRIPTION OF VOCALIZATIONS IN EXOTIC EUROPEAN RED DEER STAGS (*CERVUS ELAPHUS*) DURING THE RUT IN NORTHWESTERN PATAGONIA (ARGENTINA)

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ABSTRACT

Polygynous deer are very vocal species, producing calls in various contexts. Male rutting calls in red deer (*Cervus elaphus*) have been most studied in captive and/or free-ranging European populations. The recent application of “source-filter theory” has identified the independent roles of fundamental frequency (F0) and formants in the production of deer calls and demonstrated the relation between formant spacing and anatomical characteristics such as body size. This paper describes and characterizes the acoustical properties of male rutting vocalizations for a free-ranging red deer population located in the ecotone of the eastern Andean cordillera within the Nahuel Huapi National Reserve (Neuquén, Argentina), the first acoustic study of free-ranging red deer stags outside their historic distribution. Recordings were made of 7 identified and several unidentified stags at the peak of the rut in 2007. Calculated F0 was found to be higher for these identified stags than for Scottish red deer on the island of Rhum. The analysis of formant spacing was used to calculate the length of the vocal tract (VTL) for the 7 males studied; values were comparable to those found in stags from Rhum. The longest calculated VTLs within these males corresponded to the 4 stags with the largest antler racks and the only stags holding harems. Previous studies have shown that VTL correlates with body size, age and reproductive success and that these deer have the ability to lower their larynx and extend their vocal tracts to a maximum, which is probably under sexual selection. Our data fit with this explanation. The higher F0s recorded in this population may result from the influence of wapiti genes, known to be present in these deer, as wapiti have a much higher frequency rutting call, the bugle. Alternatively, they may be related to the mating strategy, which varies substantially from the northern European populations.

Additional keywords: roars, formants, elk, pitch, mating behaviour.

INTRODUCTION

Communication plays a crucial role in social interactions in deer, as in other group-living mammals. Although not obvious to the casual observer, polygynous deer are very vocal species. Moreover, their vocalizations are notable for their diversity. Calls have been registered in both sexes and in different contexts: social contact, mother-young interactions, and male rutting calls, which function in both male-male competition and female choice (Taylor and Reby 2010; Reby *et al.* 2010; Reby *et al.* 2005; Reby and McComb 2003b). The vocalizations emitted during mating activities have received the most attention, particularly in red deer (*Cervus elaphus*), with captive and/or free-ranging populations having been studied in Scotland, France and Italy (Favaretto *et al.* 2006, McComb 1987, McComb 1991, Pepin *et al.* 2001, Reby and McComb 2003a, 2003b, and Reby *et al.* 2001, 2005, 2010).

The “source-filter theory” of mammalian sound production hypothesizes that the vocalizations produced result from two independent processes. First the sound is generated by the vocal folds of the glottis, which produce the fundamental frequency (F0). The sound then passes through the cavities of the superior vocal tract where it is filtered by the resonant frequencies of the cavities. Some frequencies are augmented (the formants) and others are dampened (Taylor and Reby 2010). The distribution of the formants or formant frequency dispersion, unlike F0, is related to the variation in the anatomical characteristics of the caller, such as body size, since it accurately reflects vocal tract length (Fitch 1997; Reby and McComb 2003a, 2003b). Using source-filter theory, the different contributions of the source, as F0, and the filter, as formant frequency dispersion, have clarified the form of production of many animal calls, how variations in F0 can reflect hormonal or motivational changes and how formant frequency dispersion is an accurate reflection of vocal tract length (VTL) and thus of body size (Taylor and Reby 2010). In red deer and fallow deer (*Dama dama*), the use of this theory led to the discovery that stags of these species can lower their larynxes during vocalization, thereby increasing their VTLs,

decreasing the formant frequency dispersion, and increasing their apparent size (Reby and McComb 2003a, McElligott *et al.* 2006). In red deer, formant frequency dispersion is used by stags to evaluate their opponents and by hinds to select a mate (Reby *et al.* 2005, Charlton *et al.* 2007).

Here we present the first study of vocalizations of free-ranging red deer stags outside their historic distribution. Exotic red deer were first introduced to Argentina in the period 1902-10 by Pedro Luro to the La Pampa Province. The records from the company importing the red deer showed them all to be of Austrian/Hungarian stock from the mountains of the Alps and Karpaten (Wollenhaupt 1983). In the early 1920s, Robert Hohmann brought 20 individuals from Parque Luro to the 'estancia' Colluncó near San Martín de los Andes, from where they were liberated in 1922. In 1924, individuals from Colluncó were translocated to an enclosure on Peninsula Huemul in Nahuel Huapi National Park to be later released in 1926 (reviewed in Flueck and Smith-Flueck 1993 and Smith-Flueck 2003).

The population from which our males were sampled originated from the animals released at Peninsula Huemul. Today's distribution of free-ranging red deer includes a wide north-south stretch of the Andean-cordillera ecotone, including the lenga forest to the west and reaching out into the steppe to the east (Flueck *et al.* 1995, 2003), with numbers estimated at more than 100,000 individuals in Patagonia (Flueck *et al.* 2003). In the favourable ecotonal habitat, densities recently reached a high of 100 deer/km² (this study area) and 40–50 deer/km² in the steppe (Flueck *et al.* 2003), several fold higher than densities recorded for Scottish deer on the Island of Rhum (Clutton-Brock *et al.* 1982, p 268).

Besides an Austrian-Hungarian heritage, the red deer in this area have also been shown to contain genes identified specifically with N. American wapiti or elk (*Cervus elaphus canadensis*) which would have already been present in the first red deer imported from Europe one hundred years ago. The importation of American wapiti to Europe is well documented, with individuals brought to Austria, Poland and Hungary over 250 years ago to improve trophies for sport hunting. For one example, several hundred wapiti were said to have been introduced to Austria by the Emperor Franz Josef (1830-1916) where they hybridized with the native red deer (Whitehead 1993). Introductions of wapiti were primarily into deer parks from where subsequent introductions were made into wild populations (Perez-Espona *et al.* 2010). Wapiti, although currently classified as the same species as red deer (Nowak and Walker 1999), have a few phenotypic characteristics that make them easily discernible from their European relative: larger body size (several fold larger than Scottish red deer); an antler rack of greater size, weight and a different form; and easily distinguishable male rut calls. Whereas an elk is commonly known to *bugle* during the rut (Feighny *et al.* 2006), the red deer male *roars*. The bugle is a much higher frequency vocalization (F0 0.41 – 1.90 KHz) than the roar (F0 70-162 Hz).

The aim of this paper was to describe and characterize the acoustical properties of male vocalizations produced during the rutting period for a free-ranging red deer population located in the ecotone of the eastern Andean cordillera within the Nahuel Huapi National Reserve (Neuquén, Argentina). We also wanted to compare our results with those obtained from Scottish stags on Rhum (Reby and McComb 2003a) in order to determine whether red deer calls in this part of the world have different acoustical properties, which might be attributed to different environmental conditions or genetic origins.

MATERIALS AND METHODS

The study population inhabits the mountainous ecotone and steppe habitats of the eastern Andean cordillera in Patagonia where the dominant climate is temperate with main precipitation occurring between April and September, with an average of 1200 mm annual precipitation. The breeding season, occurring sometime between early March and late April, lasts 3 to 6 weeks, and peaks around the last 2 weeks of March. The study area was located on private land within the Nahuel Huapi National Reserve, (40°58"S; 71°12"W), Argentina at 950 m altitude, less than 20 km from the 1926 release site on Peninsula Huemul. The area over which the rut takes place is characterized by a mosaic of habitats, with the open mallins being the predominant habitat used by prime stags to set up their territories for retaining harems:

- (i) **forest patches:** represented by a variety of pure and mixed stands of *Nothofagus antarctica*, *Austrocedrus chilensis*, *Lomatia hirsuta*, *Maytenus boaria* and *Schinus molle*.
- (ii) **brush patches:** represented predominately by *Berberis spp.*, *Colletia spinosissima*, and *Rosa mosqueta*.
- (iii) **mallins:** a type of wet grassland; common throughout the eastern cordillera of the Andes, which are defined as exhibiting running water or inundated soils all year round. Cyperaceae, Juncaceae and other wetland species dominate the wet central sections of the mallins. Of the graminoides, *Carex*

canescens and *Carex macloviana* (Cyperaceae) and *Juncus depauperatus* are the most prevalent species. The dryer outer edge of the mallin have more xeric graminoid and forb species, such as *Festuca pallescens*, *Hordeum chilense*, *Poa spp.*, and *Ranunculus peduncularis*.

(iv) **grass-dominated steppe:** represented by *Stipa speciosa* var. *major* and *Festuca pallescens* with variable occurrence of brush species like *Mulinum spinosum*, *Berberis spp.*, and *Colletia spinosissima*.

(v) **riparian:** a habitat with *Salix sp.* and *Nothofagus antarctica* the predominant species.

The recordings were conducted in 2007 during the peak of the rutting period (20-26 March), predominately during the height of daily activity (approximately sunrise to 1100 hours, and 1630 hours to sunset). For the 7 identified males recorded, behavioural records were taken as well as photographs: four of these were dominant breeders, each of which held a territory with 7 or more hinds present. Throughout the peak period of the rut, focal stags remained the entire day in their established territories in the open mallins, leaving only if disturbed. While these dominant stags often bedded down in the open in the heat of the day, the females, in contrast, often left to bed in the cover of the brush and forest patches on the nearby hillsides. Throughout the day, the males would continue to call, even while bedded, though sporadically and with less intensity. An occasional intruding male was cause for the bedded stags to rouse, concomitantly increasing the frequency of their calls. The roaring activity picked up again in the late afternoon, stimulated by hinds returning to the mallin, though timing varied daily according to the meteorological conditions. (Smith-Flueck and Flueck 2006).

The recordings of the vocalizations were made with a Sony TCD 5M portable recorder, type II cassettes and a unidirectional microphone (Audio-technica AT835b, frequency response=20 – 20,000 Hz) with a wind damper. The maximum recording distance was 100 meters. The tapes were then digitalized with Spectrogram 14 (sample rate: 11 kHz, 16 bit, FFT 512, freq. resolution 21.5) and acoustic characteristics (duration, low frequency and high frequency) analysed with Raven Pro. For formant and pitch analysis we selected 37 common roars from the 7 identified males and we extracted filter formant related acoustic features with Praat 5.1.37 DSP package (P. Boersma and D. Weenink, University of Amsterdam, The Netherlands). To characterize the filter, we measured the lowest frequency values for the first eight formants and the minimum spacing between them (Min Δ F), using the formant command in Praat (edit, formant, show formants). Formant analysis parameters were: maximum formant frequency = 2000 Hz, number of formants = 8, window length (s) = 0.1. In some cases the lowest frequency formants were not visible or the values obtained seemed too high. In these cases we compared the formant values from Praat with the formant means and ranges obtained by Reby and McComb (2003a) and then we decided whether this value was the first or second formant: eg. if the first formant indicated by Praat was 390 Hz, this value is closer to the second formant in Reby and McComb (2003a), and thus was interpreted as such. In these cases we did not put a value for the first formant and instead used only the higher formants. We determined the relationship between Δ F and VTL by the formula given by Reby and McComb (2003a) and Fitch (1997). We plotted the formant values as a graph of frequency (kHz) v. formant spacing (Δ F); then a linear regression line was fitted and the slope of the linear regression was used as the best estimate of Min Δ F. This graphic method was obtained from Reby (Reby and McComb, 2003a).

Because the F0s in many vocalizations were not visible, we measured the frequencies at the 5th harmonic using Spectrogram 14. In harmonic sounds, the harmonics are an integer multiple of F0 and spacing values between harmonics permitted us to estimate F0. Next we divided this value by five and then, we averaged these values to estimate F0 for each male to then make comparisons with red deer data from the Island of Rhum (Scotland). We obtained F0 for the 37 common roars analysed from the 7 identified stags.

RESULTS

Of the 731 vocalizations, we were able to analyse only 91, for the others were not clear enough, containing too much noise either from wind interference or overlapping vocalizations from nearby males. From the spectrograms, three principal types of vocalizations were identified. These were mainly distinguished by their structure and total duration in seconds (Fig. 1):

(i) *Common roar:* a harmonically structured sound with well-defined tones and formant frequencies. This vocalization had the greatest spectrographic variability, since some common roars had many harmonics and others had atonal parts (both at the beginning and at the end of the call); these vocalizations were emitted both in bouts and in isolation. N = 37, duration = 1.43 ± 0.19 (all means $\pm$ s.e.) sec, low frequency = 183 ± 16 Hz, high frequency = 2828 ± 125 Hz. These values represent the frequency range of the calls.

(ii) *Harsh roar*: has a chaotic structure with some areas of greater intensity within its frequency range. This vocalization was produced both in bouts and in isolation. N= 31, duration = 0.65 ± 0.07 sec, low frequency = 89 ± 17 Hz, high frequency = 2706 ± 140 Hz.

(iii) *Bark*: the shortest vocalization, showed an atonal structure, and was produced either in a rapid series, or as single barks. N= 23, duration= 0.23 ± 0.01 sec, low frequency = 133 ± 26 Hz, high frequency = 2538 ± 249 Hz.

The most common vocalization was the common roar (47%), followed by the harsh roar (28%) and the least common was the bark (25%).

In addition to these parameters, for the 37 common roars sampled from the 7 identified males, we present the average values for mean fundamental frequency, Min ΔF and MaxVTL compared with values obtained by Reby and McComb (2003a) in Table 1. Although we do not know the ages of our recorded males, the calculations of ΔF and MaxVTL obtained allowed us to identify them as adult males by comparing them with values of Reby and McComb (2003a), in which the ages and weights of the Scottish stags were recorded. The four males with the highest VTL values (73.5 cm, 71.6 cm, 73.1 cm and 72.1 cm) were also the males with the largest antler racks, containing 11 to 14 points (M2, M4, M6, and M7) and had harems with hinds at the time of recording. The remaining 3 males had adult, but smaller, values of VTL (69.3, 70.4, and 71.3 cm), and lacked hinds at the time of recording.

Table 1. average of variables measured in our study for each male and comparison with values obtained for Reby and McComb (2003a)

Individual (n)	Fundamental frequency		Formant frequency	
	MeanF0 (Hz)	MinF0	MaxF0	MaxVTL (cm)
1 (7)	140.4			69.3
2 (6)	139.6			73.5
3 (4)	142.6			70.4
4 (5)	123.0			71.6
5 (3)	138.6			71.3
6 (3)	116.8			73.1
7 (9)	120.7			72.2
Mean	131.7			71.6
Reby and McComb 2003a	64.4	111.7	142.2	70.6

DISCUSSION

From our recordings of the vocalizations produced by red deer stags (*Cervus elaphus*) in the study area of Nahuel Huapi, the reproductive calls were found to be similar to their native European counterparts in that we could identify the same 3 main vocalizations as described for the deer of the Island of Rhum in Scotland (Reby and McComb 2003a, 2003b). Although some red deer from Nahuel Huapi have a genetic component from wapiti (*Cervus elaphus canadensis*) with the superoxide dismutase allele typical for wapiti found to occur in 11% of animals in this study population (Flueck and Smith-Flueck 2011), these deer have roars typical of red deer. The bugle, which is the rut vocalization characteristic of wapiti (Feighny *et al.* 2006), was never distinguished in any of the vocalizations recorded. Instead, during our study period, which fell during the rut peak (Smith-Flueck, unpublished data), the common roar was the most frequently produced vocalization. Harsh roars during this time period were mainly observed in agonistic interactions between stags and alternated with common roars.

Reby and McComb (2003b) described two types of barks distinguished by their behavioural context: the isolated bark, or single bark, which is directed to a stationary female and the serial bark, which corresponds to the chase bark and is emitted by stags when chasing a female or a competitor. In addition, in the Nahuel Huapi population, the single bark has been observed in males in cases such as (i) when trying to get a stationary female in his harem to move, and (ii) when suddenly faced with an

unknown disturbance. The chase bark is frequently used by a territorial male chasing a juvenile male out of his territory (Smith-Flueck, unpublished data).

Common roars are the vocalization most used in previous acoustical and statistical analyses (McComb 1987, Reby *et al.* 2001, Reby and McComb 2003a, Charlton *et al.* 2007, Charlton *et al.* 2008, Reby *et al.* 2010). Bouts of common roars permit individual identification (Reby *et al.* 2006). The application of source-filter theory to red deer common roars showed that minimal formant frequency dispersion in general is negatively correlated with body size and thus an honest indicator of fighting ability (Reby and McComb 2003a; Reby *et al.* 2005). In red deer, minimal formant frequency dispersion is also a good indicator of age (decreasing with age) and is positively correlated with reproductive success (Reby and McComb 2003a). During the emission of the common roar, the stag raises its head and stretches its neck, inducing a descent of the larynx, which decreases the spacing between the formants (ΔF), the minimal formant frequencies, and increases the VTL.

We found that the 4 males with the lowest ΔF and the longest calculated VTLs were the only identified males holding harems, which is in agreement with the findings of Charlton *et al.* (2007). Using playbacks of common roars re-synthesized to indicate longer VTLs and thus larger body size, those authors determined that hinds prefer males whose roars have formants with lower minimum frequency dispersion and ΔF . Another study of male behaviour during the rut on this same Nahuel Huapi population showed only males of larger body mass and antlers with a greater number of points defending and holding territories and females who chose to remain in those territories (Smith-Flueck and Flueck 2006).

In our data, the male with the lowest F0 is not the male with the highest value of VTL (and therefore largest body size), because F0 is not a good indicator of body size (Reby and McComb 2003a). Although mean F0 and male quality are not correlated, F0 is higher in the roars of subadults than adult stags (Reby and McComb 2003a) and thus potentially can act as an additional cue to the maturity of the stags (Charlton *et al.* 2008). Recent studies have also found that estrous red deer hinds prefer stags with higher F0s, so that sexual selection could be acting to raise the F0 (Reby *et al.* 2010). The mean values of F0 for our population (Table 1) are higher than the values obtained previously for adult males (7-13 years old) and closer to the values for subadult red deer (5-6 years old, Reby and McComb 2003a).

These results show that an exotic European red deer population separated from Europe for over a century has vocalization patterns almost indistinguishable from native red deer. The fact that the F0s observed in the Patagonian red deer are somewhat higher than those observed in the red deer from Rhum (Reby and McComb 2003a) may indicate a genetic influence of wapiti on the roars, given that Patagonian red deer do have some wapiti genetic markers (Flueck and Smith-Flueck 2011). Wapiti, however, have much higher F0s (0.41-1.9 kHz) (Feighny *et al.* 2006). Although wapiti have also been introduced in the past two centuries to Great Britain (Whitehead 1982), deer from the Scottish highlands were shown to have little introgression of haplotypes from wapiti when analysing a paternally inherited genetic marker on the Y chromosome (Pérez-Espona *et al.* 2010), indicating that exotic wapiti there seem to have had little impact on the genetic make-up of the Scottish Highland red deer. In contrast, we might explain the notably stronger wapiti influence on the genetic make-up of the Nahuel Huapi population by the fact that the founder population started with 20 individuals that likely had a high genetic make-up of wapitoid species, reflecting the history of numerous wapiti introductions to Austria. Also with few founders in Patagonia, substantial dilution over the past century would not be expected.

Another explanation for the higher F0s in the calls of the Patagonian red deer might be related to the different mating strategies utilized, which in turn could be influenced by environmental factors (Carranza *et al.* 1995). The dominant males of this study population display territoriality (Smith-Flueck and Flueck 2006) in contrast to prime males in Scotland defending mobile harems (Clutton-Brock *et al.* 1982). Factors that alone or in combination could affect mating strategies include habitat quality, population density, sex and age composition, and genetic make-up of the population.

ACKNOWLEDGEMENTS

We thank David Reby for his helpful comments on the methodology used and on an early review of the manuscript and for the constructive criticism of three anonymous reviewers. This study was made

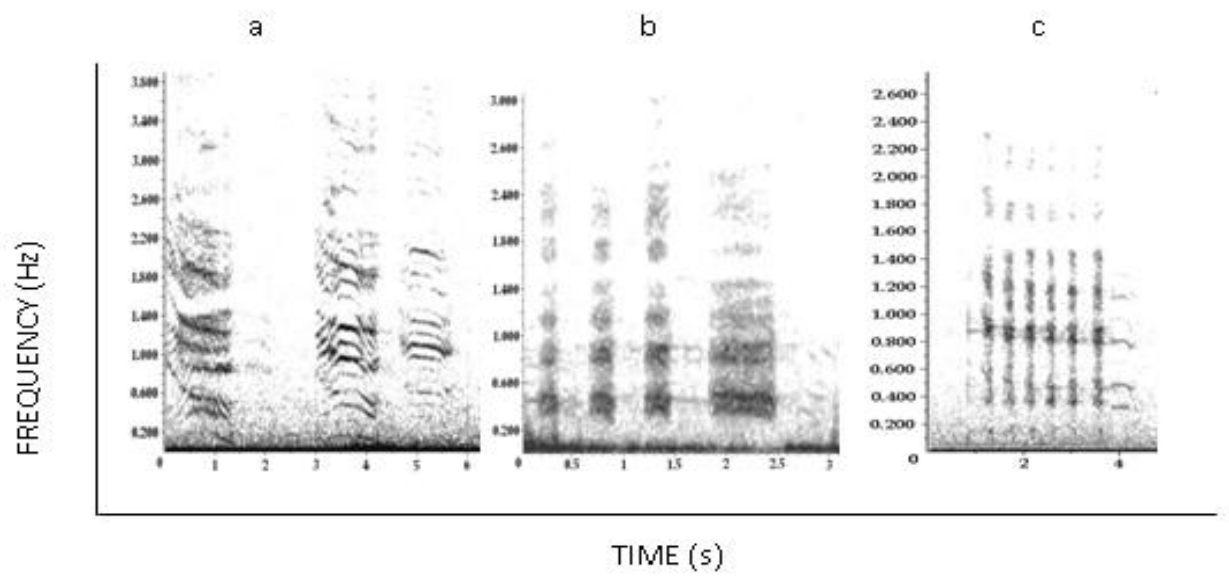
possible only through the generosity of the Jones family in permitting us to camp and study the red deer rut on their Tequel Malal ranch.

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Figure 1. Spectrogram of calls [(a) bout of common roars, (b) bout of harsh roars (c) bout of chase barks] recorded during the rut in Patagonian red deer.
Spectrogram from RavenPro



EFFECT OF SELECTING THAWED EPIDIDYMAL SPERMATOZOA WITH DIFFERENT DENSITY GRADIENTS ON SPERM QUALITY IN IBERIAN RED DEER STAGS

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ABSTRACT

The aim of this work was to study the effect of selecting thawed epididymal spermatozoa from Iberian red deer by means of different discontinuous density gradients (Percoll[®], Puresperm[®] and BovipureTM) on sperm quality after selection procedure. Sperm samples were collected from epididymides of eight Iberian red deer being extended with Triladyl[®] (20% egg yolk) and frozen in nitrogen vapors for 10 min. After thawing, to evaluate the effect of thawed sperm quality on the efficiency of selection methods, males were classified as with high sperm quality after thawing when sperm motility (SM) $\geq 70\%$, or as with low sperm quality after thawing when SM $\leq 69\%$. Sperm samples from males belonging to the same sperm quality group were pooled. An aliquot from each pool was used in the following discontinuous density gradients: Percoll[®], Puresperm[®] and BovipureTM. After centrifugation and washing, several sperm parameters were evaluated. The percentage of recovered spermatozoa was similar between selection treatments. Likewise, there were no differences in NAR, VAP, LIN and %DFI for these treatments. However, the values of SM, YO-PRO-1+/PI- and MT+/PI- were higher for thawed epididymal sperm samples selecting with Percoll[®] and Puresperm[®] (72.50% and 66.04%; 7.71% and 6.31% and 64.27% and 71.78%, respectively) in relation to BovipureTM (55.83%; 5.90% and 55.91%, respectively), being higher the value of YO-PRO-1-/PI- for Puresperm[®] (83.37%). There was a interaction between the density gradient and sperm quality group for YO-PRO-1-/PI- and YO-PRO-1+/PI-. As expected, the values for most parameters assessed after selection procedures were higher for the group of males classified as with high sperm quality in relation to those with low sperm quality, although there were no significant differences between both groups for VAP, LIN and %DFI. In conclusion, our results can be useful for developing other assisted reproductive technologies, as *in vitro* fertilization or sexing sperm, in the cervid species.

Additional keywords: Iberian red deer, sperm selection, density gradient, epididymal spermatozoa.

INTRODUCTION

In different assisted reproductive techniques, as *in vitro* fertilization or sperm sexing, the sperm separation methods have a very important role. Selection procedures are able to separate motile from nonmotile spermatozoa, remove seminal plasma, cryoprotective agents, other background materials and debris (Zavos 1992). Density gradient centrifugation remains the most popular method (Mortimer 2000). Density gradients separate spermatozoa according to their density and the most used is Percoll[®] based upon colloidal silica coated with polyvinylpyrrolidone (PVP). Percoll[®] has been withdrawal from clinical use in humans by its manufacturer (Pharmacia Biotech, Uppsala, Sweden) and its use has been restricted to research purposes (Mortimer 2000). Percoll[®] has been replacement for other products as Puresperm[®] used in human and BovipureTM used in bovine species. The main difference between these selection methods is that Percoll[®] is based in PVP-coated silica which can have deleterious effects on sperm membranes (Strehler *et al.* 1998), whereas Puresperm[®] and BovipureTM contain silane-coated silica particles. Nevertheless, these colloids seem to be as good as Percoll[®] in human and bovine species (Centola *et al.* 1998; Samardzija *et al.* 2006). To date, there are no studies about the efficacy of Puresperm[®] and BovipureTM in the cervid species. Thus, in different reports about *in vitro* fertilization in deer, the method used to enrichment the sperm samples based in density gradients, has been Percoll[®] (Comizzoli *et al.* 2001; Berg *et al.* 2002; García-Álvarez *et al.* 2011).

In addition, it should be taken into account that males with different sperm quality could respond differently using different density gradients. Therefore, it would be interesting to use the best density gradient methods according to the initial sperm quality.

With this background, the objective of our work was to assess the efficacy of different density gradients methods (Percoll[®], Puresperm[®] and BovipureTM) in males of Iberian red deer classified as with high and low sperm quality after thawing.

MATERIAL AND METHODS

Reagents

Chemicals were reagent grade and were purchased from Sigma (Madrid, Spain). Fluorescence probes were purchased from Invitrogen (Barcelona, Spain), except propidium iodide (PI), from Sigma. Chromatographically purified acridine orange was purchased from Polysciences Inc. (Warrington, PA, USA). Triladyl[®] was purchased from Minitüb (Tiefenbach, Germany) and Percoll[®] (Ref. P1644), Puresperm[®] and Bovipure[™] from Sigma (Madrid, Spain) and from Nidacon (Göteborg, Sweden) respectively.

Sperm collection and cryopreservation

Sperm samples were collected from epididymides of 8 Iberian red deer (*Cervus elaphus hispanicus*) hunted during the rutting season (September-October) between 1995 and 1998 according Spanish Harvest Regulation, Law 2/93 of Castilla-La Mancha, which conforms to European Union Regulation. Testes were transported to the laboratory at 20°C within 3 to 6 hours after death. Sperm samples were collected from distal portion of epididymides by cuts and diluted in the Triladyl[®] extender (20% egg yolk and 6% glycerol) to concentration $\sim 400 \times 10^6$ spermatozoa/mL determined with Bürker chamber. After that, sperm motility in diluted fresh samples was assessed subjectively and the sperm samples with a minimum of 70% motile spermatozoa were processed. Extended sperm samples were cooled to 5°C for 90 min, holding them to this temperature for 2 h more and freezing them in nitrogen vapours in 0.25 mL straws.

Sperm evaluation

Thawing was performed to 37 °C for 30 s. After that, sperm motility (SM) was assessed. To evaluate the effect of thawed sperm quality on the efficiency of selection methods, males were classified as with high sperm quality after thawing when $SM \geq 70\%$, or as with low sperm quality after thawing when $SM \leq 69\%$. Sperm samples from males belonging to the same sperm quality group were pooled. The pool was split and aliquots from one of the parts were used to assess different sperm quality parameters: sperm motility (SM) assessed subjectively, acrosome integrity (NAR) by phase-contrast microscopy as described by García-Álvarez *et al.* (2009), objective motility by computer-assisted semen analysis (SCA[®]) assessing average path velocity (VAP) and linearity (LIN) as described by Domínguez-Rebolledo *et al.* (2009), and membrane stability (YO-PRO-1-/PI-: intact spermatozoa and YO-PRO-1+/PI-: apoptotic spermatozoa), mitochondrial membrane potential (MT+/PI-: viable spermatozoa with active mitochondria) as described by García-Álvarez *et al.* (2009) and DNA fragmentation index [%DFI: percentage of spermatozoa with DNA fragmentation index (DFI) > 25] using the sperm chromatin structure assay (SCSA[®]) as described Evenson *and* Jost (2001) assessed by means of flow cytometry analysis.

Sperm selection

The remainder of the pool was used for the sperm selection process. The selection procedures were carried out at room temperature. For sperm selection the Percoll[®], Puresperm[®] and Bovipure[™] density gradients were used. Percoll[®] and Puresperm[®] density gradients were diluted with bovine gamete medium (BGM) to obtain 90% and 45% Percoll and Puresperm solutions. For 90% Percoll, 9 parts of Percoll[®] and 1 part of BGM 10 X were mixed, whereas for 90% Puresperm, isotonic BGM was used. Solutions of 90% Percoll and Puresperm were mixed v:v with isotonic BGM to obtain solutions 45% Percoll and Puresperm. Bovipure[™] was prepared by using ready-to-use solutions. Each density gradient was made with two layers of 1 mL in 15 mL centrifuge tubes. Aliquots from sperm samples from males with high and low sperm quality were layered (0.8 mL) over each discontinuous density gradient and centrifuged at 700 g for 15 min. After centrifugation, the supernatant above the pellet was carefully removed. Pellet (0.6 mL) was washed in 5 mL of Boviwash[™] by centrifugation (300 g for 10 min) leaving 0.2 mL of final pellet after removing the supernatant. Then, all sperm parameters previously described were assessed. Also, the percentage of recovered spermatozoa (RS) was calculated as $(\text{Volume} \times \text{Concentration})_{\text{after centrifugation}} / (\text{Volume} \times \text{Concentration})_{\text{before centrifugation}}$.

Statistical analysis

Statistical analysis was performed using SPSS for windows v. 15 (SPSS Inc, Chicago, III). A GLM-ANOVA was carried out to test the effect of male group (high and low sperm quality after thawing) on sperm quality after thawing to assure a correct classification of the sperm quality group. After thawing, there were significant differences between males classified as with high and low sperm quality for most sperm parameters except for %DFI (Table 1). As expected, all sperm parameters were higher for the males with high sperm quality in relation to low ones. Since these results were not the aim of this work

they were not discussed and were only showed in the Table 1. In addition, the effects of density gradient (Percoll[®], Puresperm[®] and Bovipure[™]) and the male group (high and low sperm quality) and the interaction density gradient $\times$ male group on sperm quality after sperm selection were tested by GLM-ANOVA. Significance was set to $P \leq 0.05$ and the comparisons were made by using the Bonferroni test. The experiment was replicated three times.

RESULTS

After sperm selection treatment, both males with high and low sperm quality improved the values for most sperm parameters in relation to sperm quality after thawing (Table 1). Males with high sperm quality obtained higher significant values after selection for RS, SM, NAR, YO-PRO-1-/PI-, YO-PRO-1+/PI- and MT+/PI- in relation to males with low sperm quality (Table 1). However, the values for objective motility parameters (VAP and LIN) and %DFI were similar for both quality groups (Table 1).

Regarding to the effect of density gradients on sperm quality, RS was similar when sperm samples were selected with Percoll[®], Puresperm[®] and Bovipure[™] (Table 1). For Percoll[®] and Puresperm[®] higher significant values of SM, YO-PRO+/PI- and MT+/PI- were obtained in relation to Bovipure[™] and higher significant values of YO-PRO-1-/PI- for Puresperm[®] in relation to Percoll[®] and Bovipure[™] (Table 1). There were no significant differences between selection methods for NAR, VAP, LIN, and %DFI (Table 1).

There was a significant interaction between type of density gradient and sperm quality group for YO-PRO-1-/PI- ($P = 0.04$) and YO-PRO+/PI- ($P = 0.003$) parameters. Thus, for the males with high sperm quality there were no differences between density gradients for both parameters. However, for the males with low sperm quality, the value of YO-PRO-1-/PI- was higher with Puresperm[®] and Bovipure[™], and the value of YO-PRO+/PI- was higher with Percoll[®] (Fig. 1).

DISCUSSION

In the different studies about *in vitro* fertilization carried out in red deer the methods used for selecting spermatozoa have been swim-up and density gradients (Comizzoli *et al.* 2001; Berg *et al.* 2002; García-Álvarez *et al.* 2010). According to the density gradients, the only method employed to date in this cervid species has been Percoll[®]. In this study we have assessed the chance to replace Percoll[®] by others density gradients as Puresperm[®] and Bovipure[™] since, using Percoll[®] exists the risk of contaminations with endotoxins (see review by Henkel and Schill 2003).

Our results have showed that there were no differences between Percoll[®] and Puresperm[®] for most sperm parameters studied after sperm selection, except for YO-PRO-1-/PI- with a higher value for Puresperm[®]. These results agree with those reported by Centola *et al.* (1998), Claassens *et al.* (1998) and Söderlund and Lundin (2000) who did not find differences between selecting with both density gradients. In our work, the best result obtained with the Puresperm[®] in relation to YO-PRO-1-/PI- could be due to the osmolality registered in the lower layer of density gradient (Percoll: ~ 265 mOsm/kg and Puresperm: ~ 305 mOsm/kg). Membranes of thawed spermatozoa show sublethal damages being susceptible to the osmotic difference between intra- and extracellular osmolalities and to the water movement through the membrane (Holt, 2000). Osmotic shock should be higher when thawed spermatozoa are recovered in lower layer from 90% Percoll and later washed with the isotonic solution Boviwash[™] (~ 300 mOsm/kg). In addition, our results have showed that the detrimental effect of Percoll[®] for YO-PRO-1-/PI- and YO-PRO+/PI- was more evident in males with low sperm quality. It is possible that these samples could have a higher sublethal damage in relation to sperm samples from males with high sperm quality and therefore are more susceptible to osmotic differences through the membrane.

However, in this work the worst sperm quality was obtained after selecting the spermatozoa with the isotonic solution Bovipure[™] in relation to Percoll[®] and Puresperm[®]. Our results are not in agreement with those reported by Samardzija *et al.* (2006) who did not find differences between used Bovipure[™] or Percoll[®] for selecting sperm samples in bovine. Since Puresperm[®] and Bovipure[™] are very similar (both isotonic salt solutions containing colloidal silica particles coated with silane), it is possible that the Bovipure[™] procedure used by us has not been the optimum for this density gradient method.

Our results have showed that after selection procedure most sperm parameters improved for males with high and low sperm quality in relation to thawed sperm samples. After selection, males with high sperm quality showed higher values for most sperm parameters in relation to low sperm quality ones, except for velocity parameters and %DFI. These results agree with those reported by Gellert-Mortimer *et al.* (1988) who found a relation between the initial sperm motility and final sperm motility following selection. It is possible that sperm samples with poor sperm quality are more susceptible to the selection and centrifugation procedures and for this reason never reach the values of sperm samples with high quality.

In conclusion, the results from this study show that Puresperm[®] can be used to replace Percoll[®] when density gradient is performed for selecting epididymal spermatozoa from Iberian red deer. Nevertheless, more studies are necessary to know the fertilization ability from spermatozoa selected by Puresperm[®] and Percoll[®].

ACKNOWLEDGEMENTS

This work was supported by the Spanish Ministry of Science and Innovation (Project AGL2010-21487). Olga García-Álvarez, Pilar Jiménez-Rabadán and Alejandro Maroto-Morales were recipients for scholarships from INIA and JCCM, respectively.

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Table 1. Sperm quality after selection procedure with different density gradients in males of Iberian red deer with high and low sperm quality after thawing

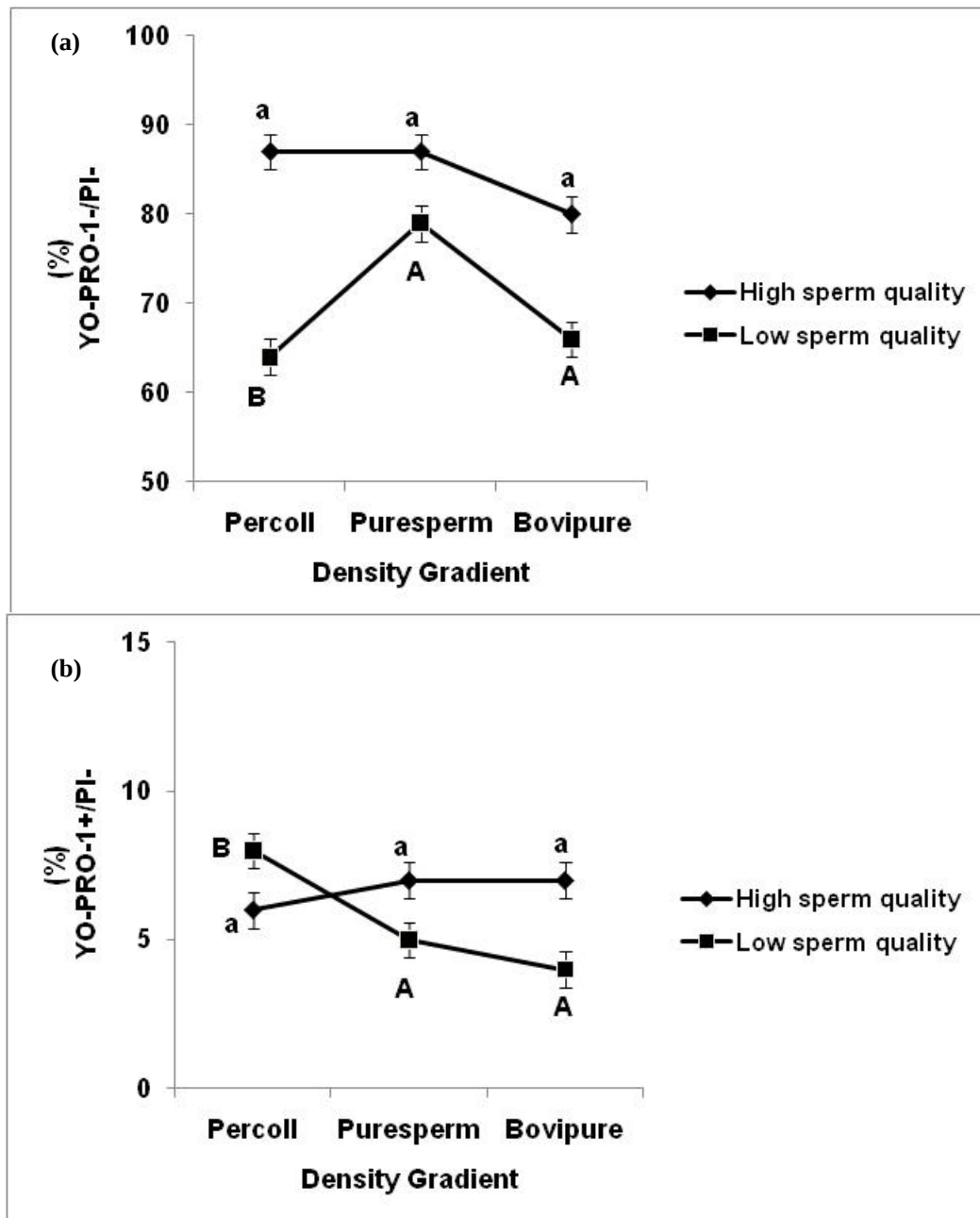
Data are least square mean $\pm$ s.e.m.

RS, recovered spermatozoa; SM, sperm motility; NAR, acrosome integrity; VAP, average path velocity; LIN, linearity; YO-PRO-1/-PI-, intact spermatozoa; YO-PRO-1+/PI-, apoptotic spermatozoa; MT+/PI-, viable spermatozoa with active mitochondria; %DFI, percentage of spermatozoa with DNA fragmentation index > 25 .

Within columns, least square means followed by different letters are significantly different

Treatment		RS (%)	SM (%)	NAR (%)	VAP ($\mu\text{m}/\text{sec}$)	LIN (%)	YO-PRO-1-/PI- (%)	YO-PRO-1+/PI- (%)	MT+/PI- (%)	%DFI
Sperm Quality After thawing	High	-	77.50 $\pm$ 0.71a	85.00 $\pm$ 2.38a	74.37 $\pm$ 2.58a	28.49 $\pm$ 0.77b	40.93 $\pm$ 3.13a	38.06 $\pm$ 1.98a	32.30 $\pm$ 3.38a	15.82 $\pm$ 3.16a
	Low	-	41.25 $\pm$ 0.71b	49.25 $\pm$ 2.38b	61.10 $\pm$ 2.58b	41.09 $\pm$ 0.77a	21.80 $\pm$ 3.13b	22.24 $\pm$ 1.98b	14.49 $\pm$ 3.38b	13.45 $\pm$ 3.16a
	<i>P</i> -value	-	0.001	0.001	0.002	0.001	0.001	0.001	0.001	0.846
Sperm Quality After selection	High	23.32 $\pm$ 1.48a	70.14 $\pm$ 2.09a	85.36 $\pm$ 3.18a	107.63 $\pm$ 6.67a	62.33 $\pm$ 2.38a	85.10 $\pm$ 1.65a	7.25 $\pm$ 0.38a	74.43 $\pm$ 3.46a	14.64 $\pm$ 4.26a
	Low	14.75 $\pm$ 1.48b	59.44 $\pm$ 2.01b	63.78 $\pm$ 3.06b	105.79 $\pm$ 6.67a	57.24 $\pm$ 2.38a	70.06 $\pm$ 1.65b	6.03 $\pm$ 0.38b	53.55 $\pm$ 3.46b	16.58 $\pm$ 4.26a
	<i>P</i> -value	0.001	0.003	0.001	0.849	0.157	0.001	0.032	0.001	0.749
Density gradient	Percoll [®]	15.16 $\pm$ 2.13a	72.50 $\pm$ 2.45a	76.83 $\pm$ 3.75a	106.46 $\pm$ 8.17a	64.54 $\pm$ 2.12a	76.01 $\pm$ 2.02b	7.71 $\pm$ 0.47a	64.27 $\pm$ 4.24ab	13.01 $\pm$ 5.22a
	Puresperm [®]	18.37 $\pm$ 2.13a	66.04 $\pm$ 2.30a	79.96 $\pm$ 3.50a	110.43 $\pm$ 8.17a	55.21 $\pm$ 2.12a	83.37 $\pm$ 2.02a	6.31 $\pm$ 0.47ab	71.78 $\pm$ 4.24a	15.56 $\pm$ 5.22a
	Bovipure TM	16.57 $\pm$ 2.13a	55.83 $\pm$ 2.75b	66.92 $\pm$ 4.19a	103.24 $\pm$ 8.17a	59.60 $\pm$ 2.12a	73.36 $\pm$ 2.02b	5.90 $\pm$ 0.47b	55.91 $\pm$ 4.24b	18.27 $\pm$ 5.22a
	<i>P</i> -value	0.210	0.003	0.089	0.826	0.119	0.005	0.028	0.046	0.778

Figure 1. Interaction between density gradient and sperm quality group for (a) YO-PRO-1-/PI- and (b) YO-PRO-1+/PI-
Different letters on the same line indicate significant differences between means



RECOVERY OF EPIDIDYMAL SPERMATOZOA FROM BULL AND RED DEER, STORED AT DIFFERENT TIMES AND TEMPERATURES BEFORE FREEZING-THAWING

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ABSTRACT

As a first trial, epididymal spermatozoa from slaughtered bulls (*Bos taurus*) were recovered at 30, 54, 78 and 102 h after death. The scrotal contents were stored at both 5 and 20°C. The sperm cells of each treatment (time + temperature combinations) were frozen with Triladyl (T) or Triladyl + Trehalose (TT) diluents. In order to assess the sperm viability and integrity, post-thawing evaluation included individual motility, sperm viability, hypoosmotic swelling test, acrosome status and sperm chromatin structure assay.

At raw and post-refrigerated states, the sperm motility rate was higher in the 5°C stored group, compared with the 20°C stored group for all times, maintaining a motility of 20% at 5°C for 102 h (raw state). When comparisons were carried out at post-thawing state, motility was higher in the 5°C group, achieving, at 102 h the best results with TT diluent (7.5%). However, when supravital stain and HOSTest were observed, viability and membrane integrity were well preserved even at 102 h post-mortem (30% and 36% with TT diluent at 5°C, respectively). These results may confirm that low motility rate could be observed in frozen-thawed epididymal spermatozoa while most of them remain alive. Acrosome status was not greatly affected by storage time.

As a second trial, epididymal spermatozoa from hunted red deer stags (*Cervus elaphus*) were recovered at 4 and 30 h after death. The scrotal contents were stored at 20°C. The sperm cells were frozen with Triladyl + Trehalose (TT) diluent. Post-thawing evaluation included the same parameters indicated for bull spermatozoa. The assessment of 30 h frozen-thawed spermatozoa may confirm that in this time, an acceptable motility rate (35%) and viability (39.7%) were achieved. *In vivo* preliminary trial showed that, after artificial insemination, the pregnancy rate decreased when 30 hours post-mortem elapsed.

According to these results, Triladyl + Trehalose preserve viable and fertile sperm cells after the freezing-thawing process.

Additional keywords: deer, frozen-thawed, sperm cells, hypertonic extenders, epididymal spermatozoa.

INTRODUCTION

Some species offer opportunities for reproduction studies, which can be applied to different contexts such as farming, hunting, and conservation of endangered species or populations. At present, many ruminant species are used as experimental models for studies of epididymal spermatozoa cryopreservation, recovered after death. For the first step, bull was selected due to the possibility to determine the exact time elapsed after death. For the second step, red deer was used, because the results obtained can be applied in conservation of other cervids. Thus, both species result useful experimental models for assisted reproduction programs applications.

The aims of this work were: (i) to determine how the time *post mortem* and storage temperature affect the cryopreservation of bull epididymal spermatozoa using two extenders; (ii) to evaluate the application of some results obtained in bull (time post mortem) on cryopreservation of red deer epididymal spermatozoa using a hypertonic extender.

MATERIALS AND METHODS

As a first trial, spermatozoa from eight epididymis (caudal section) of four slaughtered bulls (*Bos taurus*) were recovered (Goovaerts *et al.* 2006) and the experiment was designed as follows: time after death – 30; 54; 78 and 102 hours (Nichi *et al.* 2007); storage temperature – refrigerated (5°C) and room (20°C); diluents – (i) Triladyl (T), containing Tris, citric acid, fructose, glycerol and egg yolk (final osmolality =

1430 mOsm) and (ii) Triladyl + Trehalose (TT), containing T and 100 mOsm trehalose (final osmolarity = 1565 mOsm).

As a second trial, spermatozoa were recovered from ten epididymes (caudal section) of five hunted red deer stags (*Cervus elaphus*) at 4 and 30 h after death. The mean storage temperature was 20°C, varying according to field and shipment conditions (scrotal content wrapped with paper, and put into a cell foam box). The sperm cells were diluted in Triladyl + Trehalose (TT) diluent.

Epididymal spermatozoa from both trials (bull and deer) were pooled and evaluated at 30°C. The cells were diluted to obtain 400 x 10⁶ cells/mL, cooled to 5°C at -1°C/min cooling rate, loaded into 0.25 mL straws, frozen and stored in liquid nitrogen.

Thawing was performed in a bath at 37°C during 20 s. Then, the samples were diluted in PBS (1:10), and equilibrated for 20 min before evaluation. The *in vitro* parameters for sperm cells assessment were: individual motility (M), by videomicroscopy and subjective evaluation; Hypoosmotic Swelling Test (E+), with sodium citrate mOsm solution; Acrosome status (AS), by phase contrast videomicroscopy; sperm viability (NE), by nigrosin-eosin test, and Sperm Chromatin Structure Assay (SCSA), using the method of Martins *et al.* (2007).

An *in vivo* test was performed only in red deer, under laparoscopic artificial insemination (AI) of 20 synchronized does with CIDR and eCG (Larreguy *et al.* 1999). Pregnancy rate was determined by ultrasonography at 35 days post insemination.

In vitro results were analyzed with ANOVA test, and *in vivo* data by heterogeneity test (χ^2).

RESULTS AND DISCUSSION

At raw state, bull spermatozoa showed a gradual decrease in M values as time *post mortem* increased, being higher at 5° C than at 20° C. At cooled state, M values decreased drastically compared with raw sperm only with spermatozoa kept at 20°C. At 5°C sperm was not affected significantly by this process before 78 h. We did not find significant differences between diluents at post-thawing state (Table 1). Motility showed a similar behavior to that observed in cooled state, the values with TT diluent were higher than those with T diluent, from 54 h at 5°C. E+ values at 5°C were higher with TT than with T at 30 and 54 h, whereas AS showed no significant differences between times *post mortem*.

At post-thawing, NE -in accordance with M- was better for 5°C, and TT diluent shows better protection capacity than T from 54 h. SCSA showed a significant damage from 78 h *post mortem*, 5°C proving to be a better temperature of conservation. It was demonstrated that trehalose has two effects on cells: by dehydrating them, as a non-penetrating disaccharide (Crowe *et al.* 1990), and specifically by interacting with membrane phospholipids during freezing (Bakás *et al.* 1991). That confers better cryoprotection and post-thaw fertilizing ability (Aisen *et al.* 2002).

In red deer (Fig. 1), M and AS parameters were higher at 4 h than 30 h *post mortem*, whereas NE and E+ showed not differences among time elapsed. SCSA values demonstrated that at 30 h the cell chromatin damage increased.

In vivo preliminary trial showed that, after laparoscopic insemination, the pregnancy rate decreased (not significantly) when 30 h *post mortem* elapsed compared with 4 h (28.6 % v. 38.5 % respectively).

To conclude, a low motility rate can be observed in frozen-thawed epididymal spermatozoa while most of the cells remain alive. The best storage temperature of bull scrotal content was 5°C in most parameters. TT diluent showed higher cryoprotective activity according to membrane integrity (both in head and tail domains) and motility, especially at 54–78 h *post mortem*. In those cases when the sperm cells were recovered at 30 h *post mortem*, the best recommended diluent was Triladyl + Trehalose. The chromatin damage increased strongly after 78 h *post mortem* (especially for 20°C).

In red deer, the observations in all evaluated parameters were similar to bull's parameters at 30 h *post mortem* (frozen state). Motility and viability percentages at 4 h *post mortem* were similar to other authors (Martínez Pastor *et al.* 2005; Martínez Pastor *et al.* 2009). The pregnancy rate decreased when 30 h *post mortem* elapsed compared with 4 h. These results are slightly higher than those obtained with AI with sperm cells recovered at 20 h after death (Garde *et al.* 1998; Larreguy *et al.* 1999).

Obtaining viable and fertile sperm cells from epididymis of dead males permits the preservation of genetic material for the creation of cryopreserved germoplasm banks (Locatelli 2008). These banks could be useful to maintain the variability of vulnerable species, with several assisted reproduction techniques (AI; IVF; ICSI; MOET).

ACKNOWLEDGEMENTS

Pablo Malmoria (COPROMANEU, Senillosa, Neuquén) and Alejandra Dabrowski (Facultad de Lenguas, Universidad Nacional del Comahue).

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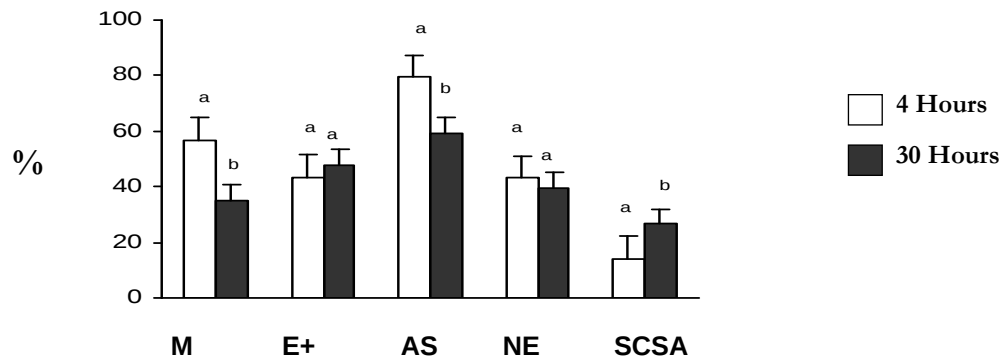
Table 1: Bull epididymal spermatozoa parameters (post-thawing state) under different treatments (Time post mortem, Temperature and Diluent)

T, Triladyl; TT, Triladyl + Trehalose; M, individual motility; E+, Hypoosmotic Swelling Test; AS, Acrosome status; NE, Sperm viability; SCSA, Sperm Chromatin Structure Assay
n.s., not significant. * $P<0.05$. ** $P<0.01$.

Values are expressed as mean $\pm$ s.e.

Time post mortem (h)	Treatment Temperature (°C)	Diluent	M (%)	E+ (%)	AS (%)	NE (%)	SCSA (%)
30	5	T	60 $\pm$ 7.2	48 $\pm$ 11.3	50 $\pm$ 2.8	44 $\pm$ 2.8	5 $\pm$ 1.4
30	5	TT	57.5 $\pm$ 3.5	54 $\pm$ 12.7	61.5 $\pm$ 2.1	35 $\pm$ 1.4	4 $\pm$ 2.8
30	20	T	22.5 $\pm$ 4.2	33 $\pm$ 1.4	60.5 $\pm$ 12	34 $\pm$ 8.4	12 $\pm$ 4.2
30	20	TT	17.5 $\pm$ 3.5	33 $\pm$ 2.8	56 $\pm$ 5.6	18 $\pm$ 8.5	4 $\pm$ 1.4
54	5	T	40 $\pm$ 7.1	41 $\pm$ 7.1	55 $\pm$ 7.1	34 $\pm$ 2.8	6 $\pm$ 2.8
54	5	TT	45 $\pm$ 7.1	54 $\pm$ 8.5	58.5 $\pm$ 19	40 $\pm$ 7.1	7 $\pm$ 4.2
54	20	T	5 $\pm$ 1.4	28 $\pm$ 5.6	54.5 $\pm$ 13.4	22 $\pm$ 2.9	12 $\pm$ 1.4
54	20	TT	1 $\pm$ 0	33.5 $\pm$ 2.1	62 $\pm$ 2.8	24 $\pm$ 5.6	7 $\pm$ 2.8
78	5	T	7.5 $\pm$ 3.5	36.5 $\pm$ 2.1	68.5 $\pm$ 4.9	38 $\pm$ 2.8	16.5 $\pm$ 2.1
78	5	TT	10.5 $\pm$ 2.8	40 $\pm$ 11.3	62 $\pm$ 2.8	46 $\pm$ 2.9	13.5 $\pm$ 0.7
78	20	T	5 $\pm$ 2.8	35 $\pm$ 1.4	48 $\pm$ 16.9	26 $\pm$ 14.1	28 $\pm$ 4.2
78	20	TT	0	35 $\pm$ 7.7	56 $\pm$ 14.1	14 $\pm$ 8.5	24.5 $\pm$ 2.1
102	5	T	3.5 $\pm$ 2.2	37.5 $\pm$ 3.5	58.5 $\pm$ 9.2	24 $\pm$ 1.4	46 $\pm$ 4.2
102	5	TT	7.5 $\pm$ 3.5	36 $\pm$ 5.6	57 $\pm$ 1.4	30 $\pm$ 14.1	30 $\pm$ 2.1
102	20	T	0	25.5 $\pm$ 13.4	50.5 $\pm$ 13.4	8 $\pm$ 2.9	82 $\pm$ 4.2
102	20	TT	0	21 $\pm$ 2.8	58 $\pm$ 2.8	4 $\pm$ 1.4	59 $\pm$ 2.8
Time			**	*	n.s.	**	**
Temperature			**	**	n.s.	**	**
Diluent			n.s.	n.s.	n.s.	n.s.	**
Time*Temperature			**	n.s.	n.s.	n.s.	**
Time*Diluent			n.s.	n.s.	n.s.	n.s.	**
Temperature*Diluent			*	n.s.	n.s.	*	*

Figure 1: *In vitro* parameters in red deer epididymal spermatozoa with Triladyl+Trehalose diluent (post-thawing state), under different times *post mortem*. Values expressed as mean $\pm$ s.e. M, individual motility; E+, Hypoosmotic Swelling Test; AS, Acrosome status; NE, Sperm viability; SCSA, Sperm Chromatin Structure Assay. Similar letters indicate that values are not significant.



RED DEER INTRODUCED TO PATAGONIA

1. DISEASES AND IMPLICATIONS FOR NATIVE UNGULATES

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ABSTRACT

The red deer (*Cervus elaphus*) invasion in Patagonia has been continuing for nearly a century. Red deer are found in all habitats between 34-55°S, occupying >51,000 km², and reaching densities of 100 deer/km² in ecotones and 40-50 deer/km² in steppes. Their distribution, movement patterns and density raise concerns over their potential epidemiological role (e.g. Foot and mouth disease, brucellosis, tuberculosis). Adult females collected by rifle and radiocollared deer that died naturally were necropsied in the field to determine gross pathology, and whole specimens were also examined in a national pathology laboratory. No ectoparasites were found ($n = 73$). *Fasciola hepatica* was encountered at prevalences of 9-50% ($n = 108$). *Taenia ovis krabbei* was identified, and *Cysticercus tenuicollis* was found at a prevalence of 8% ($n = 12$). *Ostertagia* sp., *Bunostomum* sp., and *Dictyocaulus* sp. had a prevalence of 75%, 25% and 13%, respectively. Testing for Foot and mouth disease was negative ($n=41$). A suspect case of tuberculosis had substantial parietal pleural adhesions, granulomatous mediastinal lymph nodes, nodules covering the costal pleura and liver, lesions in the intestinal tract including lymph nodes covering the rumen. Antler damage was found on 73% of shed antlers, with 36% having major breaks of tines and main beam, possibly indicating mineral imbalances. One male not only had broken tips and tines, but both entire antlers, including pedicles with portions of frontal, parietal and occipital bones, were broken off the skull, causing his death. Regarding native huemul (*Hippocamelus bisulcus*), several gastrointestinal parasites found at low prevalence in Chile are common in livestock and considered commensals in ruminants. Sympatry of huemul with livestock is commonplace, whereas with red deer it occurs in <2% of known populations. In one area of sympatry, per huemul there were 1.2 red deer, but 25.2 livestock, making livestock the determining epidemiological factor. Considering that red deer have been coexisting with livestock for >100 years in Argentina, both red deer and livestock play epidemiological roles for shared diseases. Research, conservation and management efforts should be directed towards livestock herd health programs or restriction of free livestock movements, particularly if diseases are shown to impact on recruitment of threatened natives. Livestock, being routinely researched and inspected at slaughtering time can provide a proxy for diseases afflicting co-existing ungulates.

INTRODUCTION

Argentina has a long tradition of animal introductions, beginning with livestock that reached millions of feral cattle, horses and goats shortly after Spanish settlement (Torrejon 2001). Exotic wildlife were introduced later and included red deer (*Cervus elaphus*), which are considered to be one of the world's 14 worst mammalian invasive species (IUCN 2002, www.issg.org). Red deer expansion into the southern cone of Latin America began less than a century ago, and has yet to reach a state of equilibrium (Flueck and Smith-Flueck 1993; Flueck *et al.* 2003).

Red deer have established themselves in all mayor habitats and currently are established in most forested habitat types encountered between about 34-55°S. The area occupied in 2003 was estimated >51,000 km², consisting of 29% forest habitat, 57% Patagonian steppe habitat, and 14% of non-forested habitat such as wet meadows and riparian habitat, brush or grasslands of anthropogenic origin, and high altitude vegetation above the tree line (Flueck *et al.* 2003). In Chile, they were estimated to occupy about 7700 km². The present distribution of red deer has the following environmental characteristics: it covers the latitudes between 37°42'S and 54°55'S (noncontiguous); longitudes between 73°36'W and 69°50'W (noncontiguous); and altitudes from sea level to > 2450 m (Flueck *et al.* 2003). Within the present distribution, red deer may number >100,000 animals at an average density of about 2 deer/km². This appears to be a conservative estimate considering that favorable ecotonal habitats have revealed densities around 100 deer/km² whereas in steppe areas they reached 40-50 deer/km² (J. Amaya pers. comm.; Flueck *et al.* 1995). Initially forming resident

populations, red deer now also have migratory segments with individuals traveling 25 km, and cases of dispersal going 40 km (Flueck 2005). In the study area reported here, red deer are commonly sympatric with cattle, horses, sheep, goats and wild species like fallow deer (*Dama dama*), wild boar (*Sus scrofa*), and guanaco (*Lama guanicoe*).

The distribution, movement behavior, and uncontrolled national and transnational shipments of red deer (Flueck and Smith-Flueck 2006; Flueck 2010a) raise concerns over their potential epidemiological role for various diseases such as foot and mouth disease (FMD), brucellosis and tuberculosis (Flueck 2005; Flueck and Smith-Flueck 1993; Flueck *et al.* 2003, 2006). Here we report for the first time on various diseases encountered in red deer in Patagonia and evaluate their potential to impact native ungulates.

MATERIALS AND METHODS

The study area is centered in the Patagonian ecotone (40°58'S, 71°12'W), Argentina. The topography is primarily mountainous with most features formed by glacial processes. The dominant climate is temperate with most precipitation occurring between April and September. There is an abrupt precipitation gradient from west to east due to the Andean orography which results in a strongly defined vegetation structure and floristic composition. The study sites are between 900-1200 m elevation and represent grasslands, or ecotones between forests and grasslands. Patches of forests are characterized by false beech (*Nothofagus antarctica*) and cypress (*Austrocedrus chilensis*) at lower elevations and are replaced by deciduous lenga (*Nothofagus pumilio*) at higher elevations. Forest patches at lower elevations alternate with wet grasslands with abundant growth of herbaceous plants whereas at high elevation they are replaced by grass-dominated steppe containing *Stipa speciosa* var. *major* and *Festuca pallescens*, with variable occurrence of brush species like *Mulinum spinosum*, *Berberis* spp. and *Colletia spinosissima*. Riparian areas also contain galleries of *Lomatia hirsuta*, *Maytenus boaria* and *Schinus patagonicus* trees.

Females older than one year were collected randomly by rifle between 1991 and 2009 and radio collared deer that died naturally were evaluated between 2001 and 2009. All individuals were necropsied in the field according to techniques described by Salwasser and Jessup (1978) and Wobeser and Spraker (1980). Time, location and morphometric measurements were recorded (Mitchell *et al.* 1976). The focus of deer collections varied, relating either to issues of reproduction, development, seasonal body condition, population dynamics, genetics or gross pathology. The examination for the presence of diseases was thus not equally intense in every situation, and prevalence is only indicated where an adequate number of individuals had been examined for that purpose (Davis and Anderson 1971; Davis *et al.* 1981; Jones *et al.* 1997; Williams and Barker 2001; Samuel *et al.* 2001). Additionally, in 1994–95 we collected whole female deer, which were examined exhaustively, including the gastrointestinal tract for parasitology, in the pathology laboratory of the National Institute for Agricultural Technology, INTA. The four study areas where animals were collected were: A (41°00'S, 71°17'W), B (40°59'S, 71°11'W), C (40°57'S, 71°11'W), and D (40°29'S, 70°59'W). Considering possible nutritional deficiencies, the damage on antlers and breakage were analyzed. Damage on antlers was classified as broken tips of tines, tines completely broken off, and breakage of the main beam.

RESULTS

Ectoparasites were not present upon gross examination in study area A ($n = 21$) (Flueck *et al.* 1993), nor in the 1995 study area C ($n = 9$, Flueck *et al.* unpublished data), nor in the study site D ($n = 43$) (Smith-Flueck and Flueck 1998). *Fasciola hepatica* were encountered regularly among deer: at site A, the prevalence in the population was 50% ($n = 20$) (Flueck *et al.* 1993, 1995 unpublished data), whereas at site D it was 9% ($n = 43$) (Smith-Flueck and Flueck 1998). Additional deer examined between 1998–2009 in area A exhibited a prevalence of 13% ($n = 45$). *Taenia ovis krabbei* was identified based on hook characters, size, appearance, and anatomical location of a cysticercus. A sylvatic cycle involving red deer appears to exist (Flueck and Jones 2006). On several occasions we found taenid larval stages attached to liver, omentum and peritoneum, with the typical gross appearance of *Cysticercus tenuicollis*, which were deposited and identified by the state agency INTA. In the study area A their prevalence was 8% ($n = 12$) (Flueck *et al.* 1993). *Ostertagia* sp. were found at a prevalence of 75%, with two individuals carrying 800 and 900 adult parasites, respectively; *Bunostomum* sp. were found at a prevalence of 25%; and *Dictyocaulus* sp. occurred at 13% prevalence (Flueck *et al.* 1995 unpublished data). FMD was tested for by the Federal agency SENASA in deer from site B ($n = 41$) in 1994–95 to allow export of the tissue as part of a genetic study (Flueck and

Smith-Flueck 2011): all samples were negative (there had been an outbreak in livestock in 1991). Among an additional 393 specimens collected for reproductive and physical condition studies, the only notable finding was a suspected case of tuberculosis from 1996 in area D. We submitted fresh tissue from this specimen to INTA but no cultivation was done and histological sections were negative. There were substantial parietal pleural adhesions, granulomatous mediastinal lymph nodes, nodules covering the costal pleura, and additional lesions in the intestinal tract, including lymph nodes covering the rumen and nodules in liver (Fig. 1).

Broken antlers

Damage was found on 73% of shed antlers collected between 2006–2009 (Table 1, $n = 132$). Antlers with broken tips had on average 2 broken tips, and 36% of antlers had major breaks of tines and main beam (Fig. 2). An extreme case was a male that died during the rut from breakage of the skull and antlers remaining solely attached to skin. Not only were 2 tines and 7 tips broken off, but both pedicles with portions of frontal, much of the parietal and antero-dorsal portion of occipital bones were broken out such that the animal lost most of the upper brain case (Fig. 3). Incidentally, deer and cattle have been observed eating bone or antlers. One ranch reported in 2009 on 3 free-ranging domestic cows that continued to deteriorate, and when eventually captured for checking, all had bones stuck sideways in the mouth preventing them from foraging.

Table 1. Breakages on shed red deer antlers found between 2006-2009

Type of damage	<i>n</i>
Main beam only	2
Tines only	10
Main beam and tips	3
Main beam, tines and tips	1
Tines and tips	31
Tips only	50
No damage	35

DISCUSSION

The general absence of ectoparasites upon gross examination refers foremost to groups like ticks, fleas and lice. Similarly, it is common for wild ruminants like guanaco living in cold Patagonia to be devoid of ectoparasites (Karesh *et al.* 1998), although contact with sheep may lead to cross contaminations. Infections with *F. hepatica* and *C. tenuicollis*, both cosmopolitan parasites, as well as the exotic *T. o. krabbei*, are considered trivial for otherwise healthy cervid hosts. For the same reason, *Ostertagia* sp., *Bunostomum* sp. and *Dictyocaulus* sp. are also trivial, but the latter two can be a concern when deer are weak for other reasons. These parasites have been noted in Argentine Patagonia in livestock and wildlife including red deer (Flueck and Jones 2006; Suarez *et al.* 2007; this study).

Although it is not possible to differentiate between *T. o. ovis* and *T. o. krabbei* on morphological grounds with absolute certainty, red deer have been reported to be refractory to *T. o. ovis* infection whereas other potential intermediate hosts like cattle, goats, pigs and sheep have been shown to be refractory to *T. o. krabbei*. Possible or known definitive hosts in the study area include native felids like *Puma concolor*, *Felis colocolo*, *F. guigna* and canids like *Dusicyon griseus*, *D. culpaeus*, and domestic dogs. The adult stage of *C. tenuicollis*, the tapeworm *Taenia hydatigena*, is also found commonly in domestic and wild canids and felids in the region.

In Chile, *Ostertagia* sp., *Capillaria* sp., *Bunostomum* sp., *C. tenuicollis* and *Dictyocaulus* sp. have all been found at low levels in free-ranging exotic fallow and red deer, whereas fecal exams from 9 native huemul deer (*Hippocamelus bisulcus*) showed only very low levels; and in only 3 animals *Moniezia* sp. was confirmed and also considered to be non-significant to the hosts (Rioseco *et al.* 1979). Most all of these parasites, considered in general to be nonthreatening, are common in livestock and thus occur in most areas with livestock (Love and Hutchinson 2003). The suspected case of tuberculosis is a concern more for animal production systems with high densities of animals. The present case stemmed from a high density population of red deer at site D (about 50 deer/km², Flueck and Smith-Flueck unpublished data). As a zoonosis it requires caution by people involved with wild deer. Similar lesions (costal pleural, pulmonary, rumenal) have been described for white-tailed deer (*Odocoileus virginianus*) and red deer infected with *Mycobacterium bovis* (O'Brien *et al.* 2001; Glawischnig *et al.* 2006; Martin-Hernando *et al.* 2010).

There are frequent claims in the literature of huemul being highly susceptible to cattle diseases such as *C. tenuicollis*, FMD, coccidiosis, 'parasites', or actinomycosis (Povilitis 1978; Thornback and Jenkins 1982; Schuerholz 1985; Redford and Eisenberg 1992; Simonetti 1995; Wemmer 1998; Lord 2007). According to Simonetti (1995), "*Cysticercus tenuicollis*, when transmitted by livestock is fatal to huemul", citing Texera (1974). However, Texera stated that he did not consider the presence of *C. tenuicollis* to be the cause of death, rather that the condition of the female deteriorated after a premature parturition, aggravated by very little space and little variety of food provided. Furthermore, in other cervids and ungulates the presence of *C. tenuicollis* is considered of little significance (Leiby and Dyer 1971). Then, high susceptibility of huemul to coccidiosis is frequently mentioned and based on the only report on coccidiosis by Texera. However, Texera (1974) questioned whether it was the cause of death, because there existed many *other* problems, besides again referring to limited space and poor nutrition. A sick sheep was brought to the same pen later on, and huemul which subsequently died also had, among many other problems, coccidia. Anecdotal accounts by settlers are cited to claim that FMD via livestock was responsible for decimating huemul over huge areas 60-70 years ago. In contrast, recent FMD outbreaks in the UK resulted in experimental studies of 5 cervid species that were all susceptible to FMD to some degree. Based on natural behavior of these free-living deer in the UK, they are considered unlikely to be an important factor in the maintenance and transmission of virus during an epidemic of FMD in domestic livestock (Thrusfield and Fletcher 2002; Fletcher 2004). Earlier concerns about FMD in deer during an outbreak in 2001 in the UK proved to be unfounded (Davies 2002): numerous samples from deer showing lesions suggestive of FMD were sent in to the Pirbright laboratory but all proved to be negative. There has been no evidence of wild deer being implicated in this epidemic despite the fact that the deer population in Great Britain was 10 times greater than it was in 1967 (Davies 2002). At normal densities of cervids, FMD is considered a self-limiting disease (Morgan *et al.* 2003). The very low densities of huemul and reactions of other cervids to FMD renders those early anecdotal accounts by settlers doubtful. Additionally, even if FMD would reduce the population, after the outbreak is over and at the rate of increase of 1.21 documented in Chile for huemul, a population would have recovered by 300% in only 6 years. Thus, claims that FMD decimated huemul populations in the last century are unfounded. Lastly, a recent review on FMD found no reports of any previous disease nor outbreaks in wildlife populations under field conditions among several South American wild animal species susceptible to FMD (Pinto 2004).

The prevalence of antler breakage reported here was similar to that found in *C. elaphus* in California by Johnson *et al.* (2005), who suggested that it related to Cu and/or P deficiencies (Johnson *et al.* 2007). There are no reports of Cu deficiency in this Andean region, however, P is low in part due to volcanic soils (Wittwer *et al.* 1997; Thomas *et al.* 1999). Given that the area has been exploited for >100 years without fertilizer replacement, P levels might have continued to diminish. The harvest of red deer alone represents an export of P of about 1.8 kg P/female, whereas removing from the system the antlers shed by a male that is harvested later at age of 8 years exports about 7.2 kg of P (Flueck 2009). Removing livestock from the systems exports additional substantial P, and the estimated overall export rate for P compares to rates measured in other extensive production systems which, in contrast, receive 10-50 kg/ha.year of P as fertilizer to compensate the losses from biomass exports.

In conclusion, red deer were found to harbor a few diseases commonly associated with livestock and wildlife, with the only exception of *T. o. krabbei*, which though is a trivial parasite. Having been in Argentina for >100 years and in coexistence with livestock, both red deer and livestock play roles in the epidemiology of the various diseases they share. Regarding huemul, whereas livestock are commonly sympatric with them (probably 100% of huemul populations), spatial overlap with red deer is exceptionally rare, occurring in <2% of known populations, and is of recent time (Flueck 2010b). Even in this latter case, livestock presence is the determining epidemiological factor, since in the one (of two) population, for each huemul there were 1.2 red deer, but 25.2 livestock (Pastore and Vila 2003). Thus, for most if not all huemul populations the primary factor regarding infectious and parasitic diseases are feral and free-ranging livestock. Research, conservation and management efforts should thus be directed towards finding appropriate solutions, including livestock herd health programs or restriction of free livestock movements, particularly where huemul still occur in protected areas. Livestock, being regularly researched and inspected at slaughter provide a good proxy for the parasite community and other diseases afflicting sympatric red deer. Although sympatry between red deer and guanaco is common and substantial, including the formation of mixed groups, and has been suggested to affect the epidemiology of shared diseases (Flueck 1996; Flueck *et al.* 2003), we found no other reports expressing sanitary concerns. Lastly, uncontrolled importations of wild ungulates is of special

concern if it involves cervids, due to transmissible spongiform encephalopathy of cervids. The appearance of this disease could be disastrous for South America due to the large variety of endemic cervids.

ACKNOWLEDGEMENTS

We thank several landowners for access to their properties and evaluate red deer. We also like to acknowledge valuable comments by two anonymous reviewers. The study was funded in part by the Argentine government through FONCYT Pict99, CONICET grant 87/99 and 327/04, Ministry of Foreign Affairs through NZAID, and Swazi, New Zealand.

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Figure 1. Female red deer: (a) Lesions in rumenal lymph tissue, (b) Pulmonary adhesions and costal pleural and pulmonary lesions, (c) nodules in liver.

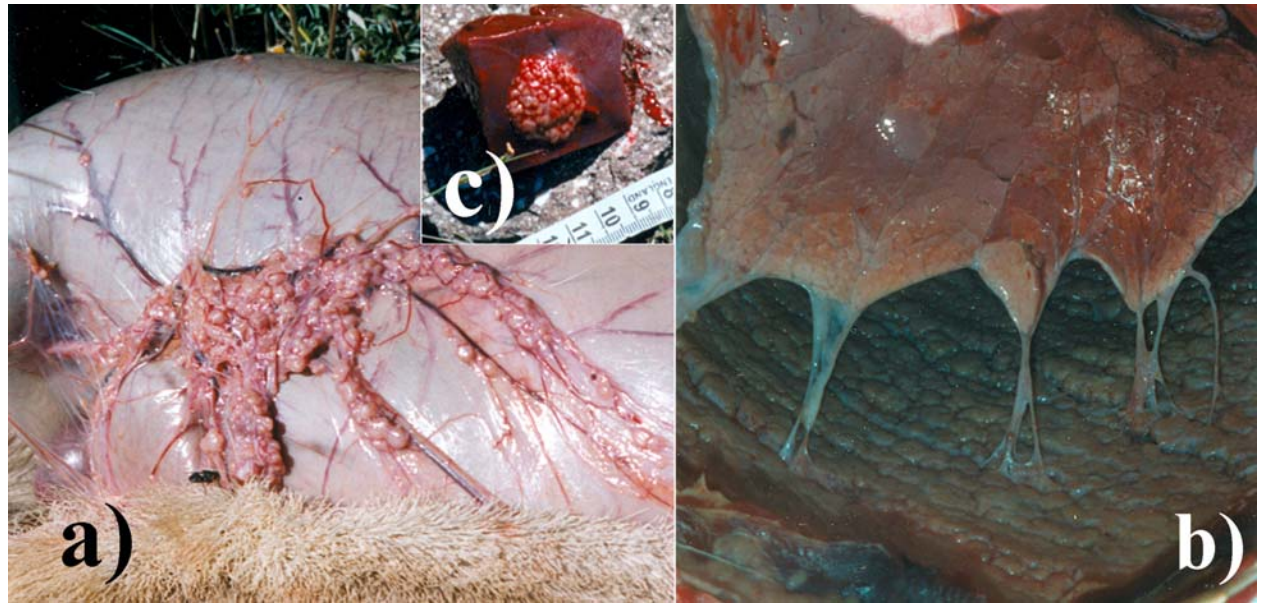
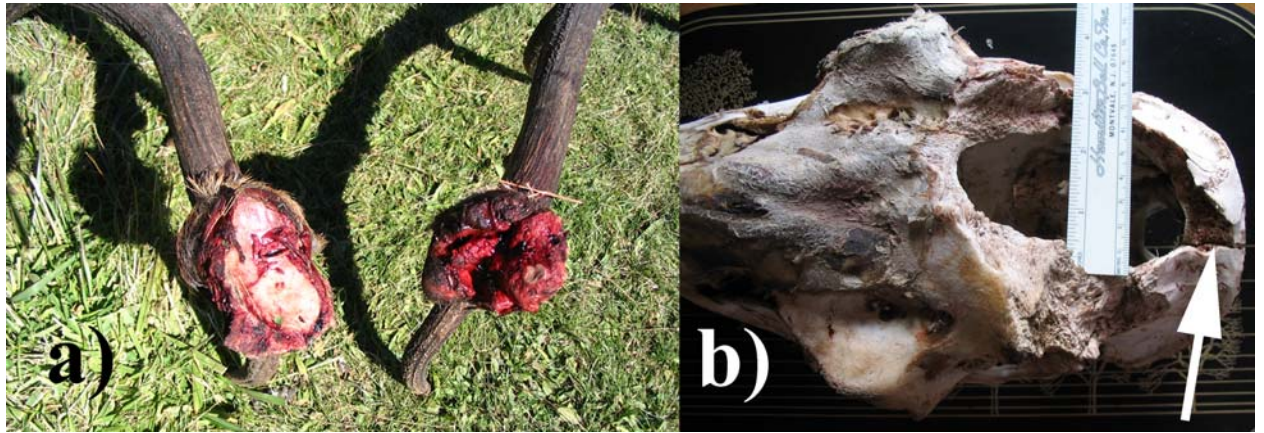


Figure 2. Antler damage in red deer: (a) broken tines, (b) broken crowns, (c) broken main beams.



Figure 3. Breakage of antler and skull: (a) bases of antlers as found, (b) dorsal view of cranium with posterior part of frontals and most of parietal broken out. Note that the occipital is cracked and has the antero-dorsal portion broken out (arrow).



RED DEER INTRODUCED TO PATAGONIA

2. CAMPYLOGNATHIA OR BENT-NOSE DISEASE

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ABSTRACT

Red deer (*Cervus elaphus*) introduced to southern Latin America now occupy over 51,000 km² and maybe numbering >100,000 animals. Of 776 specimens, campylognathia (facial scoliosis) was found in 2 females and 5 males, which were all otherwise healthy individuals. The low prevalence indicates that the disease unlikely is inheritable, considering that the founding stock consisted of 20 animals and prevalence would have been at least 5%. Handedness of scoliosis reported in a total of 131 cases related significantly to the hemisphere where the specimen originated. Handedness was according to that expected from the coriolis force arising from the earth's rotation. These effects are large enough that humans subjected to an artificial coriolis force cannot reach out and hit a target with their finger, until repeated attempts have corrected central nervous system responses and motor compensations. Furthermore, coriolis forces are known to affect early stages of development such as the innervation pattern of the mammalian vestibular system, the plane of bilateral symmetry, or the orientation of microtubules in the vegetal pole region of embryos. It is therefore conceivable that the networks processing these environmental cues, or the mechanisms responsible for compensation, are malfunctioning and thus result in a preponderance of facial scoliosis in accordance to the earth's rotation.

INTRODUCTION

Cases of campylognathia or bent-nose have been observed repeatedly among cervids. The etiology is unknown and may have multiple origins. We describe the phenomena in red deer (*Cervus elaphus*) from Patagonia. The free-ranging animals were descendants from a group of European red deer introduced to an enclosure in central Argentina in 1906, from where a small group was taken to an enclosure in the Neuquen province in 1922 (Flueck and Smith-Flueck 1993). The liberation occurred a few years later and has resulted in an extensive distribution in Patagonia, deer having become established in all mayor habitats between about 34-55°S, maybe numbering >100,000 animals, and having yet to reach a state of equilibrium (Flueck and Smith-Flueck 1993; Flueck *et al.* 2003). As handedness of campylognathia in cervids has been correlated to the coriolis effect (Suttie and MacMahon 1993), we analyzed this relationship in our and other reported cases.

METHODS

The study area is centered in northwestern Patagonia (40°58'S, 71°12'W), Argentina (for details see Flueck and Smith-Flueck 2011). Cases of campylognathia stem from our collecting deer by rifle and revisions of specimens hunted by the public. We also performed a literature search on ISI Web of Knowledge and analyzed cases of campylognathia reported in cervids with respect to geographical location and handedness using a chi square goodness of fit to test the expected versus observed frequency of cases (Zar 1996).

RESULTS

We have found cases of campylognathia in two female and five male red deer (our specimens $n = 522$, from hunters $n = 254$). **Case 1** was a female about 4 years old collected in 1996. She was in good condition, average size, reproductively active, and there were no indications of anatomical aberrations in the postcranial skeleton or other gross pathological changes. The scoliosis in the upper skull affected the nasal, maxillary and premaxillary bones, resulting in the frontal portion to bend to the left as well as down (Fig. 1a). The magnitude of the down bend resulted in the lower incisors extending considerably beyond the palate which is evidenced by the wear pattern on incisive teeth, and the biting capacity must have been very limited. Additional asymmetry was prominent on the anterior sphenoid which bent to the right whereas the following vomer bent again to the left. It resulted in the distance between the hamulus and the maxilla tuberosity to be 21% larger on the left side. The mandible exhibited 12 mm

more growth on the left side and was also bending, such that the incisor arcade deviates about 25 degrees from the normal plane, but in accordance with the position of the palate and showing additional changes, possibly as secondary complications from misalignments (Fig. 1b). The left P4 appears normal in form but is in a much displaced position. The right M3 has a normal form, but its position is displaced, possibly due to bone restructuring from periodontal problems related to the M2. The mandibular bone at the level of the right M2/3 is 38% wider and height is reduced by 33%, compared to the left mandibular bone. The right M2 is not only displaced but is abnormally grown. **Case 2** was a 1 year old female collected in 1998. She was in good condition, and there were no indications of anatomical aberrations in the postcranial skeleton or other pathological changes. The head presented scoliosis in the upper skull and mandibles, resulting in a bend to the left, but not as pronounced as in case 1. Although the body measurements show that the female was slightly larger than average, the head length was shorter than average due to the aberrant growth. **Case 3** was a male hunted in 2000 and about 12-14 years old, with the anterior portion of nasals, maxillae and premaxillae involved in the scoliosis, resulting in the frontal portion to bend to the left. The maxillary teeth had all normal appearances. **Case 4** was a male hunted in 2007 and about 6-8 years old, with the anterior portion of nasals, maxillae and premaxillae involved in the scoliosis, resulting in the frontal portion to bend to the left. **Case 5** was a male hunted in 2008 and about 5-6 years old, with the anterior portion of nasals, maxillae and premaxillae involved in the scoliosis, resulting in the frontal portion to bend to the right and strongly downward such that the lower incisors extended considerably beyond the palate (Fig. 2). **Case 6** was a 4-5 year old male with the nasals, maxillae and premaxillae involved in the scoliosis, resulting in the frontal portion to bend to the left (Fig. 3a). Lastly, **case 7** was a 4-5 year old male with the anterior portion of nasals, maxillae and premaxillae involved in the scoliosis, resulting in the frontal portion to bend to the left (Fig. 3b).

Table 1. Reported cases of campylognathia among cervids with respect to hemisphere and direction of facial scoliosis

No. of cases	Hemisphere	Direction	Reference
<i>Cervus elaphus</i>			
6	South	Left	This study
1	South	Right	This study
1	North	Right	MacNally (1989)
4	North	Right	MacNally (1992a)
1	South	Left	Suttie & McMahon (1993)
1	South	Left	Suttie & Pearce (1994)
1	North	Right	Suttie & Pearce (1994)
1	North	Left	Campbell (1995)
<i>Cervus nippon</i>			
1	North	Right	MacNally (1989)
<i>Cervus duvauceli</i>			
1	North	Left	Banwell (1999)
A mixture of <i>C. elaphus</i> , <i>C. unicolor</i> , <i>C. nippon</i> , <i>C. timorensis</i>			
95	South	Left	Banwell (1999)
13	South	Right	Banwell (1999)
<i>Capreolus capreolus</i>			
1	North	Right	MacNally (1992a)
1	North	Right	MacNally (1992b)
1	North	Right	Prior (1993)
<i>Rangifer tarandus</i>			
1	North	Right	Suttie & McMahon (1993)
<i>Dama dama</i>			
1	North	Right	Horsefield (1993)

Campylognathia with respect to hemispheres: Table 1 summarizes the reported cases of campylognathia in cervids. Of a total of 131 cases, 12% defy the expected directional asymmetry according to the coriolis effect, however, the correlation is significant ($x = 0.8$, d.f. = 1). Lutz (1988), citing further literature not available for our consultation, mentioned additional cases among cervids.

DISCUSSION

Different causes are known or suggested to result in campylognathia which has been described for cervids since the 19th century (MacNally 1992a). Atrophic rhinitis can result in campylognathia, but is restricted to suids (Lutz 1988). A literature search on ISI Web of Knowledge did not reveal descriptions of rhinitis or osteodystrophy in cervids. Abnormal positioning of the fetus or intrauterine pressure, and mineral deficiency have been suggested as possible causes (MacNally 1989, 1992a). Other possibilities include injuries from physical impacts, but all cases in cervids described here were from apparently healthy individuals. Arthrogryposis (inherited, viral or from teratogenic factors) in domestic ruminants often is associated with campylognathia (Bähr *et al.* 2003; de la Concha-Bermejillo 2003). Although wild deer in North America are often sero-positive to viral infections known to cause campylognathia in livestock (Blackmore and Grimstad 1998), we found no reference of sero-positive deer exhibiting campylognathia. Furthermore, presence of Cache Valley or Potosi virus as causative agents has not been reported for southern Argentina (Camara *et al.* 1990). Suttie and McMahon (1993) mentioned a red deer calf born with a straight rostrum which began to bend at 6 months of age. They excluded nutrition as a factor as deer were well fed during a study, and as upper as well as lower jaw bent together, they suggested it to be a developmental abnormality. Another case involved an otherwise healthy female (with facial scoliosis to the left) which was exported from New Zealand to Canada. There she produced a daughter which also exhibited campylognathia but to the right, and thus suggestive that there might be genetic inheritance involved (Suttie and Pearce 1994). However, it could have also occurred through inheritable epigenetic effects. Nonetheless, there are many confounding variables which might be involved from deer living together and presenting similar pathologies. Genetic inheritance as a primary cause seems unlikely given that the extensive red deer population in Patagonia (Flueck *et al.* 2003) resulted from 20 initial individuals (Franke 1949), and thus the original frequency would have had to be at least 5% (one out of the 20) and expected to remain so. Yet there have been no reports of such frequent cases among deer hunted in this population since 1920's, and the seven cases found represent <1% of deer revised by us. Many specimens reported by others came from deer farms and were either considered healthy or lacked a comment, and probably all deer shot in the wild made it to adulthood, and also were either considered healthy or lacked comments. Thus, the origin of campylognathia in deer appears to related to a non-inheritable developmental disorder. Primary causes are not known and might include congenital genetic aberrations, teratogenic compounds, infections, and other epigenetic factors affecting the developmental process. The fact that there is a pronounced difference in the developmental pattern with respect to hemispheres, suggests that the disturbed developmental process may be influenced by geophysical factors like the coriolis force as proposed by Suttie and MacMahon (1993).

Coriolis forces are inertial forces that arise when an object moves linearly within a rotating spatial reference frame like the earth. Any body movement that is not parallel to the earth axis will be affected by a transient coriolis force that arises as the movement begins and decays as the movement ceases. Humans subjected to an artificial coriolis force for instance cannot reach out and hit a target with their finger, until repeated attempts have corrected central nervous system responses and motor compensations (Lackner and DiZio 2000). Similarly, coriolis forces are constantly acting on arms or legs from limb movements made during voluntary body rotation: although they are not perceived, motor compensation for their presence are made, otherwise the movements would be inaccurate (Lackner and DiZio 2000). Furthermore, coriolis forces are known to affect early stages of development, Bruce *et al.* (2006) for instance found that movements of embryos within the dam's uterus, and as the dam herself moved about, causing coriolis effects which affected the innervation pattern of the mammalian vestibular system. Even subcellular effects of geophysical forces are known, such as gravity influencing both the plane of bilateral symmetry and the orientation of microtubules in the vegetal pole region of embryos (Kochav and Eyal-Giladi 1971; Fluck *et al.* 1998). These various known systems affected by coriolis forces all play roles in mammalian ontogeny, yet normal individuals grow symmetrical within the range of geophysical forces encountered on the globe. It is therefore conceivable that the networks processing these environmental cues, or the mechanisms responsible for compensation, are malfunctioning and thus result in a preponderance of facial scoliosis in accordance to the earth's rotation.

ACKNOWLEDGEMENTS

We thank several landowners for access to their properties and evaluate red deer. We also like to acknowledge valuable comments by two anonymous reviewers. The study was funded in part by the Argentine government through FONCYT Pict99, CONICET grant 87/99 and 327/04, Ministry of Foreign Affairs through NZAID, and Swazi, New Zealand.

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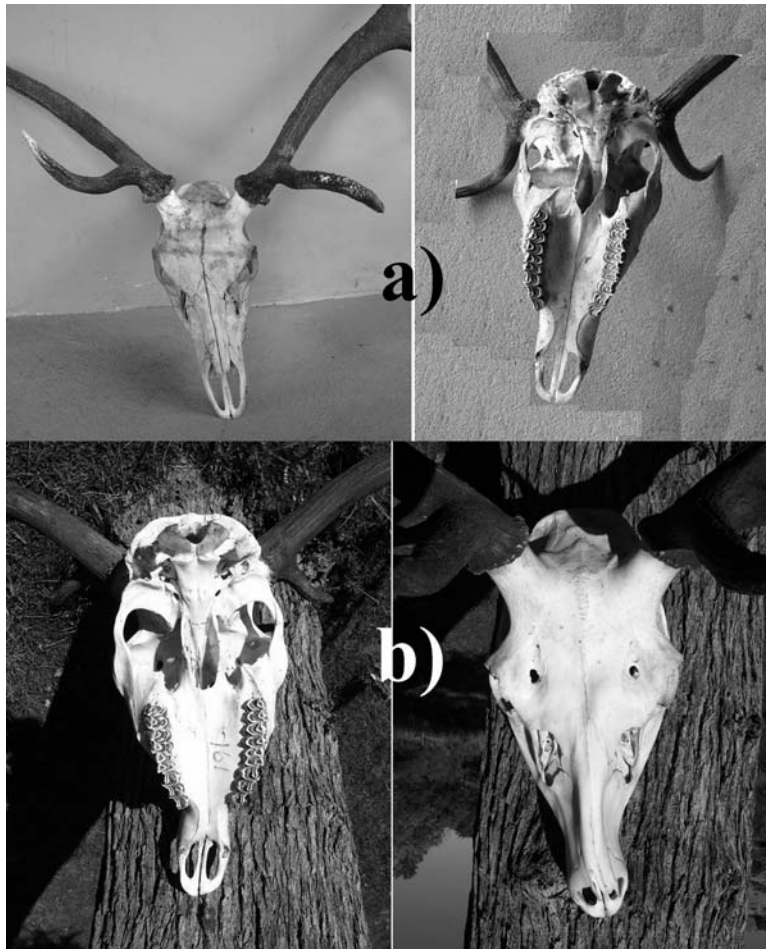
Figure 1. Campylognathia of an adult female red deer with (a) lateral and (b) ventral view of scoliosis of the skull.



Figure 2. Adult male red deer with (a) lateral and (b) ventral scoliosis.



Figure 3. (a) Adult male red deer with pronounced scoliosis at the level of eye sockets and (b) adult male red deer with lateral scoliosis of anterior rostrum.



HUEMUL HERESIES: BELIEFS IN SEARCH OF SUPPORTING DATA

1. HISTORICAL AND ZOOARCHEOLOGICAL CONSIDERATIONS

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ABSTRACT

Patagonian huemul (*Hippocamelus bisulcus*) were eliminated from many former ranges before arrival of early explorers who already acknowledged their rareness. Considering ungulates in mountains back home analogous, huemul was called a mountain deer, which is repeatedly cited without validation and remains the orthodox interpretation. Yet species considered analogous also use lowlands, flatlands, deserts and grasslands, and *H. antisensis* uses habitat with high affinity to Patagonian grasslands. Recent comparative analyses of postcranial morphologies show that huemul cannot be associated with rock climbing species, but fall within ranges of other cervids. Analyses of past human utilization rely on one study of economic anatomy and bone remains, frequently concluding that huemul were unimportant to hunter-gatherers. However, considering only bone fat and omitting easy removable fat is erroneous. Total energy of deer in autumn/winter partitions into 53% as fat and hunter-gatherers elsewhere focused on deer during the peak fat cycle, using all easy removable fat (>1200% more energy than bones) and consuming fat/marrow while butchering. Natives likely influenced huemul distribution and density in winter ranges due to high incentives (fat) and easiness to kill. Sparse evidence likely stems from surveys in Patagonia being biased toward caves, leaving out transient movements and camps.

Generalist cervids passed the Panama land bridge filter to reach South America. Considering paleoclimate, *Hippocamelus* dispersed east of the Andes, pushed by glaciations even to northeastern Brazil. *Hippocamelus* would only reach and cross Andes after deglaciations. As mixed feeder, huemul utilized Patagonian steppe. Hunter-gatherers arrived after the last glaciation and influenced the local distribution of *Hippocamelus*, especially in northern and central Chile after early adoption of agricultural lifestyle. Introductions of horses and livestock converted native economies through an equestrian lifestyle and based on millions of introduced animals, which thus affected early writings. Only few records indicate presence of large groups of huemul far from forests and substantial killings. Human-caused range contractions of northern ungulates affected mainly losses at low elevations and most species persisted in the marginal periphery, including high elevation refuges. Paleoeology, zoogeography, and land use history in southern South American indicate that mountain huemul are secondary relicts created by impacts of European settlements. We caution against the rigid application of modern huemul habitats in interpreting past habitat use, and simply considering the few extra-Andean accounts as abnormal outliers and thus ignoring them.

Additional keywords: *Hippocamelus bisulcus*, *H. antisensis*, zooarcheology, biogeography, morphology, range contractions, human influence, historical condition

INTRODUCTION

Huemul (*Hippocamelus bisulcus*) were often compared by early explorers and naturalists to fauna confined to remote mountains back in their home country, namely ibex (*Capra ibex*), chamois (*Rupicapra rupicapra*), mountain goat (*Oreamnos americanus*), and bighorn sheep (*Ovis canadensis*) (Flueck and Smith-Flueck 2011a). These and other observations which appeared in old reports have been mentioned repeatedly throughout the years, but without any rigorous scrutiny of their validity. Interpretations based on such nonmetric traits contain a high degree of inter- and intraobserver subjectivity of qualitative descriptions and thus proneness to false dichotomies. As securing reliable data is difficult on species highly reduced in numbers and remaining mainly in remote refuge areas, the continuing lack of well-substantiated information on the biology and ecology of huemul results in the reliance on old sources and hearsay. Due to their uncritical application, this belief system presents important obstacles in current conservation efforts, and in part might explain the absence of successful recovery of huemul during the last three decades. Similar impediments were reported for panther

(*Puma concolor coryi*) where unreliable inferences have appeared in prominent, peer-refereed scientific journals and have been repeatedly cited and miscited, thus compromising panther conservation (Conroy *et al.* 2006). Here we review historical and zooarcheological information in relation to some aspects of the biology and ecology of huemul being utilized dogmatically, even in scientific publications, but that do not appear to hold up under scrutiny. This analysis is presented in three separate papers and aims at improving our understanding of huemul and its conservation. While the second part reevaluates antlers, group size and density, predation, diet, diseases and competition, and the third part covers reproduction, resource defense, genetic isolation and sexual segregation, in this first part we reevaluate analogies made to other ungulates, body shape, human utilization of huemul, and their natural distribution.

ANALOGY TO OTHER UNGULATES OCCURRING IN MOUNTAINS

Early descriptions come largely from initial naturalists exploring interior Patagonia, with only rare accounts from Pacific and Atlantic coastal areas. Even before the arrival of early explorers, huemul had been eliminated from much of the former range (Diaz 1993; Diaz and Smith-Flueck 2000). Thus early encounters with huemul were often in remote and barely accessible Andean mountains, and initial writings already acknowledged the prevailing rareness, process of disappearing, or even referred to the endangerment of huemul (Flueck and Smith-Flueck 2011a). Since explorers were mainly finding huemul in mountain refuge areas, they compared them with ungulates found in mountains back home: these, however, were just subjective statements. Yet this interpretation that huemul were synonymous with *mountain deer* persists even today (MacNamara 1982; Eisenberg 1987; Redford and Eisenberg 1992; Webb 1992; De Nigris 2004; Merino *et al.* 2005; Iriarte 2008; Guérin and Faure 2009; Muñoz-Pederos and Valenzuela 2009; Cruz *et al.* 2010; Vila *et al.* 2010).

Species claimed analogous to ‘mountain huemul’ do of course utilize mountain habitats. Bighorn sheep, however, are well known to also inhabit low, flat, deserts and grasslands (Geist 1985; Fairbanks *et al.* 1987). Chamois as an ‘Alpine’ species is an artefact of past human-induced displacements (Senn and Suter 2003): currently they are re-occupying forest-covered rolling hills, and referred to as ‘forest chamois’ (Molinari-Jobin *et al.* 2002; Baumann *et al.* 2005). Lastly, even Alpine ibex is a misnomer: extirpation was much easier and much earlier at low and medium altitude, resulting in a prejudice in early conservation efforts whereby reintroductions were made to high elevations because that’s where the last remaining groups had been seen (Choisy 1994). These reintroductions actually created an artificial model of an animal of high altitude, living there all year above the tree line (Choisy 1994; Phoca-Cosmetatou 2004; Lyman 2006). Yet many remains have been found down to sea level, sometimes as dominant prey species in archeological sites. Thus altitude clearly was not a factor in ibex ecology (Phoca-Cosmetatou 2004). Accordingly, recent reintroductions to very low elevation areas were successful. There are even cases of natural recolonization, from high elevation populations to low elevation areas (Choisy 2001). Historical comparisons of huemul to ibex and chamois were therefore done under the misbelief that both latter species were specialists for high altitudes and extreme rocky areas: instead, the only link between these three species appears to be historical anthropic displacement from favorable habitats. Moreover, the only sister species, taruca (*H. antisensis*) - considered by several authors osteologically indistinguishable and thus possibly a mere subspecies (Philippi 1857; Dabbene 1911; Krieg 1925; Diaz 1995) - currently also utilizes treeless grassland habitat with high affinity to Patagonian grasslands (Fernández and Busso 1997), and coexists with several camelid and other cervid species (Jungius 1974; HersHKovitz 1982; Miller and Burger 1995; González *et al.* 2006; Nunez 2006; Barrio and Ferreyra 2008). However, taruca as well has been displaced very early by human pressures (Dabbene 1911; Horkheimer 1960). They used to also occupy Prosopis forests down to the Pacific coast based on several different lines of evidence (Horkheimer 1960; Sinclair 2009). Although now mainly found above tree line, some populations are still found, even exclusively, in Polylepsis and yungas forests, also due to continued hunting pressure (Aldenderfer 1998; Tarifa and Yensen 2001), or in foothill areas (Siefeld and Guzmán 2011). Also historically, populations occurred to just west of the Mato Grosso with one area disconnected from the Andes, the Sierra Pampeana, still containing remnant taruca populations (Diaz 1995). Lastly, taruca were bred successfully in the Berlin zoo at sea level for over a decade, and several zoos since 1890 reported that taruca was one of the easier species to maintain, including regarding forage (Vidal *et al.* 2011).

BODY SHAPE

Huemul has foremost been considered a mountain deer based on subjective qualitative descriptions of its physiognomy and historic distribution. The external appearance of *Hippocamelus*, like stockiness and short legs, was compared to ibex by Heck already in 1897 (Frädrich 1978), and to ibex and

chamois by Kurten (1979), who referred to them as *mountain deer*. Kurten's conclusion unfortunately was based on an erroneous construct of a new fossil 'mountain deer' which was later declared a *nomen nudum* (Morejohn and Dailey 2004), and without having any type of data on *Hippocamelus* (Kurten 1975). Similarly, without providing data, huemul were claimed to have body proportions reminiscent of bighorn sheep (Eisenberg 1987). There is no lack of further references to stocky and massive huemul resembling mountain goats, chamois, bighorn sheep and ibex right up to today (Krieg 1925; Heck 1969; Kolliker Frers 1969; Kurten 1979; MacNamara 1982; Redford and Eisenberg 1992; Frid 1999; Nowak and Walker 1999; De Nigris 2004; Lord 2007; Cruz *et al.* 2010; Vila *et al.* 2010).

In contrast, a recent study, analyzing for the first time the postcranial morphology of huemul and comparing it to ungulates claimed to be analogous to huemul in the use of rugged mountains, found no supporting evidence (Flueck and Smith-Flueck 2011a). Comparing leg morphometrics of huemul and 12 other ungulates revealed that huemul cannot be associated with rock climbing species, and the morphology does not overlap with species previously considered analogous, but falls within the range of other cervids (Fig. 1). Rather, the alert 'horseshoe' stance and long-haired coat may have influenced early description (Flueck and Smith-Flueck 2011a).

NATURAL DISTRIBUTION

The pre-Columbian distribution of huemul has its roots in founding stock, likely of the *Odocoileus* line (Morejohn and Dailey 2004), which dispersed through the Panama land bridge during the Great American Interchange. Species had to pass this equatorial filter, presumably a continuous savanna habitat, and the ones succeeding were generalists and predominantly savanna-adapted (Webb 1978). *Hippocamelus* were established by the Pleistocene, and considering the paleoclimatic conditions, dispersed south on the eastern side of the Andes through continuous savanna habitat (Hoffstetter 1963; Hershkovitz 1969; Frailey *et al.* 1980; Markgraf and Kenny 1997). Several periods of glaciation kept ancestral *Hippocamelus* repeatedly away from the Andes, and fossil *Hippocamelus* are even known as far north as 8°9'S, 36°22'W in the most eastern tip of Brazil, and from the plains of Argentina, Uruguay and southeastern Brasil (Castellanos 1944; Magalhaes *et al.* 1992; Eisenberg 2000; da Silva *et al.* 2006; Guérin and Faure 2009).

During glacial periods, the Andes were covered with ice even at northern latitudes to near the equator (Clapperton 1993; Coltorti *et al.* 2007), and a continuous sheet covered the mountains from about 33 to 56°S during the last glacial maximum. Glaciers south of about 42°S dipped into the Pacific, and were overlaying the Andes about 1600–1800 m thick, which intensified this orographic wind barrier. Glaciers reached hundreds of kilometers into eastern Patagonian plains where only treeless habitat existed (Fig. 2), with Patagonia-like grasslands reaching way into Brazil, and much of South America covered by savannah and grasslands (Marshall 1988; Clapperton 1993; Rasmussen 1994; Markgraf and Kenny 1997; Tatura 2002; Rabassa and Coronato 2009). Moreover, the sea level was about 120 m lower and the Atlantic coastline located 300 km or more to the east of the present coastline in some latitudes, which greatly extending the flat paleosteppe region eastwards (Fig. 2).

During the last glaciation, Fireland was connected to continental Patagonia when guanaco (*Lama guanicoe*) reached this southernmost area (González *et al.* 2006): huemul likely arrived simultaneously. Fireland had been separated from the continent only around 8000–10000 years BP, though the Beagle channel, as narrow as 2 km, would not constitute a barrier to good swimmers like huemul. Although extinct today, past presence of huemul on Fireland is indicated by Waterhouse reporting in 1834 on Darwin's collections and Ramón Lista mentioning in 1881 the occurrence of huemul on Fireland (Diaz and Smith-Flueck 2000), by Lacroix's (1841) account of its zoology, and corroborated by remains found in a zooarchaeological site (Mansur and Piqué 2009). Pressure from hunter-gatherers could have led to its local extirpation (see next section).

During glaciations, *Hippocamelus* thus persisted in eastern treeless lowlands, with fossils from the plains of Uruguay, northern Argentina and southern Brazil (Eisenberg 2000). As mixed feeders, huemul are known to be able to incorporate notable amounts of grass in the diet (Smith-Flueck 2003; Prothero and Foss 2007). Furthermore, besides graminiae, Patagonian steppe contains a large component of shrubs, maintains important green grass production throughout winter (Fernandez *et al.* 1991; Adler *et al.* 2005), and deer are known to make much use of seed heads (Takatsuki 2009), which further corroborates past and even historic distributions of huemul in non-forested habitat.

Once eastern regions became glacier-free, huemul were able to reach Andean habitat and when

deglaciation allowed for it, eventually cross the Andes (Hershkovitz 1969; Moreno *et al.* 1994). Faunal exchanges from the east were foremost across low Andean passes and explain the occurrence of late Pleistocene huemul in Chile as far northwest as 30°S by the Pacific coast (Moreno *et al.* 1994; Simonetti 2000; Frassinetti and Alberdi 2001). With the last glacial retreat, forests spread from the few western refuges, and eventually covered the southern Andes again, reaching their current extent only 2-3000 ya (Armesto *et al.* 2010; Markgraf and Kenny 1997; Rabassa and Coronato 2009).

Humans arrived with the last interglacial period, about 10-12'000 ya in the southern Andes. Having been nomadic hunter-gatherers (Latorre *et al.* 2007), they would have had some influence on local distribution of *Hippocamelus* (Dabbene 1911; Horkheimer 1960; Sincaire 2009). However, in northern and central Chile, humans adopted sessile and agricultural lifestyles long before arrival of the Spanish. They reached very high densities, completely changed the habitat through slash and burn, and regionally extirpated several species including huemul (Miller 1980; Simonetti 2000; Armesto *et al.* 2010). The same pattern was also confirmed for pudu deer (*Pudu puda*) (Saavedra and Simonetti 1991). Further south, humans became established mainly along the Pacific coast and focused on marine resources. Consequently, early explorers still found coastal areas abundant with huemul: it quickly became an established fact that such areas indicated low passes across the Andes from which to reach the eastern slopes via expeditions. The logical interpretation was that coastal huemul stemmed from source populations on the eastern side of the continental divide (Moreno 1899; Steffen 1904). East of the Andes, huemul also existed in zones between the Andean foothills and the Patagonian mesas (Finn 1909; Lydekker 1925). Onelli (1905) described huemul regularly occurring in flat grasslands about 120 km east of the Andes (44°55'S, 70°7'W), and as still, although already rarely, occurring up to another 140 km further east. It coincides with Prichard (1902) still collecting huemul at 200 km from the Andes and other historic records of this species at 270 km east of the Andes (Flueck and Smith-Flueck 2011c). However, this species also reached the Atlantic coast (Lydekker 1915). Reports from the 16th, 18th and 19th centuries mention huemul near Port San Joseph and Port Desire (de la Piedra n/d; MacDouall 1833; Moreno 1899; Diaz 1993; Diaz and Smith-Flueck 2000), old shed antlers were still found in steppes by Serret (1990), and Cruz *et al.* (2010) recently found a prehistoric human-modified antler near the Atlantic, although it cannot be ascertained that it stemmed from that locality.

The Spanish arrival resulted in highly significant changes brought about by the introduction of horses, which created an equestrian lifestyle for native people and profoundly changed their economies. Horses first arrived in Buenos Aires in 1537 and became feral, but by 1580 they already reached the Strait of Magellan (Darwin 1839). Introduced livestock immediately became feral and soon roamed by the millions (Darwin 1839; Behm 1880; Ljungner 1959; Torrejon 2001). Native tribes prevented colonization of Patagonia and dominated the region for some 300 years until displaced by wars. Only some intrusion by settlers from Chile was possible. Accordingly, the major industry in these forests was livestock raiding by nomadic intruders and eastern tribes, with 47% of these forest areas having been burnt rather recently or shortly before studies by Willis (1914). Darwin (1839) found that native people knew to use knives, forks, spoons and relished sugar, and having had so much contact with sealers and whalers, that most of the men spoke some English and Spanish. He further noted that these natives traveled up to 750 km inland during summer to hunt in the foothills, each man having 6-7 horses. After overpowering the tribes, fencing and ranching occurred rapidly throughout Patagonia (von Siemiradzki 1893; Torrejon 2001) which further affected the remaining huemul distribution.

As a result of the above mentioned history, the first early writings were posterior to significant anthropogenic changes in the distribution of huemul, with explorers therefore largely unaware of previous history. Their descriptions of huemul often were from remnant populations living in remote and inaccessible places. Subsequent naturalists found an even more reduced distribution, but as locations coincided with the few early accounts, it lead to dogmatic descriptions. Thus, decades have gone by further ingraining the notion that huemul are exclusively of Andean forests and not part of lowland central Chile (Osgood 1943; Povilitis 1978; Thornback and Jenkins 1982; Redford and Eisenberg 1992; Corti *et al.* 2010; Vila *et al.* 2010); specially adapted to precipitous rocky terrain, and forest habitats of the Andes (Thornback and Jenkins 1982; Belardi and Otero 1998; Nowak and Walker 1999; Dolman and Wäber 2008; Fernandez 2008); a mountain deer living above tree line (National Research Council 1991; Prothero and Schoch 2002; Guérin and Faure 2009); living between 1300-1700 masl or high elevation mountains (Nowak and Walker 1999; Merino *et al.* 2005); or as preferring steep, rocky, north-facing slopes (Thornback and Jenkins 1982; Redford and Eisenberg 1992; Lord 2007). Preference to the high Andes, principally near the international border along the continental divide, was considered to be due to the conditions in that area being the most favorable to huemul, as

explicitly stated by e.g. Osgood (1943). The problem of basing ecological interpretations on current distribution is illustrated by the suggestion that huemul habitat use moves down about 107 m in elevation for every 11° latitude further south (Gill *et al.* 2008). The conclusion was based on regressing the median elevation of upper and lower elevations of signs registered for some populations with latitude. The upper elevation of habitat use simply coincides with the tree line which progressively diminished at increasing latitude, a trivial observation. The relationship between lower elevation and latitude however is an artefact by leaving out historic and zooarcheologic data. Thus, whereas it is correct to conclude that the upper elevation of huemul habitat use diminishes with lower latitude (parallel to the diminishing tree line), it is fallacious to conclude the same for the lower boundary of current habitat occupation. The lower limits are determined by the altitude of adequate interior winter ranges and sea level on both sides of the continent (Flueck and Smith-Flueck 2011b), and absence of huemul is due to having been displaced. These generalized descriptions of the species, implying preferences, exclusiveness, or most favorable conditions are strong terms with specific ecological meaning, yet such casual statements are not enough to make these inferences. More recently another explanation was offered, namely that “huemul has been reported in all habitats associated with steep, irregular and rough slopes, with rocky terraces or around cliff edges” (Vila *et al.* 2010). While the statement is correct, the authors completely omitted mentioning other types of habitats used by huemul in historic times and even today. Miller *et al.* (1973) already cautioned that characterization the optimal habitat of huemul is difficult due to most huemul having only survived in dense cover. Being well documented (Diaz 1993; Diaz and Smith-Flueck 2000; Smith-Flueck 2003; Flueck and Smith-Flueck 2011a), ignoring these facts distorts the interpretation of basic ecological traits of huemul (Fig. 3).

HUMAN USE OF HUEMUL

Based on a low quantity of zooarcheological remains and the only study on economic anatomy, Belardi and Otero (1998) concluded that huemul were not important to hunter-gatherers. Their interpretations relied on an analysis of two fresh carcasses from late autumn that had no fat in the meat, and bone marrow was considered as the only source of fat. Unfortunately, these authors neglected the fact that their samples were particularly incomplete regarding fat reserves because these animals had been skinned and eviscerated before being sent for analysis, and thus the most important portions were not available. Other studies on huemul also focused on bones, emphasizing the low utility of meat due to lack of fat during the annual cycle, while omitting the issue of dissectable fat (e.g. De Nigris 2004). However, these recent interpretations about huemul utility are in stark contrast to fat reserves in other cervids collected during the same season: McCullough and Ullrey (1983) found that *total energy content* of deer was partitioned in up to 47% as dissectable fat, 1% as marrow, 5% within bone, and 32% in muscle. Fats have the highest energy density and were partitioned on average (by weight) into 65% dissectable fat (up to 76%, most occurring with visceral organs), 8% in high-density fatty visceral tissue, 4% in bone tissue, and 2% in marrow. As dissectable fat averages 12% (up to 16%) of total body weight, but represents up to 47% of the deer energy content, it explains observations on hunter-gatherers elsewhere who focused on deer during the autumn/winter peak of fat. Cracking long bones takes little effort compared to energy return, and boiling bones for further fat extraction also does not take much effort (Church and Lyman 2003), although it requires fuel. Marrow and bone grease extraction is well documented for many hunter-gatherers, including in Patagonia (Bourlot 2006). Although this practice may render maximally 6% of total fats, it is erroneous to ignore that professional hunter-gatherers would certainly have taken advantage of easy accessible high-density fatty visceral tissue and dissectable fat which present >1200% more energy content than that obtained from bones. Price (1939) noted that northern Natives emphasized eating fatty visceral tissue, used marrow for various preparations and as milk substitute for children, but gave much of the muscle to their dogs. Moreover, human foragers generally ignored poor or starved animals for their lack of fat (Lipo 2007; Morin 2007), as documented from narratives by Natives regarding huemul hunting: “in stormy days, when herds of huemul came down from the Andes, we chose the fattest one to eat” (Diaz and Smith-Flueck 2000).

In autumn, 20-30% or more of body weights of cervids may consist of fat (McCullough and Ullrey 1983; Cook *et al.* 2007), and therefore north American hunter-gatherers preferentially killed deer in autumn/winter for being the easiest period and because cervid fat accumulation was maximal (Thomas and Toweill 1982; Lipo 2007). More importantly, while butchering they consumed dissectable fat, fatty visceral tissue and marrow, and then transported remaining bones for marrow and tallow extraction at camp (Price 1939; Thomas and Toweill 1982; Morin 2007; Speth 2010). The same traditions existed with Patagonian hunter-gatherers utilizing guanaco, where accessible fat including marrow was the favored part, and together with organs were consumed raw at butchering (Steward 1946; Tavener 1955;

Bourlot 2006). Guanaco also have annual fat cycles peaking in autumn/winter (Raedeke 1979), which is likely more pronounced in migratory populations.

Models of reconstructed hunter-gatherer diets have frequently assumed that muscle tissue was the sole animal tissue consumed; however, many ethnographic reports of various hunter-gatherer societies show that nearly all of the edible carcass was consumed. Moreover, analyzing 229 hunter-gatherer societies showed that virtually all potential fat contained in animal carcasses would generally be consumed (Cordain *et al.* 2000). Similarly with guanaco, no selective transportation operated in the past, instead all body parts were utilized and no anatomical units were abandoned unused (De Nigris and Mengoni Gonalons 2005), indicating that kills could be done near the shelter. This was suggested for huemul and guanaco based on bone assemblies also in other sites, where bone processing was fat-oriented (Fernandez 2008).

Hunter-gatherers commonly moved according to seasonal movements of prey, and such seasonal logistic mobility has been documented in several instances. Distances up to 150 km for hunting, particularly in autumn and early winter, best explained logistic mobility in low-density hunter-gatherers in northern environments (Lovis *et al.* 2005). From hunting camps, groups of young men would make roundtrips of >100 km in about three days. Moreover, these hunters have been able to portray detailed maps covering 240,000 km² and animal movements within (Lovis *et al.* 2005). Deer being preferred, a temporary camp would remain if there were individuals within 50 km (Lovis *et al.* 2005; Lipo 2007). Considering historic reports of winter concentrations of huemul, foraging conditions in the pre-Colombian era were likely even superior in terms of hunter-gatherers.

Bones of guanaco and huemul regularly coexist in archeological sites in Andean foothills between ca. 2500-6500 years BP, based on four stratigraphic layers (De Nigris 2007). The domination of guanaco in excavations has been explained by the supposed range-wide rarity of huemul and by hunter-gatherers being able to exploit ungulates most effectively where they are territorial, which is the case for guanaco (Muñoz and Mondini 2008). Alternative explanations include: (a) the well-documented ease to kill huemul which could easily result in local overhunting and produce rareness through exploitation depression (see Lipo 2007), (b) being non-territorial (Flueck and Smith-Flueck 2011*d*), huemul will temporarily evade hunting parties by moving to other sites, resulting in more mobile hunting which leaves little or no signs, and (c) given more mobile hunting, bones may be left behind as bone grease would only present about 4% additional fat compared to easily recoverable fat (Price 1939).

If tactics to hunt huemul differed from hunting guanaco, which is almost certainly the case, then most of the kill and butchering sites of huemul may remain undescribed. Similarly, Fiedel and Haynes (2004) found no descriptions of kill sites east of the Plains where large mammal like elk, deer, bear, or woodland bison were utilized by paleoindians during the past 12,500 years. If archaeologists in eastern North America have not yet stumbled upon the remains of any such butchered carcasses – 12,500 years with no discoveries - what is the likelihood of finding a butchered huemul? Empirical evidence, from analogous modern cases, show that even overkill would leave few fossil remains behind to identify the very processes involved in the resulting extinctions (Fiedel and Haynes 2004). Although there is no doubt that huemul had been used by hunter-gatherers all along, the sparse evidence from a few caves representing camps and settlements may not allow confident reconstruction of early hunting behaviors by men towards huemul and the associated impacts on huemul distribution. Borrero (2008) acknowledges that so far there had been a bias in surveys in Patagonia which focused on caves that represent more permanent sites. Transient hunting camps and movements are thus under-represented and difficult to document anyway.

GENERAL DISCUSSION

Horses and livestock brought by the Spanish soon became feral and roamed by the millions, which transformed lifestyles and economies of native tribes and allowed them to resist the Spanish for >300 years. Highly mobile, the Native's impact on the vegetation augmented as they increasingly hunted wildlife and feral livestock to trade with the colonies. As early writings by explorers were posterior to these significant anthropogenic changes, they described remnant huemul populations in remote and inaccessible places. References of huemul being analogous to other ungulates using mountainous terrain was intuitive in such historic settings and for apprehensible reasons: mainly because huemul had already been eliminated from much of their former range, were rare and mainly found in mountain refuge areas. Understandably, they were labeled as *mountain deer*. Yet to be known was the fact that

these other ungulates were stereotyped mountain dweller that too had experienced anthropogenic displacements. Now it is shown that these once existed -and currently exist again- in non-mountainous habitats.

Historic remarks used huemul body shape as circular reasoning for labeling it a mountain deer. These nonquantitative assertions were dismissed by a recent analysis of postcranial morphology showing that huemul are not analogous to rock climbing species, but fall within the range of other cervids (Flueck and Smith-Flueck 2011a). Using mountains is not a unique trait of huemul among cervids. For instance, Allen (1900), of the famous Allen's rule, observed that Mountain caribou used the same range occupied by the rock specialist Stone sheep (*Ovis dalli stonei*) and had habits much like those of sympatric Stone sheep. Strikingly, the literature abounds with casually labeled mountain ungulates: Alpine reindeer, Mountain caribou, Mountain mule deer, Mountain roe deer, Alpine roe deer, Alpine red deer, Alpine chamois, mountain bison, mountain guanaco, or mountain vicuña. However, whereas huemul is often claimed to be exclusively a mountain deer, other ecotypes are accepted unequivocally for other ungulates like Plains reindeer, Forest reindeer, Woodland caribou, Plains mule deer, Forest chamois, Field roe deer, Steppe red deer, etc. None of these labels constitute a scientific concept and merely refer to local adaptations. Most extant huemul then may be considered as satellite subpopulations that utilize mountains and have assumed some "mountain mannerisms" as an expression of intraspecific variation (reviewed in Putman and Flueck 2011). Still, modern circular reasoning continues to invoke remnant huemul as having primarily survived in remote areas like upper drainages in inaccessible mountains due to: a) being prime habitat, b) being specialized and selecting to live in steep and broken terrain, and c) having short legs and stocky build as evidence for specialization. By necessity this construct only holds up by marginalizing historic evidence and insights from zooarcheology, paleontology and paleoclimate.

Understanding past utilization of huemul by humans has implications for interpreting the prehistoric distribution. Although it is frequently claimed that pre- and post-Columbian Natives did not hunt huemul much, presumably due to their leanness, this contrasts comparative data from other cervids. Deer in seasonal environments accumulate fat by autumn/winter such that >50% of total energy is in fat, mostly in highly accessible deposits and often consumed at butchering, as also practiced by Patagonians. Accordingly, and due to substantial hunter mobility, camps were maintained if deer were within 50 km. Natives in upland Peru used 53% camelids and 31% cervids like the congeneric taruca, before domesticating camelids (Miller and Burger 1995). In contrast to common belief, due to extreme ease to kill huemul particularly in autumn/winter, their large winter concentrations in the past, and strong incentives due to their high energy density, Natives likely influenced huemul distributions and density in winter ranges, particularly once Natives had access to horses for mobility. Given the productivity of ecotones and adjacent steppes based on current biomass of exotic herbivores, densities of other *Odocoileus* in similar habitat elsewhere, and the few indicative records about huemul density in those areas before extirpation, we posit that huemul abundance was substantial. As Cabrera and Yepes observed in 1940: in the past huemul was one of the most frequently hunted by Patagonians who traded their skins to Carmen and even Bahia Blanca (Atlantic ports).

A necessary look at evolutionary history provides further leads regarding past distribution. To reach South America, cervids, as generalists, were able to pass through the Panama land bridge filter. Considering paleoclimatic conditions, huemul dispersed south on the eastern side of the Andes, also because glaciations pushed them eastward with fossils encountered in northeastern Brazil. As the ice reached the Pacific, huemul could only enter Andean habitat and cross the Andes when deglaciation allowed for it. As a mixed feeder, huemul could utilize 'Patagonian' steppe with its many shrubs and important green grass production throughout winter, being far removed from isolated small forest refuges in midwestern Chile coasts. During glaciations, Patagonian' steppe reached way into Brazil and down to Fireland. Evidence of huemul presence exists for Fireland and areas near the Atlantic even historically. Regardless, interpretation of a prehistoric human-modified huemul antler recently found near the Atlantic followed orthodox descriptions of huemul: the current huemul distribution was considered more valid than historic accounts, and thus it was concluded that more likely, paleoindians had walked the 250 km to the Andes and received the antler that way (Cruz *et al.* 2010).

Knowledge of historical ranges is important regarding endangered species, because recovered or recovering species have been found to occupy a greater percentage of their historic range than declining species. Instructively, anthropogenically-caused range contraction of ungulates with broad former historic distributions were heavily skewed toward loss of low elevation ranges (Laliberte and

Ripple 2004). Often it is assumed that when a species becomes endangered, its geographical range should contract inwards, with the core populations persisting until the final stages of decline. However, analyzing patterns of range contraction of many cases, Channell and Lomolino (2000) found that most species persist in the marginal periphery of their historical geographical ranges. Populations that persisted the longest were those last affected by the crawling spread of historic extinction forces; that is, those along the edge of the range, on an isolated and undisturbed island, or at high elevations (Channell and Lomolino 2000).

CONCLUSION

Past distribution and morphological studies show that huemul fit comfortably within the ranges for other deer and ungulate species using sub-alpine habitats. Previous use of Patagonia steppe areas far from forests is similar to habitat use by the only congeneric taruca and other *Odocoileus*. The seasonal fat cycle and congregations likely made huemul a prime candidate for hunter-gatherers, who would have therefore influenced their distribution and density. Several other ungulates had mainly lost their low elevation habitats from anthropogenic pressures and range contraction allowed them to persist only in marginal peripheral habitat, concentrated at high elevations or otherwise inaccessible sites. Historical ranges are thus an important consideration for conservation strategies of endangered species like huemul. Paleocology, zoogeography, and history of land use in the southern cone of Latin American indicate that mountain huemul are secondary relicts created by post-Columbian anthropogenic impacts. We caution against the rigid application of modern huemul habitats in interpreting past habitat use and against simply considering the few extra-Andean accounts as abnormal outliers that one can thus ignore. Although adopting a uniform conservation program over a large geographical area is attractive to policy-makers and conservation planners, the large range of past geographical and ecological sites used by huemul indicate that conservation programs could benefit from broadening strategies accordingly.

ACKNOWLEDGMENTS

We like to thank NI Díaz for providing several new sources, and J. Barrio and two anonymous colleagues for all the constructive reviews.

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Figure 1. Examples of male and female huemul showing body proportions while in summer coat.



Figure 2. Continuous ice sheet covering the Andes down to 56°S. Glaciers south of about 42°S dipped into the Pacific, and reached at times hundreds of kilometers into the Patagonian plains east of the Andes such that only treeless habitat existed east of the Andes. The coast line also was shifted eastwards by hundreds of kilometers (from Auk 111:143, 1994).

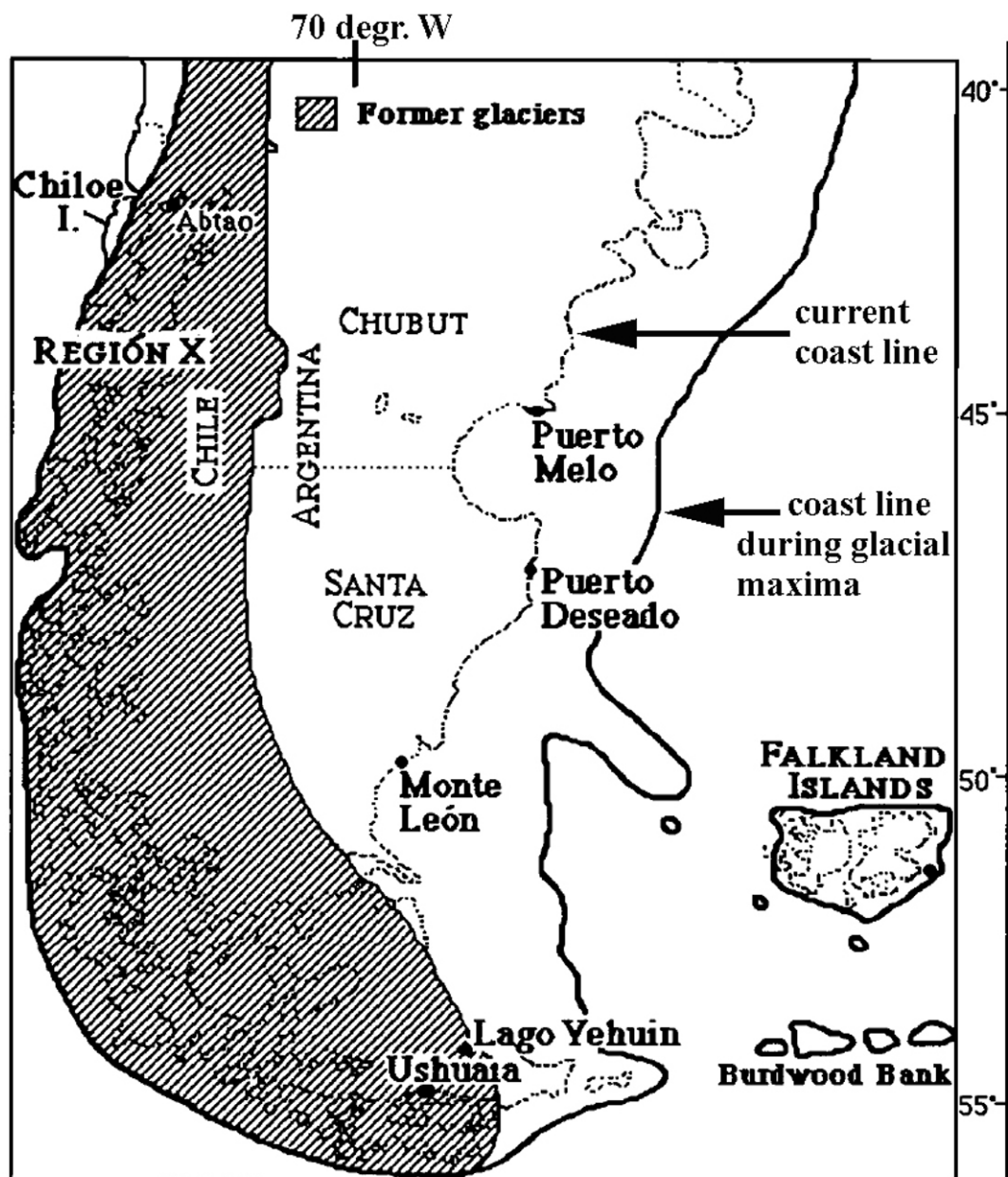
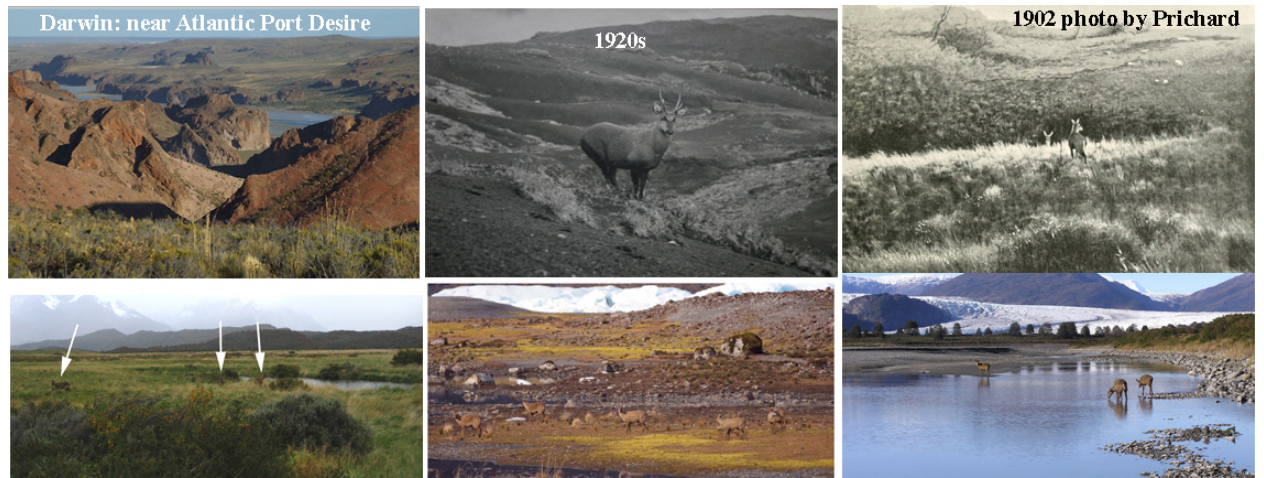


Figure 3. Past and current use of open and flat habitat by huemul, far from forests and cover. Note that use of such habitat occurs during full daylight hours.



HUEMUL HERESIES: BELIEFS IN SEARCH OF SUPPORTING DATA

2. BIOLOGICAL AND ECOLOGICAL CONSIDERATIONS

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ABSTRACT

The continuing lack of well-substantiated information about huemul (*Hippocamelus bisulcus*) results in reliance on early sources of interpretations. The repeated citing of such hearsay is scrutinized here for their validity. Huemul antlers provide clues about well-being and past changes as up to 5 times have been documented historically. Antlers are misinterpreted by erroneously considering >2 times as abnormal. The question is: “What conditions in the past allowed many tines, and allowed antler expressions to be closer to the species norm?” Significant past changes resulted in only few early records of large groups, abundance and killing many huemul. Current orthodox descriptions of huemul are based on little data from remnant populations in marginal habitats. Relying on such biased information results in circular reasoning when interpreting zooarcheology, paleodiets, prehistoric distribution, and huemul ecology in general. Claims of inadequate antipredator response due to evolutionary absence of cursorial predators is unsupported as several *Canis* species arrived together with cervids, overlapping with dogs having arrived with paleoindians. Huemul reactions toward dogs are similar to other Odocoilines. However, any predation event in severely reduced huemul subpopulations may be important due to dynamics of small populations. There is no evidence that livestock or red deer (*Cervus elaphus*) have caused declines or prevented recovery of huemul. In contrast, huemul have disappeared in numerous areas lacking livestock or red deer, but have persisted elsewhere with livestock for several hundred years. Recent sympatry with red deer affects <2% of huemul populations and in one case livestock outnumber red deer 2100%. Cervids including huemul exhibit great variation in feeding behavior, irrespective of dental and gastrointestinal features, due to flexibility in behavior, physiology and morphology. Although presence of other herbivores will shift spatio-temporal habitat use and affect performance, persistence of multi-species assemblages is the rule, like huemul coexisting with guanaco (*Lama guanicoe*), pudu (*Pudu puda*) and livestock. However, impacts from management of livestock, particularly the presence of people and dogs exert heavy pressure on wildlife and create incompatibilities. Concerning diseases from exotic ungulates, huemul coexisted with livestock for several centuries, but only recently in a few cases with red deer (but outnumbered 21-fold by coexisting livestock). Parasites found so far in huemul were only at very low levels. These are mostly considered nonthreatening, are found in red deer but commonly in livestock and therefore occur in most areas with livestock. Thus, the primary factor regarding contagious diseases is feral and free-ranging livestock, and being regularly researched and inspected at slaughter, these provide a good proxy for diseases afflicting sympatric red deer.

Additional keywords: *Hippocamelus bisulcus*, antler, group size, density, predation, diet, competition, diseases.

INTRODUCTION

In the accompanying paper about historical and zooarcheological considerations (Flueck and Smith-Flueck 2011a), we analyzed information regarding historical conditions and how it has influenced current interpretations of the biology and ecology of huemul (*Hippocamelus bisulcus*). Together with the current paper, the reports aim to improve our understanding of huemul and its conservation. Here we focus on issues related to antlers, group size and density, predation, diet, competition, and diseases to reveal inconsistencies in currently accepted beliefs that do not stand up under closer scrutiny.

Securing reliable data is difficult because huemul populations contain few individuals and, those remaining, are located in remote refuge areas. However, a detailed search was conducted in ISI Web-of-Knowledge and 17 external databases, three books and three dissertations on huemul, historic and

grey literature available in a collection containing 286 entries on huemul, plus publications on other related deer species to allow a comparative approach to analyze and interpret huemul.

ANTLERS

Antlers are deciduous secondary sexual characteristics, and principle factors influencing their phenotypic expression are recognized as age, nutrition, animal condition and social environment. Antlers provide a phenomenal tool for interpreting a myriad of biological and ecological relationships due to being a luxury tissue regrown annually, the only such regenerating appendage found among mammals. The correct interpretation of antlers therefore is not a trivial issue. Yet the literature on huemul is replete with erroneous interpretations which fail to appreciate the informational content of antlers.

Past literature commonly considered huemul antlers to consist only of simple forks (e.g. Frick 1937). Kurten (1975) even claimed that huemul differed from *Navahoceros* by having two, and not three antler tines. More recently, the same claim continued (Redford and Eisenberg 1992; Webb 2000; Lord 2007; Guérin and Faure 2009), or that forks are typical and 20-25 cm long (Povilitis 1985; Nowak and Walker 1999; Vila *et al.* 2010). However, antlers with three tines are still common in some localities, representing 43% of samples we measured in Chile and Argentina ($n = 47$), while antlers with four tines have been found many times with lengths >34 cm (Fig. 1) (Philippi 1892; Prichard 1910; Krieg 1925; Bubenik and Bubenik 1990; Serret and Borghiani 1998; Díaz and Smith-Flueck 2000). Although in the past antlers with five tines were documented (Osgood 1923), antlers beyond forks were, and still are, labeled *abnormal* (Philippi 1892; Rusconi 1936; Cabrera and Yepes 1940; Vila *et al.* 2010). Larger antlers with multiple tines found historically likely resulted from bucks still having been able to fully develop by reaching older ages and having been described from areas used formerly that provided access to good nutrition. In contrast, some extant remnant populations have very young age structures with no individuals encountered older than four years old (Smith-Flueck and Flueck 2001), and are restricted to small fractions of ranges used previously (e.g. Fig. 6 in Flueck and Smith-Flueck 2011b).

GROUP SIZE AND DENSITY

Group sizes and densities of huemul are often stated categorically when in reality these data only represent particular remnant subpopulations studied recently and occurring in a subset of habitats used formerly. The range for group size is commonly stated as solitary, or 1-5, and to 8 during winter (Redford and Eisenberg 1992; De Nigris 2004; Fernandez 2008; Vila *et al.* 2010). In contrast, groups of 10 huemul in summer (Grosse 1949) and 11 in autumn (Díaz and Smith-Flueck 2000) occurred even recently; they used to form wintering groups of 100 or more (Prichard 1902). Larger groups were reported from more open, and particularly treeless landscapes, in concordance to behavior of other cervids (reviewed in Putman and Flueck 2011).

Similarly, the range of density is stated from 0.002-1.2 huemul/km² (Redford and Eisenberg 1992; Belardi and Otero 1998; Fernandez 2008). However, even some extant populations have densities of about 9 huemul/km² (Wensing 2005), and although there are no density estimates for historic times, we can deduce that densities commonly must have been substantial. For instance, Juan Ladrillero first reported huemul from Chile in 1558, as he commented "...and then we went up to the place known as the Deer Point, where in just one hour two of our men shot fifteen of them with the arquebus..." (Díaz and Smith-Flueck 2000); and they were using weaponry which was less accurate than bow and arrow! Early naturalists regularly mentioned that huemul occurred in great numbers together with guanaco (*Lama guanicoe*) in the "Patagonian pampa" (Burmeister 1873; Steffen 1900; Prichard 1902; Hatcher 1903; Osgood 1923). Natives in Argentina knew huemul well and hunted them frequently, and preferentially where feasible, for food and skins, which were traded at the Atlantic coast (Burmeister 1873; Cabrera and Yepes 1940). Based on shooting many huemul, Prichard "could have very easily shot ten huemul in a day": yet Natives assured him that these deer were at one time even more numerous in that region (Prichard 1902; Hatcher 1903). Crews of early expeditions still found numerous groups of huemul such that hunting was easy and provided large crews with fresh meat every day while three weeks traversing the region (Martin 1899; Steffen 1900; Osgood 1923). Grosse (1949) saw groups every day and so many tracks that he concluded that there were huge herds ("riesige Herden"). Later during colonization however, huemul were killed by the thousands/year and at rates of up to 1-2 deer/km² (Anon. 1936; Grosse 1949; Iglesias 1965), which is about the average density where they often occur now (Díaz and Smith-Flueck 2000). Huemul were not only used for people, but also to feed dogs, chicken and pigs, and skins were used to construct shelters for people and domestic animals (Gigoux 1929; Anon. 1936; Krieg 1940; Madsen 1948; Iglesias 1965). At the same time there were

already huge herds of feral livestock using open areas, huge post-fire areas previously forested, forested areas and open areas above tree line (Veblen and Lorenz 1988): unfortunately, there are no data on their densities. Instructively in terms of capacity, good habitat in eastern foothills and grasslands was quickly filled with a large biomass of livestock (Table 1), reaching a maximum during the 1950s (von Thüngen and Lanari 2010). Today, former huemul range in the ecotone produces 3000–5000 kg/km² of exotic ruminant biomass (Flueck 2010), equivalent to about 40-60 huemul/km² if they would forage similarly, as expected from mixed feeders. Differences in extant and historic group sizes and densities foremost relate to the type localities of reported observations and thus provide important insights about huemul and for planning strategies towards potential recovery.

Table 1. Estimates of livestock, from Willis 1914, Fernández and Busso 1997, and von Thüngen and Lanari 2010

Year	Country and Area	Sheep	Cattle	Horses	Mules
1900	Chile	1,340,000	830,000	?	?
	Argentina	120,000,000	28,000,000	?	?
1908	Argentine Patagonia	10,000,000	834,000	490,000	15,000
1912	Argentine Patagonia	22,000,000	1,400,000	?	?
		Stocking rate is 16-60/km ²			
1952	Argentine Patagonia	25,000,000	?	?	?

PREDATORS

Predation events in severely reduced subpopulations, as now found with huemul, are very important due to dynamics of small populations (Caughley 1994). Nonetheless, it is equally important to understand the underlying causes of supposedly *excess* predation on cervids. Overabundant predators and inappropriate antipredator behavior to novel predators have been implied as major general impediments for huemul (see below), but alternative explanations include elevated morbidity, modified habitat structure, other undetected causes of mortality unrelated to predation, and methodological errors regarding differentiating predation from scavenging and carnivores involved. Even puma (*Puma concolor*) scavenge appreciably, treating scavenged carcasses as they would their own kills: between 8-12 puma scavenged 19 of 44 placed deer carcasses during a mean of 5 days even when rotting and maggot-infested (Bauer *et al.* 2005); 64% of monitored puma scavenged at least once and spending up to 50% of feeding time on carrion (Knopff *et al.* 2010); or a healthy female puma feeding exclusively on four carcasses during at least 22 days (Nowak *et al.* 2000). Interestingly enough, this behavior was already documented by puma scavenging a huemul that was killed earlier by Prichard (1910).

Frequently, predation by dogs is implied as a major factor causing declines or preventing recovery (e.g. Lord 2007), yet only occasional kills are reported and the impact on recruitment has not been quantified conclusively in any population. The assumed highly effective predation from dogs has been asserted to stem from huemul having evolved in, and still inhabiting, areas lacking large cursorial predators (Saucedo and Gill 2004; Corti *et al.* 2009, 2010; Vila *et al.* 2010). However, the cervid radiation to South America was accompanied with members of the Canidae, including at least three species of *Canis* (HersHKovitz 1972; Prevosti 2009). Dire wolf for instance co-existed with huemul into the Holocene (Marshall and Sempere 1991; Hunt 1996; Frid 1999), and existed until after man arrived with domestic dogs (Steward 1946; Orquera 1987; Miotti and Salemme 1999; Simonetti *et al.* 1999; Muñoz and Mondini 2008). Identifiable domestic dogs in Eurasia date back to >14,000 years, and paleoindians likely crossed Beringia already with dogs (Fiedel 2005; Napierala and Uerpmann 2010). By providing humans with hunting assistance, transport as well as emergency food, dogs likely arrived in South America together with paleoindians, with records from late Pleistocene (Miotti and Salemme 1999). Pre-Columbian hunter-gatherers clearly hunted huemul with dogs, and one tribe, with dogs but no horses, was called ‘huemules’ due to their clothes being made from huemul skins (Steward 1946; Díaz and Smith-Flueck 2000). The large fox (*Lycalopex culpaeus*) is sympatric with huemul,

considered analogous to *Canis latrans* in appearance and habits (HersHKovitz 1972), and also is a cursorial species pursuing large prey (Novaro *et al.* 2009). Direct observations of dogs and huemul (Jimenez *et al.* 2008) include: a) a persecuted mature huemul buck bounding side to side to navigate logs apparently attempting to out-run the dogs, b) a female with fawn out-running 2 dogs and escaping, not running to water but contouring a hill for about 1.5km and climbing to higher ground, c) a radio-collared female with a bedded fawn interceding several approaching dogs to distract them from the fawn; she then ran away, making a large circle, to return later to nurse and then take the fawn some 500m away. These observed huemul did basically what has been observed with red deer, Sika, and fallow deer when encountering dogs (Jimenez *et al.* 2008). Considering that huemul have had continuous exposure to cursorial predators for millennia, they likely have retained appropriate antipredator behavior. Furthermore, prey-predator interactions do not appear to become specialized due to prevailing multiprey-multipredator systems (Flueck 2000), and reintroductions of large predators has resulted in rapid readjustments of prey behaviors (Breitenmoser and Haller 1993; Molinari-Jobin *et al.* 2002; Flueck 2004).

Other members of the Odocoile line besides huemul employ the same escape strategies: they hide and freeze, bolt or run off at close encounter, or take to water. Mule deer tend to bound uphill, imposing a heavy cost on predators, whereas white-tailed deer bolt down and along hillsides (Geist 1981). Huemul are known to snort, stomp the ground, run, trot or race away uphill or downhill and also bound like mule deer (Gigoux 1929; Texera 1974). Their tendency to take to water has been used to capture them (Geist 1981; MacNamara 1982).

With regard to puma predation, severely reduced huemul populations might not be able to sustain additional losses, and temporary predator control might allow recovery to sustainable numbers as documented for bighorn sheep (Williams 2010). On the other hand, a huemul population in Torres del Paine increased despite of foxes, feral dogs and a high-density puma population of 6/km² (Flueck 2010). This is possible when habitat is adequate, puma is the sole main predator, and antipredator responses are adequate as evidenced by huemul evading attacks by a puma with offspring (Prichard 1902) or chasing puma into trees (Murillo and Ramb 1975), similar as has been documented for female red deer (*Cervus elaphus*), and notably, this was a species that did not evolve with puma (Flueck 2004).

Ungulates may form larger mixed-species groups due to foraging advantages and predator avoidance (Stensland *et al.* 2003). In open areas for instance, huemul (Prichard 1902; Osgood 1943; Díaz and Smith-Flueck 2000; Guineo *et al.* 2008) or red deer (Flueck 1996) mix with guanaco, and historically huemul commonly ended up in corrals with livestock having been herded down to winter ranges (Flueck and Smith-Flueck 2011b). Recently, a young female huemul in southern Chile, found among a ranch's cattle, was lassoed and kept in a staple until confiscated by authorities a month later (Vidal *et al.* 2011). Moreover, open habitats with shallow slopes offer the additional benefit of diminished risks from puma predation as it provides little hiding cover for puma to hunt (Atwood *et al.* 2007). Thus, open lowlands east of the Andes likely presented source areas of huemul, because puma as the only main predators had limited impact due to prey forming large group sizes in such habitat, including mixed-species groups, and the large biomass of herbivores sustained in such habitat.

DIET

Circular reasoning is used to claim that huemul needs forests and browse by referring to their molars as being brachyodont, because such teeth supposedly indicate a browser (Vila *et al.* 2010). However, most all cervids have brachyodont teeth, yet even small species thrive exclusively in treeless grasslands, like Pampas deer (*Ozotoceros bezoarticus*) or roe deer (*Capreolus capreolus*) (Perez *et al.* 2008). Many cervids, including *Odocoileus*, are extremely versatile in food exploitation and successfully utilize grasslands, steppes and deserts (Putman and Flueck 2011), and quite independently from their cranial and dental features (Ozaki *et al.* 2007; Codron and Clauss 2010). For instance, mule deer will eat forage of unexpected low digestibility, but by compensating with faster gut passage rate: however, variance in diet between individual deer was greater than between elk, sheep and deer (Hobbs *et al.* 1983). Moreover, there is strong support that leaf-grass mixed feeding was the original feeding style of cervids (DeMiguel *et al.* 2008). Yet importantly, deer existing in steppes or grasslands do not solely rely on the fibrous portion of grasses. Besides other available plant species, deer can heavily use seed heads, particularly in winter (Wright and Kelsey 1997). When huemul still occurred at 200 km (Fig. 2, Prichard 1902) and even 270 km east of the Andes (Fig. 3, Díaz pers. comm.), Allen (1905) described huemul as grazing there. Furthermore, besides Gramineae, Patagonian steppes contain a large component of shrubs, forbs, and maintain important green grass production throughout winter

(Fernandez *et al.* 1991; Adler *et al.* 2005). Extant huemul had 16% of grass in diet (Sierralta 2003), whereas the sister species taruca (*H. antisenensis*) had about 60% of grass in diet, composed of 15 species, during the rainy season (Gazzolo 2006). In comparison, similar sized *Odocoileus* also live in broken table grassland (<300mm precipitation) and deserts (74mm precipitation) (Fig. 4, Dusek 1975; Marshal *et al.* 2006). Irrespective, based on an unpublished report, huemul are still claimed to avoid grasslands and steppes and thus would not be expected there according to Cruz *et al.* (2010): huemul antler they found near the Atlantic consequently was suggested to have resulted instead from paleoindians walking 250 km to the Andes.

The biogeographic origin of ancestral *Hippocamelus* indicates that they were savanna-adapted in order to pass the Panama bridge filter, and subsequent periods of glaciation kept them repeatedly away from the Andes and forests, with fossils even known from northeastern Brazil as treeless Patagonia-type habitat then reached way into Brazil (Flueck and Smith-Flueck 2011b). According to Codron and Clauss (2010), gut morphophysiology does not impede feeding in alternate niches because a range of different adaptations may serve for utilizing the same dietary niche. Ingestion of other resources is a physical and physiological possibility and only translates into slight differences in chewing efficiency. It is possible, for instance, to maintain a concentrate selector-type ruminant on a purely grass-based diet, or a cattle-type ruminant on a purely browse-based diet - at least for a period of several weeks to months. Within the adaptive capacity of a ruminant, nutrient acquisition might be such that diet choice is largely random, which could explain why ruminants are so often observed to feed, apparently, suboptimally (Codron and Clauss 2010).

COMPETITION

The decline in distribution and abundance, as well as lack of recovery of huemul are frequently attributed to conflicts with exotic herbivores. Especially red deer is claimed as outcompeting and displacing huemul (Thornback and Jenkins 1982; Miller *et al.* 1983; Lever 1985; Schuerholz 1985; Navas 1987; Saizar 1987; Redford and Eisenberg 1992; Nowak and Walker 1999; Jaksic *et al.* 2002; Lord 2007; Dolman and Wäber 2008; Urrutia and Ojeda 2008; Vila *et al.* 2010). However, Díaz and Smith-Flueck (2000) questioned these assumptions, particularly red deer being responsible for declines of huemul, as no examples have ever been documented (also see Jimenez *et al.* 2008; Flueck 2010). In contrast, huemul were found to have disappeared in several areas lacking cattle, sheep, or exotic red deer (Smith-Flueck 2003). Moreover, feral cattle have co-existed with huemul several hundred years, and over 112 years with ranched cattle in one documented case (Jimenez *et al.* 2008). And although exotic red deer in southern forest habitats ate many of the same plants and had similar dietary preferences as huemul (Smith-Flueck 2003), this is relevant only if it reduces the population growth rate of huemul to <1. Also, considering huemul diet behavior from all studies, it is highly likely that huemul can shift diet without necessarily affecting recruitment (Flueck 2003; Codron and Clauss 2010), as is known for other cervids (Putman and Flueck 2011). Then, considering the impressive densities of exotic domestic and wild herbivores on former huemul habitat, it is considered unlikely that such areas were, or still would be, limiting to huemul in terms of energy and major plant nutrients (Krieg 1940; Flueck 2001, 2003). Moreover, mammalian herbivore communities commonly are multi-species assemblages, and the presence *per se* of other herbivore species is unlikely to be problematic for huemul, as evidenced by documented coexistence with livestock, pudu (*Pudu pudu*) and guanaco. On the other hand, the ecosystem can be affected if overabundance of herbivores occurs, be it through livestock or exotic wild herbivores.

Many remaining huemul populations share habitat with some livestock (exceptionally with red deer), but no studies show conclusively that there is spatial displacement from avoidance. In contrast, huemul used to mingle with livestock and were commonly driven together to winter ranges (reviewed in Flueck and Smith-Flueck 2011b). However, spatial avoidance could also result from activities related to livestock production, such as human harassment and hunting, and from accompanying unleashed dogs. Lastly, one common difference in habitat use is that only livestock have access to lower lands and valley bottoms, as the anthropogenic pressure does not allow huemul to persist there anymore (Flueck and Smith-Flueck 2011b).

DISEASES

There are frequent claims of huemul being highly susceptible to cattle diseases: *Cysticercus tenuicollis*, foot-and-mouth disease (FMD), coccidiosis, 'parasites', or actinomycosis (Povilitis 1978; Thornback and Jenkins 1982; Schuerholz 1985; Redford and Eisenberg 1992; Simonetti 1995; Wemmer 1998; McCallum and Dobson 2002; Uhart and Chang Reisig 2006; Lord 2007). However, such assertions

were based on hearsay, guesses, or misquoting of original sources. For instance, "*C. tenuicollis* when transmitted by livestock is fatal to huemul", yet the original source stated that presence of *C. tenuicollis* was not considered to be the cause of death. McCallum and Dobson (2002) wrote that *C. tenuicollis* is highly pathogenic to huemul, livestock being the reservoir, and increased habitat fragmentation unequivocally bad as it might maintain high infection rates and hence lead to declines or extinction of huemul. In contrast, in other cervids and ungulates the presence of *C. tenuicollis* is considered trivial (Leiby and Dyer 1971), which appears to be the same for huemul judged by several centuries of coexistence with livestock, millennia with guanaco that also harbor this parasite, and absence of direct evidence. Similarly, the only report on coccidiosis (Texera 1974) was subsequently misinterpreted, or FMD claimed to have wiped out huemul when cervids are currently considered unlikely to be an important factor in the maintenance and epidemiology of FMD in livestock outbreaks. Besides, FMD is self-limiting at normal densities of cervids (reviewed in Flueck and Smith-Flueck 2011c). Furthermore, a recent review of FMD in wild populations of susceptible South American wild species found no reports of any previous disease nor outbreaks (Pinto 2004).

Parasites found so far in huemul occurred only at very low levels. These and other parasites found in livestock and red deer are mostly considered nonthreatening, and being common in livestock occur in most areas where these are found (Love and Hutchinson 2003). Although red deer are suggested to present a special disease threat to huemul, they harbor diseases commonly associated with livestock. Having coexisted with livestock for >100 years, both red deer and livestock play roles in the epidemiology of the various diseases they share. While livestock are commonly sympatric with huemul (nearly 100%), spatial overlap with red deer is exceptionally rare, occurs in <2% of known populations, and occurred in recent time. Even in these latter cases, livestock presence is the determining epidemiological factor, since for each huemul there are 1.2 red deer in contrast to 25.2 livestock. Thus, for huemul the primary factor regarding contagious diseases are feral and free-ranging livestock (Flueck and Smith-Flueck 2011c).

DISCUSSION

Huemul antlers provide clues about changes in historic times and actual well-being. As luxury appendages they represent many biological and ecological relationships. Interpretations about huemul antlers are misguided and the informational content of antlers underappreciated. To consider >2 times as abnormal is erroneous and clearly incompatible with modern understanding of antler biology. The question rather is: what conditions in the past allowed larger antler growth with up to 5 times, and thus antler expression to be closer to the species' norm? Of plausible historical changes, the strongest effect likely stems from preventing access to nutritionally superior places (Flueck and Smith-Flueck 2011a, 2011b), followed by few males reaching prime age. It is reminiscent of red deer in primeval Europe when Lords forced the farmers to let deer feed in fertile fields and poaching was punished with death: body and antler sizes were much larger than those from deer now forced to live in closed forest tracks and high mountains (Beninde 1937:164).

Historical and current cases show that group sizes and density can be substantially larger than orthodox descriptions of huemul which are based on remnant populations in marginal habitats. Relying on such biased information results in circular reasoning when interpreting zooarcheological data, paleodiets, prehistoric distribution, and the ecology of huemul in general. For instance, prehistoric expansion of agriculture in central Chile led to much clearing of forests with the spread of weeds and other indicators of open habitats, with fires peaking 12000-6000 years BP, and causing local extirpation of several species including huemul (Flueck and Smith-Flueck 2011a). The Spanish colonization rapidly reduced indigenous people due to disease and slaughter, which was followed by a transient expansion of forests into abandoned land. Similarly, war and disease reduced the native population east of the Andes and may have allowed some temporary recovery of huemul in eastern lowlands. This may explain why early explorers again could easily hunt many huemul each day, and hundreds of kilometers from Andean forests (Prichard 1902). However, the subsequent colonization extirpated all huemul in most lowlands and open areas. The expansion of livestock ranching and settlements in most favorable lowlands and valley bottoms resulted not only in the inaccessibility to that part of former range for huemul, but also in the loss of their migratory traditions with likely nutritional consequences (Flueck and Smith-Flueck 2011b).

As marginal habitat results in fragmentation and reduced subpopulations, any mortality factor becomes more important, including predation. Determining the cause of death becomes a central concern and challenge, as predation has to be differentiated from scavenging (and even puma scavenging

appreciably), predator/scavenger species have to be differentiated, and lastly the possibility of underlying debilitating factors (physical condition, disease, etc) have to be evaluated, as these might be concealed by predation events. Claiming that huemul lack adequate antipredator behavior towards cursorial predators due to their absence in the evolutionary history is unwarranted. To the contrary, the cervid radiation to South America was accompanied with several cursorial predators including *Canis* lasting into the Holocene. Furthermore, paleoindians likely arrived in South America together with dogs. Earliest records date to late Pleistocene and Pre-Columbian hunter-gatherers clearly hunted huemul with dogs. Direct observations showed that huemul antipredator tactics towards dogs frequently resulted in preventing death of offspring or adults. Furthermore, cervids can rapidly readjust their behavior to reintroductions of large predators. All cervids can suffer losses from dogs, and although such predation is claimed to be causing declines or preventing recovery in huemul, the impact on recruitment from occasional kills is unknown. Most studies on effects of feral dog predation on *healthy* deer populations suggested that the influence is minimal (Reed 1981). However, predation events in severely reduced subpopulations, as now found with huemul, may be important due to dynamics of small populations. Thus, even puma predation in such circumstances might call for temporary predator control to allow huemul recovery to sustainable numbers. Importantly, of the two huemul populations documented to have increased, one recovered despite a high-density puma population (Guineo *et al.* 2008; Flueck 2010), whereas the other recovered despite puma and frequent incursions by dogs from nearby settlers and a town only 3km away (Díaz and Smith-Flueck 2000). The lack of recovery in other populations therefore might indicate other ongoing processes including excess morbidity, or other more important causes of mortality unrelated to predation. Lastly, certain areas in the past allowed large aggregations of huemul, forming mixed groups with guanaco in areas which today produce a large amount of herbivore biomass, and therefore likely represented source areas for huemul.

Cold-temperate ecosystems contain several ungulate species, with competition expressed as adjustments in spatio-temporal habitat use and feeding behavior, and modulated by factors like predation. Competition thus affects parameters like: distribution, recruitment, morphology and physiology, yet persistence of multi-species assemblages is the rule. There is no data supporting claims that competition, particularly from red deer, have caused declines in distribution and abundance in the past century, or prevented recovery of huemul. In contrast, huemul have disappeared in areas lacking livestock or exotic red deer (Vogel 1969; Smith-Flueck 2003). Moreover, huemul have also remained in coexistence with livestock over several hundred years, besides coexisting with guanaco and pudu. Intra- and interspecific food habits in cervids vary greatly and quite independently from dental and gastrointestinal features, due to numerous adjustments which can be made in behavior, physiology and morphology. This is even true for small deer (*Ozotoceros*, *Capreolus*), but also *Odocoileus* and taruca persist in grasslands and steppes. Huemul exhibit very flexible feeding behavior considering the use of at least 145 plant species, apart from species used historically in Patagonian steppe far from forests, and exotic food received for several years in subtropical Buenos Aires (Flueck 2010). In Chile, captive huemul accepted fruits, concentrates for heifers, ground oats, alfalfa hay, exotic willow and poplar, but given the choices they did not consume native *Nothofagus* (lenga, coihue) (Rottmann 2003). Hershkovitz (1972) recognized *Hippocamelus* as pastoral and only secondarily adapted to sylvan habitats, which accords with habitats associated with past glacial events and with historic evidence of huemul still living far from Andean forests.

There are no data supporting claims that huemul is exceptionally susceptible to livestock diseases, nor that these affect population dynamics or have caused extinctions. Parasites found in huemul were at very low levels and are generally considered nonthreatening. Regarding epidemiology, emphasizing red deer is not warranted as they share diseases with livestock, having coexisted for >100 years. Livestock in turn have coexisted with huemul for several hundred years. Currently, most all huemul populations are exposed to livestock, but practically none are sympatric with red deer, in which cases livestock still determine the epidemiology since they outnumber red deer by 2100%. Thus, for huemul the primary factor regarding contagious diseases are feral and free-ranging livestock.

CONCLUSION

Huemul antlers frequently are simple forks, but rather than claiming it to be the norm, we need to determine what conditions in the past allowed much larger antlers. Orthodox descriptions of density and group size stems from biased data. To avoid circular reasoning when interpreting zooarchaeology or past distribution, historical data on abundance need to be considered. Having co-evolved with cursorial and ambush predators, huemul have adequate antipredator tactics, and any current predator impacts

would relate to dynamics of small populations or to other underlying problems. Although competition from other herbivores can shift spatio-temporal habitat use and affect performance, persistence of multi-species assemblages including huemul/guanaco/pudu is the rule. There is no evidence that livestock or the red deer in particular have caused declines or are preventing the recovery of huemul. In contrast, huemul have disappeared in numerous areas lacking livestock or red deer, while persisting in other areas with livestock for several hundred years. Cervid feeding behavior varies greatly due to flexibility in behavior, physiology and morphology. Thus, competition *per se* appears to be of minor importance, more likely incompatibilities relate to management associated with livestock production, particularly the presence of people and their dogs which exert heavy pressure on wildlife. Concerning contagious diseases from exotic ungulates, the overwhelming primary factor would be livestock given their numbers and degree of overlap with huemul. Regular research and slaughter inspections of livestock provide a good proxy for pathogens afflicting sympatric red deer or huemul.

Whereas active management should aim to increase recruitment with all possible means for the short-term to prevent extinction of highly reduced remnant subpopulations, sustained recovery may depend on re-establishing source populations on more productive habitats, guided by zooarcheological and historical data.

ACKNOWLEDGMENTS

We like to thank several anonymous reviewers for extensive comments and constructive critics which helped improve this manuscript.

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Figure 1. Huemul antlers still commonly have three tines, and up to five in the past.



Figure 2. Past and current use of open and flat habitat by huemul, far from forests and cover. Note that use of such habitat occurs during full daylight hours.

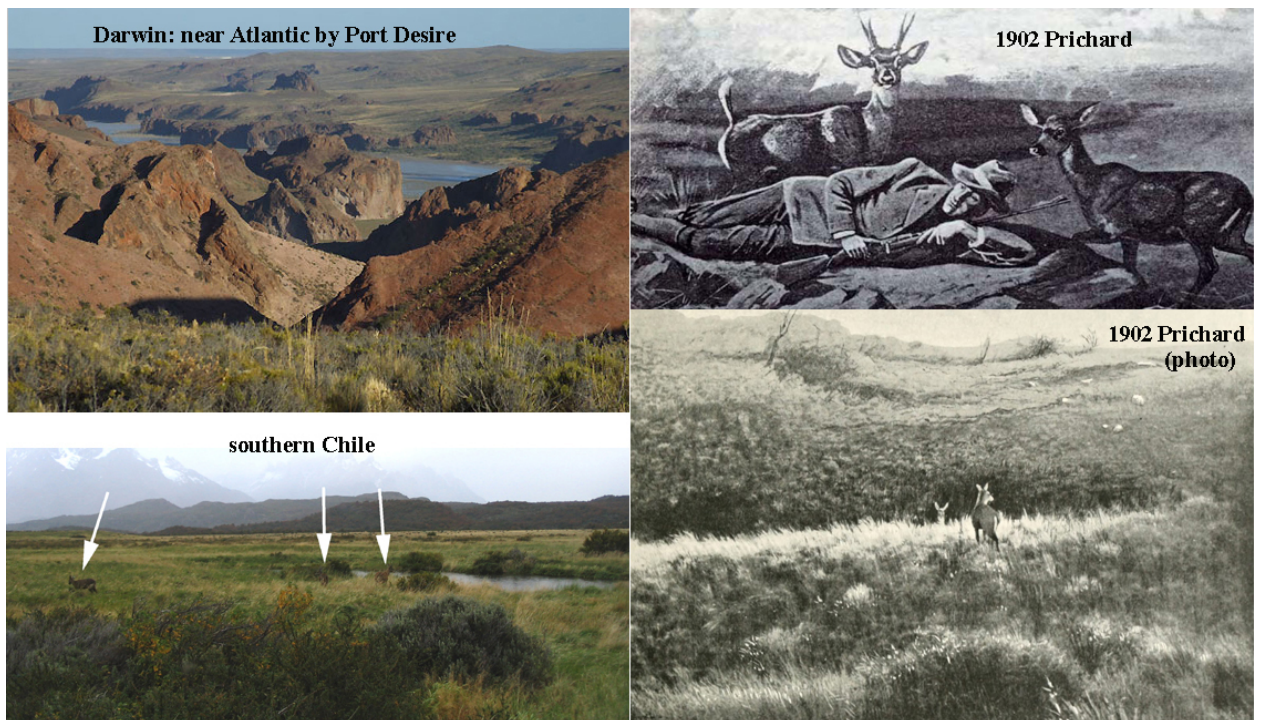
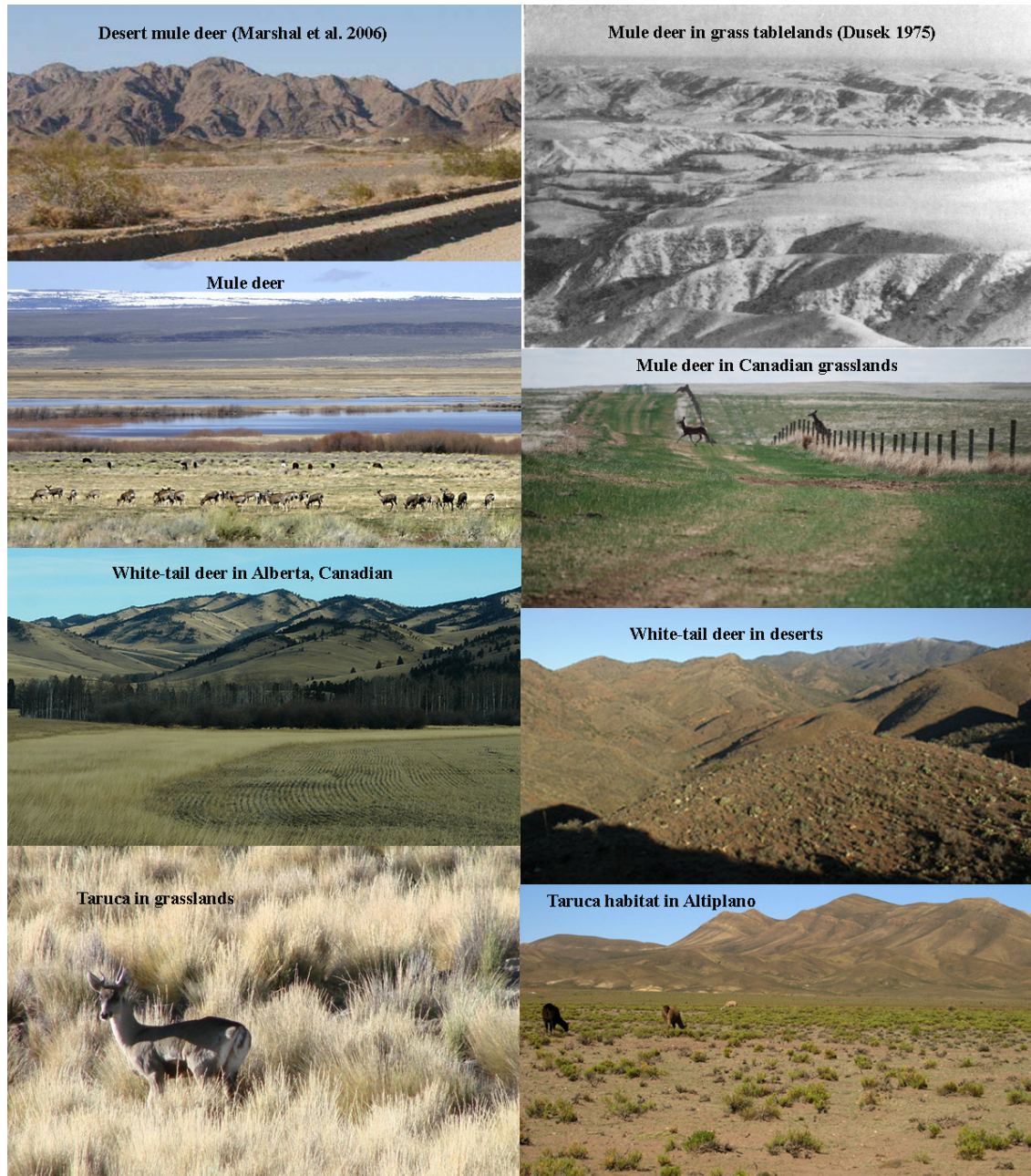


Figure 3. Huemul shot at 270 km from the Andes at Sierra Piré Mahuida (with courtesy of N. Díaz).



Figure 4. Similar sized *Odocoileus* and taruca (*H. antisensis*) also live in broken table grassland (<300mm precipitation) and deserts (74mm precipitation).



HUEMUL HERESIES: BELIEFS IN SEARCH OF SUPPORTING DATA.

3. REPRODUCTION

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ABSTRACT

The general absence of recovery of remnant huemul populations (*Hippocamelus bisulcus*) hinges on factors affecting the population dynamics. We analyze recent published findings about reproduction and behavior with regard to their possible implications. The life history of huemul is characterized by age at maturity of one year with evidence that fawns may also breed. Reports of twinning need confirmation, but it occurs in congeneric taruca (*H. antisensis*) and other Odocoileines. Huemul breed every year and frequently raise fawns successively. Life cycle calculations should apply these parameters because unrealistic parameters may cover up ongoing processes and lead to spurious conclusion. Sexual segregation in huemul is facultative, similar to many other cervids and reflects relationships between populations and their environment. Dominance group breeding systems were described several times without indications of territoriality, as with taruca. Recently, huemul bucks were characterized as territorial all year, life-long, defending and monopolizing female groups and two 'territorial' bucks sired most offspring. These two bucks though sired only 26% of fawns in their respective social groups. In their area, 'territorial' bucks bred about as many wandering females than resident females, whereas 32-45% of fawns from resident females were sired by outside males. Furthermore, when applying one year as the age at maturity rather than three years, there were unaccounted fawns and possibly <50% of all neonates were sampled. Overall, these data do not support territoriality in huemul. Regarding movements, extant huemul occupy flat grasslands, and at times nearly exclusively so, while historically huemul occurred up to 270 km from forests and in rolling topography. The claim that 5 km of open valley inhabited with guanaco (*Lama guanicoe*) present a barrier to huemul causing genetic isolation is unwarranted: moreover, huemul coexist with guanaco currently and historically. Erroneously considering landscape features as barriers and underestimating reproductive capacity may distract from discovering the factors underlying the lack of recolonizations generally observed in currently reduced huemul populations.

Additional keywords: *Hippocamelus bisulcus*, reproduction, sexual segregation, territoriality, resource defense, genetic isolation.

INTRODUCTION

Securing reliable data is difficult on species highly reduced in numbers and remaining mainly in remote refuge areas. In the accompanying paper (Part I. Historical and zooarcheological considerations, this issue) we analyzed information regarding historical conditions which influence the current interpretation of the biology and ecology of huemul (*Hippocamelus bisulcus*). Part II addresses antlers, group size and density, predation, diet, competition, and diseases. These papers together aim to improve our understanding of huemul and its conservation. Here we analyze older and more recent sources and focus on issues related to reproduction, sexual segregation, resource defense, and genetic isolation to reveal discrepancies. We show that current beliefs do not stand up under close scrutiny.

METHODS

We reviewed literature via Cross-Search of ISI Web-of-Knowledge and 17 external databases, three books and three dissertations on huemul, historic and grey literature available in a collection containing 286 entries, plus publications on other related deer species to allow a comparative approach to analyze and interpret huemul literature, including questionable information, which continues to be used to describe huemul.

RESULTS

Reproduction

The general lack of recovery among huemul populations raises immediately the question about population dynamics including vital rates, and other parameters which are essential to diagnose factors limiting recovery. Age at maturity of huemul has been asserted to be three years, although without supportive data (Corti *et al.* 2009). However, irrefutable evidence exists that huemul breed as yearlings (Texera 1974; Guineo *et al.* 2008; Vidal *et al.* 2011). Furthermore, there is good evidence to show that even fawns can breed (Anon. 1936; Texera 1974; Vidal *et al.* 2011), as is known for other *Odocoileus* including *Odocoileus* and *Rangifer*. Given the above evidence, it is therefore unrealistic to classify females < 3 years old as juveniles and base calculations of intrinsic population growth rates on 'adult' female huemul (Wittmer *et al.* 2010). Other parameters misrepresented regard claims by Garcia *et al.* (2008) that available information indicates that females have only a fawn every second year, unless the fawn dies early on. However, indisputable evidence shows that huemul breed every year (at least in six consecutive years; Aldridge 1988) and frequently raise fawns successfully during several successive years (Guineo *et al.* 2008; Vidal *et al.* 2011). Lastly, huemul have been reported to have twin fawns (Housse 1953; Whitehead 1993), as is known to occur in the sister species taruca (*H. antisensis*) under good conditions (Barrio 2010), as well as in *Odocoileus*. No recent cases are known and twinning needs to be confirmed.

Sexual segregation

Huemul social structure is said to differ from other cervids. In contrast to other cervids, it is commonly stated that mixed-sex groups occur not only during the rut, but continuously and throughout the whole year (Povilitis 1983, 1985; Vila *et al.* 2010). However, in addition to mixed-sex groups, sexual segregation, including single animals, and multiple male (Fig. 1) and female groups, has also been documented (Serret and Borghiani 1997; Frid 1999; Wensing 2005). In one population, the sexes were mainly segregated with adult males and females associating only twice out of 104 group sightings (Frid 1994).

The degree of segregation between the sexes (social or spatial separation) is highly variable between different cervid populations (reviewed in Putman and Flueck 2011 as follows). In red deer (*Cervus elaphus*), with sexes usually considered to be highly segregated outside the rut, in some populations even among mature animals, only 18-20% of stags and 50-56% of hinds were seen in segregated parties. In other populations some males leave the rutting area but migrate to areas that contain females all year and loose groups may form. In some fallow deer (*Dama dama*) populations, males remain in female areas long after the rut and in largely open landscapes, or in populations with few adult males; aggregations containing adults of both sexes remain frequent throughout the year. In white-tailed deer (*Odocoileus virginianus*), some populations formed mixed groups year round; among mule deer (*Odocoileus hemionus*), mixed groups of adults also occurred all year, making up 13-30% of all groups encountered. In roe deer (*Capreolus capreolus*), home ranges of adult males and females overlap all year and mixed-sex groups can be common all year. Moose (*Alces alces*), considered a rather solitary species, may nonetheless occur in mixed groups throughout the year, with mixed-sex groups being more in populations with proportionally more females. Thus, the pattern of mix-sex groups described in huemul as being so unusual, in actuality also occurs in numerous other cervids, and conversely, several cervids including huemul form single-sex groups, with several known factors accounting for this variation.

Territoriality

Breeding systems are particularly important in small populations (Stephens and Sutherland 1999) and thus we evaluate contradictory evidence about the huemul mating strategy. The dominance group mating system is the only one which has been observed for huemul until recently (Povilitis 1985; Diaz and Smith-Flueck 2000; Wensing 2005; Fundación RA Philippi 2009). This consists of multi-male aggregations in which one male is dominant over all others and achieves most matings. Subordinate males are tolerated in the group, male aggression is relatively low compared to other systems (Aldridge 1988; Serret 2001), and territoriality is not exhibited. During a 10 year study, no indications of huemul being territorial with active defense were found (Guineo *et al.* 2008). The sister species taruca also exhibits a dominance system and lack of territoriality (Barrio 2010).

In contrast, huemul bucks were recently claimed to be strongly territorial, thus displaying behavioral characteristics unusual for most cervids (Corti *et al.* 2009, 2010). According to Maher and Lott (1995), territoriality is achieved by expelling potential competitors from a defended area, with defense behaviors taking place at the boundaries, which includes scent marking, displays, retreats, chases and fights, and agonistic or aggressive behavior in general. Corti *et al.* (2009) considered huemul bucks as

being territorial, defending their area during all seasons, and life-long. Furthermore, they asserted that these territorial bucks defended and monopolized female groups in specific areas and sired most offspring, while non-territorial bucks rarely sired offspring (Corti *et al.* 2009). Specifically, eight of 16 sampled sexually mature bucks present during 2005–2007 sired no offspring, and 44% of fawns were sired by only two bucks. Thus, a few bucks sired most of the fawns for several consecutive years with the same groups of females (Corti *et al.* 2009).

Territorial behavior as described by Corti *et al.* (2009) for huemul would indeed be unusual among cervids. Instances of territoriality described in cervids so far relate to male behavior during the rut in only a few species (reviewed in Putman and Flueck 2011). Red and fallow deer can adopt a strategy of defending very small mating territories, which is determined by population density and resource patchiness. In the only cervid with delayed implantation, roe bucks often maintain larger breeding territories, especially in forests, but not in open-field habitats. Territorial roe bucks will mark the periphery and interior of their area. However, adult males of all these facultatively territorial cervids also may occur in mixed groups year round with highly variable group sizes depending on density, habitat and sex structure, which determine the type of breeding system adopted (Putman and Flueck 2011). However, no other cervid is known to exhibit territoriality during the whole year as claimed for huemul (Corti *et al.* 2009).

Male behavior makes up only part of the mating strategy. Female roe deer commonly acquire extra-pair matings (Foerster *et al.* 2003). Although roe bucks often maintain breeding territories, up to 55% of females in one study performed excursions outside their usual home ranges during the peak of the rut, ranging from 0.6 to 3.0 km, and lasting for several days (Lovari *et al.* 2008; Richard *et al.* 2008). Moreover, Vanpé *et al.* (2009) found 14% of polytocous litters sired by more than one buck. Multiple paternity also occurs in *Odocoileus virginianus* (DeYoung *et al.* 2002), where recent studies refuted the prevailing assumption that male reproductive success is highly skewed toward a small number of mature, dominant bucks (DeYoung *et al.* 2009). In fact, physically immature males (1.5 and 2.5 years of age) collectively fathered 30–33% of offspring in all studied populations, even where mature males were present. The same flexible mating strategies also occurs in huemul, where subordinate males did all the siring in consecutive years in areas of supposedly territorial males, females being bred by neighboring males, and females moving into areas of other males to return after mating (Corti 2008; Povilitis 1983, 1985; Wensing 2005).

Evidence for territoriality in huemul bucks

Huemul bucks being territorial during the entire year (Corti *et al.* 2009) is unusual among cervids, with implications for population genetics. Based on the original source, 41 marked huemul (20 with radio-collars), observed at least once per month between late March 2005 to December 2007, were the basis to determine interactions and group membership (Corti 2008). These observations resulted in 25.7 spatial points on average from 18 males and 23 females to determine home range sizes and spatial overlap. In the following we analyze several lines of arguments to claim territoriality among huemul bucks.

Territoriality based on dominance. According to (Corti 2008), “adult males were considered territorial if they appeared dominant to other males and held a specific area. Non-territorial adult and juvenile males were considered subordinates”. However, dominance was not defined and there was no information as to how it was measured.

Territoriality based on spatial overlap. Observations of males resulted in 7–20 spatial points/male, therefore, not all individuals were seen every consecutive month (Corti 2008). The 50% core area of dominant males (based on 15.6 spatial points on average) was considered to represent the defended territory or exclusive area of mating, averaging 55.7 ha (range 25–114 ha). However, as these spatial points were collected during a period of 2.5 years and with large time intervals in between readings, the delimited core areas and overlaps do not indicate spatio-temporal relationships. Moreover, little or no overlap of home ranges does not constitute evidence of territorial defense behavior (Grant *et al.* 1992). In addition, 50% of the ‘territorial’ males had their core areas overlapping with another ‘territorial’ male (Corti 2008). Notably, the claim of life-long territoriality was based on 3 adult males remaining in the same area until death (2–3 years).

Territoriality based on behavior. Very few interactions among ‘territorial’ males were seen, and therefore “cannot establish conclusively that they defend areas against other males” (Corti 2008).

Instead the degree of home range overlap was used to deduce territorial behavior. However, home range overlap in cervids is strongly tied to animal density and vegetation structure (Jepsen and Topping 2004; Kjellander *et al.* 2004; Tufto *et al.* 1996). Actual interactions between huemul bucks during the rut were documented as ‘territorial’ males chasing subadults 5 times, and one observation of parallel walking of two ‘territorial’ males at the apparent boundaries of two territories. Parallel walking, however, is common among many cervids, unrelated to territoriality, and interpreted as assessment of the opponent, leading either to fighting or withdrawal (Clutton-Brock *et al.* 1979). A ‘territorial’ male with a broken forelimb, and a juvenile male with both a broken rear leg and antler were considered evidence of aggressive territorial behavior during the rut. This seems unlikely given that no direct interactions between ‘territorial’ males were ever seen (Corti 2008), that juveniles do not engage in fights with mature males (Thomas *et al.* 1965; Ozoga 1972; Geist 1981; Anderson and Wallmo 1984; Povilitis 1983, 1985; Guineo *et al.* 2008), and the general absence of such reported injuries from rutting in other cervids. Although accidental falls of rutting males might result in broken legs, a more plausible scenario would be a pursuit by dogs (Packard 1947), which frequently occurred in the study area (Corti 2008). The fraying of trees and shrubs was interpreted as territorial marking, referring to behavior of roe deer during the mating season (Johansson and Liberg 1996). However, the common behavior of non-territorial cervids to rub antler and forehead against stems of trees and bushes has been described as innate extra-rut behavior, velvet shedding, marking behavior, and conspecific communication. *Odocoileus*, and likely the related huemul, have much less glands in the forehead skin than territorial *Capreolus* (Quay and Müller-Schwarze 1970, 1971). Intensive rubbing and thrashing, also called horning, is known for huemul (Geist 1998), and is very common in *Odocoileus*, yet this genus is non-territorial.

Territoriality based on skewed parentage. According to Corti (2008), the huemul mating system was polygynous, with males defending several estrus females sequentially, and 13% of mature males siring 44% of all fawns. This highly skewed male reproductive success was implied to support the existence of a territorial breeding system (Corti *et al.* 2009). Highly skewed reproductive success would indicate that few ‘territorial’ huemul were able to monopolize breeding by maintaining strong associations with many females within their reduced core mating area. However, simple ratio association indices were very weak and ranged from 0.00 - 0.03 for males and 0.01 - 0.03 for females, indicating that most huemul spent only short periods of time in small groups or were solitary, especially adult males (Corti 2008). Furthermore, at only 1.7 huemul/km², the supposedly exclusive 50% core areas (56 ha on average) would require a clumped distribution, where groups of females would have to remain principally in these reduced areas of a given ‘territorial’ buck. In contrast, in other cervids at low density and few males, females started to wander in search of breeding opportunities (Labisky and Fritzen 1998; Lovari *et al.* 2008; Richard *et al.* 2008), multiple fathers are common (DeYoung *et al.* 2002; Vanpé *et al.* 2009), and non-dominant males frequently have breeding success (DeYoung *et al.* 2009). Huemul actually exhibited similar variations as the deer in these other studies: a) one of seven ‘territorial’ males never sired any fawns; b) one subordinate male entered the area of a ‘territorial’ male in two consecutive years and sired all fawns; c) five females had offspring sired by males inhabiting neighboring areas; d) and three females were directly observed leaving their areas and moving into areas of other males before returning to their ranges after mating (Corti 2008). Huemul groups in other areas (Povilitis 1983; Wensing 2005) and taruca (Barrio 2010) were also found to be very fluid with members coming and going, and Povilitis (1985) described a female getting courted by two or three males intermittently, another female being mainly courted by one male but mounted by another male, a dominant male chasing a subordinate male after the latter had mounted a female, and dominant males leaving the females for prolonged times in apparent search for other estrus females. Thus, the skewed breeding success among male huemul reported in Corti *et al.* (2009) seems at odds with these other observations and is evaluated below.

Determination of skewed reproductive success. Several possible biases need to be considered when determining the reproductive success of ‘territorial’ huemul bucks. Due to limited genetic variability, incomplete sampling, and probably genotyping error, it was not possible to assign paternity at high confidence, and was considered to possibly generate a large bias (Corti 2008). This bias was reduced as much as possible by assigning only putative fathers as those that were near the area of a mother; yet indices of overlap of fathers' 50% core area with mothers' home range were low (average of 0.29, range 0.00- 0.67) (Corti 2008).

Based on Corti (2008), relative success of ‘territorial’ males breaks down to siring only about 45% of observed fawns in their social group, while siring another 41% of fawns to wandering females, and

importantly, their social groups also 'lost' 32-45% of the observed fawn crop to outside males. Moreover, one of seven 'territorial' males did not sire any fawns during the 2 years: all fawns stemmed from a subordinate male coming in from another social unit. Within social units, 22% of females got bred by other males, 11% of females were either bred by the 'territorial' or other males, and 33% of female had no data. The 13% of mature males (two 'territorial' bucks) siring 44% of all fawns (Corti *et al.* 2009) were responsible for only 26% of the fawns in their respective social groups.

However, when applying the age at maturity of one year (Guineo *et al.* 2008; Vidal *et al.* 2011) instead of 3 years, there are potentially 28 fawns unaccounted for, apart from the 23 sampled ones (Table 1). Thus it is probable that less than 50% of all neonates were sampled which adds another source of bias.

Table 1. Offspring production during the 2 years, based on the age at maturity of 1 year and assuming successful breeding each year (capture data are from Corti 2008)

	<i>n</i>	Sampled fawns	Missing fawns
Females resulting in fawn captures each year	6	13 ^A	
Females resulting in only one captured fawn	10	10	10
Females resulting in zero captures	9	0	18
Total		23	28

^A One female resulted in 3 sampled fawns over 3 years.

Genetic isolation

Mate finding or genetic isolation can be affected by physical isolation of subpopulation. Genetic isolation requires either absolute barriers, or an absence of immigration due to other factors, including lack of conspecifics through a discrepancy between maximal dispersal distance and separation between neighboring populations. It can also result from neighboring populations being too small to produce dispersers as this is influenced by the Allee effect on behavior (Stephens and Sutherland 1999) and population density (e.g. Kokko and Lopez-Sepulcre 2006).

To support claims of genetic isolation, a valley 5 km wide was asserted to present a barrier to huemul (Corti *et al.* 2009), due to being flat and open (Fig. 2). Yet the habitat shouldn't present an obstruction to huemul movements. Extant huemul are known to occupy grasslands, and coastal populations used open bottom grasslands 48% of the time, and males nearly exclusively so (Frid 1994, 1999). Data from historic huemul distribution show that they still occurred up to 270 km away from forests and in flat or rolling topography (see Part I. Historical and zooarcheological considerations, this issue), and during glacial maxima huemul occupied flat and rolling areas void of forest to the east of the Andes (Armesto *et al.* 2010). Furthermore, the valley contains guanaco (*Lama guanicoe*), a species that coexists with huemul adjacent to that study area and also further south (Guineo *et al.* 2008). Commonly the two species were sympatric in the past; such that Prichard collected a guanaco and huemul with two consecutive shots (Prichard 1902; Hatcher 1903; Osgood 1923). In contrast to the claim regarding a barrier, a description of the nature reserve containing this valley states that a "population of huemul deer [that] occupies the valley and neighboring Huemul Reserve" (www.conservacionpatagonica.org/patagonia_huemul.htm).

To further support supposed genetic isolation, a very limited dispersal capacity of huemul was asserted (Corti *et al.* 2009). Dispersal of huemul has been determined to be 8 km during a 2-3 year study, based on one subadult female (Gill *et al.* 2008), which was used to claim that the study population cannot receive dispersers from a population 10 km away, and gene flow thus was absent (Corti *et al.* 2009). However, given low densities, very small groups and short-termed studies (Gill *et al.* 2008), this one dispersal distance might not present the norm for huemul dispersal behavior and unlikely represents a maximum. Huemul certainly disperse effectively considering their rapid postglacial occupancy of a huge region of South America (30-55°S) (Diaz and Smith-Flueck 2000). *Odocoileus virginianus* disperse 50 km on average (but may exceed 200 km, Brinkman *et al.* 2005); the small *Capreolus capreolus* disperse 120 km and 51 km on average in northern Sweden and in interior areas of Norway, respectively; and *Alces alces* disperse a minimum of 20 km and up to 150-200 km (reviewed in Hjeljord 2001).

DISCUSSION

Age at maturity of wild and captive huemul is undoubtedly at one year, with good evidence that fawns may breed as well. Several well known factors affect the age at maturity, usually through food limitation, as reflected in proportionally smaller offspring and juvenile weights below the norm (reviewed in Putman and Flueck 2011). The low-density huemul population claimed to have females sexually mature at three years does not appear to be food-limited as evidenced by a several-fold increase during 3-4 decades and sizes of male skulls ($n = 8$, Blue Thomas, pers. communication 2006). Reports of twinning in huemul, as known for the congeneric taruca and other *Odocoileus*, needs confirmation. Also, huemul clearly breed every year and frequently raise fawns in successive years. For calculations of intrinsic growth rates or life tables we recommend these parameters be used. Applying unrealistic parameters may cover up underlying processes and lead to spurious conclusions, such as underestimating reproductive potential and consequently, underestimating mortality events, or overvaluing other processes.

Mixed-sex groups during all seasons, segregation of sexes, single or multiple male and female groups are all expressed in several cervids including huemul. In one huemul population, adult sexes associated in <2% of all group sightings (Frid 1994). Similarly, populations of mature red deer, fallow deer, moose, roe deer, mule deer, or white-tailed deer also have mixed groups all year, at times up to 30% of all groups, most likely reflecting relationships between populations and their environment. Mixed-sex groups in huemul all year are neither a consistent trait nor unique among cervids, instead, variation in social group composition is common, which will assist in reinterpreting the historically much larger group sizes and densities of huemul, use of other habitat types, and potential variations in behavior and ecology (see Part 1 for past biogeography, and Part 2 regarding group size and density).

The huemul breeding system has several important implications. Although only dominance male group systems were described, equivalent to the only congeneric taruca, recently huemul bucks were characterized as strongly territorial during all seasons and life-long, defending and monopolizing female groups and siring most offspring, while non-territorial bucks rarely sired offspring (Corti *et al.* 2009). Spatial overlap was the surrogate for territoriality, but is considered inappropriate (Grant *et al.* 1992). Also, the few spatial points covering 2.5 years do not reveal spatio-temporal relationships, and for huemul it took 600% more spatial points before the estimated home range size became asymptotic (Gill *et al.* 2003). 'Life-long' territoriality was based on three bucks remaining in their area until death (2-3 years), and broken legs in a juvenile and a mature male considered evidence for territoriality, interpretations that we reject. Furthermore, elsewhere another young male was found with a broken forelimb more than 2 months before the rut (Cerdeña *et al.* 2011). No behaviors commonly associated with territoriality were observed, and 50% of 'territorial' bucks had their core area overlap with other 'territorial' males. The two 'territorial' bucks siring most fawns were though responsible for only a quarter of the fawns in their respective social groups, with many more fawns sired by outside males. Then, 14% of 'territorial' bucks never sired fawns, a subordinate male sired *all* fawns for two consecutive years in the area of a 'territorial' buck, and several females were bred by bucks from neighboring areas or they moved into areas of other males to be bred there before returning. Such fluid membership has been described in several other huemul populations. Additionally, when applying the more realistic age at maturity of one year (instead of 3 years), there are potentially 28 fawns unaccounted for, apart from the 23 sampled ones. Thus it is probable that less than 50% of all neonates were sampled, the remainder may have been lost unnoticed during the perinatal period. Overall, these data do not support territoriality in huemul.

Genetic isolation requires either absolute barriers, or an absence of immigration due to other factors. A valley 5 km wide claimed to result in genetic isolation has a landscape similar to areas which have been and still are used by huemul elsewhere. Limited dispersal capacity was also implied to justify this 5-km wide barriers, based on a single subadult female dispersing only 8 km. However, additional documented movements include a male which moved about 8.5 km for 3 months before returning (Gill *et al.* 2003). These two sole records unlikely represent maximal capacity of huemul which disperse effectively considering their rapid postglacial occupation of a huge region of South America (30-55°S). In comparison, mountain goats (*Oreamnos americanus*) which are true rock specialists with extremely short legs, will still travel up to 25 km during spring and summer to get to low elevation mineral licks, crossing large stretches of habitat considered completely unsuitable for mountain goats, like flat forests (Brandborg 1955; Poole *et al.* 2010). Visits of such highly specific places, and lasting <2 days are clearly a result of vertical traditions which was considered vulnerable to anthropogenic impacts (Poole

et al. 2010). This may also indicate that reduced movements in huemul may be due to past elimination of huemul which by tradition crossed areas later settled by man.

CONCLUSION

Available confirmed reproductive parameters should be used for life cycle calculations to avoid spurious conclusion. Until recently, dominance group mating systems with fluid group memberships, lacking territoriality, was described for several huemul and taruca populations. Recent claims of territoriality during all seasons, where territorial males defend and monopolize female groups and sire most offspring, is not supported by the data. The assessment of potential barriers to huemul movements needs to consider past and current habitat use. Particularly, neither open space nor gentle topography constitute an impediment. Erroneous considerations of landscape features as barriers may distract from discovering the factors underlying the lack of recolonizations generally observed in currently reduced huemul populations.

ACKNOWLEDGEMENTS

We like to thank three anonymous reviewers for many constructive comments to improve this paper.

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Figure 1. Groups of multiple huemul bucks. The scene is from the book 'Der Kampf der Andenhirsche' (with courtesy of Milada Krautmann).



Figure 2. Valley of 5 km width containing guanaco (*Lama guanicoe*), a species which coexists with huemul adjacent to this area and elsewhere.



DISTRIBUTION, REPRODUCTION AND GROUPING PATTERNS IN THE TARUCA DEER (*HIPPOCAMELUS ANTISENSIS* D'ORBIGNY, 1834) IN THE EXTREME NORTH OF CHILE

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ABSTRACT

The taruca is a sturdy and robust cervid species distributed in the Andes between Perú, Northwest of Argentina and Northern of Chile, countries that face serious conservation problems. Our study aimed at assessing the grouping patterns, presence of fawns and juveniles, and antler growth and development of taruca populations of northern Chile. Various fieldtrips were carried out during the last three decades (1986-1987, 2004-2005, and 2008-2010) mainly to the Parinacota Province but also in the Tamarugal Province. The distribution of this species in Chile is located in the Parinacota province and extends south to Quebrada Blanca in the Tamarugal province (21°S). The taruca lives in social groups represented by family aggregations, female groups and male groups. The family groups were the most frequent and largest (mean 5.13 ± 2.46). The female groups included one or more females and their fawns. The males tended to be more solitary, forming smaller groups than did females (mean 1.35 ± 0.53). The taruca breeds seasonally, with the first fawns observed at the beginning of April (after the rainy season). The antler-shedding of males was observed between end of August and end of September (dry season) and the first antlers in velvet were observed at the end of September, which was lost at the beginning of January. From 1986 to 2009 it has suffered a dramatic decrease. The irrigation channels, extension of fenced agricultural land, introduction of goat led and the construction of the Arica-Tambo Quemado highway and other secondary roads resulted in animals being run over, causing injury or death, and in general a critical situation of the taruca populations in northern Chile. Therefore, respective decisions should be taken urgently to prevent further habitat loss and local populations to go extinct.

INTRODUCTION

The taruca or *Hippocamelus antisensis* d'Orbigny, 1834 (Figure 1) inhabits Andean environments from Peru to NW Argentina; it reaches as far south as the province of La Rioja in Argentina and the locality of Tignamar in Chile (Cajal 1983; Redford and Eisenberg, 1992; Pearson 1951; Pine et al. 1979; Regidor et al. 1997). Its populations are in evident recess; it is apparently extinct in Ecuador (Cabrera 1961) and has serious conservation problems in Argentina (Díaz and Ojeda 2000; Ferreyra 2007), Bolivia (Jungius 1974; Yensen et al. 1994; PAHS 1995; Nuñez 2005) and Chile (Glade 1987; Carrasco et al. 1991; Cofre and Marquet 1999). According to Weber and González (2003), its original distribution has been reduced by 60%. For these reasons the taruca is included in the vulnerable category in Appendix I of CITES (Barrio and Ferreira 2008) and was declared a Natural Monument in Argentina by National Law N° 24.702 (Ferreyra 2007).

The taruca ranges from 1800 m to 5200 m altitude, associated with the Andes Range (Wemmer 1998; Roe and Rees 1976). In Chile it prefers the western slopes in the Parinacota province (Sielfeld et al. 1999) and foothill areas of the II Region (Spotorno et al. 1998); it is probably not present in the III Region of Atacama (González et al. 2000). For a number of reasons, information on the ecology and behavior of Neotropical cervids is scarce and fragmented (Roe and Rees 1976; Duarte and González 2010); in the case of the taruca this information is fundamental for its conservation and management (Cowan and Halloway 1973), since it is considered to be the least known species of deer (Nuñez and Tarifa 2006). Grimwood (1968) provided general information on the situation of the species in Bolivia. The preliminary observations of the Peruvian population made by Roe and Rees (1976) were later complemented by ecology and behaviour studies by Merkt (1987), who described reproductive aspects and grouping patterns. Recently Nuñez and Tarifa (2006) reported on group sizes, age structure and the radius of action by sex in a population of tarucas in the province of La Paz in Bolivia, and Ferreyra (2007) estimated the potential habitat and total population for Argentina. In Chile, the agricultural

development of the last decade promoted by the Instituto de Desarrollo Agropecuario (INDAP) in the province of Parinacota has turned into a violent advance of agriculture on the areas inhabited by this deer and other native species such as the guanaco *Lama guanicoe*. Other threats include the construction of a highway with zero environmental consideration (Arica-Tambo Quemado road), access roads to formerly isolated areas such as the roads to Aroma and Coca canyon where taruca pasture, and browsing areas due to canalization of water with cement and PVC pipes and intervention in the springs of the watercourses. The construction of inadequate storage tanks and extensive perimeter fences around agricultural areas (localities of Putre, Socoroma, Lupica, Murmuntani and Tignamar, financed by SAG, INDAP, CONADI, FNDP and IMP) has eliminated access to water and generates accidents, lameness and deaths. In addition, we must add the introduction of milk goats (Saanen and Anglo-Nubian varieties, among others) which overgraze the slopes of the mountains that formerly constituted the forage for the native fauna during the driest period, just before the beginning of the summer rains. This situation has been indicated on a number of occasions by Sielfeld *et al.* (e.g. 2004) by means of technical reports and meetings with appropriate government entities (SAG, CONAMA, CONAF) without positive results, since up to now no remedial actions have been taken nor policies established to protect the taruca in this area; thus its future is uncertain. The above is a cause of concern, since the Chilean population is apparently restricted to the province of Parinacota. There the population size was estimated in 715 animals in 1986 and 832 individuals in 1988; the latter number showed small variation during the yearly cycle, indicating that the species does not migrate. Later census (1988 and 1990; CONAF 2008) counted 730 and 666 individuals, and others using satellite images in the years 2005 2007 recorded 587, 585 and 560 individuals, respectively (CONAF 2008). These last figures confirm the estimate of 466-545 individuals for the year 2004 reported by Sielfeld *et al.* (2004). In consequence, own datas on the anthropic activities identified above and associated with the destruction of the habitat, the advance of the agriculture frontier, isolation of sources of water and introduction of goats appear as possible causes of the 23.3% decrease that the population has registered since 1990 and 29.4% since 1988.

This Short Communication intends to characterize grouping patterns, aspects related to reproduction (fawns and juveniles) and the sexual cycle (growth and development of the antlers) and the altitudinal distribution of the taruca in the north of Chile.

MATERIALS AND METHODS

The present data set comes from the projects “Study of the taruca in the First Region of Chile”, CONAF/PNUD/FAO-CHI/83/017 (Carrasco *et al.* 1992; Galáz 1998; Sielfeld *et al.* 1988; 1999), “Population study of the taruca and the guanaco associated with agricultural production and farming in the foothills of the province of Parinacota”, SAG/UNAP 2003-04 (Sielfeld *et al.* 2004); “Registry of biodiversity information for priority sites, I Region of Tarapacá, GORE/FNDR/BIP:30064517 2008; “Characterization of the wetlands of Lirima and Caya”, SAG/2009 (Sielfeld in: CONAMA 2010) and Population Diagnosis of Guanaco and Taruca, Province of Parinacota, CONAF/BIP 20193099-0 2008 (CONAF 2008).

Period, study area and habitat of the taruca

The data come from two time periods (1986-87 and 2004-09), principally from the province of Parinacota (9,801.5 km²) (currently the XV Region, in the extreme north of Chile). It borders the republics of Peru and Bolivia (Fig. 2). However, the surveys included as far south as Quebrada Blanca and Quebrada Maní in the El Tamarugal Province (I Region, 20° S), and also included the contiguous area of the Altiplano. The elevation of the studied area ranges from 1500 m in the western limit of the distribution of the taruca to 6342 m (the top of the Parinacota Volcano) in the eastern sector. The scarce precipitations in the area come from a tropical regime, and vary from 5 mm/year to 400 mm in the highest parts (di Castri 1969; Contreras *et al.* 1986). The habitat of the taruca is characterized by great irregularity of the land, including steep sided canyons and steep slopes of the Andes and their foothills. The preponderant vegetation is composed of perennial shrubs; the most abundant are *Coreopsis suaveolens*, *Diplostephium meyenii*, *Bacharis santelisis*, *Baccharis boliviensis*, *Tarasa operculata*, *Ephedra breana* and *Balbisia microphylla*. In the higher parts in areas protected from the wind there is also *Polylepis besseri*, and in areas with much exposure to wind are grasses such as *Deyeuxia nardifolia* and *Festuca orthophylla*. Pisano (1956) called this area “spiny sub-Andean matorral”.

Field observations

In the field we covered trails and secondary roads, from which animals were observed with 7x50 binoculars from distances which ranged from 15 to 1500 m. Sexes were recognized by the presence of

antlers, and in their absence by observation of external genitalia. Individuals with adult coloration but smaller size were counted as juveniles. Fawns were individuals born in the season and distinguishable from juveniles by their grayish color, which according to Merkt (1987) is replaced by the ochre brown color of adults at about age 30 days. The antler development of males was classified as: without antlers, antlers completely covered by velvet, partially covered by velvet and without velvet. Seasonal variation in group size was analyzed using three periods of the year: December-February, the rainy season when forage is mainly composed of grasses; March-May, the post rain period with abundant forage and June-November, the cold, dry period with absence of rain and scarcity of forage mainly composed by shrubs.

RESULTS

Distribution

A total of 859 individuals in 208 groups were recorded through the 23-year period (1986-2009). These were found mostly in the Parinacota province, in which they were associated with the higher sectors of the watersheds of (from north to south) Ancolacaya/Putre, Socoroma/Guanacagua, Murmuntani/Chapiquiña, Belén/Lupica, Belén/Lupica and Timalchaca/Marqués. Overall south of Parinacota, and despite intensive and extensive surveys, individuals were sighted only at three sites: Coscaya: Quebrada de Tarapacá 10/09/2009), Chiapa: Quebrada de Tarapacá (three individuals) (14 October 2010) and Quebrada Blanca (one individual) (07 July 2005). The taruca was observed on mountain slopes from 2600 to 4250 m in Parinacota (Fig. 3). Family groups (10.3%) were observed mostly at 3500-3550 m; 89.9% were found between 3000 and 3900 m. Solitary males were apparently displaced by the dominant males of family groups to the lower and upper distribution ranges: below 3000 m and above 3450 m. 14.3% of them were found between 2851-2900 m and 64.3% between (3450-4150 m.). Thus 78.6% of solitary males were found outside the altitudinal range preferred by family groups.

Reproduction

Births. In 1986-87 fawns were observed from 10 January to 28 May 1987 (Fig. 4). For the groups of Socoroma and Chapiquiña, 80% were first observed in the first half of May. The information from 2009 is concordant; fawns were observed in the Chapiquiña sector on 3 May 2009 and in the Quebrada Aroma on 29 April 2009. The birth of fawns was not observed, but was probably in the month preceding the first sighting (late March-April).

Antler growth. Since the animals often fled, not all observations included a register of the characteristics of the antlers. The information obtained for some individuals indicates that the antlers were lost from August to September and began to grow a new; they had velvet until the end of January and the beginning of February. Loss of velvet occurred in a few cases at the beginning of January, and by March 100% of males had antlers without velvet. In August 50% had lost their antlers; this process finished in September (Fig. 5).

Grouping patterns

Types of groups. In all observation points and dates there was a clear grouping of the individuals of *H. antisensis*: family groups, groups of males, groups of females with offspring, groups of females alone and groups of juveniles (Fig. 6). In 1986-87 159 family groups ($n = 806$ individuals) were counted; 28 groups of males ($n = 39$ individuals); and six groups of females with offspring ($n = 6$). Groups of juveniles were not recorded; these formed part of family groups and/or females with offspring. Females were the most abundant ($n = 415$) followed by males ($n = 290$), juveniles ($n = 139$) and fawns ($n = 23$) (Fig. 6). The same groups were maintained in 2004-2009, however, the abundances were quite different from 1986-1987. In 2004-2009, only five family groups were found ($n = 18$ individuals), 9 groups of males ($n = 11$), 2 groups of females ($n = 3$), and one solitary juvenile. Females with offspring were not observed in this period. Adult males were the most frequent ($n = 16$), followed by females ($n = 10$), juveniles ($n = 4$) and finally fawns ($n = 3$) (Fig. 6).

Group sizes. For the whole area over the entire study period (1986-2009) family groups averaged 5.1 individuals ($SD = 2.46$) and were the largest aggregations, while males (1.35 ± 0.53) and females (1.17 ± 0.37) were often found as solitary individuals (Table 1). There was a decrease in the size of groups between the two study periods; the size of family groups diminished from 5.0 to 3.6, although the decrease was not significant (ANOVA: $F=3.89$; d.f.= 1; $P>0.05$). Males and females continued to be mostly solitary, and females with offspring were not observed from 2004 to 2009 (Table 1).

Table 1. Size groups and significance values for the taruca in the extreme north of Chile

Types of groups	1986–1987		2004–2009		ANOVA <i>P</i> -value ^A	1986–2009	
	<i>N</i>	Mean ± (SD)	<i>N</i>	Mean ± (SD)		<i>N</i>	Mean ± (SD)
Family groups	159	5.18 ± 2.47	5	3.60 ± 1.36	0.15	164	5.13 ± 2.46
Males	28	1.39 ± 0.56	9	1.22 ± 0.42	0.41	37	1.35 ± 0.53
Females-offspring	6	3.00 ± 0.00	—	—	—	6	3.00 ± 0.00
Females	4	1.00 ± 0.00	2	1.50 ± 0.50	0.17	6	1.17 ± 0.37
Total	197	4.51 ± 2.64	17	1.94 ± 1.34	—	208	4.30 ± 2.66

^ASignificance value (ANOVA) for the periods 1986–1987 and 2004–2009.

Seasonal variation in group sizes. From December to February it rains and forage grows, mostly composed of gramineae. In this period only a few family groups and groups of males were seen (3 and 5 groups, respectively); no female-offspring groups or groups of only females were observed (Fig. 7a). This tendency began to change in the March-May post-rain period when there was abundant forage; this is when the fawns of the year appeared. There was an increase in the number of family groups ($n = 8$) and a decrease in groups of males, who were incorporated into the family groups. Two solitary females were also observed. Breeding occurred during the June-November period, in which there are low temperatures, absence of precipitation and scarcity of forage. In this period there was an abundance of family groups (142) and groups of males (19), as well as a few (2) groups of females; female offspring pairs began to appear (6 groups) (Figure 7a). In the period from December, 1986 to November, 1987 there was a clear and sustained increase in the number of males and females in the surveyed area, probably as result of immigration from adjacent areas; no juveniles or fawns were observed from December to February. Fawns appeared in the period March-May ($n = 22$), which later advanced to form part of the cohort of juveniles which were observed in abundance from June to November (Fig. 7b).

DISCUSSION

Our data (1986-2009) confirm that in Chile the taruca is distributed principally in the Province of Parinacota (Sielfeld *et al.* 1999); however, we also report three new localities to the south in the adjacent Province of Tamarugal. The farthest south was Quebrada Blanca (21°), where one indeterminate individual was seen in 2005. Although Spotorno *et al.* (1998) cited this species close to 22° S (Antofagasta Region, at 3000 m), their record was based on part of a foot and not on live animals. In Chile the taruca is distributed from 2600 m to 4150 m, however, most individuals prefer to live between 3000-3900 m, where 89.9% of the groups were families, the most numerous aggregations in Parinacota. In Bolivia the taruca has been found from 2500 m to 5200 m (Wemmer 1998; Nuñez and Tarifa 2006); and in the south part of its range in Argentina from mountain grasslands at 1800 m to above 5000 m (Galaz 1998) in the puna steppe, the prepuna and high Andean steppe (Cabrera and Yepes 1960; Redford and Eisenberg 1992, Regidor *et al.* 1997). The altitudinal distribution in Chile coincides with zones which have benign characteristics of vegetation and availability of water; unfortunately, these areas are those most used for agriculture and livestock, thus producing a high level of interaction between the native fauna and the indigenous communities of the sector (Carrasco *et al.* 1992; Sielfeld *et al.* 1999). Births occur at the end of the rainy season, in the summer-autumn transition; they probably occur in March and April, since the first fawns were observed from April to May (Fig. 4). This is considerably later than the Peruvian populations of this species, for which Pearson (1951) and Merkt (1987) reported that births occurred in February and March. Probable reasons for this difference include the difference in altitude in the two areas and the differences in precipitation. With respect to altitude, both Pearson (*op. cit.*) and Merkt (*op. cit.*) studied animals near Puno and Cusco at altitudes above 4000 m. There the warm summer period is shorter, and thus it seems adequate to have births earlier. The mean annual precipitation is around 900 mm (O.N.E.R.N. 1965), with a rainy period which begins in August and has its maximum from January to March (Merkt *op. cit.*). In the Province of Parinacota there is significantly less precipitation, and this commences in December. This delay of one or more months in the growth and flowering of shrubs and herbs, whose maximum for the area is from February to April, should also be considered as a probable factor in the delay of births. Antler development was directly correlated with the climatic conditions of the area; growth began at the end of September and antlers were covered with velvet until the middle of March. Loss of velvet began in January; antlers without velvet were observed from March to July. By August half of the males had lost their antlers; this process finished in September. These results are similar to those reported by Merkt (1987) for Peru, in which antlers are shed in and some males begin to grow new ones in September; the

period of velvet loss also coincided with our results. As is the case in the populations in neighboring countries, the tarucas of the north of Chile tend to form groups according to age and sex. In Parinacota we observed family groups, groups of males, females with offspring, solitary females and groups of juveniles. The largest group recorded had 13 individuals, which contrasts with the group sizes of up to 31 and 41 reported by Merkt (op. cit.) for Puno. These differences may be due to the fluctuating structure and size of social aggregations, as well as to the greater amount of hunting which the population of Parinacota has apparently suffered and the alteration of the habitat by intense agriculture, the introduction of milk goats and the building of new roads. In terms of the population dynamics of the aggregations, during autumn (March-May) there was an increase in the family groups as a result of the beginning of the breeding period, in which solitary males were incorporated into groups of females. The parallel increase in the number of females without males appears to be related to the segregation of gravid females, which increased the "groups of females" in this period and the groups of males in the period before the births, as mentioned by Merkt (1987) for the Peruvian population. This behavior also produced a reduction in the number of solitary males observed, while family group size was at its maximum. In the spring there was some separation of males from the family groups, which was reflected in an increase in the number of solitary males and groups of females. The size changes in groups during the year mainly affected the family groups, and were related to the addition of males in the breeding season and fawns in the birth season. Although the difference in group size was not significant (Table 1), there was a decrease in the number of groups observed in 1986-87, before the construction of the Arica-Tambo Quemado road and the installation of perimeter fences in agricultural areas, and in 2004-2009, after these changes had occurred. There was also a reduction in the number of sightings, which we interpret as a result of reduction in population number and displacement to other isolated sectors.

CONCLUSIONS

In Chile the taruca is found as far south as 21° in Quebrada Blanca, in the I Region. However, the main population is in the Province of Parinacota (XV Region), where they prefer altitudes between 3000 m and 3900 m. The social and reproductive behavior of the populations of Parinacota was generally similar to that described for Peruvian populations (Roe and Rees 1976; Merkt 1987), except for some differences in group size and birth times. Reproduction in Parinacota was correlated with the climatic conditions of the area; births occur during April and May, in which there is maximum growth of vegetation, high temperatures and abundance of water. The taruca has a strong tendency to form social groups. "Families" are the most common and numerous, especially from March to May because of the addition of males and fawns. Only few animals were solitary and mainly wandering males. Finally, agriculture, livestock and roads are producing habitat destruction, isolation of water, accidents and death of animals. This is a cause of concern, since although Chile subscribed to the Biodiversity Convention and thereby agreed to protect 10% of its relevant ecosystems, up to now there is no protection plan for this endangered species, it does not receive sufficient attention, has no protected areas and its future in the country is seriously threatened.

ACKNOWLEDGEMENTS

Fieldwork assistance of Marcos Ferrú, Pamela Valdés, Nelly Ledesma, Marcela Gaete, Robinson Herrera and Marcia Solis is appreciated. To Juan Torres Santibañez by the picture of taruca.

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Figure 1. Male adult of *H. antisensis* from Parinacota Province in the extreme north of Chile. (photo by Juan Torres Santibañez).



Figure 2. Geographic location of study area in the extreme north of Chile (only Putre, Socoroma, Chapiquiña, Belen, Tignamar, Coscana, Quebrada Blanca) Arica, Pisagua and Iquique are places of references.

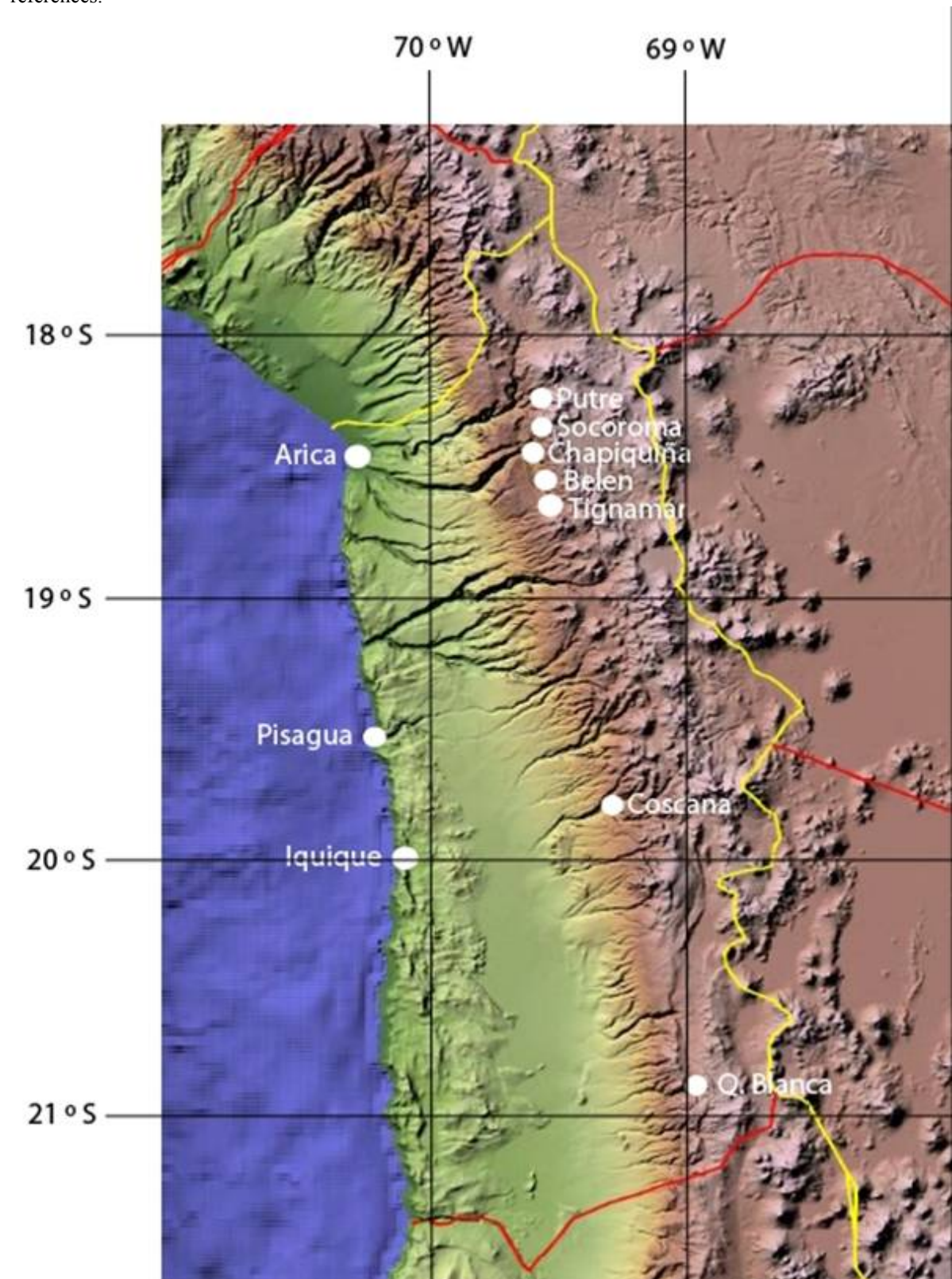


Figure 3. Altitudinal distribution in the taruca in the extreme north of Chile. (A) number of groups; (B) number of specimens.

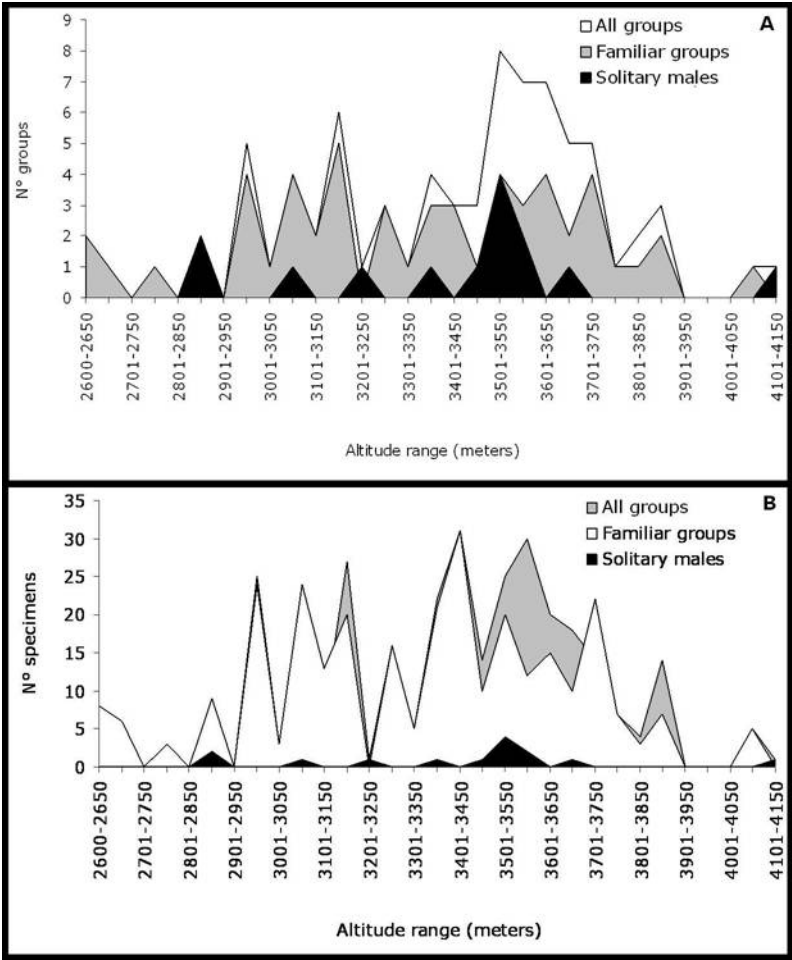


Figure 4. Seasonal distribution of fawns in the taruca during 1987 in the extreme north of Chile.

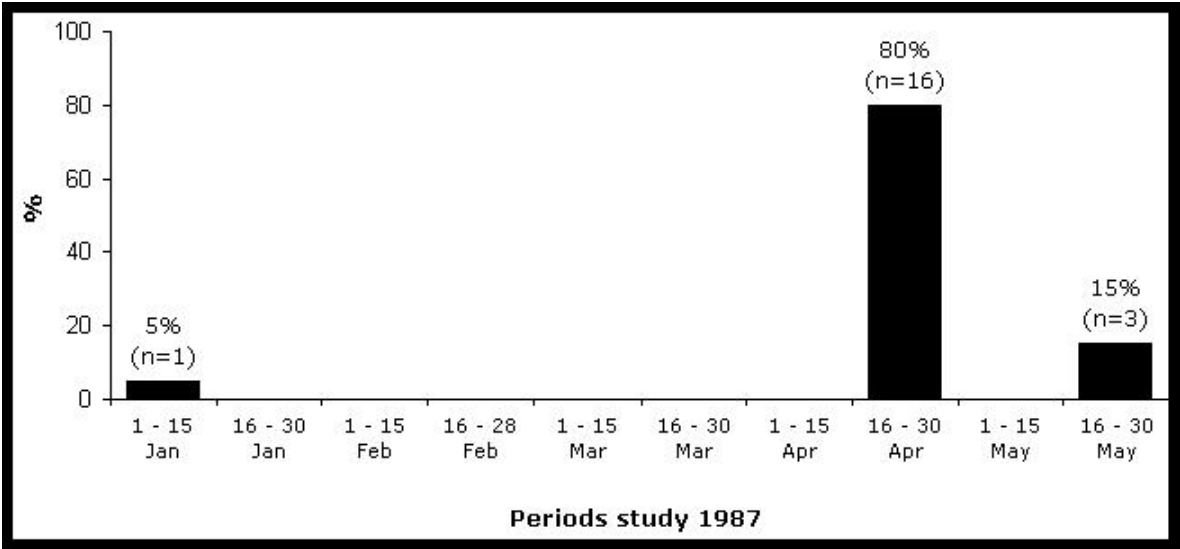


Figure 5. Antler growth (n / %) of *Hippocamelus antisensis* observed during 1986–2009 in the extreme north of Chile.

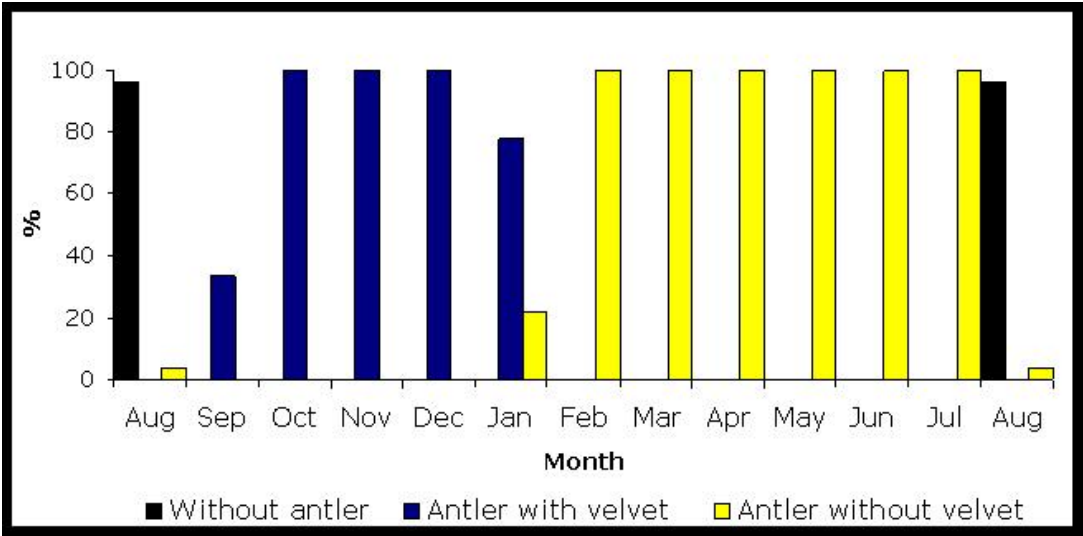


Figure 6. grouping patterns in the taruca a) number of groups by sex and age; b) number of specimens in each group, c) number individuals by sex and age (types of groups).

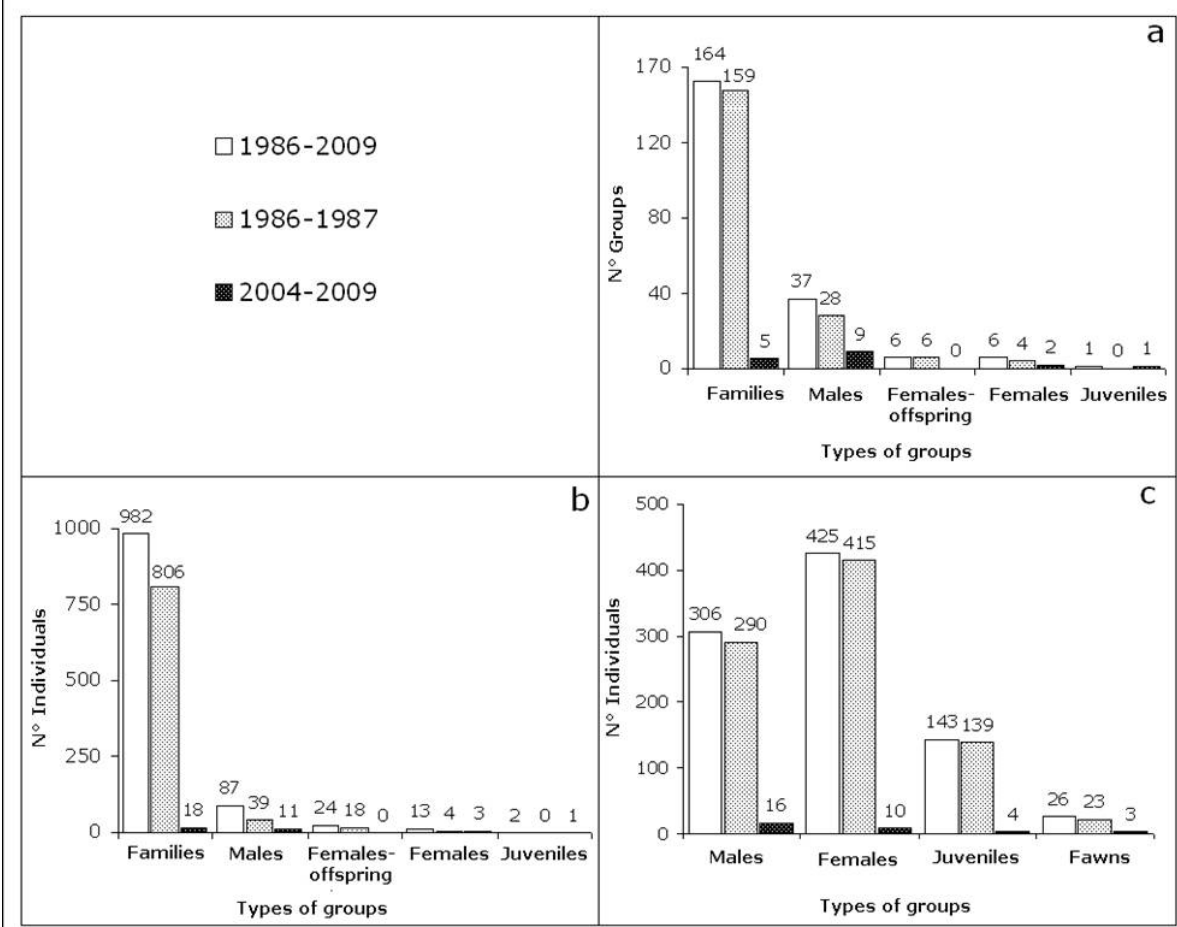
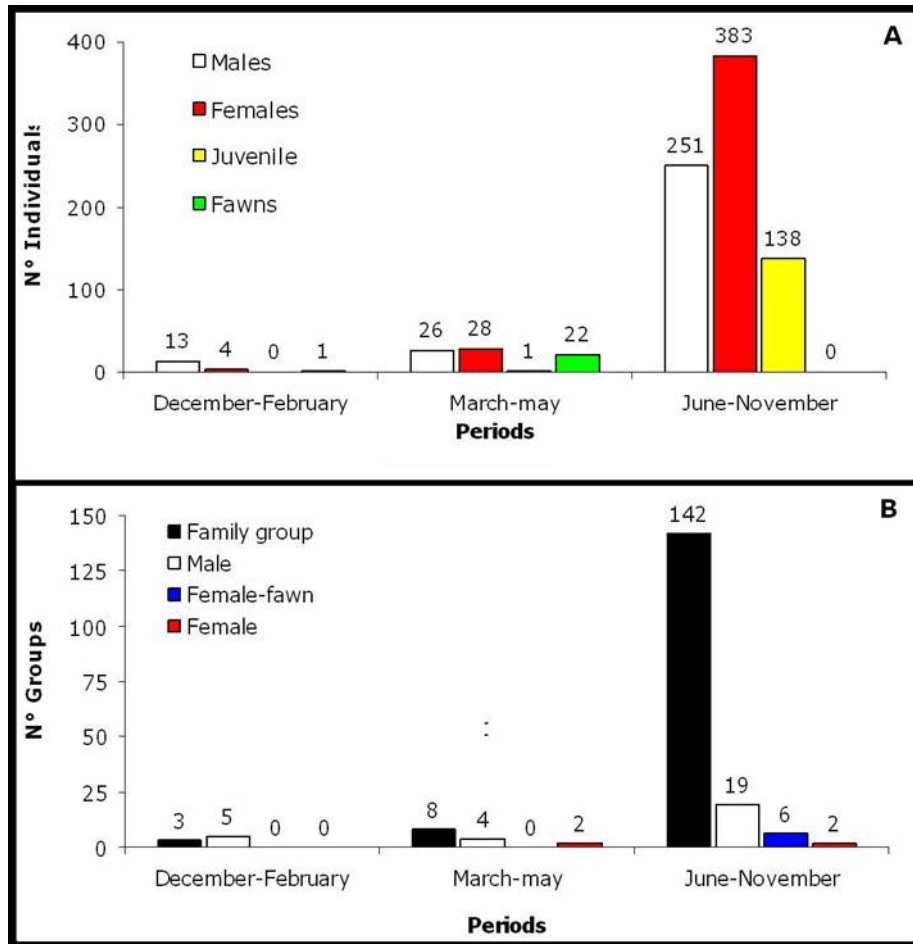


Figure 7. Types of groups and individuals by groups in the taruca during during 1986–1987 in the extreme north of Chile. (A) number of individuals; (B) number of groups.



POSSIBILITY OF TWO REPRODUCTIVE SEASONS PER YEAR IN SOUTHERN PUDU (*PUDU PUDA*) FROM A SEMI-CAPTIVE POPULATION

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ABSTRACT

Pudu (*Pudu puda*), occurring in the southern cone of Latin America, has been classified as vulnerable by the IUCN, yet little is known about this animal in the wild, with most knowledge on the breeding behavior coming from captive animals. For this second smallest deer in the world, delayed implantation has been suggested to explain the two peaks in the annual cycle of male sexual hormones based upon the accepted tenet that the breeding period occurs only once a year between March and June. However, in this study, birth dates from fawns born at the Los Canelos semi-captive breeding center in Chile and male courting behavior revealed possibility of two rutting periods: autumn and spring. To our knowledge, this is the first time that late fall/early winter births (May through early June) have been recorded for the southern pudu; two of these four births were conceived by females in the wild. From available zoo and captive birth records ($n = 67$), no fawns were born in the winter. For all births combined ($n = 91$), 64% occurred in spring. The roe deer (*Capreolus capreolus*) and Pere David deer (*Elaphurus davidianus*) have been considered the only two temperate cervids in which sexual activity is initiated by increasing day length and which breed in early summer. Yet, the present results indicate a similar response from the southern pudu if under a wild or semi-captive environment, with breeding taking place in spring. These results suggest that this species may either have two reproductive periods per year or retained the capacity to be a breeder for a much more extended period of time than documented by earlier studies. Pudu, like other temperate deer, are responsive to photoperiod for timing their breeding period, but may further optimize their production of offspring by also responding to other environmental cues such as seasonal variation in food supply when the climatic conditions are favorable.

Additional keywords: aseasonal reproduction, parturition, subtropical breeding, Valdivian rainforest, austral, Argentina

INTRODUCTION

The southern pudu (*Pudu puda*) occurs only in southern Chile and southwestern Argentina (Fig. 1). The species' distributional range continues to diminish in size due to anthropogenic impacts, with populations today mainly found in the temperate rainforests along the southern Andes. In southern Chile, the species also inhabits the coastal mountain range and scattered forest patches in the valleys (HersHKovitz 1982; Jimenez 2010). Although classified as vulnerable by the IUCN (2008) and being a unique species in that it is ranked as the second smallest deer in the world (HersHKovitz 1982; Whitehead 1993; Geist 1998), weighing in at less than 15 kg (Eldridge *et al.* 1987; Geist 1998), little has been published on the biology of this species. Even less is known about the only other species to share its genera, the northern pudu (*P. mephistophiles*), separated by about 3200 km (Whitehead 1993). The cryptic behavior of southern pudu in the wild makes it a particularly difficult animal to study in its habitat of dense vegetation along its altitudinal range from sea level to 1700 masl (meters above sea level). The individuals are often solitary or in small family groups, coming out to feed at the forest edge in undisturbed areas (Jimenez 2010). Given their elusive nature, most information on the southern pudu has been acquired through studies on physiology and behavior of captive animals, and predominately on investigating hormonal profiles in males (reviewed in Bubenik *et al.* 2000). A Web of Science search of original studies published on southern pudu since 1923 revealed 35 hits, of which 91% were of captive populations.

Gestation period in captivity has been recorded to last from 197 to 223 days (Vanoli 1967 in Jimenez 2010; Reyes *et al.* 1988; HersHKovitz 1982). Normally females have one fawn but twins also occur (HersHKovitz 1982; Whitehead 1993). For captive breeding populations, the data have indicated that a monomodal breeding pattern exists in pudu, with the breeding season generally assumed to be between April and June (Whitehead 1993; MacNamara and Eldridge 1987) and for one captive population (Concepción, Chile) between March and April (Reyes *et al.* 1988). Recorded births from captive females have indicated one birthing period between October to February in the southern hemisphere (MacNamara and Eldridge 1987; Reyes *et al.* 1988), with the fawning period shifted by six months in northern hemisphere zoos (HersHKovitz 1982; Blanvillain *et al.* 1997); individuals translocated to the northern hemisphere synchronize quickly to the local photoperiod. Females remained receptive for about 48 h, during which time they could be bred by multiple males (Reyes *et al.* 1988).

Results from a study on captive females in Europe suggested that the southern pudu is a seasonal polyestrous breeder with a reproductive cycle cued to a seasonal factor and a cycle length of about 11 days (Blanvillain *et al.* 1997). Given the wide variation of the estrous cycle length of three mature females (16, 18 and 33 days), Blanvillain *et al.* (1997) proposed that pudu females might respond to seasonal cues with less rigidity than northern temperate deer. Bubenik *et al.* (2000) on the other hand, in comparing the length of gravidity in pudu (average of 203 days, Reyes *et al.* 1988) with that of red deer (average of 232 days) – an animal 10 times heavier than the pudu – considered the pudu's gestation to be enormously long and speculated that this species might exhibit delayed implantation of the embryo, unique only to the roe deer (*Capreolus capreolus*) among artiodactyls (Aitkens 1974; Semperé 1990; Lambert *et al.* 2001). To our knowledge, this hypothesis has never been investigated. Here we provide observations of reproductive behavior for the first time from a semi-captive population of pudu that challenges this hypothesis and suggests yet another strategy unique to a temperate deer.

METHODS AND STUDY AREA

The current population consists of 28 pudu at the semi-captive center operated by Fauna Andina - Los Canelos (39°16' S latitude), in the central valley near Villarica, Chile, Araucania Region, within the natural distributional range of the species. The climate is mild and humid with average min-max temperatures in summer and winter of 9-26°C and 4-15°C, respectively, with main precipitation as rain between May and July (late autumn and winter with 517 mm average, and a total annual average precipitation of 1130 mm). The 13 ha enclosure at 330 masl consists of dense native vegetation common in the Valdivian temperate rainforest ecosystem, including approximately 2.5 ha grassland pastures, with *Nothofagus* and *Festuca* species predominating inside and surrounding the enclosure. All animals are free-roaming inside the enclosure. Disturbance and contact is minimal so that animals retain their elusive behavior and natural fear of man to facilitate their subsequent reintroduction. No one but the caretaker (F. Vidal) and authorized researchers and government inspectors under Vidal's supervision are allowed into the enclosure. Fresh water is available year round from streams that do not come in contact with any domestic animal or livestock before reaching the pudu.

Data were collected on births dates of pudu born at the Los Canelos Center between 2000 and 2010, either by directly observing the parturition or the neonate within two to three days after birth. This data set of a semi-captive population was compared to parturition dates of captive pudu in the northern and southern hemisphere and included: 20 births at western European zoos (HersHKovitz 1982); 22 births at the captive breeding center of La Victoria Island (41°05' S latitude) in the Argentine Nahuel Huapi National Park (MacNamara and Eldridge 1987); four births in the province of Neuquén (ca. 40°40' S latitude), Argentina (Schmidt 1944 in HersHKovitz 1982); two births in Osorno-Llanquihue (40°34' S latitude) region of Chile (Vanoli 1967 in HersHKovitz 1982); one birth in an unspecified Chilean location (Hick 1967 in HersHKovitz 1982); and six births at two breeding centers of the University of Concepción (36°50' S latitude) in Chile (Reyes *et al.* 1988). Recent births ($n = 12$) of the last five years at the North American zoos of Detroit (42°20' N latitude) and Woodland Park (Seattle, 47°36' N latitude), and the United Kingdom's Belfast Zoological Gardens (54°35' N latitude), Bristol Zoo Gardens (51°27' N latitude), Edinburgh Zoo (55°57' N latitude), Hamerton Zoo Park (52°24' N latitude), Marwell Wildlife (51°01' N latitude), and Paignton Zoo (50°26' N latitude) were obtained from the zoos' electronic news briefs. Given that the fawning period shifts by six months in northern hemisphere zoos, the comparisons are made by season. From the European zoos, three were conceived in Chile: for the analysis, these were included in the data set for captive centers in the S Hemisphere. A lone winter birth (January) at Germany's Cologne zoo was not included in the data set as no history was available regarding this individual's conception site (HersHKovitz 1982). Despite some of the

record books being stolen, we have 23 dates from a total of 30 births at the center to include in this analysis, plus an observation of a wild fawn in the Huilo Huilo Reserve (39° 48' S latitude). For the analysis, we consider the meteorological season of winter to begin 1 June and 1 December for the southern and northern hemisphere, respectively.

On a daily basis at Los Canelos Center, animals were observed for health condition and any behavioral changes. Whenever opportunities arose to record mating copulations and parturition behavior, *ad libitum* sampling was conducted on those individuals.

RESULTS

A characteristic autumnal rutting period, occurring between 24 March and 20 April was observed at Los Canelos Center. A second rutting period was observed to take place in the spring occurring between 17 October and 20 November.

For the entire data set, the majority of births fell in spring (64%), mostly falling on the last month of spring (52%), signifying May and November for the northern and southern hemisphere, respectively (Fig. 2). The births at the northern hemisphere zoos ranged from 27 April and 3 September (129 days). Captive center birth dates in Argentina and Chile ($n = 38$) were between October and February. Birth dates for the 20 fawns born in the spring/summer at the Los Canelos (LC) semi-captive center ranged from 19 October to 17 February (121 days), with 46% in November. Late fall/winter births (May and first week of June) were observed in 4 cases: of the three of these born at the LC centre, one was conceived in the wild; the fourth was a wild fawn seen in July at the Huilo Huilo Reserve observed from a distance of 10 m (F. Vidal and E. Arias, personal observation). A May birth date was estimated based on the individual's size and vivid spots, which, for this species generally begin to fade at 6 weeks of age and disappear by 3 months of age (Reyes *et al.* 1988; F. Vidal unpublished data). Births were absent for five months of the year with the exception of one at the initiation of autumn on 3 September at Germany's Erfurt zoo (Czernay 1977 in Hershkovitz 1982).

DISCUSSION

Observations of females and their fawns ($n = 4$) during parturition were similar to those of Reyes *et al.* (1988). Lying on her side, the female gives birth to a fawn with the eyes already open. The mother stands up almost immediately after her new born is dropped and begins to clean it intensively. Suckling was observed to take place from 10 to 60 minutes after birth.

During both rutting periods, males were observed to sire the females successfully, and courtship behavior was as described in MacNamara and Eldridge (1987). In addition, just prior to mounting an estrous female, the males in our study population gently nudged her hind legs. Their attempts were persistent over several hours.

The endocrine cycle of adult male pudu is rather unique among the deer species (Bubenik *et al.* 2002). Unlike most other temperate deer, the male of this species exhibits two seasonal peaks of equal magnitude for the reproductive hormones, FSH and testosterone, spaced about 6 months apart (Bubenik *et al.* 1996). Although two seasonal peaks can also be detected in various other deer species, coinciding with the solstices and equinoxes (Bubenik 1982; Rolf and Fischer 1990), the peak outside the rutting season is much smaller than that found for pudu. The non-rutting period for these deer is characterized by a small reactivation of reproductive function and hence, a testosterone pulse (Bartoš and Bubenik 2011), while in other species only one seasonal peak of testosterone coinciding with the reproductive period has been detected (Bubenik *et al.* 1982; Suttie *et al.* 1984). The seasonal variation of reproductive hormones in male pudu most closely resembles that of roe deer (Semperé 1990; Bubenik *et al.* 1996; Reyes *et al.* 1997). This may not be coincidental because of similar phylogenetic roots of pudu and roe deer. Randi *et al.* (2001) analyzed Cervinae using mitochondrial DNA. Their study indicates *Capreolus* being closest to *Mazama*, a south American cervid (Pudu was not included in that study). Subsequently Ruiz-García *et al.* (2007) found close roots between *Pudu* and *Mazama* (*Capreolus* was not included). Yet even though both roe deer and pudu have two circannual peaks of reproductive hormones, in contrast, the two hormonal peaks of the blood plasma levels of LH and testosterone in the roe deer occur much closer together, with the spring peak being considerably smaller than the summer one (Semperé 1990; Semperé *et al.* 1992).

Whereas most tropical and subtropical species exhibit asynchronous reproductive cycles independent of the photoperiod, most temperate and boreal cervids exhibit annual rutting seasons, synchronized by

photoperiod (Bubenik 2006). To our knowledge, with the exception of Pere David's deer, *Elaphurus davidianus* (Li *et al.* 2004), until now the roe deer has been considered the only other temperate cervid in which sexual activity is initiated by increasing day length and which breeds in early summer, with the onset of seasonal pituitary activity occurring in January and the beginning of testicular function following in spring (Semperé 1990). All other cervids of temperate and boreal regions are short-day season breeders (Bubenik 2006). However, the observations made at Los Canelos Center indicate that the southern pudu has a second breeding season in spring, shortly following the vernal equinox with males demonstrating full mating behavior. For roe deer, the first smaller peak in testosterone was associated with mineralization of antlers, and the second summer peak was related to the rut (Semperé 1990). However, for the Los Canelos pudu population, the observed rut activity in the austral spring and fall coincides with the two annual testosterone peaks, suggesting therefore, that the first peak is associated not only with mineralization, but also rutting behavior. This concurs with Bubenik *et al.* (1982) that maximal levels of testosterone appear to be essential for rutting behavior.

Comparably, the roe deer and southern pudu are both small-bodied temperate deer (Lincoln 1992), historically found at latitudes ranging from 19° to 70° N and at least 33° to 50-53° S, respectively. Delayed implantation in roe deer may have allowed it to inhabit extremely seasonal environment, whereas in general, smaller species with shorter gestation periods inhabit mostly tropical and subtropical regions; in contrast, larger cervids, exhibiting longer gestation periods, more commonly live in the temperate and boreal regions (Bubenik 2006). Evidence suggests that ancestors of temperate cervids may have displayed two rutting periods per year (Bubenik 2006). Therefore, the bimodal rhythm could be a relic behavior of such or a vestige of some ancestral reproductive pattern such as aseasonal breeding that still persists in some extant tropical and temperate cervids (Bubenik *et al.* 2002). Regardless of their similarities, the roe deer is mostly monestrous (Semperé *et al.* 1992; Semperé *et al.* 1998), and occasionally polyestrous (Strandgaard 1972), whereas the pudu appears to be seasonally polyestrous (Blanvillain *et al.* 1997). It remains to be determined to what degree endogenous rhythms and photoperiod are driving the sexual cycle of pudu.

To our knowledge, this is the first time that late fall/early winter births have been recorded for the southern pudu. Although these results suggest that pudu have 2 distinct seasonal reproductive periods per year, future observations may reveal births occurring throughout the current autumn gap, thus necessitating an alternative explanation. The measurements of testicular parameters indicate a prolonged period of gonadal activity in southern pudu (Reyes *et al.* 1997) than what should be the result of the hormonal levels only. From the study of Reyes *et al.* (1997) it is not clear whether or not spermatozoa were absent outside of the March rut. In September, they detected, only infrequently, few precursor cells (most probably spermatocytes) inside the lumen of epididymal tubules of pudu males. Therefore, unless investigated, we cannot reject the possibility that the males maintain the ability to produce spermatozoa for much longer time than currently believed. Males of many deer species are fertile for a prolonged part of the year and are capable of mating if the females are in the heat. For example, in one of the most studied temperate species, the red deer, *Cervus elaphus*, the rutting season occurs in September or October (according to region) in the Northern Hemisphere. After the rut, the testosterone level decreases almost immediately, while spermatogenesis remains very active in November and December, and then declines until June (Lincoln 1971). Under certain circumstances it is possible to incite complete male's sexual behavior to obtain vital spermatozoa up to June (Krzywiński and Jaczewski 1978). Females can follow similar seasonal pattern. In the absence of conception, estrous cyclicity in red deer can persist at least for four to six months with a gradual increase in estrous cycle length being evident with later cycles (Guinness *et al.* 1971; Asher *et al.* 1993; García *et al.* 2002). That reproduction in this extended period may occur is documented by occasional delayed parturitions, as late as October and November instead of the typical May and June births, in many red deer populations across Europe (Bartoš unpublished data). Additionally up to six estrous cycles for black-tailed deer, *Odocoileus hemionus* (Wong and Parker 1988), and seven for white-tailed deer, *Odocoileus virginianus* (Knox *et al.* 1988), have been observed.

Today's extreme reduction in distributional range likely has resulted in a diminished variety of habitat types utilized, along with concomitant reduction in variability of behavioral expressions (Putman and Flueck 2011). For instance, migratory behavior of the past has been eliminated in some areas. Older inhabitants from the Inferior Valley of the El Manso River immediately outside the southern boundary of the Argentine Nahuel Huapi National Park reported having seen solitary pudu on various occasions in the past during winter months, while in a nearby, less disturbed area within the same park, fresh tracks and pudu remains found in summer at 1200 masl indicate migratory behavior as snow levels

would not allow pudu to remain at that elevation during winter (Smith-Flueck unpublished data). Abbe Molina noted (1782, cited in Hershkovitz 1982) that pudus descended in flocks from the mountains to the warmer coastal plains of the southern provinces in winter. Gay (1847, cited in Hershkovitz 1982) mentioned them living in small flocks in the central valley of Chile between the Cordilleras. Such large formations of social groups and movement patterns no longer occur. Some historic habitats with no snowfall, particularly those towards the Pacific coast (within the temperate Mediterranean and Temperate oceanic climatic zones), may have been sufficiently productive all year, such that two breeding seasons or aseasonality could have been a viable tactic for pudu. Accordingly, pudu respond to photoperiod like other higher latitude cervids in order to time the optimal breeding season, but may also have a pattern of conception directly influenced by the seasonal change of food quality, similar to deer adapted to tropical conditions (Lincoln 1985).

Although Bubenik *et al.* (2000) speculated that pudu might show delayed implantation similar to roe deer, the breeding and parturition dates of those at the Los Canelos population and the wild fawn indicate instead that the species breeds twice annually or possibly even aseasonally. The tropical northern Andean pudu also seems to have two rutting periods: one in March-April and another in Oct-Nov (Montulet 1984 in Whitehead 1993; Bubenik 2006). Furthermore, nearly full-term fetuses were taken from northern pudus killed in April and November (Grimwood 1969 cited in Hershkovitz 1982). These observations on northern pudu would concur with our hypothesis that the southern pudu also can breed twice per year. The pudu adults in the Chilean wild and at La Canelos semi-captive centre may be responding to internal, environmental and/or social factors than are not available to individuals in captive centers and zoos. Considering that the females are seasonally polyestrous, one can speculate that those which don't conceive in the fall are stimulated by some environmental or social cue to ovulate again in the spring, which coincides to when males enter their second annual peak in testosterone levels. Thus this species might be more flexible than most other temperate deer in terms of their ovarian activity and are not bound to one annual breeding season. Research should concentrate on the reproductive physiology of the female pudu to reveal the factors responsible for regulating a spring estrous and therefore elucidate further the unique physiological characteristics of this deer in relation to other temperate deer. Comparative studies with its tropical cousin can provide us with insight about their ancestors, and perhaps further demonstrate the flexible nature of cervids.

ACKNOWLEDGMENTS

We would like to thank Bruno Behn T and Camila Bentjerodt for their generous support of this pudu center and for the protection, conservation and research on a variety Chilean wildlife. We also thank Patricia Black-Decima and two anonymous reviewers for suggestions that greatly improved the paper. We especially would like to thank the Fundacion Fauna Andina Los Canelos and the Secretaria de Agricultura y Ganadería (SAG) for granting the permits to realize the Los Canelos Center.

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Figure 1. Pudu male (*Pudu puda*) in August (winter, photo by Smith-Flueck).



Figure 2. Birth dates for southern pudu fawns born in northern hemisphere zoos, captive breeding centers in South America, and the Los Canelos semi-captive center in Chile (LCC). Two of the four fawns born in winter were conceived in the wild, and included one wild fawn observed at Huilo Huilo Reserve, Chile. Northern spring: March-May; southern spring: Sept.-Nov.

