ACUTE PHASE PROTEINS IN WILD BOAR, PYRENEAN CHAMOIS AND SPANISH IBEX: METHOD VALIDATION AND REFERENCE VALUES

ESTER BACH i RAICH

Directors
Rafaela Cuenca Valera
Josep Pastor Milán

Tesi Doctoral
Departament de Medicina i Cirurgia Animals
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El Doctors **Rafaela Cuenca Valera** i **Josep Pastor Milán**, Professors Titulars d'Universitat de l'Àrea de Coneixement de Medicina i Cirurgia Animals de la Facultat de Veterinària de la Universitat Autònoma de Barcelona,

INFORMEN:

Que la memòria titulada "ACUTE PHASE PROTEINS IN WILD BOAR, PYRENEAN CHAMOIS AND SPANISH IBEX: METHOD VALIDATION AND REFERENCE VALUES", presentada per **ESTER BACH i RAICH** per a la obtenció del grau de Doctor en Veterinària per la Universitat Autònoma de Barcelona, s'ha realitzat sota la nostra direcció i, un cop considerada satisfactòriament finalitzada, autoritzem la seva presentació per tal que sigui avaluada per la comissió corresponent.

I perquè així consti als efectes que siguin oportuns, firmem el present informe a Bellaterra, el 26 de setembre de 2012.

Firmat: Rafaela Cuenca Valera

Firmat: Josep Pastor Milán

Firmat: Ester Bach i Raich

Concerning all acts of initiative (and creation) there is one elementary truth,
the ignorance of which kills countless ideas and splendid plans:
that the moment one definitely commits oneself,
then providence moves too.
All sorts of things occur to help one that would not otherwise have occurred.
A whole stream of events issues from the decision,
raising in one's favor all manner of unforeseen incidents and meetings and
material assistance which no man would have dreamed would come his way.

I have learned a deep respect for one of Goethe's couplets:
"Whatever you can do, or dream you can, begin it.
Boldness has genius, magic, and power in it.
Begin it now"

W.H.MURRAY
The Scottish Himalayan Expedition, 1951

Il·lustracions i disseny de la portada originals de David Pradas

AGRAÏMENTS

A pocs dies del dipòsit de la tesi arribo a la part final d'aquest treball, però no per això menys important, els agraïments.

Als meus directors de tesi,

Rafi, por haberme guiado y acompañado a lo largo de este camino largo y tortuoso, desde los días en la Universidad de Murcia donde estuvimos horas y horas en el laboratorio, hasta estos últimos meses de escritura y correcciones de la tesis. Gracias.

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A la Montse Mesalles, per haver-me aguantat tots els anys que vaig estar al servei. I a la Nuria i la Elisabet, amb les que també va ser un plaer compartir hores de laboratori.

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SEGUR, SEGURÍSSIM, QUE EM DEIXO PERSONES IMPORTANTS PER AIXÒ VULL FER UN AGRAÏMENT ESPECIAL A TOTS ELS QUE EM DEIXO I QUAN ME N'ADONI EM SABRÀ GREU HAVER-ME DEIXAT.

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1. ABSTRACT

1. ABSTRACT

Acute phase proteins (APPs) are a group of plasma proteins that change in concentration after any tissue injury induced by infection, inflammation, trauma or stress. Their main function is to restore homeostasis and limit microbial growth. The quantification of APPs can be used as a tool for diagnostic and prognostic as well as to monitor treatments. In the literature there are many articles describing the APPs patterns against different diseases in domestic animals, mainly livestock. However, the usefulness of APPs and their dynamics in diseases typical of wild populations has not been characterized. Analytical validation of the laboratory methods in use for the determination of APP should be assessed before the report of any value. This is especially important in wild animals, in which often the techniques in use in domestic animals are extrapolated without prior validation, so that the reliability of the results is unknown.

The main goals of the present thesis are to validate analytical methods available for determination of APPs in domestic ungulates for its use in wild ungulates, to provide reference values and to verify the utility of several APPs for the species studied.

In STUDY I six different APPs in wild boar were studied: serum haptoglobin (Hp), serum amyloid A (SAA), C-reactive protein (CRP) and porcine major acute phase protein (Pig-MAP) concentrations were determined using commercial kits available, the last two were porcine-specific methods; Acid soluble glycoprotein (ASG) and ceruloplasmin (Cp) were analyzed using assay methods described previously in literature. All the methods demonstrated good precision (CVs<15%), except inter assay CVs for ASG and SAA with the low concentration pool. Hemolysis affected all the proteins studied, mostly the SAA. There were significant differences between healthy and diseased animals. Reference ranges obtained for healthy wild boars were similar to those reported previously in literature for domestic pigs.

In STUDY II a human CRP turbidimetric immunoassay (TIA) was compared to the porcine specific enzyme linked immunosorbent assay (ELISA) CRP and validated for its use in wild boar. Passing bablok regression demonstrated that there was a

proportional error between TIA and ELISA which was reduced using a porcine in-house calibrator. The validation of TIA CRP with the porcine in-house calibrator showed good precision and accuracy. Important interference was observed in the study of hemolysis.

The method validation of Hp, SAA, ASG and Cp conducted in STUDY III in Pyrenean chamois demonstrated good precision and accuracy of all the proteins studied. Hp and SAA yielded many values below the limit of detection when samples from healthy animals were analyzed. ASG was significantly affected by hemolysis. The values obtained from healthy and diseased animals were significantly different from each other.

Hp and SAA methods were evaluated for Spanish ibex in STUDY IV with good intra-assay precision and accuracy. An important inter-assay imprecision was observed for both proteins. Inflammation induced by turpentine injection produced a significant change in the concentration of Hp and SAA. No significant changes in any of the proteins studied were observed when a experimentally bluetongue virus infection was induced.

APPs methods validated in these studies demonstrated to be reliable in the species studied, except for some parameters that should be kept in mind when implementing these methods. Likewise, significant differences between healthy and disease animals were observed for all the proteins studied in wild boar and Pyrenean chamois. In Spanih ibex, haptoglobin and serum amyloid A discriminated well before and after the establishment of an aseptic inflammation induced by turpentine injection.

2. RESUM

1. RESUM

Hi ha un grup de proteïnes plasmàtiques, anomenades proteïnes de fase aguda (PFAs), la concentració de les quals canvia quan es produeixen lesions tissulars induïdes per traumes o estrès i per condicions infeccioses i inflamatòries. La funció principal de les PFAs és recuperar la homeòstasi i limitar el creixement microbià. La quantificació de la concentració de les PFAs pot ser utilitzada com a eina pel diagnòstic i pronòstic, així com pel seguiment de tractaments. A la bibliografia hi ha molts articles que descriuen els diferents patrons de les proteïnes enfront diferents malalties. No obstant, la utilitat de les APPs i la seva dinàmica en malalties pròpies de poblacions salvatges no ha estat determinada. Els mètodes de laboratori que s'utilitzen actualment per mesurar les APPs s'haurien de validar abans de proporcionar cap resultat. Això és especialment important en les espècies salvatges, on les tècniques dels animals domèstics s'extrapolen sense validació prèvia, i per tant la fiabilitat dels resultats es desconeguda.

Els objectius principals d'aquest treball són validar els mètodes analítics disponibles per la determinació de proteïnes de fase aguda en ungulats domèstics per al seu ús en ungulats salvatges, així com proporcionar valors de referència per a les espècies estudiades i verificar la utilitat d'aquestes proteïnes de fase aguda.

En l'ESTUDI I, realitzat en el senglar, es va determinar la concentració sèrica de sis APPs: l'haptoglobina (Hp), el sèrum amiloide A (SAA), la proteïna C-reactiva (CRP) i la proteïna de fase aguda major de porcí (Pig-MAP) utilitzant kits comercials. Els kits de les dues últimes proteïnes mencionades dissenyats específicament per la seva aplicació en mostres de porcí. També es van determinar la glicoproteïna àcida soluble (ASG) i la ceruloplasmina (Cp) mitjançant mètodes analítics descrits prèviament. Tots els mètodes utilitzats van demostrar una bona precisió (CV <15%), excepte per les proteïnes ASG i SAA que van presentar coeficients de variació (CVs) entre anàlisis més alts. Aquests dos CVs van ésser obtinguts pel pool de concentració baixa. L'estudi

d’interferència va demostrar que l’hemòlisi produeix interferència en totes les proteïnes estudiades, sobretot el SAA. Totes les proteïnes estudiades van presentar diferències significatives entre les concentracions d’animals sans i malalts. Els valors de referència obtinguts pels senglars sans van ser similars als descrits prèviament en altres treballs realitzats en el porc domèstic.

En l’ESTUDI II es van comparar dos mètodes diferents per la determinació de la CRP en el senglar. Un mètode immunoturbidimètric (TIA) específic de humana amb un mètode immunoenzimàtic (ELISA) específic de porcí. També es va validar el mètode immunoturbidimètric per al seu ús en els senglars. La regressió Passing-Bablok va demostrar que hi havia un error proporcional entre TIA i ELISA. Aquest error es va reduir quan es va utilitzar un calibrador de porcí fabricat en el laboratori. La validació del TIA amb el calibrador de porcí obtingut al laboratori va demostrar tenir una bona precisió i una bona exactitud. Es va demostrar també que l’hemòlisi produeix interferències importants en l’anàlisi de la CRP amb mostres de senglar amb el TIA.

La validació dels mètodes de la Hp, SAA, ASG i Cp pel seu ús en l’isard es va realitzar en l’ESTUDI III. En general, tots els mètodes van presentar bona precisió i exactitud. Les mostres procedents d'animals sans van produir molts valors per sota del límit de detecció establert per les proteïnes Hp i SAA. La ASG es va veure afectada significativament per l'hemòlisi. Els valors obtinguts d'animals sans i malalts van ser significativament diferents entre sí.

Els mètodes de determinació de la Hp i el SAA van ser avaluats pel seu ús en la cabra salvatge en l’ESTUDI IV. Aquests mètodes van demostrar tenir bona precisió intra-assaig i bona exactitud. En l’estudi inter-assaig es va observar una gran imprecisió per ambdues proteïnes. La inflamació induïda amb la injecció de trementina va produir un canvi significatiu en la concentració de Hp i SAA. En canvi, la infecció experimental amb llengua blava no va produir canvis significatius en cap de les proteïnes estudiades.

Els mètodes validats per la determinació de les PFA en aquests quatre estudis van demostrar que els resultats que produeixen son fiables i que per tant es poden aplicar en les espècies estudiades, amb l'excepció d'alguns paràmetres que caldria tenir en compte quan s'utilitzin aquests mètodes. De la mateixa manera, hi va haver diferències significatives entre els animals sans i malalts en totes les proteïnes estudiades en el senglar i l'isard, i en l'haptoglobina i l'amiloide A sèric en la cabra salvatge. En aquesta última espècie les dues proteïnes estudiades van produir canvis de concentració suficients com per poder discriminar bé entre abans i després de l'establiment d'una resposta inflamatòria induïda per la injecció de trementina.

3. RESUMEN

1. RESUMEN

Hay un grupo de proteínas plasmáticas, llamadas proteínas de fase aguda (PFAs), la concentración de las cuales cambia cuando se producen lesiones tisulares inducidas por traumas o estrés y por condiciones infecciosas e inflamatorias. La función principal de las PFAs es restablecer la homeostasis y limitar el crecimiento microbiano. La cuantificación de la concentración de las PFAs puede ser utilizada como herramienta para el diagnóstico y pronóstico, así como para el seguimiento de tratamientos. En la bibliografía hay muchos artículos que describen los diferentes patrones de las proteínas frente distintas enfermedades. Sin embargo, la utilidad de las APPs y su dinámica en enfermedades propias de poblaciones salvajes no ha sido determinada. Los métodos de laboratorio que se utilizan actualmente para medir las APPs deberían ser validados antes de proporcionar ningún resultado. Esto es especialmente importante en las especies salvajes, donde las técnicas de los animales domésticos se extrapolan sin validación previa, y por tanto la fiabilidad de los resultados es desconocida.

Los objetivos principales de este trabajo son validar los métodos analíticos disponibles para la determinación de proteínas de fase aguda en ungulados domésticos para su uso en ungulados salvajes, así como proporcionar valores de referencia para las especies estudiadas y verificar la utilidad de estas proteínas de fase aguda.

En el ESTUDIO I, realizado en el jabalí, se determinó la concentración sérica de seis APPs: haptoglobina (Hp), el amiloide A sérico (SAA), la proteína C-reactiva (CRP) y la proteína de fase aguda mayor de porcino (Pig-MAP) utilizando kits comerciales. Los kits de las dos últimas proteínas mencionadas diseñados específicamente para su aplicación en muestras de porcino. También se determinaron la glicoproteína ácida soluble (ASG) y la ceruloplasmina (Cp) mediante métodos analíticos descritos previamente. Todos los métodos utilizados demostraron una buena precisión (CV <15%), excepto para las proteínas ASG y SAA que presentaron coeficientes de variación

(CVs) entre análisis más altos. Estos dos CVs fueron obtenidos para el pool de concentración baja. El estudio de interferencia demostró que la hemólisis produce interferencia en todas las proteínas estudiadas, sobretodo el SAA. Todas las proteínas estudiadas presentaron diferencias significativas entre las concentraciones de animales sanos y enfermos. Los valores de referencia obtenidos por jabalíes sanos fueron similares a los descritos previamente en otros trabajos realizados en el cerdo doméstico.

En el ESTUDIO II se compararon dos métodos diferentes para la determinación de la CRP en el jabalí. Un método immunoturbidimètrico (TIA) específico de humana con un método immunoenzimàtico (ELISA) específico de porcino. También se validó el método immunoturbidimètrico para su uso en los jabalíes. La regresión Passing-Bablok demostró que había un error proporcional entre TIA y ELISA. Este error se redujo cuando se utilizó un calibrador de porcino fabricado en el laboratorio. La validación del TIA con el calibrador de porcino obtenido en el laboratorio demostró tener una buena precisión y una buena exactitud. Se demostró también que la hemólisis produce interferencias importantes en el análisis de la CRP con muestras de jabalí con el TIA.

La validación de los métodos de la Hp, SAA, ASG y Cp para su uso en el rebeco se realizó en el ESTUDIO III. En general, todos los métodos presentaron buena precisión y exactitud. Las muestras procedentes de animales sanos produjeron muchos valores por debajo del límite de detección establecido para las proteínas Hp y SAA. La ASG se vio afectada significativamente por la hemólisis. Los valores obtenidos de animales sanos y enfermos fueron significativamente diferentes entre sí.

Los métodos de determinación de la Hp y el SAA fueron evaluados para su uso en la cabra montés en el ESTUDIO IV. Estos métodos demostraron tener buena precisión intra-ensayo y buena exactitud. En el estudio inter-ensayo se observó una gran imprecisión para ambas proteínas. La inflamación inducida con la inyección de trementina produjo un cambio significativo en la concentración de Hp y SAA. En cambio, la infección experimental con lengua azul no produjo cambios significativos en ninguna de las proteínas estudiadas.

Los métodos validados para la determinación de las PFA en estos cuatro estudios demostraron que los resultados que producen son fiables y que por tanto se pueden aplicar en las especies estudiadas, con la excepción de algunos parámetros que habría que tener en cuenta cuando se utilicen estos métodos. Del mismo modo, hubo diferencias significativas entre los animales sanos y enfermos en todas las proteínas estudiadas en el jabalí y el rebeco, y en la haptoglobina y el amiloide A sérico en la cabra montesa. En esta última especie las dos proteínas estudiadas produjeron cambios de concentración suficientes como para poder discriminar bien entre antes y después del establecimiento de una respuesta inflamatoria inducida por la inyección de trementina.

4. LITERATURE REVIEW

4. LITERATURE REVIEW

4.1. Wild ungulates

Ungulates (Ungulata) are the most diverse group of mammals that exists today and can be found throughout the world except Antarctica. Within this group we find wild ungulates, the largest terrestrial mammals occurring. Their influence stretches across nearly every biome and in most European countries they play an important role in the ecological dynamics. Most domestic ungulates share common ancestors with wild species, to which they are related phylogenetically. For this reason in many cases domestic and wild animals may be carriers of the same pathogens as well as suffer the same diseases, which may be transmitted from one to another (Pastoret *et al.,* 1988).

Next we present a brief introduction to the wild boar, Pyrenean chamois and Spanish ibex, the wild ungulate species included in this work.

4.1.1. Wild boar

The wild boar (Figure1), *Sus scrofa*, is an artiodactyl belonging to the Family Suidae with a wide distribution in Europe, Asia and North Africa (Figure 2). Some authors have stated that exist up to 17 subspecies (Étienne and Pijoan, 2004), two of which are found in Spain; *S. s. castilianus* distributed in the north of the Iberian Peninsula and *S. s. baeticus* in the South. It has even been suggested that the subspecies *S. s. castilianus* could coincide with European *S. s. scrofa* (Rosell-Pagès, 1998). Today, the wild boar is on an upswing after declining steadily for many years, and the proof is that 30.000 animals were hunted in 1980 in Spain, which is practically the same number of animals hunted in 2009 in Catalonia (Noth-Eastern Spain) (Closa-Sebastià, 2009).

Figure 1: Wild boar (*Sus scrofa*) adult male (left) and female with piglets (right). Photo courtesy of Natural Park Sant Llorenç del Munt i l'Obac (Diputació de Barcelona, Spain).

In the Iberian Peninsula the wild boar is adapted to almost all habitats, and can be found from coastal to high mountain areas, as long as they find the conditions of food and water availability, and quiet places to hide and rest. However, it is becoming more common to see wild boars near urban areas in times of low food availability, and even set up their beds close to these places. The wild boar is an omnivorous animal that feeds from dusk to dawn. He eats mainly the products of flowering of various species, especially the oak (*Quercus rotundifolia*), and also grass, roots, mushrooms and truffles. Some of the animals included in its diet are reptiles, snails, insects, small mammals, eggs or birds (Fernández-Llario, 2006).

Adult males are usually solitary animals, but sometimes remain near a family group of females and their offspring, which is the wider social unit with a matriarchal structure. Zeal occurs once a year during the months from October to January, when mating occurs. Pregnancy usually lasts 114 days and birth gives 4 or 5 piglets (Closa-Sebastià, 2009).

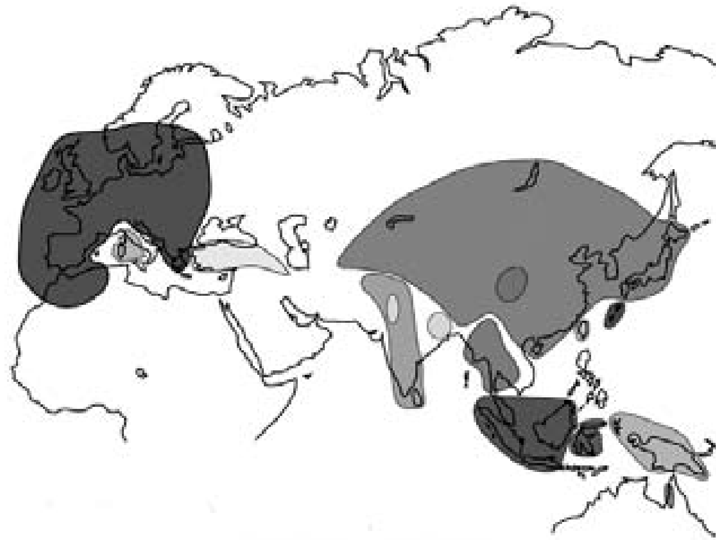

Figure 2: *Sus scrofa* world distribution. Different grey tones indicate different phylogenetic subgroups. Adapted from Larson *et al.,* 2005.

The increase in wild boar population and distribution over the past decades, has led to a growing interaction between these animals and humans, livestock or wildlife. Wild boars can cause crop damage, traffic accidents and may become a potential health risk as a reservoir and transmitter of a long list of diseases such as toxoplasmosis, brucellosis, trichinosis, circovirosis or parvovirosis (Closa-Sebastià *et al.,* 2010; 2011). In some areas management measures such as high hunting pressure throughout the hunting season and even, if necessary beyond, have been carried out.

4.1.2. Pyrenean chamois

The chamois is an artiodactyl belonging to the family Bovidae, subfamily Caprinae, tribe Rupicaprini and genus *Rupicapra*. In the most recent literature two species are considered: the Pyrenean chamois, *Rupicapra* pyrenaica, with three subspecies, and the northern chamois, *Rupicapra rupicapra*, with seven subspecies.

The Pyrenean chamois (Figure 3), *Rupicapra pyrenaica*, is a wild ungulate endemic to south-west Europe, where it occurs as three subspecies: *R. p. parva* in the Cantabrian Mountains, *R. p. pyrenaica* in the Pyrenees and *R. p. ornata* in the Apennine Mountains (Shackleton, 1997; Pedrotti and Lovari, 1999) (Figure 4).

R. p. parva and *R. p. pyrenaica* are listed as Least Concern by the IUCN (International Union for Conservation of Nature) Red List. However, *R. p. ornata* is assessed here as Vulnerable. The 2003 estimate for the total number of *R. p. pyrenaica* was around 53,000 (Herrero *et al.*, 2008a). This is now likely to be an overestimate of the population, as many chamois populations have locally declined since then due to outbreaks of disease associated to pestivirus infection (Marco *et al*., 2009).

Figure 3: Adult male of Pyrenean chamois (*R. p. pyrenaica*). Photo: I.Marco.

Pyrenean chamois are found at elevations of 400-2.800 m (Palomo and Gisbert, 2002) in alpine meadows, rocky areas, and forested valleys and lower slopes in mountainous regions. This species generally stays above 1,800 meters in alpine meadows during the warmer months of the year (Herrero *et al.,* 2008a). In late fall chamois descend, while usually staying on steep slopes, and in forested areas. Home range of Pyrenean chamois oscillates from 20 to 100 hectares in males to 50 to 500 hectares in females (ANCGG, 1992). Pyrenean chamois feeds predominantly on herbaceous plants (grass

and pulses), although up to 300 different plant and several lichen species have been described in its diet (Catusse *et al.,* 1996).

Pyrenean chamois is a gregarious species, and females, kids and young up to two years form herds. Young males also can form groups, although normally males older than two years are solitaries (Catusse *et al.,* 1996). During rut, males join female groups, and try to keep females grouped in a small area which they defend from other males (ANCGG, 1992). Pyrenean chamois reaches sexual maturity at 18-20 months of age (Catusse *et al.,* 1996). Rut takes place from the end of October to the beginning of December. Gestation takes 160 to 185 days and births are singles (Pflieger, 1982).

Figure 4: Natural distribution range of *Rupicapra* spp. *Rupicapra pyrenaica*: (1) *parva*, (2-red) *pyrenaica*, (3) *ornata*. *Rupicapra rupicapra*: (4) *cartusiana*, (5) *rupicapra*, (6) *tatrica*, (7) *carpatica*, (8) *balcanica*, (9) *caucasica*, (10) *asiatica.* Adapted from Coraltti *et al.,*, 2011.

Today, the most important threat for Pyrenean chamois is disease. Since 2001, the disease outbreaks associated to pestivirus infection have caused important declines in different populations from the Pyrenean subspecies. On the other hand, in the Cantabrian subspecies, sarcoptic mange outbreaks periodically cause local declines (Fernández-Morán *et al.,* 1997).

Most Pyrenean and Cantabrian populations are hunted (with the exception of those within the National Parks). Chamois is a major game species in Spain, France and

Andorra and it is considered as an important economical source for rural communities (Herrero *et al.,* 2008a).

4.1.3. Spanish ibex

The Spanish ibex (Figure 5), *Capra pyrenaica*, comprises four subspecies: *C.p. pyrenaica*, *C.p. lusitanica*, *C.p. victoriae*, and *C.p. hispanica*. This species is endemic to the Iberian Peninsula. The subspecies *C.p. pyrenaica* and *C.p. lusitanica* are extinct. *C.p. victoriae* occurs in the central Spanish mountains (Sierra de Gredos), and has been re-introduced to a number of additional sites in Spain and northern Portugal (Palomo and Gisbert, 2002; Herrero *et al.,* 2008b). *C.p. hispanica* occupies the mountains that run along the Mediterranean coast, from the Ebre river mouth (Tarragona, Spain) to the rock of Gibraltar (southern end of the Iberian Peninsula)(where it no longer occurs), as well as the Sierra Morena (Castilla-La Mancha and Andalusia, Spain). It has been reintroduced in Montserrat (Barcelona, Spain) and in Sierra de Guara (Huesca, Spain) (García-González and Herrero, 1999). The most recent updated distribution of the Spanish ibex is shown in Figure 6 (Acevedo and Real, 2011).

Figure 5: Spanish ibex (*Capra pyrenaica*) adult male (left) and female (right). Photo: I. Marco.

The species is found from sea level to 3,400m (Palomo and Gisbert, 2002). It shows big habitat plasticity, but it invariably occurs in rocky habitats. Rocky areas on the coast

may be used, although cliffs and screes interspersed with scrub or pine trees are the most typical habitats. The Spanish ibex is a mixed feeder (browser and grazer). This species shows high feeding plasticity, and the percentage of each type of resource consumed may vary altitudinally (Martínez, 1994), geographically (Granados *et al.,* 2001) and seasonally (García-González and Cuartas, 1992).

The sexes are segregated for most of the year and there are male-only groups and mixed groups of females, juveniles and subadults. During the rutting season adult males and females come together (Granados *et al.,* 2001). However, this pattern may vary (Acevedo and Cassinello, 2009). Ibex altitudinal dispersion occurs according to resources availability, e.g. heading to rich, high altitude areas in summer, which are usually covered by snow in winter (Gonçales, 1982; Escos, 1988; Travesi, 1990).

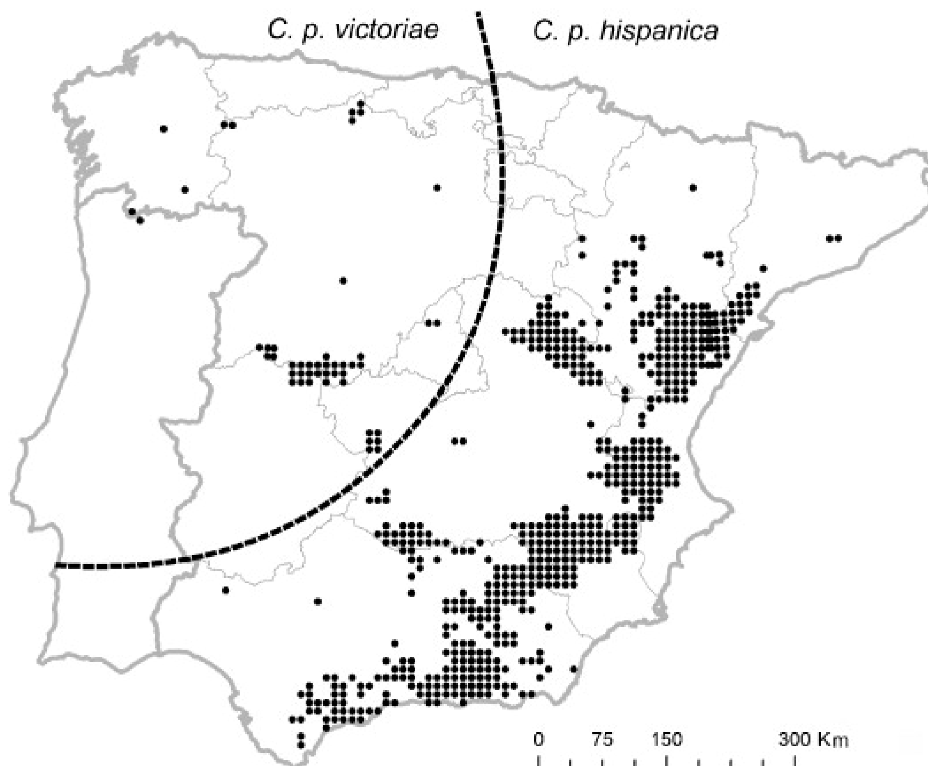

Figure 6: Current distribution of the Spanish ibex. The discontinuous line represents the distribution of the two subespecies, *C. p. victoriae* in the north-west, and *C. p. hispanica* in the south and east. Adapted from Acevedo and Real, 2011.

Mating occurs in November-December. Dominant males do most of the courting. Gestation lasts 175-185 days, and usually one kid is carried per pregnancy.

The major disease threat for Spanish ibex is sarcoptic mange, caused by *Sarcoptes scabiei* (Shackleton, 1997; León-Vizcaíno 1999; Herrero and Pérez, 2008). Alteration and fragmentation of the habitat may also impact negatively upon certain Spanish ibex populations. The founder effect also represents a threat for the Spanish ibex populations (Pérez *et al.*, 2002). The impact of hunting (predominantly for trophies) has not been scientifically assessed (J. M. Pérez, personal communication), but poaching of large dominant males might alter gene flow.

4.2. Acute phase proteins

4.2.1. Systemic reaction to inflammation

Local inflammation is the first immune response against harmful stimulus like trauma, infection or neoplasia, amongst others. This response includes platelet aggregation, increased vascular permeability and cell activation, mainly inflammatory. When these mechanisms are not sufficient to counteract the established damage a systemic response starts (Gabay and Kushner, 1999). The main functions of the systemic response are designed to provide substrates and energy to combat invading pathogens, prevent the transfer of metabolites necessary for pathogens, limit damage caused and as far as possible remove and restore damaged tissue (Whicher and Westacott, 1992; Ebersole and Cappelli 2000).

Systemic inflammatory response activation is initiated by both external and internal stimuli; pathogen associated molecular patterns (PAMP) and damaged associated molecular pattern (DAMP) respectively. PAMP are molecules recognized by blood and cell associated proteins and shared by pathogens like bacteria, fungi and viruses (Brodsky and Medzhitov, 2009). DAMP are molecules released when there is cell death or damage and are recognized by receptors from the innate immune response (Krysko *et al.,* 2011). Sometimes, PAMP and DAMP can share pattern recognition receptors. The production of pro-inflammatory cytokines is the final step of the activation sequence of the systemic response, which starts with the recognition of PAMP and DAMP. The main pro-inflammatory cytokines produced in this process are interleukin-1β (IL1β), tumoral necrosis factor-α (TNFα) and interleukin-6 (IL6) (Gauldie *et al.,* 1987).

The release of the pro-inflammatory cytokines induces a series of systemic changes including fever, leukocytosis and changes in concentration of a series of proteins named acute phase proteins (APPs) (Murata *et al.,* 2004; Ceciliani *et al.,* 2012). The liver is the main site of synthesis of these APPs, although concentration increases in nonhepatic tissues such as mammary gland, lungs, adipocyte and intestine after stimulation have also been described (Eckersall, 2008a). Generally, the production of

these proteins is designed to defend the animal form pathological damage and to restore homeostasis.

4.2.2. Classification

There are different classifications of acute phase proteins according to different criteria either based on the variation of the APPs levels or based on APPs physiological function.

It must be said that there are important differences between species in the change of concentration of APPs during systemic reaction to inflammation. Moreover, different pathophysiological challenges will lead to different patterns in the production of proinflammatory cytokines, which in turn will produce different changes in proteins. Thus, the same protein can behave differently depending on the disease (Eckersall, 2008a; Ceciliani *et al.,* 2012,).

According to level variation APPs can be classified as (Murata *et al.,* 2004; Eckersall, 2008a; Ceciliani *et al.,* 2012):

<u>Negative APPs</u>: in this group are included those proteins that decrease in concentration during the systemic reaction to inflammation. Some examples of negative APPs are albumin, prealbumin, transferrin or apolipoprotein-1 (APO-1); Nevertheless, albumin has been demonstrated to be a positive APP when synthesized in the mammary gland during mastitis (Shamay *et al.,* 2005).

<u>Positive APPs</u>: are those which level increase during the systemic reaction to inflammation. This group can also be divided in three subgroups depending on the magnitude of change in concentration. Thus we can find major, moderate or minor responses. Major APPs concentration increases over 100- or 1000-fold on stimulation; moderate APPs increase 5- to 10-fold after stimulation and minor APPs show a gradual increase and only increases in concentration by 50% to 100%.

According to physiological function APPs can be classified as (Kushner and Mackiewicz 1987; Eckersall, 2008a):

APPs with defensive function: These proteins are involved in the adaptation or defense of the host to face the pathogen. They have antibacterial activity and the ability to influence the course of the immune response. In this group we can find C-reactive protein (CRP), serum amyloid A (SAA) and alpha-1-acid glycoprotein (AGP).

Binding APPs with antioxidant activity: These proteins bind the metabolites released from phagocytic cells during inflammation. Thus they avoid that pathogens use these metabolites and also recycle them for metabolic processes of the host. Ceruloplasmin (Cp), haptoglobin (Hp) and SAA are proteins included in this group.

APPs with antiprotease activity: These proteins inhibit the enzymes released by phagocytes and other cells, protecting or minimizing damage on host tissues. $Alpha_1$-antitripsine and $alpha_2$- macroglobulin are included in this group.

4.2.3. Biochemistry, patophisiology and function of main APPs

4.2.3.1. Haptoglobin (Hp)

Haptoglobin is a plasma glycoprotein with a tetrameric structure consisting of two alpha subunits combined with two beta subunits. The subunits combine to form a beta-alpha-alpha-beta chain and Hp structure differs notably among species. In ruminants, the tetramers form polymers binding to other Hp tetramers and macromolecular complexes. This protein is practically not present in serum from healthy ruminants (Eckersall, 2008a; Ceciliani *et al.,* 2012).

The main function of Hp is to bind free hemoglobin (Hb) in the plasma and reduce oxidative damage associated with hemolysis and the inherent peroxidase activity of Hb. The complex Hp-Hb cannot pass through glomerular filter and is removed by the circulation by monocyte macrophages which receptor CD163 recognizes the complex (Graversen *et al.,* 2002). The union of the Hp-Hb complex to the monocyte/macrophages CD163 upregulates a number of imunomodulatory activities (Murata *et al.,* 2004). Additionally, haptoglobin binds to the neutrophil surface causing a downregulation of these inflammatory cells by inhibiting the cycloxygenase and

lipoxygenase activities (Saeed *et al.,* 2007). Thus haptoglobin also has an anti-inflamatory role during the systemic reaction to inflammation.

Additionally, the formation of the Hp-Hb complex also confers to haptoglobin bacteriostatic properties, since the binding to free hemoglobin causes unavaiblability of iron which is needed for bacteria for its growth. However, there are pathogens that can use iron even when it is attached to the Hp-Hb complex (Eaton *et al.,* 1982; Zakaria-Meehan *et al.,* 1988; Otto *et al.,* 1994; Lewis and Dyer 1995).

4.2.3.2. Serum amyloid A (SAA)

Is a small apolipoprotein associated with high density lipoproteins (HDL) in serum. This protein is the precursor of amyloid A, thus it is a protein implicated to the amyloidosis pathogenesis. Four isoforms of SAA have been described, each one encoded by a different gene. These forms are classified in two groups: the acute phase SAA (A-SAA) composed by SAA-1, SAA-2 and SAA-3 and the constitutive SAA (C-SAA), SAA-4 which is not affected by the acute phase reaction. SAA-1 and SAA-2 are the major circulating proteins and its liver synthesis increases with the release of pro-inflamatory cytokines IL6 and TNFα during the systemic reaction to inflammation. SAA-3 is also expressed during the systemic reaction although unlike the SAA-1 and SAA-2 it is synthesized in non-hepatic tissues like lungs, adipose tissue, ovarian granulose and mammary gland (Eckersall, 2008a).

A large number of functions have been attributed to SAA. Maybe, the most well described function of this protein is the uptake and removal of cholesterol from the inflammatory site to the hepatocytes, preventing the formation of atherosclerotic plaques due to its accumulation (Banka *et al.,* 1995; Liang and Sipe 1995; Hayat and Raynes 1997; Manley *et al.,* 2006). Additionally, SAA has also been demonstrated to modulate the migration, infiltration and tissue infiltration of monocytes, as it acts as a chemoattractant of monocyte and neutrophils (Badolato *et al.,* 1994). Finally, it has been shown that SAA can bind to bacteria Gram– targeting them for opsonization. It

has been demonstrated that SAA-3 synthetized in the mammary gland (M-SAA3) binds either with Gram- or Gram+ bacteria in bovine (Molenaar *et al.,* 2009).

4.2.3.3. C-reactive protein (CRP)

Is a pentraxin composed of five units that contain a binding site for ligand. CRP is synthesized in the liver and it was the first pattern recognition receptor (PRR) to be identified. This protein is so named because it was first discovered as a substance in the serum of human patients with acute inflammation that reacted with the C-(capsular) polysaccharide of pneumococcus (Pepys and Hirschfield, 2003; Mantovani *et al.,* 2008).

CRP increases dramatically in concentration during the systemic reaction to inflammation due to a rise in the plasma concentration of IL-6, which is produced predominantly by macrophages as well as adipocytes (Pepys and Hirschfield, 2003).

The physiological role of CRP is to bind phosphocholine expressed on the surface of dead or dying cells and pathogenic bacteria, thus helps to the opsonization of altered self and foreign molecules. CRP also has other sites that activate the classical complement pathway and is involved in the regulation of leukocyte and platelet activity, as it contributes to the induction of pro-inflammatory cytokines and inhibits chemotaxis and respiratory brust of neutrophils (Murata *et al.,* 2004; Eckersall, 2008a).

4.2.3.4. Alpha 1-acid glycoprotein (AGP)

Is a highly glycosylated protein synthesized and secreted mainly by hepatocytes. Further variation in glycosylation occurs during inflammation, with observed differences between acute and chronic inflammation, and between early and late phase of acute inflammation. AGP has been classified as a immunocalin, a lipocalin subfamily that modulates immune and inflammatory responses (Lögdberg and Wester, 2000).

AGP has been thoroughly studied in human medicine because of its ability to bind drugs, bacterial compounds and a variety of endogenous components including inflammatory mediators such as heparin, histamine or serotonin (Israili and Dayton 2001; Eckersall, 2008a). Other suggested AGP functions are the inhibition of phagocytosis, neutrophil activation and platelet aggregation as well as the involvement in T- and B- lymphocyte maturation (Israili and Dayton 2001; Eckersall, 2008a). Its role in the innate defense against infections acting as a non-specific anti-microbial agent by the inhibition of some bacteria and viruses has also been described (Maeda *et al.,* 1980; Friedman 1983).

4.2.3.5. Ceruloplasmin (Cp)

An α_2-glycoprotein, is a cooper-containing ferroxidase member of the multicopper oxidase family of enzymes. It is synthesized in the liver although extrahepatic induction has also been reported (Hellman and Gitlin, 2002).

Cp is a major protein that circulates in the plasma and functions as a copper transporter that is able to couple and transport 90–95% of serum copper (Giurgea *et al.,* 2005). The increase in the hepatic copper pool results in a sustained increase in the serum Cp concentration, whereas a decrease as occurs in nutritional copper deficiency, results in a marked decrease in serum Cp (Hellman and Gitlin, 2002).

Other known functions of Cp include iron metabolism, antioxidant defense, and involvement in angiogenesis and coagulation. It has been shown that CP catalyzes the oxidation of Fe2+ to Fe3+, and this activity as ferroxidase is increased during inflammation, infections, and other conditions, and these observations seem to suggest that there is a possibility that CP acts both as an antioxidant and an acute-phase reactant. Additionally, in human medicine, it has been established that there is a relationship between the CP concentration in serum and the incidence of atherosclerosis and other cardiovascular conditions (Giurgea *et al.,* 2005).

4.2.3.6. Pig major acute phase protein (Pig-MAP)

Is an α_2-globulin that increases in concentration in pigs during the systemic reaction to inflammation induced by IL6 and is synthesized in the liver. It has been identified as porcine inter-α-trypsin inhibitor heavy chain 4 (ITIH4) and it is homologous the inter-α-trypsin inhibitor human related protein (IHRP) (González-Ramón *et al.,* 2000). Recently ITIH4 has also been identified as an APP in cattle (Piñeiro *et al.,* 2004).

The physiological function for the Pig-MAP has not yet been well described (González-Ramón *et al.,* 2000). Initially, it was classified as a complement regulatory protein (Hammer *et al.,* 1989). Later it was considered as a novel substrate for kallikreins, a group of serine proteases responsible for the regulation of blood pressure, activation of inflammation, coagulation and pain (Bhoola *et al.,* 1992; Nishimura *et al.,* 1995; Offermanns and Rosenthal, 2008). A possible role in the binding of some cell lines to the extracellular matrix has also been proposed for all the members of the inter-α-trypsin inhibitor family (Chen *et al.,* 1994; Bost *et al.,* 1998). Finally its possible role in protecting the uterus from the inflammatory response induced by conceptus attachment to the uterine epithelium has also been suggested (Geisert *et al.,* 1998).

4.2.4. APPs in different species

APPs have been investigated in recent decades as biomarkers of disease. Initially APPs were studied to understand the pathophysiology and the involvement that they have in the innate immune response. Then its application as a tool for diagnosis, prognosis and monitoring of treatments made the interest in their study grew. Now a significant number of papers about APPs in veterinary medicine is available.

In wild ungulates the literature about APPs is scarce, so when we want to study APPs in these species we must rely on the information we have on the domestic species phylogenetically closer to them. However we should take into account that in addition to the species there are other factors that may influence the change of APPs. Thus, differences between different breeds have been reported in cattle (Glass and Jensen,

2007) and pigs (Diack *et al.,* 2011). Furthermore, characteristics like the age have also been shown to influence in the variation in expression of APPs (Orro *et al.,* 2008). So although there may be similarities between phylogenetically related species each particular situation should be studied before extrapolating data and draw conclusions.

The role of APPs in domestic ruminants (bovine, ovine and caprine) and in pigs is summarized below as their phylogenetic proximity with the wild ruminants (Spanish ibex and Pyrenean chamois) and wild boar respectively.

4.2.4.1. APPs in pigs

In the pig, the literature on APPs is very extensive and the utility of these proteins as a biomarker of disease and welfare is well described. Hp, CRP, SAA and Pig-MAP are classified as major APPs whereas AGP and Cp are considered moderate (Petersen *et al.,* 2004; Parra *et al.,* 2006; Cray *et al.,* 2009).

Table 2 adapted from Gómez-Laguna *et al.* (2011) summarizes APPs reported in different disorders in pigs.

Hp has been demonstrated to increase in conditions of lamenesss, respiratory disease, diarrhea, tail bite as well as ear necrosis. The study of this protein at slaughter has also been conducted and its relation with the presence of lesions and chronic abnormalities has been described. The relation of Hp with a significant number of porcine diseases have also been studied and its increase in concentration has been reported in cases of experimental or natural infection with *Actinobacillus pleuropneumoniae, Mycoplasma hyorhinis, Toxoplasma gondii, Bordetella bronchiseptica, Pasteurella multocida* and porcine reproductive and respiratory syndrome virus (PRRSV) (Petersen *et al.,* 2004).

Increases in concentration of SAA and CRP have been reported in pigs following surgery and experimental infection with *A. pleuropenumoniae*. Further, plasma levels of CRP from animals suffering this infection also correlated with clinical findings and were reduced following antibiotic treatment (Heegaard *et al.,* 1998; Lauritzen *et al.,* 2003; Petersen *et al.,* 2004). Also, the rise of SAA has been described in infections like PRRSV, porcine circovirus type 2 (PCV2) and *M. hyopneumoniae* occurred in field

conditions (Parra *et al.,* 2006). Moreover, increases in CRP following aseptic inflammation have been reported as well (Lampreave *et al.,* 1994).

In the pig, AGP has been demonstrated to be different depending on the animal age. In the newborn pig, AGP is present at 40 times the adult level with the concentration falling more rapidly in specific pathogen-free animals than in animals exposed to the normal pathogens encountered in production (Itoh *et al.,* 1993a). Thus, in this species it is important to interpret AGP levels with regard to age as the elevated levels found at birth take about 20 weeks to fall to adult levels. In adults, AGP concentrations rose in pigs with naturally occurring pneumonia and meningitis (Itoh *et al.,* 1993b). However acute phase reaction after aseptic inflammation did not affect significantly AGP concentration (Lampreave *et al.,* 1994; Eckersall *et al.,* 1996). Although, AGP was elevated and negatively correlated to body weight in a study of the effects of stress and immune function (Grellner *et al.,* 2002).

Pig-MAP increases have been shown during infection with *A. pleuropneumoniae* (Heegaard *et al.,* 1998), in post-weaning multisystemic wasting disorder (Segales *et al.,* 2004) and following transport (Saco *et al.,* 2003).

Table 2: Acute phase proteins reported in different disorders in porcine. Adapted from Gómez-Laguna *et* al., 2012.

DISORDER		ACUTE PHASE PROTEIN	REFERENCE
INFLAMMATION	Surgery	Hp, CRP	Hernandez-Richter *et al.*, 2001
BACTERIA	*Actinobacillus pleuropneumoniae*	SAA, CRP	Skovgaard *et al.*, 2009
	Mycoplasma hyopneumoniae	Hp, CRP, Pig-MAP	Parra *et al.*, 2006
	Streprococcus suis	SAA, CRP	Sorensen *et al.*, 2009
	Bordetella bronchiseptica *Pasteurella multocida*	Hp	Magnusson *et al.*, 1999
	Mycoplasma hyorhinis	Hp	Jacobson *et al.,* 2004
	Brachyspira hyodysenteriae	SAA, Hp	Jacobson *et al.*, 2004
VIRUSES	Porcine reproductive and respiratory síndrome (PRRS)	Hp, Pig-MAP	Gomez-Laguna *et al.*, 2010
	Porcine circovirus type 2 (PCV2)	Hp, Pig-MAP	Grau-Roma *et al.*, 2009
	Influenza	Hp, CRP	Barbé *et al.*, 2011
	Aujeszky	Hp	Parra *et al.*, 2006
PARASITES	*Toxoplasma gondii*	Hp	Jungersen *et al.*, 1999
STRESS	Transport	Hp, Pig-MAP	Piñeiro *et al.*, 2007
	Housing	Hp, CRP	Salamano *et al.*, 2008
	Slaughter	SAA, Hp, CRP, Pig-MAP	Piñeiro *et al.*, 2007

4.2.4.2. APPs in ruminants: bovine, ovine and caprine.

Hp and SAA are considered major APPs whereas AGP is considered a minor APP either in bovine, ovine or caprine (Eckersall, 2008a). Cp is a minor APP both in caprine and ovine (Gómez-Laguna *et al.,* 2011) and ITIH4, a recently discovered APP in cattle, is considered a moderate APP in this species (Piñeiro *et al.,* 2004). It is also important to say that CRP is not considered a useful tool in assessing the systemic inflammatory response in any of the three species mentioned (Gómez-Laguna *et al.,* 2011).

Table 1 Adapted from Gómez-Laguna *et al.* (2011) summarizes APPs reported in different disorders in bovine, ovine and caprine.

In cattle, Hp is considered a good biomarker in cases of mastitis, enteritis, pneumonia, endocarditis and endometritis (Murata *et al.,* 2004; Petersen *et al.,* 2004; Eckersall, 2008a). Its utility in cases of fatty liver syndrome, parturition, starvation or stress of

road transport has also been described in cows (Uchida *et al.,* 1993; Nakagawa *et al.,* 1997; Katoh and Nakagawa, 1999; Ametaj *et al.,* 2005; Bionaz *et al.,* 2007). SAA has been identified as a good tool in cattle infected with *Mannheima haemolytica* and with bovine respiratory syncytial virus, as well as in animals with both experimental and natural mastitis (Horadagoda *et al.,* 1994; Grönlund *et al.,* 2003; Eckersall, 2008a). SAA increases in acute inflammatory conditions have also been reported. Besides, in chronic conditions higher values of AGP were found when compared with those obtained for SAA and Hp (Horadagoda *et al.,* 1999). Hp, SAA and Cp also increased significantly in animals with bovine tropical theileriosis (Nazifi *et al.,* 2009). Finally ITIH4 serum concentration raised in cattle with moderate to severe experimentally induced mastitis (Piñeiro *et al.,* 2004).

In sheep Hp and SAA have been recognized as markers of bacterial infection, being more sensitive than complete blood count (Skinner and Roberts, 1994). Hp can also be used in lambs to monitor inflammatory states caused by surgery, castration and tail docking (Price and Nolan, 2001; Abu-Serriah *et al.,* 2007). In reproductive disturbances like ovine dystocia and in intrauterine bacterial contaminations during the postpartum Hp has also been studied as a tool of early treatment and diagnosis, respectively (Scott *et al.,* 1992; Aziz and Taha 1997). In this species as well, the increase of Hp and SAA has been described in cases of acute caseous lymphadenitis, whereas in chronic states yielded increases in AGP.

In goats Hp and SAA have been used to differentiate between animals with and without helmint infection (Ulutas *et al.,* 2008); and a significant increase of Hp, SAA and Acid Soluble Glycoprotein (ASG) has also been described in goats undergoing experimentally induced inflammation (turpentine injection) (González *et al.,* 2008).

In Alpine ibex, sarcoptic mange demonstrated to increase SAA and AGP values rather than Hp and Cp (Rahman *et al.,* 2010).

Table 1: Acute phase proteins reported in different disorders in bovine, ovine and caprine. Adapted from Gómez-Laguna *et* al., 2011.

SPECIES	DISORDER		ACUTE PHASE PROTEIN	REFERENCE
	INFLAMMATION			
BOVINE		Lameness	Hp, SAA	Kujala *et al.*, 2010; Smith *et al.* 2010
		Postpartum	Hp, SAA	Gabler *et al.*, 2010; Humbledt *et al.,* 2006
		Chronic respiratory disease	Hp, SAA	Huzzey *et al.*, 2009;
		Metritis	Hp, SAA	Chan *et al.,* 2010; Carroll *et al.*, 2009; Tabrizi *et al.*, 2008
		Clinical mastitis	Hp, SAA, Cp	Tabrizi *et al.*, 2008; Åkerstedt *et al.*, 2008
OVINE		Peptidoglican-polysaccharide	Hp, SAA	Dow *et al.*, 2010
		Uterine involution	Hp	Regassa and Noakes, 1999
		Pneumonia	Hp, Cp	Pfeffer and Rogers, 1989
		Chronic pneumonia	SAA	Kingston *et al.*, 1982
		Subclinical mastitis	SAA	Winter *et al.,* 2006
		Intrathoracic yeast injection	Hp, Cp	Pfeffer *et al.,* 1993
		Surgery	Hp, Cp	Pfeffer and Rogers, 1989
		Castration	Hp	Paull *et al.*, 2009
		Pregnancy toxemia	Hp	Gonzalez *et al.*, 2011
CAPRINE		Turpentine oil	Hp, SAA, ASG	Gonzalez *et al.*, 2008
	BACTERIA			
BOVINE		*Escherichia coli*	SAA, Hp	Sujoala *et al.*, 2008
		Staphylococcus aureus	SAA, Hp	Eckersall *et al.*, 2006
		Pasteurella multocida	SAA, Hp, AGP	Dowling *et al.*, 2004
BOVINE OVINE		*Mannheimia haemolytica*	SAA, Hp SAA, Hp, Cp	Ulutas and Ozpinar, 2006 Ganheim *et al.*, 2003
OVINE		*Corynebacterium pseudotuberculosis*	SAA, Hp, AGP	Pépin *et al.*, 1991; Eckersall *et al.,* 2007
		Staphylococcus epidermidis	SAA	Winter *et al.*, 2003
	VIRUSES			
BOVINE		Bovine diarrea virus	SAA, Hp	Ganheim *et al.*, 2003
		Bovine respiratory syncytial virus	SAA, Hp	Heegard *et al.,* 2000
		Foot and mouth disease virus	Hp	Höfner *et al.,* 1994
OVINE CAPRINE		Lentivirus	SAA	Sack and Zink, 1992

PARASITES			
BOVINE	*Trypanosmoa congolense*	SAA	Maede *et al.*, 2009
	Theileria annulata	SAA, Hp, Cp	Nazifi *et al.,* 2009
OVINE	Myasis	SAA, Hp	Colditz *et al.*, 2009, O'meara, 1995
CAPRINE	*Sarcoptes scabiei*	SAA, Hp, AGP, Cp	Rahmann *et al.*, 2010
	Trichuris spp. *Trichostrongylidae* spp. *Fasciola* spp.	SAA, Hp	Ulutas *et al.*, 2008
STRESS			
BOVINE	Weaning	Hp	Lynch *et al.*, 2010
	Housing	Hp	Lynch *et al.*, 2010
	Transport	SAA, Hp	Lomborg *et al.*, 2008
BOVINE	Feeding	SAA, Hp Cp	Khafipour *et al.*, 2009 Arthingthon *et al.,* 2008
OVINE		SAA	Eckersall *et al.*, 2008b
OTHER			
BOVINE	Fatty liver	Hp Hp, SAA	Guzelbektes *et al.*, 2010
	Abomasal displacement	Hp, SAA	Guzelbektes, 2010
OVINE	Vaccionation	Hp, SAA	Eckersall *et al.*, 2008b

4.3. Methods of APPs determination and basic analytical validation

4.3.1. APPs method determination

In human medicine, tests for the determination of most acute phase proteins are standardized and are routinely used for the evaluation of patients. In veterinary medicine, despite the possibilities of these proteins as markers for the diagnosis of inflammation and clinical prognosis, and the development of commercial kits in recent years, there is still a lack of application of these analyses (Martínez-Subiela, 2003a).

There is a variety of methods for the determination of APPs that can be divided basically in two groups:

Spectrophotometric methods: Spectrophotometry is the quantitative measurement of the reflection or transmission properties of a material as a function of wavelength. Spectrophotometry involves the use of a spectrophotometer which is a photometer that can measure intensity as a function of the light source wavelength. The most common spectrophotometers use the UV and visible regions of the spectrum (400–700 nm visible regions are used extensively in colorimetry), and some of these instruments also operate into the near-infrared region as well. A spectrophotometer is able to determine, depending on the control or calibration, what substances are present in a target and exactly how much through calculations of observed wavelengths.

The main methods for Hp, AGP and Cp determination are based on spectrophotometry.

Immunologic methods: These methods are based on antigen-antibody reactions. The most well known and studied test based on immunologic techniques is the Enzyme-Linked ImmunoSorbent Assay (ELISA). In this technique an immobilized antigen is detected using an antibody bound to an enzyme. This enzyme is capable of generating a detectable product such as a change in color. Sometimes, in order to reduce test costs, there is a primary antibody that recognizes the antigen, which in turn is recognized by a secondary antibody that has bound the aforementioned enzyme. The dye development permits to measure indirectly the amount of antigen in the sample by means of spectrophotometry.

The immunoturbidimetry, the radioimmunoassay, the immunonephelometry or the immunoluminometry are other examples of immunologic techniques.

In immunologic techniques it is recommended the use of antibodies species specific. When this is not possible and the test contains antibodies from species different to those under study, it is necessary to validate the immunologic method in order to verify its reliability. In the same way, the test standards should also proceed from the species that are being studied (Eckersall *et al.,* 1999b).

In veterinary medicine, proteins such as SAA, CRP and Pig-MAP are measured using immunologic methods.

4.3.2. Method validation

Method validation is the process used to confirm that the analytical procedure employed for a specific test is suitable for its intended use. Results from method validation can be used to judge the quality, reliability and consistency of analytical results; it is an integral part of any good analytical practice (ICHHT Guideline, 2005).

Analytical methods need to be validated or revalidated:

- Before their introduction into routine use
- Whenever the conditions change for which the method has been validated (e.g., an instrument with different characteristics or samples with a different matrix)
- Whenever the method is changed and the change is outside the original scope of the method.

The parameters for method validation have been defined in different working groups of national and international committees and are described in the literature. Typical validation characteristics which should be considered are listed and briefly defined below (Westgard *et al.,* 1999; ICHHT Guideline, 2005).

The **specificity** is the ability to assess unequivocally the analyte in the presence of components which may be expected to be present.

Potential interfering substances in a biological matrix include endogenous matrix components, metabolites, decomposition products, and in the actual study, concomitant medication and other exogenous xenobiotics. If the method is intended to quantify more than one analyte, each analyte should be tested to ensure that there is no interference.

Interference and recovery experiments allow testing specific sources that may be cause of systematic errors. Typically, experiments are performed using interferents common in the samples analyzed. In wild animal species, hemolysis is a good example of interferent as it is frequently observed in these samples. Thus, in this species hemolysis interference experiments should be conducted.

The **accuracy** of an analytical method describes the closeness of mean test results obtained by the method to the true value (concentration) of the analyte. Accuracy is determined by replicate analysis of samples containing known amounts of the analyte. The deviation of the mean from the true value serves as the measure of accuracy.

The **linearity** of an analytical procedure is its ability (within a given range) to obtain test results which are directly proportional to the concentration (amount) of analyte in the sample. Accuracy can be measured indirectly by means of linearity.

Linearity can be determined by analyzing either a series of samples of known concentration or a series of known dilutions of a highly elevated specimen or patient pool. The expected and observed values are plotted in the x- and y-axes respectively in order to assess the linearity.

The **precision** of an analytical procedure expresses the closeness of agreement (degree of scatter) between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions.

Precision should be investigated using homogeneous, authentic samples. However, if it is not possible to obtain a homogeneous sample it may be investigated using artificially prepared samples or a sample solution.

It is usually expressed as the variance, standard deviation or coefficient of variation of a series of measurements.

Precision may be considered at three levels: repeatability, intermediate precision and reproducibility.

Repeatability

Repeatability expresses the precision under the same operating conditions over a short interval of time. Repeatability is also termed intra-assay precision.

Intermediate precision

Intermediate precision expresses within-laboratories variations: different days (inter-assay precision), different analysts, different equipment, etc.

Reproducibility

Reproducibility expresses the precision between laboratories (collaborative studies, usually applied to standardization of methodology).

The **detection limit** of an individual analytical procedure is the lowest amount of analyte in a sample which can be detected but not necessarily quantitated as an exact value.

The limit of detection is estimated as a 95% one-side confidence limit by mean value of blank plus 1.65 times the SD of the blank.

The **quantitation limit** of an individual analytical procedure is the lowest amount of analyte in a sample which can be quantitatively determined with suitable precision and accuracy. The quantitation limit is a parameter of quantitative assays for low levels of compounds in sample matrix, and is used particularly for the determination of impurities and/or degradation products.

The **range** of an analytical procedure is the interval between the upper and lower concentration (amounts) of analyte in the sample (including these concentrations) for which it has been demonstrated that the analytical procedure has a suitable level of precision, accuracy and linearity.

The **robustness** of an analytical procedure is a measure of its capacity to remain unaffected by small, but deliberate variations in method parameters and provides an indication of its reliability during normal usage.

5. OBJECTIVES

5. OBJECTIVES

APPs have been investigated in recent years as biomarkers of disease and its application as tool for diagnosis, prognosis and monitoring of treatments has been reported in domestic animals for many disorders. Nevertheless, literature about APPs use in wild ungulates is scarce. There is a need of APPs validation studies in these species, mainly when immunological methods containing antibodies non species-specific are used.

The main goals of the present thesis are to validate analytical methods available in domestic ungulates for its use in wild ungulates, to provide reference values and to verify the utility of acute phase proteins for the species studied. The specific objectives are subjected to available methods and main APPs related to the species, as follows:

Wild boar

- To perform analytical validation of methods available and provide reference values for haptoglobin, serum amiloyd A, C-reactive protein, acid soluble glycoprotein, ceruloplasmin and porcine major acute phase protein.
- Validate a human immunoturbidimetric method for C-reactive protein measurement and to compare it with a porcine specific enzyme linked immunoassay.

Pyrenean chamois

- To perform analytical validation of methods available and provide reference values for haptoglobin, serum amiloyd A, acid soluble glycoprotein and ceruloplasmin.

Spanish ibex

- To perform analytical validation of methods available and provide reference values for haptoglobin and serum amiloyd A.
- To characterize haptoglobin and serum amyloid A responses to experimentally induced inflammation.

6. STUDIES

6.1. STUDY I

Acute Phase Proteins in wild boar (*Sus scrofa*): analytical validation, reference values and major acute phase proteins

ABSTRACT

Acute phase proteins (APPs) are serum proteins which concentration changes after inflammation, infection, trauma or stress. Their main function is to restore homeostasis and limit microbial growth. The objectives of this study are to validate the measurement techniques for acid soluble glycoprotein (ASG), haptoglobin (Hp), serum amyloid A (SAA), ceruloplasmin (Cp), C-reactive protein (CRP) and pig major acute protein (Pig-MAP) for their use in wild boar, to establish the reference values for the APPs studied, and to determine the major APPs in this species. ASG and Cp were determined using assay methods described previously. Serum Hp, SAA, CRP and Pig-MAP concentrations were determined using commercial kits available. Low APP concentration specimens were obtained from healthy wild boars. Intra and inter assay coefficients of variation (CVs) were lower than 15% in all cases, except inter assay CVs for ASG and SAA with the low concentration pool. All the methods used demonstrated good linearity ($R>0.97$). The minimum values for the ASG, Hp, CRP and Pig-MAP obtained in healthy animals were below the detection limits. All the proteins were very affected by *haemolysis*, being the SAA the most affected one. SAA and Cp were the proteins that presented the highest differences between healthy and diseased animals. Ranges obtained for healthy animals were 0.02-1.28 (g/L) for ASG, 0.08-1.96 (g/L) for Hp, 17.04-60.87 (mg/L) for SAA, 0.03-0.07 (ΔAbs/min) for Cp, 4.56-137.38 (mg/L) for CRP and 0.21-1.05 (g/L) for Pig-MAP. The results indicate that the APPs studied in this work could be reliably used with wild boar samples and SAA and Cp could be classified as major APPs in this species.

INTRODUCTION

Acute phase proteins (APPs) are proteins that change their serum concentration in response to inflammation, infection and trauma; therefore many conditions can cause their elevation (positive APPs) or decrease (negative APPs) (Murata *et al.,* 2004; Tecles *et al.,* 2007a). The magnitude of change in the concentration varies between proteins, which are classified in major APPs -increases over 10 fold, reaching concentrations up to 100 or 1000 folds-, moderate APPs –increases over 5 to 10 fold- and minor APP –increases over 1,5 or 2 fold (Eckersall, 2008). There are also important patophysiological differences between species in the during the acute phase reaction (Petersen *et al.,* 2004; Eckersall, 2008).

The APPs are highly sensitive for the presence of pathological lesions while having a low specificity for a particular disease (Eckersall, 2008). Its use for prognosis and monitoring responses to therapy, for general health screening as well as for diagnosis of disease in veterinary medicine is widely described in the literature (Horadagoda *et al.,* 1999; Eckersall, 2000; Murata *et al.,* 2004; Parra *et al.,* 2006). There are many studies about APPs in domestic livestock, but there is a lack of information in this field in wildlife; thus measurement techniques have not been validated in these species (Parra *et al.*, 2006).

Research in the field of APPs has led to the development of different analytical techniques, some of them available as commercial kits. In the pig there are commercially kits for haptoglobin (Hp), serum amyloid A (SAA), C-reactive protein (CRP), and porcine major acute phase protein (Pig-MAP) (Álava *et al.,* 1997, Tecles *et al.,* 2007a). Furthermore, the techniques for acid soluble glycoprotein (ASG) and ceruloplasmin (Cp) measurement in pigs have also been studied (Eckersall *et al.,* 1996). The reliability of all these methods in pigs is well described in the literature (Tecles *et al.,* 2007a); however the validity of the results obtained through these techniques has not been established in the wild boar. The reliability of all these methods require validation in each laboratory, especially when used for species other than those for which the methods were originally designed (Lumsden, 2000). The establishment of reference values is used to describe the dispersion of variables in healthy individuals

(Geffre *et al.,* 2009). The knowledge of these values may be useful in the detection of disease states, either in individual or animal populations (Pérez *et al.,* 2003).

The expansion of wild ungulates in recent years has led to an increased density of population in certain areas; this fact is especially remarkable in the wild boar (*Sus scrofa*) that has dramatically increased in European countries in number and distribution (Artois *et al.,* 2002; Wu *et al.,* 2011). The wild boar is a wild artiodactyla species that shows high habitat plasticity. The study of tools that help to assess the health status of this species is important because wild pigs are capable of carrying numerous parasites and diseases that potentially threaten the health of humans, livestock, and wildlife (Forrester, 1992; Williams and Barker, 2001; Seward *et al.,* 2004).

The main goal of the present study was to validate the available APP measurement methods in pig for its use in wild boar. Moreover we wanted to establish the reference values for the APPs studied, and to determine which are the major APPs in this species.

MATERIALS AND METHODS

Animals and samples

Sera from eighteen wild boars, twelve from clinically healthy animals and six from animals with trauma, probably due to road accident, that arrived to the Servei de Ecopatologia de Fauna Salvatge of the Universitat Autònoma de Barcelona were used. Two different pools from the sera of the healthy animals were obtained. One (n=6) used to determine the precision of the test samples at low concentrations and the other one (n=6) used to assess interference from haemolysis. The six samples from injured animals were mixed and used as pool of high concentration to determine the precision and linearity of the techniques.

The wild boars were captured with cage-traps where they stayed a maximum of eight hours at Sant Llorenç del Munt i l’Obac Natural Park (41º 39’- 41º 42’N 1º 53’- 2º 09’E). Once captured the animals were anesthetized with dart injection using a blow pipe.

Blood was obtained from the cranial vena cava by venipuncture and placed into plain tubes containing clotting accelerator. Blood samples were centrifuged at 2000g during 10 min to obtain sera within the first six hours after collection. Specimens were separated in aliquots and maintained at -20ºC until analysed.

Additionally, to establish the reference values for the APPs studied, sera from forty clinically healthy wild boars captured in the same way above described, 27 from Sant Llorenç del Munt i l'Obac Natural Park and the other 13 from Ports de Tortosa i Beseit National Hunting Reserve (40º 43' – 40º 51`N; 0º 15' – 0º 25'E), were obtained.

Acute phase proteins

The ASG component of the serum samples was determined using the method based on that of Winzler (1955), modified by Nagahata *et al.* (1989) and Eckersall *et al.* (1996), and optimized by Tecles (2007b).

Serum concentrations of Hp were quantified using a commercial automated spectrophotometric assay (Tridelta Development Ltd, Maynooth, Ireland). The assay was performed according to manufacturer's instructions on an automated analyser (Cobas Mira Plus, ABX Diagnostics, Montpellier, France).

Serum SAA and CRP concentrations were determined by using commercial solid phase sandwich Enzyme Linked Immunoabsorbent Assays (Tridelta Development Limited, Maynooth, Ireland). Pig-MAP concentrations were determined using a non-competitive enzyme immunoassay (PigCHAMP Pro Europa SA, Segovia, Spain). Analyses were performed according to the manufacturer's instructions, and the final absorbance was measured in a microtiter plate reader (PowerWave XS, Bio-Tek Instruments Inc., Vermont, USA) at 450 nm wavelength.

For the determination of Cp values the method based on that described by Suderman and Nomoto (1970), with the modifications of Martínez-Subiela and Cerón (2005) was used.

Analytical validation

The following parameters were studied:

Precision: Intra assays coefficients of variation (CVs) were calculated after analyses of the pool with low APPs concentration and the pool with high APPs concentration, measured five times in a single assay run. Inter assay CVs were determined by analysing the same pools in five separate runs carried out on different days.

Linearity: It was evaluated by analysing serial dilutions (75, 50, 25 and 12.5%) of the pool of APPs with high concentration.

Limit of detection: It was calculated based on data from 10 replicates determinations of the zero standard and expressed as mean value plus two standard deviations.

Effects of hemolysis: Procedure was performed as previously described by Martínez-Subiela and Cerón (2005). Briefly, fresh haemolysate was prepared by addition of distilled water to packed, saline washed healthy wild boar red cells. Concentration of haemoglobin in haemolysate was determined by using automated laser analyzer (ADVIA 120, Siemens Healthcare Diagnostics, Deerfield, IL, USA) and adjusted to 200 g/L by adding distilled water to perform a stock solution. The stock solution was serially diluted with sample diluent buffer of each test and 10 µL of each dilution was added to 90 µL of the sera pool. The final haemoglobin concentrations were 10, 5, 2.5, 1.25, 0.625 and 0.0 g/L (10 µL of sample diluent buffer were added to pooled sera to give 0.0 g/L concentration). These haemoglobin concentrations could correspond to slight haemolysis (0.625 g/L), moderate haemolysis (1.25 and 2.5 g/L) and marked haemolysis (5 and 10 g/L).

Reference values

Sera from forty clinically healthy boars were analyzed by duplicate.

Statistical analyses

All data analysis was performed by using Excel 2007 (Microsoft Office, Microsoft Corp., Washington, USA) and SPSS 15.0 for Windows (SPSS Inc., Chicago, IL, USA). Significance level used in each case was $p<0.05$.

Means, standard deviations, intra and inter-assay CVs and detection limits were calculated for low and high concentration pools using routine descriptive statistical procedures.

Pearson's correlation test was used to find the correlation between the serial dilutions and the results for the APPs concentration obtained.

Data from interference study are presented in interferographs made according to previously reported protocols (Martínez-Subiela and Cerón 2005). The graphs show on the x-axes, increasing concentrations of haemoglobin, and on the y-axes, the mean percentage of change of each protein expressed as final value/original value x 100, where final value is the one obtained from sera enriched with haemoglobin and the original value is that obtained from sera without interferent.

The U-Mann Whitney test was used to compare the pools obtained from healthy and diseased animals.

For reference values, routine descriptive parameters are provided instead of a reference range due to the small sample size used (Geffré, 2009).

RESULTS

Analytical validation

Mean, standard deviation and coefficient of variation obtained for intra and inter assay analyses of the low and high concentration pools are presented in table 1. Intra assay coefficients of variation (CVs) for low concentration pool ranged from 0.343% to 12%, being lower than 5% for all the APPs except SAA (12.149%). High concentration pool yielded higher CVs, which were between 0.840 and 15.746. Again, SAA presented the highest CVs (19.981%).

Inter assay CVs for low concentration pool ranged from 8 to 16%. Hp presented the lowest (7.800%) whereas SAA had the highest (15.746%). CVs for the high

concentration pool were lower than 9% for all the APPs studied, being the Pig-MAP the protein that showed the highest value (8.221%).

Table 1: Intra and inter assay coefficients of variation (CVs) for ASG, Hp, SAA, Cp, CRP and Pig-MAP and SAA assays in wild boar. First line of each protein presents the values obtained for the low concentration pool whereas the second line shows the values for the high concentration pool.

	Intra assay			Inter assay		
	Mean	**(SD)**	**CV(%)**	**Mean**	**(SD)**	**CV(%)**
ASG (g/L)	0.876	(0.080)	8.791	0.821	(0.127)	15.492
	2.544	(0.096)	3.756	2.608	(0.146)	5.592
Hp (g/L)	0.600	(0.016)	2.635	0.558	(0.044)	7.800
	2.994	(0.080)	2.668	2.821	(0.179)	6.341
SAA (mg/L)	27.081	(3.290)	12.149	29.544	(4.652)	15.746
	386.488	(77.225)	19.981	396.739	(54.908)	0.840
Cp (ΔAbs/min)	0.033	(0.001)	0.343	0.038	(0.003)	9.967
	0.560	(0.004)	0.723	0.559	(0.039)	6.895
CRP (mg/L)	39.985	(4.243)	10.613	42.110	(5.558)	13.199
	264.765	(9.373)	3.540	270.758	(21.006)	7.758
Pig-MAP (g/L)	0.616	(0.078)	12.702	0.676	(0.081)	12.020
	4.845	(0.143)	2.947	5.108	(0.420)	8.221

SD: standard deviation. CV: coefficient of variation.

Serial dilution analyses of high pool concentration resulted in linear regression equations with correlation coefficients that were above 0.98 in all the APPs studied, except for the ASG that was 0.97. The limit of detection was 0.111 g/L for ASG; 0.084 g/L for Hp; 6.399 mg/L for SAA; 0.002 ΔAbs/min for Cp; 9.456 mg/L for CRP and 0.213 g/L for Pig-MAP.

The results obtained for the effect of haemolysis are presented in Figure 1. Initially, the value of the ASG decreased to 28%, corresponding to 2.5 g/L haemoglobin

concentration, and then recovered without getting the original value. Hp decreased up to 12% when the haemoglobin concentration was 0.625, then the value increased as the haemoglobin concentration did, so at the haemoglobin concentration of 2.5 g/L original and final values were almost the same, and with a haemoglobin concentration of 10g/L the final value was 32% higher than the original value. SAA values decreased gradually as the concentration of haemoglobin increased, with a drop of 50% in the value with 10 g/L haemoglobin concentration. For the Cp differences with the original value were about 10%. CRP decreased to 23% at haemoglobin concentration of 0.625 g/L and then recovered, remaining at values around 15% over the original value. The final value of the Pig-MAP decreased to 40% at haemoglobin concentrations of 0.625 and 2.5, and other haemoglobin concentrations yielded values that were about 20% lower than original value.

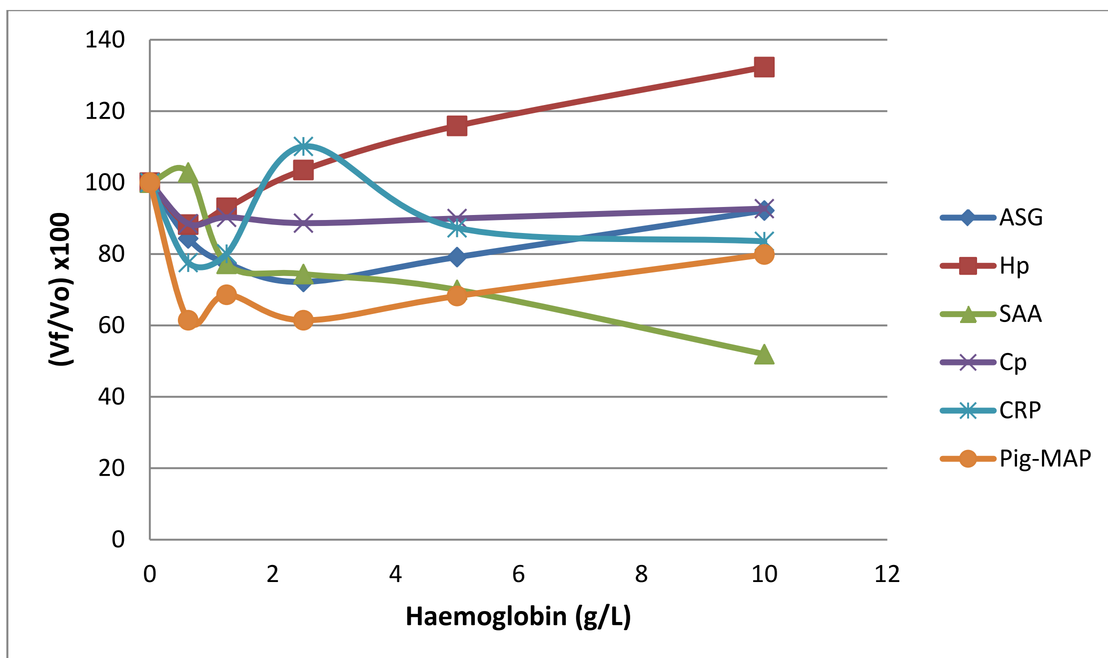

Figure 1: Haemoglobin interferographs for APPs in wild boar. Vf: final value; Vo: original value.

Significant differences between low and high pools for all the APPs studied ($p<0.005$) were obtained when compared statistically.

Reference values

Table 2 shows the mean, the standard deviation (SD), the median, the minimum and the maximum obtained when samples from forty healthy wild boars were analyzed.

Table 2: Mean, standard deviation (SD), median, minimum (Min.) and maximum (Max.) values obtained for acute phase protein studied in healthy wild boars

	ASG (g/L)	Hp (g/L)	SAA (mg/L)	Cp (ΔAbs/min)	CRP (mg/L)	Pig-MAP (g/L)
n	40	40	40	40	40	40
Mean	0.697	0.988	35.892	0.052	72.152	0.687
SD	0.412	0.590	13.695	0.011	40.981	0.235
Median	0.780	0.965	31.892	0.053	79.705	0.753
Min.	0.020*	0.080*	17.044	0.027	4.562*	0.205*
Max.	1.280	1.960	60.874	0.073	137.375	1.046

* below the limit of detection

DISCUSSION

There are many studies on the characterization of acute phase proteins in domestic pigs. Thus the validity of the tests has been demonstrated previously by several authors (Eckersall *et al.,* 1999; Petersen *et al.,* 2002; Tecles *et al.,* 2007b; Piñeiro *et al.,* 2009,) and the usefulness of these proteins has been studied in experimentally induced infections (Hall *et al.,* 1992; Heegaard *et al.,* 1998), experimentally induced non-infectious inflammation (Tecles *et al.,* 2007c) and in naturally infected pigs (Parra *et al.,* 2006) among others. Despite the problems that overpopulation of wild boar is causing in the north-eastern Spain, and the importance of knowing the health status of these populations because of the contact between wild boars and population and livestock, to the author's knowledge there are no studies that demonstrate the validity of techniques for determination of acute phase proteins, or research papers that describe their potential usefulness in assessing the health of populations in this species. This work studies thoroughly the validity of six acute phase proteins in the wild boar and provides the values for these proteins in terms of health.

For most of the studied proteins, the precision of the techniques used was acceptable, since the CVs placed below 10%. In the case of intra-assay study only the SAA, CRP and Pig-MAP showed higher CVs. These three proteins use enzyme immunoassays for its determination, and in these cases CVs up to 20% are accepted (US FDA, 2001). Nevertheless the use of species-specific calibrator as well as the automation of the techniques would probably improve these immunological tests precision (Tecles *et al.,* 2007b, 2007c). As previously reported in other APPs analytical validation studies, the intra assay CVs were lower when compared with inter assay CVs (Tecles *et al.,* 2007a, 2007b; González *et al.,* 2008; Soler *et al.,* 2011). That was valid for all the proteins studied except for SAA which presented a higher intra assay CV when compared with the inter assay CV. However, the inter assay CVs obtained never exceeded 16 %, being ASG and SAA the proteins that yielded the highest values, both for the low and high concentration pools.

To check the accuracy directly is necessary to use reference material or standards (Tecles *et al.,* 2007b; González *et al.,* 2008). Because of the lack of these reference materials in wild boar, in the present study the accuracy was tested indirectly by analyzing serial dilutions of the high concentration pool and checking the linearity of the results obtained. The correlation coefficients obtained were good for all the proteins (Westgard *et al.,* 1999) and agreed to those previously described by other authors (Martínez-Subiela *et al.,* 2007; Tecles *et al.,* 2007a).

In wildlife samples are obtained under field conditions and it is frequent to observe hemolytic samples. It is important to know the reliability of the techniques in these cases. The changes observed in the present work due to haemolysis for Hp and pig-MAP differed with those seen before by Tecles *et al.* (2007a), whereas agrees with the dramatically decrease obtained for SAA in the same work. Unlike Martínez-Subiela and Cerón (2005) in dogs, in our study Cp was the protein less affected by haemolysis. At low haemoglobin concentrations (0.63 g/lL and 1.25 g/L) important changes in CRP were observed in our work, which coincides with the results obtained in pigs (Tecles *et al.,* 2007a). These results support the fact that whenever possible haemolytic samples should be avoided.

All the proteins studied in this work demonstrated to have the potential to be used as inflammation biomarkers. Even taking in account the differences arising from the imprecision of some of the techniques employed, there were significant differences between low and high concentration pools.

Concerning to the reference values, in this study Hp, SAA and CRP yielded higher values than those reported in previous studies for pigs (Tecles *et al.,* 2007a; Diack *et al.,* 2011). Conversely wild boars showed lower values for ASG and Pig-MAP than those described previously in pigs (Tecles *et al.,* 2007a, 2007b; Diack *et al.,* 2011). The minimum values for the ASG, Hp, CRP and Pig-MAP were below the detection limits; therefore they should be taken carefully. Furthermore, the results of this study should be interpreted with caution because the capture stress could have altered some protein values as previous studies have reported increases in Hp, SAA and CRP in pigs when faced to different stressors (Petersen *et al.,* 2004; Piñeiro *et al.,* 2007).

In the pig there are discrepancies in the literature with the classification of APPs depending on the magnitude of change that occurs during the acute phase response. Thus, Petersen *et al.* (2004) classified Hp, SAA and Pig-MAP as major APPs and CRP and alpha 1-acid glycoprotein (AGP) as moderate APPs; Murata *et al.* (2004) described AGP, Hp, SAA and CRP as moderate and Cp as minor APP; and Eckersall (2008) described CRP and pig-MAP as major proteins and Hp and Cp protein as a moderate APPs. These differences could be due to the different magnitude of change between different disease states (Thomas, 2000). In most species ASG correlates well with AGP, as the glycoprotein fraction left after perchloric acid precipitation is predominant (Eckersall *et al.*, 1996).

In the present work, where the animals had suffered a trauma, SAA and Cp could be classified as major APPs as the high concentration pool was about 20 fold higher than the low pool, and ASG, Hp, CRP and Pig-MAP would be classified as moderate APPs, as the difference between pools was from three (ASG) to eight folds (Pig-MAP) higher. However the classification above mentioned should be taken carefully, as in this work there was not a monitoring of the trauma, and the measurement of the high concentration pool took place just in one point of the inflammatory process.

In conclusion, as the methods for ASG, Hp, SAA, Cp, CRP and Pig-MAP determination studied in this work showed good precision and accuracy they could be used when wild boar samples have to be analyzed. Moreover, the difference observed in the concentration between low and high pools demonstrate that APPs could be potentially used in the wild boar to detect states of disease. However more studies are needed to define the dynamics of APPs in different diseases and to establish reliable references values with largest sample size in this species.

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6.2. STUDY II

Comparison between a turbidimetric immunoassay and an enzyme linked immunosorbent assay for the heterologous determination of wild boar (*Sus scrofa*) CRP concentration

ABSTRACT

C-reactive protein (CRP) has been demonstrated to be an important acute phase protein (APP) in porcine. There is a porcine specific enzyme linked immunosorbent assay (ELISA) commercially available for its determination. The main goal of this study was to validate a human CRP turbidimetric immunoassay (TIA) for its use in wild boar (*Sus scrofa*), and to compare it with a CRP porcine specific ELISA. To conduct the comparison of methodologies CRP concentration from 32 selected sera of healthy and diseased wild boars were analyzed using both the human TIA and the porcine specific ELISA. For the TIA evaluation two different pools were obtained. The low concentration pool was from healthy animals and the high concentration pool was from animals with trauma, gunshot wounds and parasitic infestation. Inter- and intra-assay coefficients of variation (CVs), linearity under dilution and limit of detection (LD) were evaluated. Interference produced by haemolysis was also studied. Passing-Bablok regression revealed the presence of proportional error between TIA and ELISA. Porcine in-house calibrator was established to reduce the systematic error detected. The discrepancy between methodologies persisted, although the line of identity was improved. Intra and inter assay coefficients of variation (CVs) ranged from 4 to 13%. Serial dilution analyses resulted in linear regression equation with good coefficient of correlation (>0.99). The limit of detection was estimated to 0.87 mg/L. Haemolysis interfered highly with the protein concentration measurement. CRP concentration from wild boar sera samples could be reliably measured using the commercially available human CRP TIA using the porcine in-house calibration.

INTRODUCTION

C-reactive protein (CRP) has been reported to be a major acute phase protein (APP) in pigs by some authors (Eckersall *et al.,* 1996; Thomas, 2000; Diack *et al.,* 2011). In swine, this protein increases its concentration during aseptic inflammation, and in experimentally and naturally bacterial infections (Parra *et al.,* 2006; Piñeiro, M. *et al.,* 2007; Skovgaard *et al.,* 2009). Its elevation in response to physiologic stress has also been suggested by Burger *et al.* (1998). The increase of the concentration occurs within 24-48 hours after the insult and it seems that there is a correlation between severity of the inflammatory disease and the magnitude of change (Thomas, 2000). Plasma levels also fall down rapidly after recovery or treatment instauration (Deegan *et al.,* 2003). The main functions of this protein are to bind the pathogen and activate the classical complement pathway, and to induce pro-inflammatory cytokines and modulate neutrophil function (Eckersall, 2008).

In human medicine there are several techniques for measuring CRP such as immunonephelometry, immunoturbidimetry, immunoluminometry or enzyme-linked immunosorbent assay (ELISA) (Deegan *et al.,* 2003). In pigs, there is a species-specific ELISA for the measurement of CRP (Eckersall *et al.,* 1996) and some human immunoturbidmetric techniques have been evaluated for their use in this species with good results (Kjelgaard-Hansen *et al.,* 2003; Saco *et al.,* 2010). Thus although cross reactivity of anti-human CRP antibodies has been previously used for heterologous determination of porcine CRP in several studies, none of these methods has been standardized for porcine and there is a lack of species-specific standards (Saco *et al.,* 2010). In wildlife species-specific techniques do not exist and it is necessary to adapt those existing for domestic species with phylogenetic proximity. The species-specific ELISA for measuring CRP in pigs is a technique that involves many steps, long incubation times and manual work that increases the imprecision; therefore it would be preferable to validate rapid and automated techniques (Kjelgaard-Hansen *et al.,* 2003).

In recent decades, wild boars had become a social problem in its aspects both in animal welfare and public health, due to their high adaptability to different habitats (Artois *et al.,* 2002); it is therefore important to develop reliable methods that help to

study the health status of these populations. The utility of CRP as a health biomarker in wild ungulate populations has not been described, and the validity of the existing techniques for its measurement in the wild boar has not been evaluated.

The main goals of this work were, first, to compare a human turbidimetric immunoassay (TIA) both calibrated with human material provided in the kit and with a porcine-in-house calibrator established with the porcine enzyme linked immunosorbent assay (ELISA); and second to assess the reliability of the human TIA for its use in wild boar.

MATERIALS AND METHODS

Samples

Fifty wild boar sera were obtained from the sera bank of the Servei d'Ecopatologia de Fauna Salvatge (SEFaS), Universitat Autònoma de Barcelona. To conduct a comparison between TIA and ELISA methods, 32 samples including sera from healthy animals and sera from animals affected of trauma from road accidents, gunshot wound and parasitic infestations were selected.

The remaining eighteen samples were used to evaluate TIA reliability; of that twelve were from clinically healthy animals and six from animals with trauma, probably due to road accident. Two different pools from the sera of the healthy animals were obtained. One (n=6) was used to determine the precision of the test samples at low concentrations and the other one (n=6) to assess interference from haemolysis. A pool of high concentration was obtained from injured animals sera (n=6) and used to determine the precision and linearity of the technique.

Method comparison

A CRP turbidimetric immunoassay designed to measure human CRP was used (Randox Laboratories Ltd., London, United Kingdom). The principle of the assay is binding the CRP to polyclonal goat anti-human CRP antibodies to form an insoluble complex that precipitates and is measured turbidimetrically.

The 32 samples selected form the sera bank were analyzed, following the manufacturer's instructions twice; the first run was calibrated with the human calibration material provided in the kit (50 mg/L) and the second run using a porcine in-house calibrator established (121 mg/L) by means of a porcine specific CRP ELISA (Tridelta Development Ltd, Maynooth, Ireland) (Kjelgaard-Hansen *et al.,* 2007). The samples were analyzed by duplicate in each run.

Additionally, these samples were analyzed by duplicate using the porcine specific CRP solid phase sandwich ELISA and following manufacturer's instructions.

Turbidimetric immunoassay evaluation

CRP-TIA reagents were applied to Cobas Mira Plus (ABX Diagnostics, Montpellier, France) and the reliability of the method was assessed using the porcine in-house calibrator (121 mg/L). The assay was performed using the manufacturer's instructions.

Intra assays coefficients of variation (CVs) were calculated after analyses of the pool with low APPs concentration and the pool with high APPs concentration, measured five times in a single assay run. Inter assay CVs were determined by analysing the same pools in four separate runs carried out on different days. Accuracy was evaluated indirectly by analysing serial dilutions (75, 50, 25 and 12.5%) of the pool of APPs with high concentration. The limit of detection (LD) was calculated based on data from 10 replicates determinations of physiologic saline (0.9% NaCl).

The interference produced by haemolysis was performed as previously described by Martínez-Subiela and Cerón (2005). Fresh haemolysate was prepared by addition of distilled water to packed, saline washed healthy wild boar red cells. Concentration of haemoglobin in haemolysate was determined by using an automated laser analyser (ADVIA 120, Siemens Healthcare Diagnostics, Deerfield, IL, USA) and adjusted to 200 g/L by adding distilled water to perform a stock solution. The stock solution was serially diluted with sample diluent buffer of each test and 10 µL of each dilution was added to 90 µL of the sera pool. The final haemoglobin concentrations were 10, 5, 2.5, 1.25, 0.625 and 0.0 g/L (10 µL of sample diluent buffer were added to pooled sera to give 0.0 g/L concentration). These haemoglobin concentrations could correspond to

slight haemolysis (0.625 g/L), moderate haemolysis (1.25 and 2.5 g/L) and marked haemolysis (5 and 10 g/L) (Martínez-Subiela and Cerón, 2005). These samples were analyzed by duplicate.

Statistical analyses

Passing-Bablok regression analyses were used to compare both TIA calibrated with the human material and with the porcine in-house calibrator with the porcine-specific ELISA. Bland-Altman plot was used to detect the origin of any possible bias. Correlation coefficient of concordance was calculated to study the strength of the relationship between both methods (MedCalc Software Version 12.3.0, Mariakerke, Belgium).

Mean, standard deviation (SD) and inter- and intra-assay coefficients of variation (CVs) and LD were calculated with routine descriptive statistical procedures using SPSS 15.0 for windows (SPSS Inc., Chicago, IL, USA). Linearity under serial dilution was evaluated by Pearson's correlation test to find the correlation between the serial dilutions and the results for the APPs concentration obtained (Excel 2007, Microsoft Office, Microsoft Corp., Washington, USA). The percentage of variation expressed as final value/original value x 100, where final value is the one obtained from sera enriched with haemoglobin and the original value is that obtained from sera without interferent, was calculated to assess the haemolysis interference. Total error was also calculated in order to assess the acceptance limits of the technique (Jensen and Kjelgaard-Hansen, 2006).

Data from interference study are presented in interferographs made according to previously reported protocols (Martínez-Subiela and Cerón, 2005). The graphs show on the x-axes, increasing concentrations of haemoglobin, and on the y-axes, the mean percentage of change of each protein expressed as final value/original value x 100, where final value is the one obtained from sera enriched with haemoglobin and the original value is that obtained from sera without interferent.

RESULTS

Method comparison

Passing-Bablok regression detected the presence of significant disagreement with the results obtained between the TIA calibrated with human material and the ELISA ($P<0.03$) (Figure 1A), as the regression line deviated significantly from the line of identity (Intercept: 1.21 [1.03 to 2.04]; slope 0.015 [0.01 to 0.02]. Bland-Altman plot revealed the presence of differences between two methods (Figure 1B). A good correlation between methods was observed (coefficient of concordance of 0.92).

When the porcine in-house calibrator was used, the Passing-Bablok regression showed again a significant discrepancy between both methods ($P<0.03$) (Figure 2A). The Bland-Altman plot again brought to light the presence of discrepancies between both methods, mainly in samples with high CRP concentrations (Figure 2B). The correlation between both methods was however improved with the porcine in-house calibration, coefficient of concordance 0.96.

Turbidimetric immunoassay evaluation

Mean, standard deviation and coefficient of variation obtained for intra assay and inter analyses of the low and high concentration pools are presented in Table 1. The CVs ranged from 4 to 13%. The CV inter assay for the low concentration pool was the one that yielded the highest CV. The total error obtained from the within and the between run variations was 8.74%. Serial dilution analyses of high concentration pool resulted in linear regression equation with a very good coefficient of correlation (>0.99), thus the method measured the CRP in a linear model. The limit of detection was estimated to be 0.87 mg/L.

The study of haemolysis interference is presented in Figure 3. At 0.625 and 1.25 haemoglobin concentrations, CRP value was about 15% below the original value. With higher haemoglobin concentrations (between 2.5 g/L and 10g/L) the CRP values decreased between 28 and 16%, respectively compared to the original value.

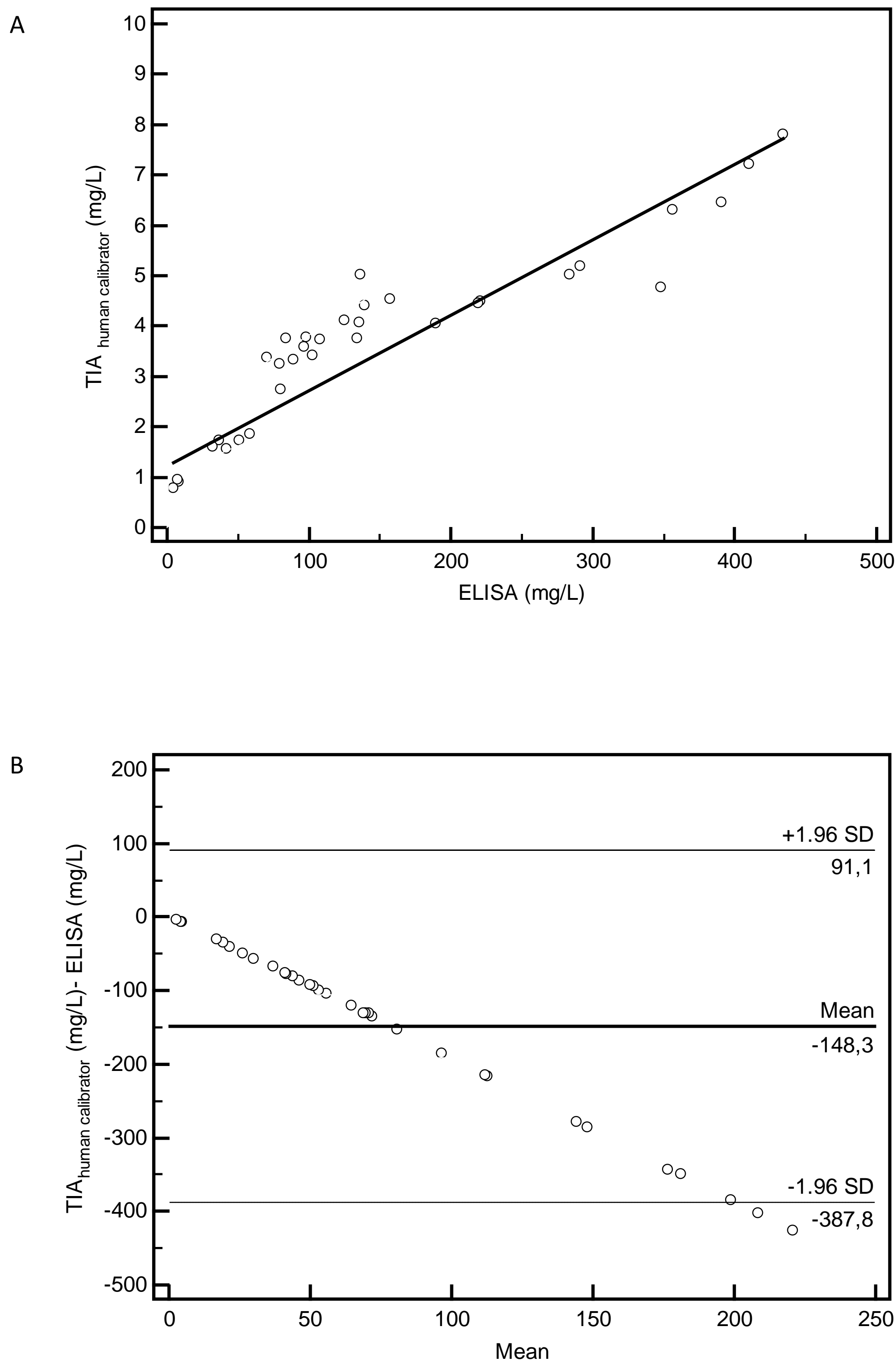

Figure 1: Comparison of methodologies for determination of wild boar C-Reactive Protein; Turbidimetric immunoassay calibrated with human material ($TIA_{human\ calibrator}$) and enzyme linked immunoassay porcine-specific (ELISA). (A) Passing Bablok regression analysis. The solid line represents the regression equation: TIA CRP (mg/L) = 0.014 X ELISA CRP (mg/L) + 1.21. (B) Bland Altman Plot revealing the presence of proportional error. Dotted lines represent mean difference and +/- 1.96 SD of the mean difference.

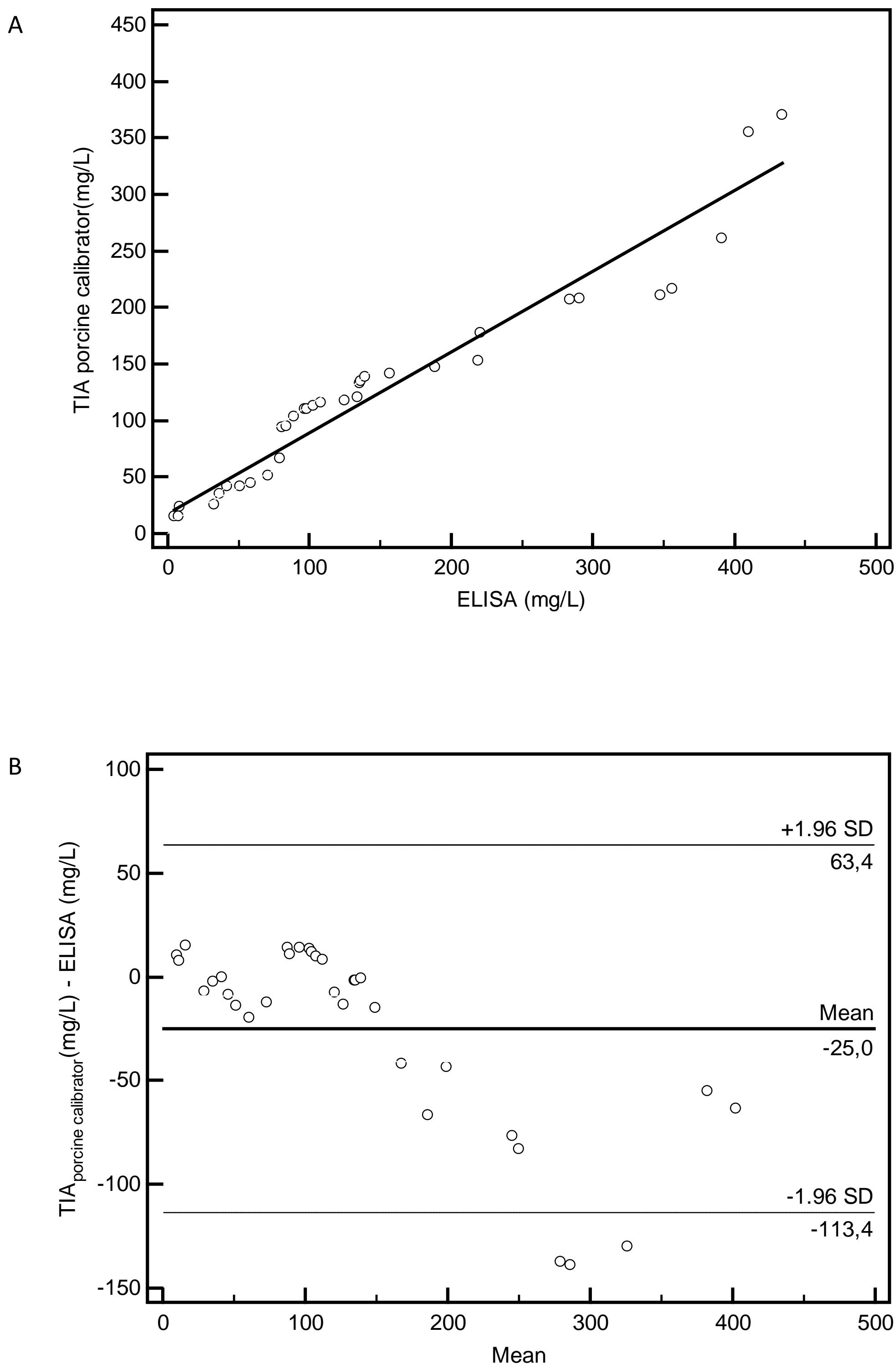

Figure 2: Comparison of methodologies for determination of wild boar C-Reactive Protein; Turbidimetric immunoassay calibrated with porcine material ($TIA_{porcine\ calibrator}$) and enzyme linked immunoassay porcine-specific (ELISA). (A) Passing Bablok regression analysis. The solid line represents the regression equation: TIA CRP (mg/L) = 0.71 X ELISA CRP (mg/L) + 17.58. (B) Bland Altman Plot revealing the presence of proportional error. Dotted lines represent mean difference and +/- 1.96 SD of the mean difference.

Table 1: Intra and inter assay coefficients of variation (CVs) CRP measured with the TIA and calibrated with locally porcine serum.

	Intra assay			Inter assay		
	Mean	**(SD)**	**CV(%)**	**Mean**	**(SD)**	**CV(%)**
Low concentration pool (mg/L)	25.93	(1.06)	4.08	25.86	(3.29)	12.73
High concentration pool (mg/L)	94.17	(7.33)	7.78	99.30	(5.06)	5.10

SD: standard deviation. CV: coefficient of variation.

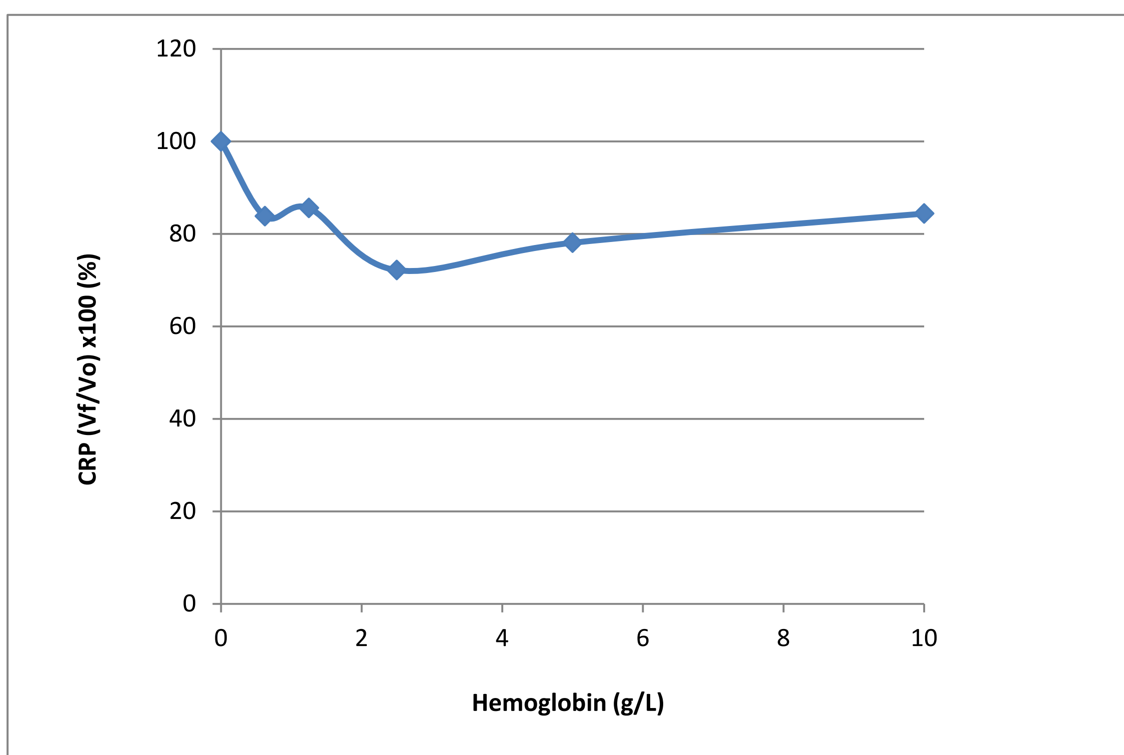

Figure 3: Haemoglobin interferographs for CRP in wild boar. Vf: final value; Vo: original value

DISCUSSION

There are several reports describing the utility of CRP in porcine. Its importance as a tool to assess health status and monitoring either the disease or the treatment has also been widely described (Parra *et al.,* 2006; Piñeiro, C. *et al.,* 2007; Salamano *et al.,* 2008). These facts altogether have lead to the development of a species-specific commercially available ELISA for the determination of CRP in serum. However, the ELISA technique for its analyzes is laborious and is difficult to find the equilibrium between cost and benefit if we want to consider CRP as a routine parameter of health screening panels in the labs (Eckersall, 2010). Human TIA, which is an automated technique, has been assessed previously for its use in pigs and dogs by several authors (Kjelgaard-Hansen *et al.,* 2007; Saco *et al.,* 2010).

When comparing human CRP TIA calibrated with human material provided in the kit with porcine CRP ELISA, the results obtained with the former were much lower than those obtained with the ELISA. Error between methods revealed by the Bland-Altman plot was proportional (Jensen and Kjelgaard-Hansen, 2006). The use of porcine in-house serum calibration improved the results, since the magnitude of the difference between the values run with the two methods were much lower. Although absolute CRP values obtained were different between methods, there was a good coefficient of correlation.

The results obtained in this work agree with those reported previously by other authors using similar methodologies in pigs (Kjelgaard-Hansen *et al.,* 2007; Saco *et al.,* 2010). The cross reactivity of the anti-human CRP antibody reagents with porcine CRP and with canine CRP was previously observed to be partial (Kjelgaard-Hansen *et al.,* 2003, 2007). Although in this work a cross reactivity assay between anti-human antibodies with wild boar sera was not conducted, the results obtained suggest that there could be a partial cross reactivity similar to that observed for porcine or canine sera samples. This could partially explain the origin of the persistent discrepancies between methods observed in our work and emphasizes the need to make species specific calibration material.

The values obtained with the calibration of the TIA using this time a porcine in-house material were closer to those obtained with porcine specific ELISA than those above mentioned. Therefore, the reliability of the TIA method for its applications in the wild boar was performed using the porcine in-house calibrator rather than the human material. The CRP TIA measured heterologous wild boar serum CRP concentration with CVs below 10% for the high concentration pool and below 13% for the low concentration pool. Thus, this method measured CRP with good precision, as the percentages obtained are considered acceptable (US FDA, 2001). The CVs obtained in this study were similar to those obtained by Kjelgaard-Hansen *et al.* (2007) in pigs, using the same commercial assay and to those obtained with a different commercial CRP TIA, in the same study, and higher to one obtained by Saco *et al.* (2010) also in pigs. In addition, the total error calculated for variations within-assay and between-assay were below the biological variation described in dogs for CRP (Jensen and Kjelgaard-Hansen, 2006; Kjelgaard-Hansen *et al.,* 2003).

These CVs were also comparable to those obtained by Tecles *et al.* (2007b) in pigs with the porcine-specific CRP ELISA and by the authors of this work with the same technique in the wild boar (see STUDY I).

Linearity under dilution showed high regression coefficient indicating that CRP was measured in a linear manner. This result indicates a good accuracy and agrees with those reported previously using CRP TIA (Kjelgaard-Hansen *et al.,* 2007). Nevertheless, species-specific material to measure accuracy in a direct way would be preferable (Kjelgaard-Hansen *et al.,* 2007; Tecles *et al.,* 2007a).

The LD was lower than the values obtained for the low concentration pool, either using the calibration material provided in the kit or the porcine in-house calibrator, which allows a reliable determination of CRP in healthy animals (Tecles *et al.,* 2007a). This LD is lower than that obtained previously in pigs by Kjelgaard-Hansen *et al.* (2003) with the same commercially CRP TIA, but higher than that obtained by the same author with a different CRP TIA.

Sampling in field conditions is difficult and is common to obtain undesirable changes in the samples such as haemolysis. The study of the haemolysis effect conducted in this

work showed that there is an important interference with the results obtained when samples are haemolytic, with changes from the original value up to 28%. In another study of the effect of haemolysis in pigs using CRP ELISA the magnitude of interference observed was not as great (Tecles *et al.,* 2007a). Therefore, non haemolytic samples of wild boar should be analyzed with the CRP TIA, as the results obtained would not be reliable.

In conclusion, the immunoturbidimmetric using a porcine in-house calibrator would be preferable to the ELISA when testing CRP wild boar serum concentration because it allows the automation, greatly improves the speed of obtaining the results and has a better equilibrium cost-benefit. Serum CRP concentration wild boar for clinical purposes can be measured reliably using Randox CRP TIA. Most likely the use of species-specific materials would improve the reliability of the results, but by now the adaptation of pig methods is the most feasible way.

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6.3. STUDY III

Acute phase proteins in Pyrenean chamois (*Rupicapra pyrenaica pyrenaica*): analytical validation and reference values in healthy animals

ABSTRACT

Changes in the concentration of plasma proteins called acute phase proteins (APPs) occur shortly after any tissue injury induced by infection, inflammation, trauma or stress. Analytical validation of the laboratory methods in use for the determination of APPs should be assessed before the report of any reliable value. The objectives of this study are to validate haptoglobin (Hp), serum amyloid A (SAA), acid soluble glycoprotein (ASG) and ceruloplasmin (Cp) concentration measurement methods for Pyrenean chamois and to establish the reference values for these APPs in this species. Serum Hp and SAA concentrations were determined by using commercial kits available. ASG and Cp were determined using assay methods described previously in the literature. High APP concentration specimens were obtained from six chamois with an inflammatory process and low APP concentration specimens were obtained from six healthy chamois. Inter and intra assay coefficients of variation (CVs) ranged between 0.08% and 14.96% the last obtained with the low concentration pool for SAA. The methods studied demonstrated good linearity (R2 >0.98). In 18 out of 30 samples and in 23 out of 30 samples from healthy animals, values obtained were below LD for Hp and SAA, respectively. ASG was significantly affected by haemolysis. Concerning reference values the minimum and maximum obtained from samples of healthy animals were 0.18-0.23 g/L for Hp, 2.52-26.86 mg/L for SAA 0.28-0.96 g/L for ASG and 0.01-0.02 ΔAbs/min for Cp respectively. The analytical methods used demonstrated to measure the concentration of proteins studied reliably, although many healthy animals results were below the LD for Hp and SAA.

INTRODUCTION

The acute phase response is the nonspecific and complex reaction that occurs shortly after any tissue injury. This response is mediated by pro-inflamatory cytokines and includes changes in the concentrations of plasma proteins, which are called acute phase proteins (APPs) (Cerón *et al.,* 2005). The APPs are very sensitive to detect pathological conditions, although they have low specificity for a particular disease (Eckersall, 2008). Its use in domestic animals as biomarkers for the detection of disease states and to evaluate the health status of a population has been extensively studied in recent years (Murata *et al.,* 2004; Gómez-Laguna *et al.,* 2011; Ceciliani *et al.,* 2012). The change in the pattern of these proteins during the acute phase response shows marked differences between species (Eckersall, 2008; Orro *et al.,* 2008).

Research in the field of APPs has led to the development of different analytical techniques, some of them available as commercial kits. Commercially kits for haptoglobin (Hp) and serum amyloid A (SAA) in bovines are available (Ceciliani *et al.,* 2012). Acid soluble glycoprotein (ASG) and ceruloplasmin (Cp) measurements have also been carried out in domestic ruminants (Young *et al.,* 1995; Ceciliani *et al.,* 2012,). The reliability of all these methods in livestock is well described in the literature; however the validity of the results obtained through these techniques has not been established in the wild ruminants and require validation in each laboratory, especially when used for species other than those for which the methods were originally designed (Lumsden, 2000).

The establishment of reference values is used to describe the dispersion of variables in healthy individuals (Geffre *et al.,* 2009). The knowledge of these values may be useful in the detection of disease states, either individual or animal populations (Pérez *et al.,* 2003).

The Pyrenean chamois, *Rupicapra pyrenaica*, is a small mountain ruminant endemic to the Pyrenees (*Rupicapra pyrenaica pyrenaica*) and Cantabrian mountains (*Rupicapra pyrenaica parva*) (North-Spain) and it is an emblematical game species whose hunting is an important economical source for rural communities (Herrero *et al.*, 2008). This species is listed as Least Concern by the IUCN (International Union for Conservation of

Nature) Red List. However, in recent years different disease outbreaks associates to Border Disease Virus infection (Marco *et al.,* 2007) and sarcoptic mange (Fernández-Morán *et al.*, 1997) have decimated several Pyrenean chamois populations.

The determination of APPs in this species could be used to assess the health status of the populations and to detect subclinical disease state prior to an outbreak like those occurred in recent years and to take management measures in advance (Cray, 2012).

The aims of the present study are to validate the APPs (Hp, SAA, ASG and Cp) concentration measurement methods for Pyrenean chamois and to establish the reference values for these APPs in this species.

MATERIALS AND METHODS

Specimens

For the analytical validation of the assays Pyrenean chamois low and high concentrations of acute phase proteins sera pools were used. Low APPs concentration specimens were obtained from six clinically healthy animals captured in drive-nets in the National game Reserves of Cadí and Freser-Setcases (42º22’N, 2º09’E). The samples were obtained within 30 minutes after capture. High APPs concentration specimens were obtained from six Pyrenean chamois from the Cadí Hunting Reserve sent to the Veterinary Faculty of the Universitat Autònoma de Barcelona with poor body condition and severe bacterial pneumonia confirmed on necropsy.

In order to provide the reference values for the different APPs studied, sera from thirty clinically healthy Pyrenean chamois captured in drive-nets in the above national game reserves were analysed in duplicated. A pool obtained from five of these animals was used to verify the effects of haemolysis in the analytical validation.

Blood samples were obtained by jugular venipuncture. Each blood specimen was collected into plain tubes containing clotting accelerator and centrifuged at 2000g during 10 min to obtain sera within first 6 hours from collection. Specimens were separated in aliquots and maintained at -20ºC until analysed.

Acute phase protein determinations

Serum concentrations of Hp were quantified using a commercial automated spectrophotometric assay (Tridelta Development Ltd, Maynooth, Ireland). The assay was performed according to manufacturer's instructions on an automated analyser (Cobas Mira Plus, ABX Diagnostics, Montpellier, France).

Serum SAA concentrations were determined by using commercial solid phase sandwich Enzyme Linked Immunosorbent Assays (Tridelta Development Ltd, Maynooth, Ireland). Analyses were performed according to the manufacturer's instructions, and the final absorbance was measured in a microtiter plate reader (PowerWave XS, Bio-Tek Instruments Inc., Vermont, USA) at 450 nm wavelength.

The ASG component of the serum samples was determined using the method based on that of Winzler (1955), modified by Nagahata *et al.* (1989) and Eckersall *et al.* (1996), and optimized by Tecles (2007b).

For the determination of Cp values the method based on that described by Suderman and Nomoto (1970), with the modifications of Martínez-Subiela and Cerón (2005) was used.

Analytical validation

The following parameters were determined:

Precision: Intra assays coefficients of variation (CVs) were calculated after analyses of pools with low and high APPs concentration, measured separately five times each in a single assay run. Inter assay CVs were determined by analysing the same pools in five separate runs carried out on different days. When the low pool concentration results were below the limit of detection of the test, a 12.5% dilution from the high concentration pool was used in order to assess the validity of the tests with samples with low concentration but above the limit of detection.

Linearity: It was evaluated by analysing serial dilutions (75, 50, 25 and 12.5%) of the pool of high APPs concentration.

Limit of detection: It was calculated based on data from 10 replicates determinations of the zero standard and expressed as mean value plus two standard deviations.

Effects of haemo*lysis*: Procedure was performed as previously described by Martínez-Subiela and Cerón (2005). Briefly, fresh haemolysate was prepared by addition of distilled water to packed, saline washed healthy Pyrenean chamois red cells. Concentration of haemoglobin in haemolysate was determined by using automated laser analyser (ADVIA 120, Siemens Healthcare Diagnostics, Deerfield, IL, USA) and adjusted to 200 g/L by adding distilled water to perform a stock solution. The stock solution was serially diluted with sample diluent buffer of each test and 10 µL of each dilution was added to 90 µL of a pooled sera from 5 healthy Pyrenean chamois. The final haemoglobin concentrations were 10, 5, 2.5, 1.25, 0.625 and 0.0 g/L (10 µL of sample diluent buffer were added to pooled sera to give 0.0 g/L concentration). These haemoglobin concentrations could correspond to slight haemolysis (0.625 g/L), moderate haemolysis (1.25 and 2.5 g/L) and marked haemolysis (5 and 10 g/L).

Reference values

Sera from thirty clinically healthy chamois were analyzed by duplicate.

Statistical analyses

All data analysis was performed by using Excel 2007 (Microsoft Office, Microsoft Corp., Washington, USA) and SPSS 15.0 for Windows (SPSS Inc., Chicago, IL, USA). Significance level used in each case was $p<0.05$.

Means, standard deviations, intra- and inter-assay, CVs and detection limits were calculated using routine descriptive statistical procedures. Pearson's correlation test was used to find the correlation between the serial dilutions and the results for the APPs concentration obtained.

Data from interference study are presented in interferographs made according to previously reported protocols (Martínez-Subiela and Cerón, 2005). The graphs show on the x-axies, increasing concentrations of haemoglobin, and on the y-axes, the mean percentage of change of each protein expressed as final value/original value x 100, where final value is the one obtained from sera enriched with haemoglobin and the

original value is that obtained from sera without interferent. The distribution of data was non parametric and results from original serum and enriched by haemoglobin were compared via paired Wilcoxon T test.

The U-Mann Whitney test was used to compare the pool obtained from healthy animals and the pool obtained from animals affected with bacterial pneumonia.

For reference values, routine descriptive parameters are provided instead of a reference range due to the small sample size used (Geffré, 2009).

RESULTS

Analytical Validation

Table 1 summarizes the results for precision. In general and for all the proteins studied, intra assay coefficients of variation (CVs) ranged from 0.08% to 12.53% and were lower than 5% for Hp, ASG and Cp when the high concentration pool was analyzed. SAA result from this pool presented the highest intra assay CVs (7.37%). The low pool concentration values were 0.05 g/L and 3.29 mg/L for Hp and SAA respectively, which were below the limit of detection of these tests. Therefore the 12.5% dilution obtained from the high APP concentration pool was used for these proteins. Low concentration pool analysis yielded intra assay CVs that were between 3 and 12%. Again, SAA presented the highest CVs (12.53%). Inter assay CVs ranged from 4.75% to 14.96%. ASG from the high concentration pool had the lowest CVs whereas SAA from the low concentration pool was the assay that showed the highest CVs (14.96%).

Dilution of high concentration pool of the different acute phase proteins resulted in linear regression equations with Pearson's correlation coefficients that ranged between 0.98 and 0.99 in all cases. The limit of detection was 0.08 g/L for Hp, 6.4 mg/L for SAA, 0.11 g/L for ASG and 0.002 Abs/min for Cp.

Table 1: Intra and inter assay coefficients of variation (CVs) for Hp, SAA, ASG and Cp assays. Low APPs concentration in the first line and high APPs concentration in the second line of each protein.

	Intra assay			Inter assay		
	Mean	**(SD)**	**CV(%)**	**Mean**	**(SD)**	**CV(%)**
Hp (g/L)	0.15*	(0.01)	2.94	0.16*	(0.01)	4.75
	1.89	(0.04)	1.94	1.85	(0.13)	7.15
SAA (mg/L)	24.88*	(3.12)	12.53	21.78*	(3.26)	14.96
	204.58	(15.07)	7.37	223.39	(17.81)	12.44
ASG (g/L)	0.44	(0.03)	7.01	0.48	(0.04)	7.96
	1.73	(0.08)	4.43	1.75	(0.11)	6.53
Cp (Abs/min)	0.0058	(0.000)	3.23	0.0064	(0.001)	13.32
	0.017	(0.00)	0.08	0.020	(0.00)	10.90

SD: standard deviation.

* Mean obtained from the 12.5% dilution of the high pool concentration.

The effect of haemolysis is presented in Figure 1. The addition of haemoglobin significantly interfered with the results for ASG, but not with those of Hp, SAA and Cp. ASG values dropped significantly when the concentration of haemoglobin in the sample was 2.5 g/L.

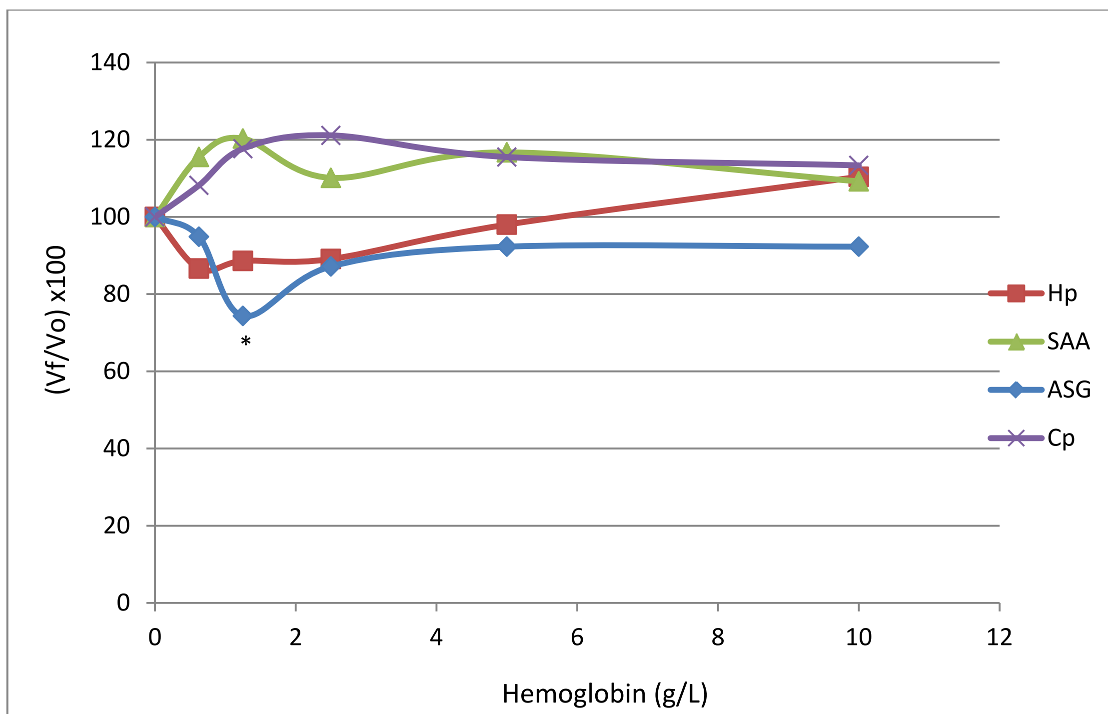

Figure 1: Haemoglobin interferographs obtained for APPs in Pyrenean chamois. Vf: final value; Vo: original value. * $p<0.05$.

Significant differences were observed between the high and low pools of APPs studied ($p<0.05$).

Reference values

Table 2 shows the mean, the standard deviation (SD), the median, the minimum and the maximum obtained when samples from thirty healthy Pyrenean chamois were analyzed. From the thirty samples analyzed, Hp yielded eighteen results below the lowest value that the Cobas Mira Plus provides, and SAA resulted in 23 values below the limit of detection obtained for this method.

Table 2: Acute phase protein values obtained for healthy Pyrenean chamois.

	Hp (g/L)	**SAA (mg/L)**	**ASG (g/L)**	**Cp (ΔAbs/min)**
N_T	30	30	30	30
N_1	-	23	0	0
N_2	18	0	0	0
N	12	30	30	30
Mean	0.18	3.29*	0.58	0.011
SD	0.03	7.14	0.19	0.003
Median	0.18	3.63*	0.54	0.010
Min.	0.18	2.52*	0.28	0.006
Max.	0.23	26.86	0.96	0.019

N_T: Number of samples analyzed.
N_1: Number of samples below detection the limit of the method.
N_2: Number of samples below the detection limit of the analyzer.
N: Number of samples used to report the reference values appearing in this table.
*Below the limit of detection.

DISCUSSION

The validity of the acute phase proteins measurement methods and their application has been extensively described and studied in domestic ruminants, such as cattle, sheep and goat (Ceciliani *et al.,* 2012). To the author's knowledge, there is no literature on APPs methods reliability in Pyrenean chamois and the usefulness that these proteins may have when assessing the health status of this species populations. The present study includes a thorough analytical validation of Hp, SAA, ASG and Cp in the Pyrenean chamois and provides reference values of these APPs in health condition.

CVs lower than 10% are considered acceptable for analytical determinations (Tecles *et al.,* 2007a). The values obtained for Hp and ASG demonstrated good precisions for either intra and inter assay assessment and the results of CVs described for these proteins were similar to those reported previously in the pig and the goat (Tecles *et al.,* 2007a, 2007b; González *et al.,* 2008).

The SAA analysis showed the highest imprecision with values in most cases above 10 percent. Nevertheless and conversely to other studies that obtained inter assay CVs up to 16.4%, data obtained in our work never exceeded 15% (Eckersall *et al.,* 2001, Martínez-Subiela *et al.,* 2003). Anyway CVs until 15 % or 20% for immunological assays are accepted (US FDA, 2001). Despite the high CVs, there is such a difference between samples with low and high concentrations that it would not have clinical significance (Tecles *et al.,* 2007a).

Cp presented good precision when intra assay analysis was performed. However, the inter assay CVs were above 10%, similar to that reported previously by Cerón and Martínez-Subiela (2004) in healthy dogs. The imprecision observed could be due to the very low values obtained in the analysis.

The development of species specific calibrators would improve the precision of the assays studied, especially in immunological tests such as the ELISA used for SAA measurement (Tecles *et al.,* 2007a; González *et al.,* 2008).

Because of the lack of reference material or standard for Pyrenean chamois, accuracy was indirectly assessed by linearity assays. The proteins studied showed very good

Pearson's regression coefficients of serially diluted high concentration pools. Both, the linearity coefficients of regression and the limits of detection obtained agree with those reported previously by other authors (Martínez-Subiela *et al.,* 2003; Tecles *et al.,* 2007a, 2007b; González *et al.,* 2008).

All the APPs tested showed significant differences between low and high pool concentration, therefore all these proteins could be potentially used to differentiate between healthy and inflammatory infectious conditions.

Hp value was 10 times higher in diseased animals (high concentration pool) than in healthy animals (low concentration pool). This difference could be due to the bacteriostatic effect and the anti-inflammatory function of haptoglobin in diseased animals (Ceciliani *et al.,* 2012). SAA has been described previously to be a major APP in domestic ruminants which agrees with the results described in the present study, as the sick animals showed a concentration 100 times greater than healthy animals (Gómez-Laguna *et al.,* 2011). The immunomodulatory activity and opsonization roles of the SAA described in the literature could explain the high values observed in the former group (Ceciliani *et al.,* 2012).

The role of haptoglobin and SAA in animals with inflammatory infectious diseases has been well described in domestic ruminants. Thus it has been shown that these APPs are a useful tool in the detection and monitoring of mastitis in bovine medicine (Eckersall *et al.,* 2001; Åkerstedt *et al.,* 2007) and acute lymphadenitis caseous in sheep (Eckersall *et al.,* 2007; Bastos *et al.,* 2011), as well as being an early biomarker of metritis in cows (Huzzey *et al.,* 2009) among others.

In most species ASG correlates well with alpha-1-acid glycoprotein (AGP), as the glycoprotein fraction left after perchloric acid precipitation is predominant (Eckersall *et al.,* 1996). In the present study ASG in diseased animals was 4 fold higher than in healthy animals which differs slightly from the response observed in other studies in which the inflammation had been experimentally induced (Lampreave *et al.,* 1994; Eckersall *et al.,* 1996; Tecles *et al.,* 2007b). However, the values observed are in accordance with those reported previously for AGP in naturally infected animals such as pneumonia and meningitis in pigs (Itoh *et al.,* 1993) or feline infectious peritonitis

(Duthie *et al.,* 1997). The immunomodulatory and the antibacterial functions of this glycoprotein could have resulted in the high values observed in sick animals (Ceciliani *et al.,* 2012).

The Cp in domestic ruminants has little importance in terms of acute phase response and is primarily considered a minor APP in these species (Murata *et al.,* 2004). The results obtained in the present study did not correspond to those previously described as the high pool concentration was 10 folds higher than the low pool concentration. These results correlate better with those obtained in dogs with leishmaniasis (Cerón and Martínez-Subiela, 2004). The influence of stress factors in ceruloplasmin elevation has been reported by Arthingthon *et al.* (2008) in calves. More studies would be necessary to determine whether the more stressful character of wild animals could be a key factor in ceruloplasmin dynamics.

Some tests are significantly altered by specimens that are haemolyzed whether if it is visible macroscopically or not (Alleman, 1990). In wild animals the sampling is usually not performed in ideal conditions and haemolytic samples are common. It is important to know the reliability of the techniques we use in the laboratory when the samples are not as clean as we would like. This study reveals that the samples slightly haemolytic interfere significantly in the analysis of the ASG, while it does not with the analysis of Hp, SAA and Cp. Similar results were reported by Martínez-Subiela and Cerón (2005) where haemolysis did not affect the analysis of SAA in dogs. However, the authors above mentioned described significant interferences for Cp in the same work as do Tecles *et al.* (2007a), for SAA and Hp in pigs, which differs from results obtained in our work.

It has been reported that AGP binds to the membranes of erythrocytes (Maeda *et al.,* 1984; Matsumoto *et al.,* 2003). In our study, the interference was created from haemolysed erythrocytes rather than pure haemoglobin, as some remnants of cellular membranes could have remained in the haemolysed, thus decreasing ASG concentration. However other mechanisms described by Alleman (1990) such as the release of erythrocyte constituents into serum, dilution of constituents in serum or

chemical interaction with the analyte by which haemolysis can affect laboratory testing cannot be ruled out.

When looking the effect of haemolysis, in all cases alterations approximately between 15 and 20% from the original value were observed (Figure 1). Thus it would be advisable to avoid haemolytic samples whenever possible.

Concerning to the reference values, healthy animals presented very low values for Hp and SAA with many of them below the detection limit, or even no detectable by the technique used. These results agree with others reported previously in domestic ruminants like cattle (Nazifi *et al.,* 2012), goats (González *et al.,* 2008) or sheeps (Bastos *et al.,* 2011). Samples with values below the limit of detection or those that the analyzer do not detect should always be treated with caution and taking into account the clinical context. This type of results highlights the importance of measuring at least two APPs that help to understand the process that is taking place (Diack *et al.,* 2011).

Like other authors have reported previously in ruminants (Ceciliani *et al.,* 2012) the values obtained for ASG and Cp were also very low in the healthy animals studied, although all the values obtained were within the detection limit.

In conclusion, the methods evaluated in this study for the determination of Hp, SAA, ASG and Cp are suitable for the analysis of samples of Pyrenean chamois, since they presented good accuracy and repeatability. In addition, the results obtained suggest that APPs could be potentially good biomarkers for inflammatory infectious diseases in this species. Additionally in this paper, the values for these proteins in healthy conditions have been established which could serve as a basis to define APPs patterns for different disease conditions. More studies with bigger sample size are needed to establish a reliable reference range, especially considering the relatively high number of samples below the limit of the detection obtained in this work for Hp and SAA.

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6.4. STUDY IV

Haptoglobin and Serum Amyloid A in Spanish ibex (*Capra pyrenaica*): method validation, reference values and characterization of acute phase protein response to experimentally induced inflammation

ABSTRACT

Body inflammation, infection or injure induce a change in concentration of a group of proteins called acute phase proteins (APPs). The quantification of APPs can be used to monitor disease and treatment, as well as being a good tool for diagnostic and prognostic. The objectives of this present study are to validate haptoglobin (Hp) and serum amyloid A (SAA) techniques, and to provide reference values for these proteins in Spanish ibex; in addition, changes in concentration of these proteins after experimentally induced inflammations (turpentine injection) and viral infection (bluetongue virus-BTV) were also studied. Serum proteins concentrations were determined using available commercial kits. The analysis of both Hp and SAA showed good intra-assay coefficients of variation (CVs) and good coefficients of correlation when serial dilutions of high concentration pool were analyzed. Inter-assay CV was good for Hp but very high for SAA. Some samples from healthy animals yielded values below the limit of detection (LD). After turpentine injection, Hp and SAA increased significantly. Hp reached its maximum at day 4 whereas SAA values peaked at day 3. BTV experimental infection induced a slightly increase in both proteins, corresponding with the viremia peak. Our study shows that the analytical methods used measure the concentration of Hp and SAA reliably, although SAA yielded a high inter-assay imprecision. Moreover, Hp and SAA can be used to discriminate between healthy and diseased animals when an acute inflammation condition has been established. Experimental infection with BTV also produced a rise in these proteins although the animals keep asymptomatic during the whole period. Thus, it would be advisable to study these two proteins when dealing with bluetongue disease as indicators of health status.

INTRODUCTION

Acute phase proteins (APPs) are a group of proteins that change in concentration as a response to body inflammation, infection or injure. Production of APPs is controlled by cytokines released from the site of pathogenic or inflammatory damage, being the liver the main site of synthesis. The functions of APPs are part of the innate immune response and are aimed to the defence of the animal against the pathological damage and restoration of homeostasis (Eckersall, 2008; Ceciliani *et al.,* 2012). The quantification of these proteins can provide diagnostic and prognostic information, as well as monitoring disease evolution and treatment (Horadagoda *et al.,* 1999; Petersen *et al.,* 2004). In cattle, APPs have been widely studied and major APPs in this species such as haptoglobin (Hp) and serum amyloid A (SAA) have been evaluated in experimental infections and natural disease (Ceciliani *et al.,* 2012). In goats, Hp and SAA have been used to differentiate between animals with and without helmint infection (Ulutas *et al.,* 2008); in sheeps acute caseous lymphadenitis increases Hp and SAA, whereas chronic caseous lymphadenitis increases alpha-1 acid glycoprotein (AGP) (Eckersall *et al.,* 2007). SAA, AGP, Hp and ceruloplasmin had been suggested in Alpine ibex (*Capra ibex*) as a tool for the identification of subclinically sarcoptic mange infection (Rahman *et al.,* 2010).

Bluetongue virus (BTV) causes bluetongue, a vector-borne disease of ruminants that has an economic impact on animal health. This virus belongs to the genus *Orbivirus* (family *Reoviridae*) and is transmitted by blood-feeding midges of the genus *Culicoides* (*Diptera*, *Ceratopogonidae*) (Muller, 1987; Mertens *et al.,* 2004). BTV is considered an emerging and re-emerging disease in Europe, where at least eight serotypes have been detected (Falconi *et al.,* 2011). In the last decades, BTV-1 emerged in southern Spain and caused several outbreaks in livestock, reaching the North of the country (García-Lastra *et al.,* 2012). The spreading of BTV-8 through Europe since its introduction in 2006 caused severe disease in cattle and sheep, causing heavy financial losses in animal industry (Thiry *et al.,* 2006; Rodríguez-Sánchez *et al.,* 2008). Recent studies have demonstrated that Spanish ibex can be infected subclinically with BTV-1 and BTV-8. (Lorca-Oró *et al.,* 2012).

Spanish ibex, (*Capra pyrenaica*), is a mountain ruminant endemic to the Spanish Peninsula, with populations widespread throughout the southern and eastern of Spain (Pérez *et al.,* 2002). In the past this species has been listed as Threatened, and currently is considered as Least concern species by the IUCN (International Union for Conservation of Nature-any) Red List of Threatened Species. However, there are a series of threats towards ibex conservation such as population overabundance, habitat fragmentation, contagious diseases (especially sarcoptic mange), and potential competition with domestic livestock and other ungulates, along with the negative effects of human disturbance through tourism and illegal hunting (González-Candela *et al.,* 2006).

The main goals of this study are to validate Hp and SAA methods for its use in Spanish ibex and to establish reference values; and to characterize the response of these two proteins after experimentally induced inflammation (turpentine injection) and experimental BTV infection.

MATERIALS AND METHODS

Animals and samples

Three groups of ibexes were used in this study.

Groups 1 and 2 were formed with Spanish ibexes from the Captive Breeding Center of Sierra Nevada National Park (El Toril, Granada, southern Spain). Permits from the study were approved by the Consejería de Medio Ambiente – Junta de Andalucía. Animals from these groups were physically restrained using handling chutes.

Group 1 consisted of six healthy ibexes which remained in the enclosure of El Toril at Sierra Nevada National Park. They were injected subcutaneously with 5 mL of turpentine at the right thoracic region. Sampling and clinical evaluation were performed at days 0, 1, 2, 3, 4, 7 and 14. Sera from this group were used for analytical validation of APPs techniques.

Group 2 was composed by seven ibexes which were transported by road to the Biosafety level 3 (BSL3) facilities of the Centre de Recerca en Sanitat Animal (CReSA, Barcelona, Spain). After an adaptation period of five days, Spanish ibexes were challenged with 106.5 TCID50/mL BTV-1 (n=3) and 106.6 TCID50/mL BTV-8 (n=4) serotypes with 2 mL of viral suspension in the jugular vein in a study aimed to evaluate Spanish ibex susceptibility to BTV inoculation and immune response vaccionation (Lorca-Oró, 2012). Sampling and clinical evaluation based on previously described signs of bluetongue in small ruminants (Backx *et al.,* 2007) were performed at 0, 2, 4, 7, 9, 11, 14, 17, 21 and 24 days post-inoculation (dpi).

Sera from groups 1 and 2 were used to characterize the response of Hp and SAA after turpentine and BTV challenges, respectively.

Group 3 was made up of forty ibexes from the National Game Reseve of Ports de Tortosa i Beseit (Catalunya, northeastern Spain). These ibexes were captured by means of box-trap and sampled once. Sera from this group were used to provide the reference values for Hp and SAA in healthy conditions.

All the samples used were obtained by jugular venipuncture. Each blood sample was collected into plain tubes containing clotting accelerator and centrifuged at 2000g during ten minutes to obtain sera within first six hours from collection. Samples were divided in 1.5 mL aliquots and maintained at -20ºC until analysed.

Acute phase protein determinations

Serum concentrations of Hp were quantified using a commercial spectrophotometric assay (Phase Haptoglobin assay, Tridelta Development Ltd, Ireland). Serum SAA concentrations were determined by using commercial solid phase sandwich enzyme linked immunoabsorbent assay (Phase SAA assay, Tridelta Development Ltd, Ireland). Analyses were performed according to the manufacturer’s instructions. The final absorbances of both techniques were measured in a microtiter plate reader (PowerWave XS, Bio-Tek Instruments Inc., Vermont, USA) at 630 nm and 450 nm wavelength for Hp and SAA, respectively.

Analytical validation

Two different sera pools were obtained from animals in group 1. A low concentration pool was obtained before inflammation induction with turpentine injection and a high concentration pool when the inflammation was evident (day 4 after turpentine injection). The following parameters were determined:

Precision: Intra assay coefficients of variation (CVs) were calculated after analyses of both pools low and high APPs concentration, measured separately five times each in a single assay run. Inter assay CVs were determined by analysing the same pools in five separate runs carried out on different days.

Linearity: It was evaluated by analysing serial dilutions (100, 75, 50, 25 and 12.5%) of the pool of samples with high APPs concentration.

Limit of detection (LD): It was calculated based on data from ten replicates determinations of the zero standard as mean value plus two standard deviations.

Reference values

Sera from animals in group 3 were analyzed by duplicate and results obtained were used to provide reference values.

Clinical evaluation

To study the dynamics of APPs, Hp and SAA concentrations were measured and compared before and after the challenge produced by turpentine injection and BTV inoculation. The results were compared to detect significant changes in Hp and SAA concentrations.

Statistical analyses

Intra and inter-assay CVs, limits of detection and ordinary regression analysis to investigate the linearity under dilution were calculated using routine descriptive statistical procedures for analytical validation (Excel 2007, Microsoft Office, Microsoft Corp., Washington, USA).

Routine descriptive statistics were calculated from sera of group 3 to provide reference values (SPSS, Version 15.0, SPSS Inc., Chicago, IL, USA).

To compare APPs concentrations in ibexes before and after the experimentally induced inflammation (turpentine) and the expermiental infection (BTV), a data analysis was performed by using a statistical software program (Version 15.0, SPSS Inc., Chicago, IL, USA). Kolmogorov-Smirnov test was used to check data distribution, which was non-parametric. Friedman and Wilcoxon signed rank tests with Bonferroni adjustments were applied. Statistical significance was set at $p<0.05$.

RESULTS

Analytical validation

Mean, standard deviation and coefficient of variation obtained for intra and inter assay analyses of the high and low concentration pools are presented in Table 1. Intra assay coefficients of variation (CVs) varied between 9.71% and 17.34%, the highest value corresponding to Hp in the low concentration pool. Inter assay CVs ranged between 4.10 and 32.92, the last obtained for the low concentration pool for SAA.

Table 1: Intra and inter assay coefficients of variation (CVs) for Hp and SAA assays. Low APPs concentration in the first line and high APPs concentration in the second line of each protein.

	Intra assay			Inter assay		
	Mean	**(SD)**	**CV(%)**	**Mean**	**(SD)**	**CV(%)**
Hp (g/L)	0.62	(0.11)	17.31	0.48	(0.13)	20.76
	1.26	(0.12)	9.74	1.20	(0.05)	4.10
SAA (mg/L)	29.73	(3.83)	12.89	56.4	(18.57)	32.92
	155.98	(25.72)	16.49	142.69	(37.35)	26.30

SD: Standard Deviation. CV: Coefficient of Variation.

Serial dilution analyses of high pool concentration resulted in linear regression equations with correlation coefficients of 0.95 for Hp and of 0.98 for SAA. The limit of detection was 0.08 g/L for Hp and 6.4 mg/L for SAA.

Reference values

Table 2 shows the mean, the standard deviation (SD), the median, the minimum and the maximum of the APPs concentrations obtained from group three sera. For reference values, routine descriptive parameters are provided instead of a reference range due to the small sample size used (Geffré *et al.,* 2009).

Table 2: Mean, standard deviation (SD), median, minimum (Min.) and maximum (Max.) values obtained for acute phase protein studied in healthy Spanish ibexes.

	Hp (g/L)	SAA (mg/L)
n	40	40
Mean	0.22	6.94
SD	0.088	7.39
Median	0.20	5.045*
Min.	0.14	0.05*
Max.	0.64	30.00

* below the limit of detection

Clinical evaluation

Figure 1 shows timecourse Hp and SAA values for the inflammation induced by turpentine injection.

Haptoglobin concentration increased progressively, reaching the peak at day 4 (mean concentration 3.31 g/L). This concentration was maintained until day 7 and then decreased. Concentrations obtained at days 4 and 7 were three-fold higher than baseline values obtained. Concentrations at days 2, 3, 4 and 7 differ significantly from day 0. At day 14 not all animals had reached baseline (Figure 1A).

SAA concentration increased faster and peaked at day 3 (mean concentration 565 mg/L), with a concentration sixteen-fold higher respect day 0 (mean concentration 36

mg/L). Days from 1 to 4 yielded values significantly different to those obtained at day 0. At day 14 all the animals had returned to basal values (Figure 1B).

On the clinical examination all the animals injected with turpentine presented ventral inflammation and subcutaneous edema of different extent.

Figure 2 shows timecourse Hp and SAA concentrations after BTV inoculation.

There were no significant changes in the concentration of Hp and SAA when BTV infection was conducted. Hp increased on 7, 9 and 11 dpi with high inter-individual variability (Figure 2A). SAA values rose at 9 dpi (Figure 2B). The increase in the concentrations of Hp and SAA observed matches with the viraemic peak and with the moment where the neutralizing antibody titers started to rise. BTV infection was mostly asymptomatic in all the infected ibexes. The only clinical sign compatible with bluetongue was a punctual increase in rectal temperature in the BTV-1 inoculated ibexes at 7 dpi (Lorca-Oró *et al.,* 2012).

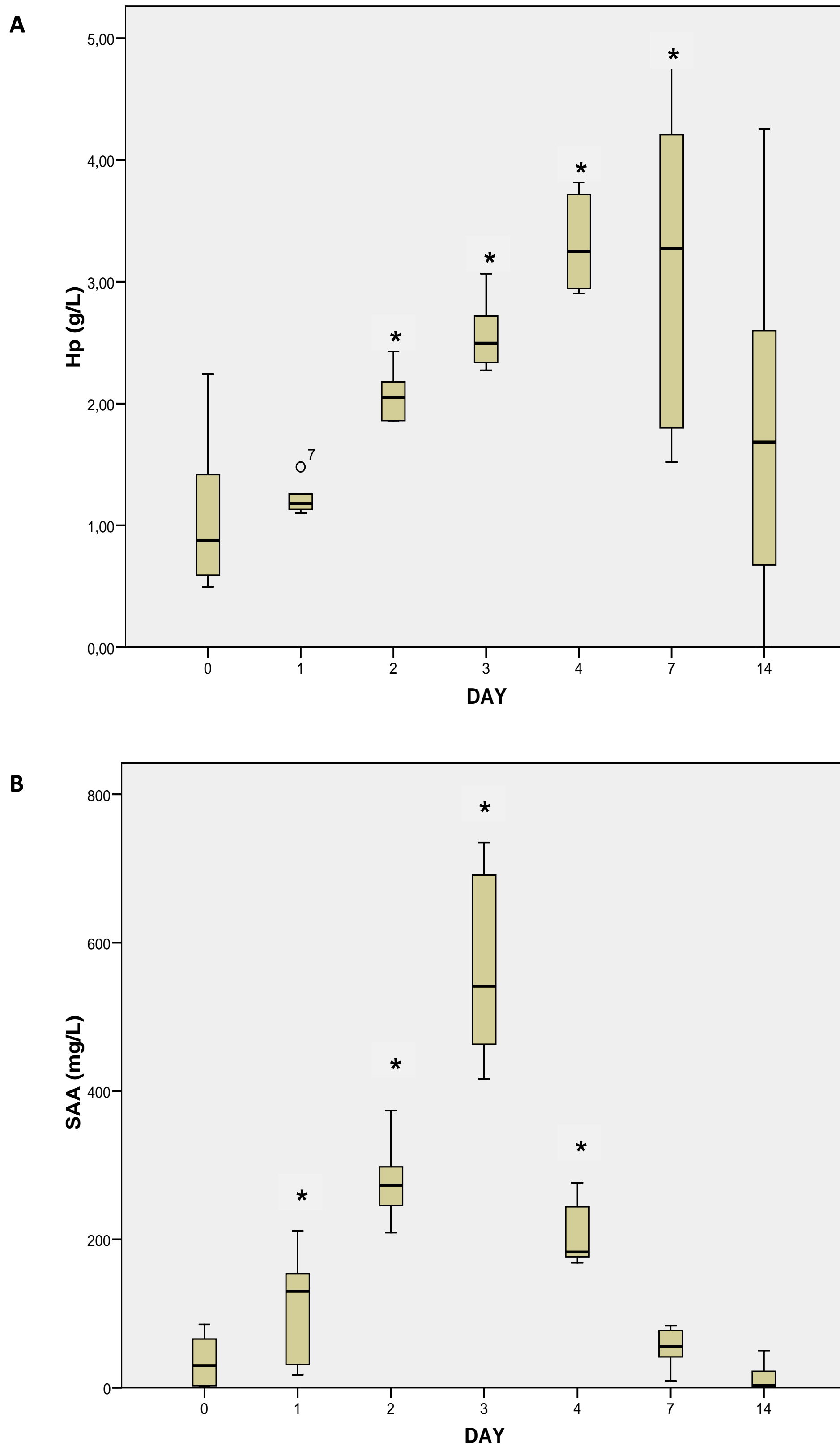

Figure 1: Box plots showing Hp (A) and SAA (B) concentrations before (day 0) and after (days 1, 2, 3, 4, 7 and 14) turpentine injection. * *P* <0.05 relative to day 0. (Friedman and Wilcoxon signed-rank tests).

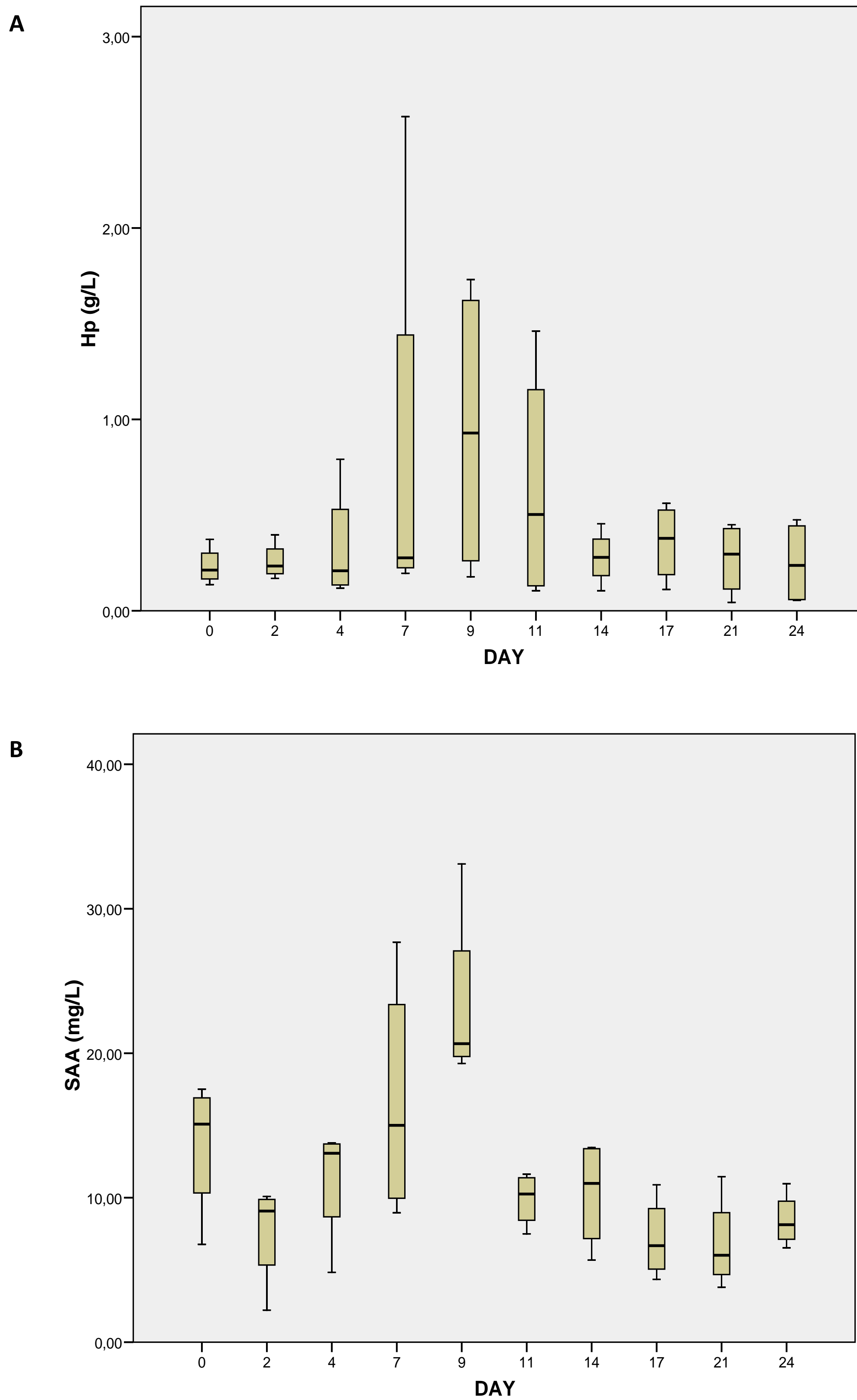

Figure 2: Box plots showing Hp (A) and SAA (B) concentrations before (day 0) and after (days 2 to 24) experimental infection with BTV.

DISCUSSION

In the present work, the techniques used for Hp and SAA determination were analytically validated. Hp and SAA responses after experimental inflammation (turpentine injection) and experimental infection with BTV were also studied. To the author's knowledge, this is the first study in analytical validation and acute phase response conducted in Spanish ibex.

In the literature there are very few studies on the use of APPs in wildlife. APPs, including Hp and SAA were used in the Alpine ibex to monitor sarcoptic mange infection. However, the analytical validation of the techniques employed was not performed by the authors, although the cross reactivity of SAA antigen in ibex serum with the anti-SAA primary antibodies provided in the SAA commercial kit was evidenced (Rahman *et al.,* 2010).

Hp showed a good precision when high concentration pool was measured. However, low concentration pool yielded high CVs, especially in the inter assay runs. In our study Hp was measured using a manual colorimetric method, which could explain the high CVs obtained, as lower CVs are described for automated method (Tecles *et al.,* 2007; Gonzalez *et al.,* 2008). Moreover, low concentration pool yielded very low values, which usually entails higher results in CVs (Lumsden, 2000).

SAA produced acceptable intra-assay CVs, as in immunological assays CVs between 15 and 20% are accepted (US FDA, 2001). Similar CVs had been obtained previously by other authors in dogs (Martínez-Subiela *et al.,* 2003) and cattle (Eckersall *et al.,* 2001) with the same technique. There was a very high inter-assay imprecision both with low and high concentration pools, with results higher than those accepted in the literature. Inter-assay CVs up to 39% have been reported previously in pigs with the same technique (Tecles *et al.,* 2007). Thus, sera samples should be analyzed in the same run whenever possible. Despite the high inter-assay imprecision obtained, both Tecles *et al.* (2007) and ourselves could distinguish between animals before and after the establishment of an inflammatory response.

In our study SAA has been measured by an ELISA designed with anti-SAA bovine antibodies. Although a previous study showed that there is cross-reactivity between

these antibodies and Alpine ibex serum antigens (Rahman *et al.,* 2010), the use of species-specific materials would probably improve the precision of the test (Eckersall *et al.,* 1999; Tecles *et al.,* 2007). Furthermore, the ELISA technique itself involves much manual work which contributes to increase imprecision (Tecles *et al.,* 2007).

Following method validation standards when there is a lack of species-specific calibration material, accuracy has to be measured indirectly by serial dilution of the high pool concentration. In our work linearity study resulted in an acceptable regression coefficient for Hp and a good one for SAA, indicating that these two proteins are measured in a linear manner (Westgard *et al.,* 1999; Lumsden, 2000).

Analysis of sera from healthy ibexes yielded many results below the LD both for Hp and SAA. Although differences between ibexes with an established inflammatory response were evident, concentrations below the LD should be interpreted with caution. These results agree with previously reported undetectable values in healthy sheep and cattle and low detectable values in healthy dogs, cats and capybaras (Cerón *et al.,* 2005; Tecles *et al.,* 2007; Gonzalez *et al.,* 2008; Bernal *et al.,* 2011).

Hp and SAA increased both with inflammation (turpentine injection) and experimentally induced infection (BTV inoculation).

Hp reached the maximum mean concentration within days 4 and 7 after turpentine injection, with significantly higher concentrations at 48 hours relative to initial values (day 0). The results agree with those reported previously in pig (Tecles *et al.,* 2007) and cattle (Conner *et al.,* 1988) but differ slightly from one reported in goats (Gonzalez *et al.,* 2008), where peak was reached 72 hours after turpentine injection. The three-fold increase observed also agrees with the study conducted in pigs (Tecles *et al.,* 2007), although it is lower than that observed in goats (Gonzalez *et al.,* 2008). However, initial values observed in Spanish ibex were much higher than those obtained in pigs and goats by the authors cited, and this affects the magnitude of change observed. In fact, the maximum absolute Hp value obtained in Spanish ibex was higher than the maximum values obtained previously in pigs and goats. A high inter-individual variability was observed at days 7 and 14, which has been reported previously (Gonzalez *et al.,* 2008).

Consistent with previous studies (Horadagoda *et al.,* 1999; Heegaard *et al.,* 2000; Gonzalez *et al.,* 2008), the SAA response after turpentine administration was faster than that observed in Hp. The sixteen-fold increase of SAA concentration also conforms well to the results described previously in pigs and goats (Tecles *et al.,* 2007; Gonzalez *et al.,* 2008,); nevertheless in goat the peak was reached earlier than in the Spanish ibex, at 48 hours.

BTV infection induced only a slight response on Hp and SAA concentrations, as the increase observed matched with viraemic peak and the onset of the acquired immune response (Lorca-Oró *et al.,* 2012). The magnitude of the change observed differ from that obtained previously in other experimental viral infections conducted in calves and cattles. In calves inoculated with bovine viral diarrohea virus both APPs increased significantly after virus inoculation, and similar results were observed in cattles undergoing experimental infection with bovine respiratory syncytial virus. In both studies high inter-individual variability was reported (Heegaard *et al.,* 2000; Gånheim *et al.,* 2003). Unlike the present work, in these two studies the animals developed severe clinical signs, including persistent fever. The magnitude of change of APPs has been related to the severity of the disease, thus more important stimulus lead to more pronounced responses (Ceciliani *et al.,* 2012). The fact that animals kept asymptomatic during the BTV infection and could explain in part the slightly increase in concentration observed in this study.

In conclusion, this study demonstrates that Hp and SAA techniques actually available can be used in Spanish ibex to discriminate between healthy and diseased animals when an acute phase response has been established and provides reference values for these two proteins. However, further studies on technique improvement and automation would be necessary to enhance the precision of the methods. The results described, also show that experimental BTV subclinical infection induces only a slight response in the two APPs studied. Further studies are needed to full characterize the APPs response in BTV disease.

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7. GENERAL DISCUSSION

7. GENERAL DISCUSSION

There are many studies on the characterization of acute phase proteins (APPs) in livestock, mainly focused on pigs and cattles. Nevertheless, works in this field on small ruminants and wildlife are scarce (Thomas, 2000; Petersen *et al.,* 2004; Eckersall, 2008a; Ceciliani *et al.,* 2012).

Part of the available literature is focused on the development and validation of APPs determination methods. Clinical interpretation of laboratory results for diagnostic approach, prognosis or monitoring of treatments, assumes that data from the laboratory are reliable and certain. This is especially important in veterinary medicine due to many species, management and disease differences encountered. Thus the introduction of a new method or technical modifications of established procedures (new instruments, reagents, ...) must be evaluated and validated with the ultimate goal of method acceptance that will allow safe clinical decisions (Lumsden, 2000). The reliability of methods in livestock species like pigs, cattle and goats has been well described (McNair *et al.,* 1995; Eckersall *et al.,* 1996; Álava *et al.,* 1997; McNair *et al.,* 1997; Kjelgaard-Hansen *et al.,* 2007; Martínez-Subiela *et al.,* 2007; Tecles *et al.,* 2007a, 2007b; González *et al.,* 2008). Studies I, II and III of the present work are focused mainly on analytical validation of techniques in wild boar and Pyrenean chamois. Additionally, reference values for all the proteins studied are also provided in these species.

Most livestock papers are focused on APPs dinamics in different diseases and conditions that involve animal welfare risks. Experimental induced infection studies have been conducted in order to study APPs response against specific infectious agents and, in the same way, research works on naturally infected animals have also been carried out. Studies on non infectious challenging situations have also been performed (Gómez-Laguna *et al.,* 2011; Ceciliani *et al.,* 2012). The Study IV of the present work evaluates the usefulness of two acute phase proteins in inflammatory conditions induced experimentally in Spanish ibex; the work includes a basic method validation for the two proteins studied.

Concerning the Analytical validation, precision studies conducted demonstrated that most proteins yielded acceptable coefficients of variation (CVs) for all species. Mainly three factors described previously in the literature and linked to the analytical validation process have influenced in our results.

First, our work highlights that the type of method used has an important influence on the repeatability of the results. Thus, we describe that the use of immunology based methods yield higher CVs than the spectrophotometric ones. This fact has been shown previously, inasmuch as CVs accepted for immunological tests (up to 20%) are higher to those reported for other methodologies (up to 15%) (US FDA, 2001). The high CVs obtained for immunological tests available have been attributed mainly to the fact that most of them do not use species-specific antibodies and species-specific calibrators. Thus, the partially cross reactivity reported previously for immunological methods like C-reactive protein (CRP) could explain the persistent errors and the high CVs observed in the analytical validation of the techniques (Kjelgaard-Hansen *et al.,* 2003; Martínez-Subiela *et al.,* 2007). Therefore studies on cross reactivity should be conducted for each species and method to well understand the origin of the errors.

Methods which involve high technician manual work like enzyme linked immunosorbent assays (ELISA) produce higher imprecision results when compared with automated methods (Tecles *et al.,* 2007a). We have confirmed this, since haptoglobin (Hp) analyzed in our study both with automated and manual procedures using the same technique, produced lower CVs in the former when compared with the last.

Sample concentration should also be taken into account when CVs are evaluated, as those ones with lower concentrations involve higher coefficients of variation, since the standard deviation is higher. That is the reason why CVs up to 20% are accepted when testing low concentration samples (Lumsden, 2000; US FDA, 2001).

Taking in mind these factors, it would be highly advisable to use species-specific materials in immunological tests and automated methodologies whenever possible.

Regarding to the specific results obtained in our work, most of the CVs obtained from proteins measured by spectrophotometry haptoglobin (Hp), acid soluble glycoprotein (ASG) and ceruloplasmin (Cp) were below 10%. The exceptions to this were the intra-assay CV for Hp in the Spanish ibex (17.31%) and the inter assay CVs for ASG in the wild boar (15.49%) and for Cp in the Pyrenean chamois (13.32%), all of them obtained for the low concentration pool. Similar CVs had been reported previously by other authors in pigs, goats, cattle, dogs and rats (Eckersall *et al.,* 1999a; Martínez-Subiela *et al.,* 2007; Tecles *et al.,* 2007a; González *et al.,* 2008).

As previously described by other authors, high CVs were obtained when immunological methods were used, thus serum amyloid A (SAA), C- reactive protein (CRP) measured both with ELISA and with a tubidimetric immunoassay (TIA) and porcine major acute phase protein (Pig-MAP) yielded CVs mostly between 10 and 20% (Eckersall *et al.,* 2001; Martínez-Subiela *et al.,* 2003; Tecles *et al.,* 2007a; González *et al.,* 2008). Exception was SAA samples analyzed in the study conducted on Spanish ibex which yielded CVs around 30%. These results match with those obtained by Tecles *et al.* (2007a) in pigs where CVs up to 39% were obtained for the same protein.

The variety of species involved in veterinary medicine represents a handicap when developing new methods of analysis. This is magnified when wild species are studied since, unlike livestock, there is not an economic interest that motivates the development of new methods. The species-specific standards, at present lacking in many species, are necessary to assess test accuracy; thereby linearity is used as an indirect manner to evaluate this parameter (Tecles *et al.,* 2007a; González *et al.,* 2008). Generally, all the proteins studied in this work presented good correlation coefficients when serial dilutions were analyzed (Westgard *et al.,* 1999) and results agreed with those previously described by other authors (Martínez-Subiela *et al.,* 2007, Tecles *et al.,* 2007a).

The LDs obtained were very similar to those reported in literature (Tecles *et al.,* 2007a; González *et al.,* 2008). Method validation guidelines recommend using samples with different concentrations to assess the repeatability of a method. The different concentrations should be selected taking in mind the limits of the method and the

medical decision limits (Lumsden, 2000; US FDA, 2001). In our work two different concentrations have been used, the low concentration pool, for healthy animals, and the high concentration pool, for diseased ones. In Pyrenean chamois the low concentration pool was below LDs for Hp and SAA. In this species a 12.5% dilution of the high concentration pool was used to assess repeatability of the method at low concentrations, an option that could be used in the future by other authors who are in the same situation.

Specimens that are hemolyzed can alter significantly some tests results either if hemolysis is visible macroscopically or not (Alleman, 1990). Wildlife sampling is usually performed under field conditions what frequently involves hemolytic samples. In the present work, interference caused by hemolysis was assessed in APPs studied both in wild boar and Pyrenean chamois.

Several interference hemolysis studies have reported changes in APPs concentration. Thus, important changes in Hp and SAA due to hemolysis have been described in pigs (Tecles *et al.,* 2007a) whereas CRP and Cp did in dogs (Martínez-Subiela and Cerón 2005). In our study, values from wild boar presented important changes when interference by hemolysis was conducted. Hp, SAA and Pig-MAP were the most affected proteins important changes in concentration whereas Cp was the less affected. An important interference with changes up to 28% from the original value was also observed when interference of the hemolysis was conducted in CRP TIA. Tecles *et al.* (2007a) observed a smaller not significant difference between the original and interfered values with the porcine specific CRP ELISA.

In the Pyrenean chamois a significant interference by hemolysis was observed for ASG and, although not significant, changes between 15% and 20% with respect the original value were observed for all the other proteins studied.

Mechanisms associated with hemolysis interferents like the release of erythrocyte constituents into serum, dilution of constituents in serum or chemical interaction with the analyte have been purposed as possible causes for the changes observed (Alleman, 1990). For ASG specifically, the capacity to bind to erythrocyte membranes of alpha-1

acid glycoprotein cannot be rule out as possible cause of the changes in concentration observed in this protein (Maeda *et al.,* 1984; Matsumoto *et al.,* 2003).

In general, the results observed for the interference study conducted, support the fact that whenever possible hemolytic samples should be avoided.

There is a species-specific ELISA for the measurement of CRP in pigs and this protein has been considered a major acute phase protein by some authors (Thomas, 2000; Eckersall, 2008a). Using ELISA techniques entails a series of drawbacks including the need of species-specific antibodies, much technical manual work and a poor equilibrium between cost and benefit (Eckersall, 2010). In human medicine there are turbidimetric immunoassays (TIA) for the CRP measurement, which have the advantage of being automated and less expensive. The use of these assays has been assessed before for its use in pigs (Saco *et al.,* 2010). In our work the use of a human CRP TIA using the calibrator material provided in the kit revealed the presence of a proportional error when compared with the porcine specific CRP ELISA. Similar results were obtained by other authors with similar methodologies (Kjelgaard-Hansen *et al.,* 2007; Saco *et al.,* 2010). This error was improved by using an in-house porcine serum calibrator, although some differences between TIA and ELISA persisted, mainly for samples with high concentration.

Concerning the reference values, in veterinary medicine it is relatively difficult to obtain the 120 samples recommended in the literature to report a reliable reference range (Solberg, 2005). This is even more remarkable in wildlife where sampling usually involves laborious animal handling and capturing either for population health screening or research purposes. In the present work the number of samples used ranges between 30 and 40, which is considered a small sample size. Thus following literature recommendations we have reported mean, median, standard deviation, minimum and maximum in order to provide extensive information about data distribution (Geffré *et al.,* 2009).

With respect to the values obtained, ruminant species included in this work yielded a relatively high number of results below the limit of detection (LD) for Hp and SAA when samples from healthy animals were analyzed. Thereby some Hp results were

below LD for the Pyrenean chamois whereas many results obtained for SAA were below LD both for Pyrenean chamois and Spanish ibex. In ruminants very low values and values below the LD have also been observed in other studies conducted in Merino lambs, Alpine ibex, goats and cattle (González *et al.,* 2008; Lepherd *et al.,* 2009; Rahman *et al.,* 2010; Ceciliani *et al.,* 2012). In the wild boar similar results were observed, with some values below the LD for Hp, CRP, ASG and Pig-MAP. A study conducted in pigs where a thorough characterization on plasma proteins in different herds was evaluated, values below LD for Hp, CRP and Pig-MAP were also obtained (Diack *et al.,* 2011).

In the literature, the influence of factors like breed and age has been demonstrated to have influence on individuals APPs concentration (Glass and Jensen, 2007; Diack *et al.,* 2011). The small sample size used in the present work impeded the study of reference values taking into account such factors.

When dealing with APPs method validation studies there is a need of using high concentration samples. In wild boar and Pyrenean chamois it was not possible to induce an inflammation response experimentally to obtain this type of samples. Thus, samples from diseased animals were used. In wild boars, samples were from traumatized animals and in Pyrenean chamois, from animals with severe bacterial pneumonia. In both cases significant differences with respect to the healthy animals were observed. Therefore, APPs could be potentially used as biomarkers of inflammatory disorders either septic or non septic in wild boar and Pyrenean chamois.

In the literature the increase of APPs has been described for many diseases, including bacterial infection and traumatic acute inflammation situations (Gómez-Laguna *et al.,* 2011). Further studies in the APPs against different diseases and conditions would be advisable in order to establish disease APPs patterns.

In the present study experimentally induction of inflammation in order to study the dinamics of APPs could only be performed in the Spanish ibex. In this species Hp and SAA responses were measured in an experimentally induced inflammation with turpentine and in an experimentally infection with bluetongue virus (BTV).

Turpentine injection has been used to induce inflammation and evaluate APPs response in other research studies with which, in general, our results conform well. As observed before, SAA response after turpentine administration was faster than that observed in Hp (Horadagoda *et al.,* 1999; Heegaard *et al.,* 2000; González *et al.,* 2008). The maximum concentration of both Hp and SAA was reached later in Spanish ibex when compared with goats and about at the same time that in pigs and bovine (Horadagoda *et al.,* 1999; Tecles *et al.,* 2007a; González *et al.,* 2008).

The experimental BTV infection was subclinical and animals kept asymptomatic. There were no significant differences before and after the virus inoculation in Hp and SAA responses although a slightly increase in concentration was observed in both APPs matching with the viraemic peak (Lorca-Oró *et al.,* 2012). To the author's knowledge this is the first study where APPs response against an experimentally induced bluetongue infection has been evaluated. Further studies on APPs response in the bluetongue disease would be advisable.

8. CONCLUSIONS

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1. The methods available in domestic pigs for determining haptoglobin, serum amyloid A, C-reactive protein, acid soluble glycoprotein, ceruloplasmin and porcine major acute phase protein, can be used reliably in wild boar samples and can be used to detect states of disease in this species.

2. Human C-reactive protein turbidimetric immunoassay (Randox) and porcine specific C-reactive protein enzyme linked immunosorbent assay (Tridelta) produce not comparable results in wild boar, however the use of an in-house porcine calibrator with the former kit can be used reliably for heterologous determination of wild boar serum C-reactive protein.

3. Haptoglobin, serum amyloid A, acid soluble glycoprotein and ceruloplasmin can be measured in Pyrenean chamois using domestic ruminant assays; these APPs are good biomarkers for inflammatory infectious disease like bacterial pneumonia in Pyrenean chamois.

4. Available methods for determining haptoglobin and serum amyloid A in domestic ruminants can be used reliably in Spanish ibex samples.

5. In Spanish Ibex, haptoglobin and serum amyloid A can detect acute severe aseptic inflammation, whereas its usefulness is limited in experimentally induced bluetongue virus infection this is not possible.

6. In APPs method validation limit of detection and inter-assay imprecision are the major concerns; therefore in order to improve the last, species-specific materials and automated methods are advisable whenever possible.

9. REFERENCES

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