HEMOGLOBIN SYNTHESIS, FUNCTION AND METABOLISM IN GREYHOUNDS

DISSERTATION

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By

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ABSTRACT

The Greyhounds’ history as racing sighthounds has resulted in a unique physiology that distinguishes them from other dog breeds. Results of routine clinical pathology tests in retired racing Greyhounds (RRGs) frequently lie outside of the reference ranges for dogs, and many of these peculiarities have also been described in other sighthounds.

Iron is necessary in the body for RBC production, but its metabolism has to be tightly regulated because when free and in excess iron is toxic. Most functional iron is found as hemoglobin (Hb). We have investigated iron metabolism in Greyhounds compared to other dog breeds, finding that UIBC and TIBC are lower and %SAT is higher. We also evaluated iron status in current blood donor dogs and effects over time: iron and %SAT are higher and UIBC is lower in blood donors when compared to controls, and over time iron and %SAT decrease and UIBC/TIBC increase. Our canine population donation intensity and iron status is comparable to human “superdonors”. Identification of the biology underlying this has important implications for human medicine.

The Hb molecule is formed by symmetric pairing of alpha and beta globin chains into a structural and functional unit with an iron ion (oxygen-binding site). Due to the high prevalence of hemoglobinopathies in people, the alpha- and beta-globin genes of
humans, mice, and chickens, are well characterized. However, despite the increasing importance of dog as models for human diseases, almost nothing is known about dog Hb genetics, and it is reported they only have one beta globin gene, and that they lack fetal Hb. Hb in Greyhounds has higher oxygen affinity, better oxygen carrying properties and same 2,3-DPG content than other dog breeds. We have defined the genetics of canine alpha- and beta-globin genes, and discovered several SNPs and haplotypes that could explain the Greyhound-specific Hb properties. Galgos Españoles (Spanish Greyhounds) have also better oxygenation parameters and higher Hb affinity than mixed breed dogs.

During intravascular hemolysis, Hb is released into the bloodstream and can cause oxidative damage to the tissues (e.g. hypertension, kidney damage, thrombosis). Haptoglobin (Hp) binds to the free Hb and removes it from circulation. Former racing Greyhounds have low Hp concentration that could partially account for the low α-globulin concentrations in the breed. We have investigated the HP gene in Greyhounds, finding that the gene is expressed in this breed, and it does not have sequence variations that can explain this anhaptoglobinemia. We also found that Galgos Españoles have similar Hp concentrations to non-Greyhound dogs.

Overall, the purpose of this thesis is to be a comprehensive investigation of hemoglobin physiology and genetic characterization in Greyhounds, including its synthesis (i.e. iron) and degradation (i.e. haptoglobin). In order to obtain a better understanding, we also studied Hb and Hp in Galgos Españoles because, although registered as a different breed, Galgos Españoles are phenotypically indistinguishable from Greyhounds, with the difference of not being under the strong selection of the racing industry.
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INTRODUCTION

The Greyhounds’ history as racing sighthounds has resulted in a unique physiology that distinguishes them from other dog breeds. Results of routine clinical pathology tests in retired racing Greyhounds (RRGs) frequently lie outside of the reference ranges for dogs, and many of these peculiarities have also been described in other sighthounds. Major clinicopathologic differences in this breed include higher RBC mass, creatinine concentration, glomerular filtration rate, activities of hepatic enzymes, and concentration of cardiac troponin as well as lower WBC, neutrophil, and platelet counts, thromboelastographic values, and concentrations of serum haptoglobin, total globulins, and T4 (Zaldivar-Lopez and others 2011b).

Iron is necessary in the body for multiple biological functions (e.g. RBC production), but its metabolism has to be tightly regulated because when free and in excess iron is toxic (Coleman 2007). Most functional iron is found as hemoglobin (Hb). Since there is no effective iron excretion mechanism, the maintenance of an appropriate balance is regulated via intestinal absorption, erythropoiesis, recycling from senescent RBCs and storage (McCown and Specht 2011). Iron deficiency causes anemia, which is common in blood donors; and iron overload causes intoxication and tissue damage. Since iron is the functional component (oxygen carrier) of Hb, it plays an important role in exercise physiology (Peeling and others 2008). Iron deficiency is
common in human endurance athletes (Beard and Tobin 2000); racing sled dogs have lower mean serum iron and higher ferritin after the race, but levels are not indicative of iron deficiency (Kenyon and others 2011). We recently investigated the iron status of Greyhounds with and without osteosarcoma (Couto and others 2011), and we found that total iron-binding capacity is lower in Greyhounds (G) than in non-Greyhound (NG) controls, consequently increasing transferrin saturation in the Greyhound group; we hypothesized that this could be a Greyhound peculiarity. Although there is copious information about other physiological parameters and peculiarities in Greyhounds (Zaldivar-Lopez and others 2011b), there is little information about iron homeostasis in this breed, and in the canine species in general.

The Hb molecule is formed by symmetric pairing of alpha-like (i.e. alpha, zeta) and beta-like (i.e. epsilon, gamma, delta, beta) globin polypeptide chains, into a tetrameric structural and functional unit (Perutz 1963). Each of the subunits (globins) has an iron ion (oxygen-binding site) held in a porphyrin ring, where the binding of the oxygen molecules leads to conformational changes (T, R or R2 states). In standard conditions, variation in Hb’s affinity for oxygen is determined mainly by the primary structure of the Hb (i.e. point mutations, nucleotide variations) and the chemical composition of RBCs (i.e. 2,3-diphosphoglycerate (2,3-DPG)). Some of the clinicopathological idiosyncrasies of Greyhounds have been hypothesized to be physiologic adaptations to racing in order to increase oxygen delivery to tissues. Hb function in Greyhounds has been evaluated by determining oxygen affinity, which was found to be higher in Greyhounds than in other breeds (i.e. left shift in the oxyhemoglobin dissociation curve -ODC-), although the RBC 2,3-DPG content was not
significantly different (Sullivan and others 1994). Based on those findings, it was proposed that decreased oxygen release to the tissues could cause increases in erythropoietin production and subsequent increase in RBC production, being the cause of the high Hb and Hct in this breed (Sullivan and others 1994).

At the molecular level, alpha- and beta-like chains are synthesized from two different gene clusters, located on different chromosomes; genes are arranged in developmental order, being turned on and off sequentially (developmental switches). Promoters and cluster-specific upstream regulatory elements (βLCR and αURE) control the expression of the globin genes (Bulger and Groudine 2011; Fromm and Bulger 2009; Tang and others 2008). Adult human Hbs are predominantly HbA (2 alpha and 2 beta chains, α2β2) and HbA2 (2 alpha and 2 delta chains, α2δ2); HbA accounts for about 97% of the erythrocyte protein molecules, HbA2 for 2% and HbF (2 alpha and 2 gamma chains, α2γ2) for 1% in normal adults. The basic globin gene structure and protein folds are similarly conserved in evolution among all placental mammals. A recent phylogenetic study of the beta-globin cluster in placental mammals showed that: the embryonic or epsilon globin (ε) gene is present in all species; fetal globins branched in two, Euarchontoglires (e.g. primates, rodents) retaining the gamma globin gene (γ), and Laurasiatheria (e.g. carnivores, ungulates, bats) the eta globin (η) gene; β-globin is present in all species studied except for order Eulipothypia (e.g. shrew, hedgehog); and δ globin is not present in orders Rodentia (e.g. mouse, rat), Lagomorpha (e.g. rabbit) or Afrotheria (e.g. lesser hedgehog tenrec) (Opazo and others 2008).
Physiologically, fetal Hbs (HbF, 2 alpha and 2 gamma chains, α2γ2) and HbA2 have higher oxygen affinity than the HbA. Hb oxygen affinity in dogs differs among breeds. Greyhounds have a lower P50 than other breeds (Sullivan and others 1994), and their Hb crystallizes in the R2 state, which supports the high affinity of Greyhound Hb (Bhatt and others 2011). This finding is counterintuitive since previous studies in exercise physiology had demonstrated that a decrease in oxygen affinity is beneficial for athletic performance (Biolo and others 2009; Cambier and others 2004). However, modern studies on hemoglobin-based oxygen carriers (HBOCs) have demonstrated that a higher oxygen affinity could be beneficial for tissue oxygenation, by suppressing the vasoconstriction elicited by early off-loading and over-oxygenating tissues at the level of the pre-capillary sphincter, and thus delivering more oxygen to the tissues (Dimino and Palmer 2007). This allows for delivery to the tissues that are in immediate need of oxygen, which should confer a benefit during strenuous exercise (e.g. racing Greyhounds) (Sullivan and others 1994).

Due to the high prevalence of hemoglobinopathies in people, the alpha- and beta-globin genes of humans, mice, and chickens, are well characterized (Fromm and Bulger 2009; Kiefer and others 2008; Schecter 2008). However, despite the increasing importance of dog as models for human diseases (Rowell and others 2011), almost nothing is known about dog Hb genetics, and it is reported they only have one beta globin gene, and that they lack fetal Hb (Brimhall and others 1977; LeCrone 1970; Seal 1969). Evidence of high affinity Hb in Greyhounds suggests that Hb genes might be mutated, affecting the physiological properties of this breed’s Hb.
The Galgo Español (Spanish Greyhound) is a dog breed closely related to the Greyhound [group 10 (sighthounds), section 3 (short-haired sighthounds)]. Similar to Greyhounds, the Galgo Español is also a sporting breed, although they are mainly used for hunting hares and lure coursing instead of sprint racing. Based on their phenotypic similarities and common origins we hypothesized that, similar to Greyhounds, blood gas parameters and oxygen carrying properties of Hb in Galgos would be different from those in other canine breeds.

RBC destruction (due to natural or pathologic causes) can be achieved by two different mechanisms: intravascular and extravascular hemolysis. During intravascular hemolysis, Hb is released into the bloodstream, with the potential of causing oxidative damage to the tissues (e.g. hypertension, kidney damage, thrombosis). The haptoglobin mechanism is the most efficient mechanism to avoid these complications of hemolysis: the Hb molecule (tetramer) is dissociated into dimers, and haptoglobin (Hp) binds very strongly to the free Hb dimers; this Hp-Hb complex is recognized by the CD163 receptor in macrophages and monocytes (blood cells) and binds to it, internalizing and degrading its components (Levy and others 2010). Hp is clinical marker of acute phase response (increased) and intravascular hemolysis (decreased) (Nielsen and Moestrup 2009).

Although in humans the HP gene has three different genotypes (Hp1-1, Hp2-1, Hp2-2), dogs only have one subtype, which is homologous to human Hp 1-1 type (Shim and others 1971). As in humans, the functional Hp unit is a tetramer formed by two αβ Hp dimers (βααβ); the β chains bind to Hb (Shim and others 1971), stabilizing the heme group within the pocket, and thus preventing release of oxidizing radicals (Levy and
others 2010). Former racing Greyhounds have low Hp concentration (Couto and others 2009) that could partially account for the low α-globulin concentrations in the breed.

Overall, the purpose of this thesis is to be a comprehensive investigation of hemoglobin physiology and genetic characterization in Greyhounds, including its synthesis (i.e. iron) and degradation (i.e. haptoglobin). In order to obtain a better understanding, we also studied Hb and Hp in Galgos Españoles (Spanish Greyhounds); although registered as a different breed, Galgos Españoles are phenotypically indistinguishable from Greyhounds, but they are not under the strong selection of the racing industry.
OBJECTIVES

1. To investigate iron metabolism in Greyhounds compared to other dog breeds, evaluating whether there are breed-specific differences.
2. To assess iron status in the canine blood donor population of The Ohio State University, which are predominantly Greyhounds.
3. To evaluate hemoglobin function and venous blood gases in healthy retired racing Greyhounds.
4. To establish reference intervals for blood gases and cooximetry in Greyhounds.
5. To define the genetics of canine alpha- and beta-globins.
6. To investigate if there are differences in the globin genes in Greyhounds compared with non-Greyhound dogs.
7. To evaluate hemoglobin function and venous blood gases in Galgos Españoles (Spanish Greyhounds).
8. To investigate if the cause of low haptoglobin in Greyhounds is due to variations in the HP gene.
9. To measure plasma haptoglobin concentrations in Galgos Españoles in order to determine if they have low haptoglobin concentrations, similarly to Greyhounds.
LITERATURE REVIEW

I. Greyhound idiosyncrasies

Since the early 1990s more than 180,000 retired racing Greyhounds (RRG, Figure 1) have been placed in adoptive homes, and this number increases every year. Therefore, practicing veterinarians are seeing more and more RRG, and consequently clinical pathology laboratories are receiving more samples from these dogs. Thus, it has become necessary for veterinarians (practitioners and clinical pathologists) to become aware of the unique hematologic and biochemical characteristics of the breed (Table 1).

Due to the selection based on performance carried out by the racing Greyhound community (e.g. owners, breeders, trainers), these dogs have acquired unique physiologic adaptations that distinguish them from other breeds. They have larger muscle mass than most breeds (Barnes 1994); higher hematocrit (HCT) (Porter and Canaday 1971);
lengthened carpal, tarsal, metacarpal, and metatarsal bones (Barnes 1994). These adaptations, among others, have likely contributed to the unique hematologic and biochemical characteristics in Greyhounds (compared to non-Greyhound breeds), which have been well documented over the last 50 years (Feeman and others 2003; Lassen and others 1986; Novinger and others 1996; Porter and Canaday 1971; Sullivan and others 1994). Results of routine clinical pathology tests in RRGs frequently fall outside of the reference ranges for dogs. Most of the hematologic differences in Greyhounds have also been described in other sighthounds (Hilppo 1986; Shiel and others 2010). Thus, reference intervals established specific to Greyhound dogs may also apply to other sighthound breeds.
Table 1. Analytes and other features characteristic of Greyhound dogs compared with those of other breeds.

<table>
<thead>
<tr>
<th>Higher Values</th>
<th>Lower Values</th>
<th>Unique Features</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCV/HCT</td>
<td>WBC count</td>
<td>Non-staining eosinophil granules</td>
</tr>
<tr>
<td>Hemoglobin concentration</td>
<td>Neutrophil count</td>
<td></td>
</tr>
<tr>
<td>MCV*</td>
<td>Platelet count</td>
<td>Higher frequency of DEA 1.1-negative dogs</td>
</tr>
<tr>
<td>MCHC</td>
<td>TEG values: K-time, angle, MA and G</td>
<td></td>
</tr>
<tr>
<td>RBC count</td>
<td>Potassium</td>
<td></td>
</tr>
<tr>
<td>Hemoglobin affinity for O₂</td>
<td>Calcium, ionized</td>
<td></td>
</tr>
<tr>
<td>Creatinine</td>
<td>Magnesium ionized</td>
<td></td>
</tr>
<tr>
<td>Glomerular filtration rate</td>
<td>Serum total protein</td>
<td></td>
</tr>
<tr>
<td>Alanine aminotransferase</td>
<td>Total globulins</td>
<td></td>
</tr>
<tr>
<td>Aspartate aminotransferase</td>
<td>α- and β-globulins</td>
<td></td>
</tr>
<tr>
<td>Sodium</td>
<td>IgA and IgM</td>
<td></td>
</tr>
<tr>
<td>Chloride</td>
<td>Haptoglobin</td>
<td></td>
</tr>
<tr>
<td>Total CO₂</td>
<td>Total T4 and free T4</td>
<td></td>
</tr>
<tr>
<td>Bicarbonate</td>
<td>Phosphate</td>
<td></td>
</tr>
<tr>
<td>Cardiac troponin I</td>
<td>Fibrinogen</td>
<td></td>
</tr>
<tr>
<td>Cardiac NT-proBNP</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Reported in only one study.

Abbreviations: PCV, packed cell volume; HCT, hematocrit; MCV, mean cell volume; MCHC, mean corpuscular hemoglobin concentration; RBC, red blood cell; O₂, oxygen; CO₂, carbon dioxide; NT-proBNP, natriuretic peptide of the B-type; WBC, white blood cells; TEG, thromboelastography; MA, maximum amplitude; IgA, immunoglobulin A; IgM, immunoglobulin M; T4, thyroxine; DEA, dog erythrocyte antigen.
Hematology

Most of the research done in Greyhounds has focused on differences in hematologic values. Hematological reference intervals for the breed have been recently published (Campora and others 2011).

Complete blood cell count

Previous studies have reported that Greyhounds have higher HCT, hemoglobin concentration (Hb), mean corpuscular volume (MCV), and mean corpuscular hemoglobin concentration (MCHC) when compared to non-Greyhound dogs (Novinger and others 1996; Porter and Canaday 1971; Steiss and others 2000; Sullivan and others 1994). Most of these studies also reported higher red blood cell (RBC) counts (Novinger and others 1996; Porter and Canaday 1971), with only one study finding a lower mean RBC count among Greyhound dogs (Sullivan and others 1994) (in that particular study, RBC count in Greyhounds was similar to previous reports, but RBC count in the non-Greyhound control group was higher). Traditionally, high HCT, Hb, and RBC count have been considered an adaptation to exercise, under selective breeding for superior track performance, resulting in dogs with higher total oxygen carrying capacity (Zaldivar-Lopez and others 2011a); however, numerous studies are currently being done to further investigate the underlying factors influencing these hematologic features in Greyhounds.
In 2007, Shiel et al. investigated the potential role of age, gender, and training on hematologic values in young pre-training Greyhounds, and the age at which previously described hematologic differences become apparent in the breed. Results of that study showed that pre-training Greyhounds between 9 and 10 months of age had higher HCT, HGB, and RBC counts, and a tendency toward higher MCV when compared to non breed-specific reference intervals (Shiel and others 2007a). This study also reported that HCT, HGB, and RBC counts all correlated positively with age. The hematologic differences were less marked in Greyhounds between 5 and 6 months of age, and there was no significant difference in HCT between dogs in the 9-10 month old group and the 12-13 month old group (Shiel and others 2007a). These findings suggest that in Greyhounds, adult hematologic values are likely reached by 9-10 months of age, which is similar to what has been described in other breeds (Bulgin and others 1970; Ewing and others 1972). At the same time, these findings suggest that training and racing are not primarily responsible for differences in hematologic values between adult Greyhounds and other breeds.

The mean erythrocyte lifespan of Greyhounds has been reported to be significantly shorter than that of non-Greyhound dogs, with reported mean values of 53.6 +/- 6.5 days versus 104.3 +/- 2.2 days, respectively (Novinger and others 1996). Possible explanations for shorter erythrocyte lifespan in Greyhound dogs include differences in erythrocyte membrane structure and hastened removal from circulation due to decreased erythrocyte membrane fluidity and/or increased membrane affinity for IgG, a trigger mechanism for the
removal of senescent RBCs (Novinger and others 1996). Another proposed mechanism for shortened RBC lifespan in Greyhounds is preferential splenic sequestration of labeled cells: Greyhounds reportedly have larger spleens than most other dog breeds, but this theory has not been tested to date. Recently, a study using the same technique (erythrocyte biotinylation) revealed no differences in RBC survival between Greyhounds and non-Greyhounds (Garon and others 2010), so this question remains unanswered, and further studies are needed to clarify this issue.

Macrocytosis has also been reported in Greyhounds (Lassen and others 1986; Porter and Canaday 1971; Sullivan and others 1994). Theoretically, if Greyhound erythrocytes have a shorter lifespan, this should result in a higher number of circulating reticulocytes that have a higher MCV; however, Sullivan reported a mean reticulocyte count of 0.2% for Greyhounds, suggesting that macrocytosis is not the result of reticulocytosis and could represent a true physiologic, breed-specific feature (Sullivan and others 1994). It has been reported that Greyhound RBCs are larger and possibly have higher HGB concentrations, leading to increases in both MCV and MCHC (Porter and Canaday 1971; Sullivan and others 1994). Other studies and data generated in our laboratory, using a Cell-Dyn 3500 hematology analyzer (Abbott, Santa Clara, CA) as a reference instrument and a LaserCyte (IDEXX Laboratories, Westbrook, ME) as a portable bench-top analyzer, have demonstrated MCVs and reticulocyte numbers in Greyhounds and other sighthounds that are within non-breed-specific reference intervals for dogs (Hilppo 1986).
Interestingly, the dog erythrocyte antigen (DEA) distribution is different in Greyhounds than in other breeds. In a recent study we reported that only 13.3% of RRG had DEA 1.1 antigen on their RBCs, in contrast with 60.6% in all other breeds combined; 2.9% had DEA 1.2 antigen (versus 0 in other breeds). Almost two-thirds (63.4%) of the Greyhounds were considered universal donors, in contrast with 18.2% in the other breeds (Iazbik and others 2010). In contrast, 45.5% of Galgos Españoles (Spanish Greyhound), are positive for the DEA 1.1 antigen (Perlado Chamizo and Viñals Florez 2010).

Previous studies have reported lower mean white blood cell (WBC) and neutrophil counts in Greyhounds compared to other breeds (Porter and Canaday 1971; Shiel and others 2007a; Steiss and others 2000). The Greyhound eosinophil has been widely studied. In the early 1960s, Jones and Paris reported that many Greyhounds had “vacuolated” eosinophils, termed “grey” eosinophils, which were void of visible stained cytoplasmic granules (Jones and Paris 1963). In most Greyhounds, eosinophils lack the typical orange granules when using Giemsa/Wright or rapid hematology stains (Jones and Paris 1963). These atypical eosinophils may be mistaken for toxic neutrophils on a routine blood smear stained with Diff-Quik, leading to an unnecessary search for a source of infection (Iazbik and Couto 2005). We recently evaluated the morphologic, ultrastructural, and cytochemical staining characteristics of Greyhound eosinophils, and found vacuolated eosinophils in >50% of Greyhound Wright’s-stained blood smears (Iazbik and Couto 2005). The granules of these vacuolated eosinophils were ultrastructurally similar to those in normal canine eosinophils,
and none of the affected Greyhounds had clinical signs of disease associated with the morphologic change, suggesting that the vacuolated eosinophils represent a change in tinctorial properties rather than a functional abnormality (Iazbik and Couto 2005). Possible causes for the staining abnormalities observed in Greyhound eosinophil granules include difference in the basic proteins that confer the pink-orange hue in normal eosinophil granules or a decrease in the pH of the Greyhound eosinophilic granules, resulting in components that do not readily bind the eosin in Romanowsky-type stains.

Platelets and hemostasis

Sullivan was the first to report lower platelet numbers in Greyhounds than in dogs of other breeds, a finding that has been supported by subsequent studies (Couto and others 2006; Santoro and others 2007; Shiel and others 2007a; Steiss and others 2000). Sullivan proposed the stem-cell competition model of hematopoiesis as a possible mechanism for the low platelet count observed in Greyhounds, suggesting that bipotential stem cells within the bone marrow are programmed to become either megakaryocytes or erythrocyte precursors. These stem cells are susceptible to stimuli for production of one cell line over the other, thus leading to an increase in one cell line and concurrent decrease in the other. Another theory for low platelet counts in Greyhounds relates to the left shift of the oxygen-hemoglobin dissociation curve described by Sullivan, leading to mild hypoxia and potentially resulting in increased production of erythropoietin, increased erythropoiesis, and a consequential decrease in megakaryocytopoiesis.
(Sullivan and others 1994). Shiel also reported a negative correlation between HCT and platelet count in Greyhounds, fitting the bipotential stem cell theory (Shiel and others 2007a).

In 2003, Shibata and others investigated the effects of high erythrocyte concentrations (i.e.; HCT, 85%) on hemostasis in a population of transgenic mice overexpressing the human erythropoietin gene. Similar to the Greyhounds, these transgenic mice also had low platelet concentrations. When the platelet concentration was adjusted based on the plasma volume fraction, the transgenic mice had platelet concentrations similar to those in wild-type mice, suggesting that the number of platelets per unit of blood is not necessarily equivalent to the number of platelets per unit of plasma in patients with high HCT (Shibata and others 2003).

Other proposed mechanisms for low platelet counts in Greyhounds include splenic or pulmonary sequestration or a chronic, low-grade, immune-mediated process leading to decreased platelet lifespan (Sullivan and others 1994). The latter mechanism was ruled out in a study that investigated the presence of platelet surface-associated IgG (PSAIgG) in Greyhounds (Santoro and others 2007). PSAIgG was not detected in any of the samples, suggesting that the lower platelet count in the breed was not attributable to immune-mediated mechanisms (Santoro and others 2007). As platelet numbers are inversely correlated with iron stores (Kadikoylu and others 2006), another possibility is that Greyhounds have higher body iron content, owing to their high HCTs, and hence, have lower platelet counts. In our experience, platelets tend to
clump more in Greyhounds than in other breeds, as is the case with feline platelets. In summary, in-depth investigation of thrombocytopenia is not warranted in healthy Greyhounds with platelet counts of $<100,000/\mu$L, as the low count likely represents a breed-related trait, or a false decrease related to platelet clumping.

The main function of the hemostatic system is to keep the blood flowing within the cardiovascular system. The term “Greyhound bleeder” has been used to describe dogs that tend to bleed spontaneously following minor trauma or after a simple surgical procedure (Lara-Garcia and others 2008). Severe postoperative bleeding 1-4 days after limb amputation for osteosarcoma or trauma has also been reported in Greyhounds, often resulting in the need for blood component therapy during this postoperative period (Lara-Garcia and others 2008). Historically, Greyhounds with spontaneous bleeding have had normal platelet counts for the breed and normal von Willebrand factor (vWF) concentration, one-stage prothrombin time (OSPT), and activated partial thromboplastin time (APTT) at the time of postoperative hemorrhage, making common bleeding disorders such as thrombocytopenia and clotting factor or vWF deficiencies unlikely causes of the bleeding.

We recently investigated platelet function in Greyhounds using the PFA-100 System (Dade Behring, West Sacramento, CA) to evaluate primary hemostasis in healthy Greyhounds compared to healthy non-Greyhounds, and in Greyhounds with post operative bleeding (Couto and others 2006). Platelet count, plasma VWF:Ag concentration, and VWF activity measured using a
collagen-binding assay were correlated with the results of the PFA-100 assays. Surprisingly, the lower platelet counts in Greyhounds were not associated with prolongation of the closure time (CT), the time required for a platelet plug to form in the capillary aperture and halt blood flow, and Greyhounds had a shorter mean CT than did non-Greyhounds; however, CT ranges were similar to reported values for other breeds (Couto and others 2006). The shorter CTs in Greyhounds are likely explained by the higher PCV, and thus viscosity, in the breed (Porter and Canaday 1971; Steiss and others 2000) as higher PCV and whole blood viscosity lead to peripheral platelet distribution and consequent increased interaction with the blood vessel surface. Shorter CTs in the breed may be an adaptive platelet response to accommodate higher shear in Greyhounds (Couto and others 2006), as Greyhounds also have significantly higher arterial blood pressure and aortic velocity than do non-Greyhounds (Fabrizio and others 2006; Pape and others 1986; Pape and others 1984).

The most common canine hereditary hemostatic defect is von Willebrand disease (Brooks 1999). Although some VWF-deficient Greyhounds have been identified in breed surveys (Brooks 1999; Stokol and Parry 1993) and in coagulation screens, the disease does not appear to be common in Greyhounds. Of the Greyhounds tested at The Ohio State University and at the Cornell Comparative Hemostasis Laboratory, < 10% (22/216) had activities of VWF below 30%; in most dogs with low vWF activity, there was evidence of hemolysis in the plasma (unpublished data).
Thromboelastography (TEG) permits evaluation of blood coagulation through assessment of the speed and strength of clot formation. TEG is dependent on the function of primary and secondary hemostasis and fibrinolysis, all of which can be affected by certain illnesses, environmental conditions, and pharmacologic agents (Hoffman and Monroe 2007). We recently reported that Greyhounds have slower clot kinetics and weaker clot strength compared with non-Greyhounds, lending support to increased bleeding tendencies in Greyhounds following minor trauma or surgical procedures (Vilar and others 2008). The observed differences may be related to blood viscosity, as the higher HCTs in Greyhounds result in less plasma per unit volume of blood (Bodey and Rampling 1998); these hemostatic findings are similar to those reported in polycythemic mice (Shibata and others 2003). It is also known that RBCs interfere with TEG variables; for example, it has been shown in vitro that an increase in RBC mass reduces clot strength and kinetics, even when TEG analysis is performed in plasma samples without platelets (Bochsen and others 2011). In our TEG study, we found no significant differences in fibrinogen concentration between Greyhounds and non-Greyhounds, suggesting that the slower clotting kinetics and weaker clot strength cannot be attributed to hypofibrinogenemia in the former (Vilar and others 2008).

We investigated the prevalence of postoperative bleeding in RRGs, finding that 26% (23/88) of Greyhounds had moderate to severe bleeding 36-48 hours after routine gonadectomy (Lara-Garcia and others 2008). Primary and
secondary hemostasis were evaluated preoperatively by measuring platelet count; OSPT, aPTT and fibrinogen concentration (with the ACL 200); platelet function (with the PFA-100); plasminogen, antiplasmin (AP), antithrombin (AT) activities; D-dimer concentration; VWF (VWF:Ag) and VWF collagen-binding assay (vWF:CBA) activities; and Factor XIII was reported as normal or abnormal. Hemostasis assays were repeated postoperatively in those RRGs that had bleeding complications at the time of the detection of the bleeding, and in an age- and sex-matched control group of RRGs that had the same surgical procedures at the same time and did not bleed (Lara-Garcia and others 2008). Of the variables measured, the only differences found were lower AP and AT and higher vWF:CBA activities preoperatively in bleeders compared with non-bleeders. At the time of the bleeding, bleeders had a lower platelet count and HCT, shorter OSPT, and higher fibrinogen concentration. Results suggested that excessive postoperative bleeding in RRGs was not attributable to a defect in primary or secondary hemostasis, but may have been related to enhanced fibrinolysis, as the “bleeders” had lower antiplasmin concentrations than did “non-bleeders” preoperatively. Furthermore, we proposed that this could be an adaptation to racing or an evolutionary trait intended to prevent clotting of blood with high viscosity during exercise (Lara-Garcia and others 2008). The pedigrees and racing performances of “bleeders” and “non-bleeders” were not evaluated; thus, it is possible that a genetic alteration leading to defects in clotting or fibrinolysis may play a role in the observed bleeding as racing Greyhounds are derived from a small genetic pool (Lara-Garcia and others 2008).
Clinical Chemistry

Several studies reported differences in specific serum biochemical values in Greyhounds when compared to those of the general canine population (Hilppo 1986; Lassen and others 1986; Porter and Canaday 1971; Shiel and others 2007a; Steiss and others 2000). Veterinarians working with Greyhounds must consider these breed-specific differences when interpreting serum chemistry profiles, as interpretation based on standard canine reference intervals may lead to misdiagnoses. These differences have been confirmed in a recent study with a large number of healthy animals, where even narrower, and therefore more breed-specific reference intervals, have been established for Greyhound biochemistry values (Dunlop and others 2011).

Blood Urea Nitrogen and Creatinine

Previously published Greyhound-specific reference ranges for BUN and creatinine are 10-22 mg/dL and 0.8-1.6 mg/dL, respectively (Steiss and others 2000). The BUN was similar to that in other breeds. However, the mean creatinine concentrations were significantly higher in Greyhounds than in non-Greyhound dogs (1.6 mg/dl and 1.03 mg/dl, respectively) (Feeman and others 2003). Greyhounds have considerable muscle mass and predictably have higher body stores of phosphocreatine, which may result in higher serum creatinine concentrations. Because creatinine is well absorbed from the intestinal tract, high-creatine diets containing animal tissues fed to many racing Greyhounds
may also contribute to the high serum creatinine concentration in the breed. However, high serum creatinine concentrations also occur in retired racers, years after they have left the track (Feeman and others 2003). Another possible reason for high serum creatinine is low glomerular filtration rate (GFR). We investigated the GFR of normal Greyhounds and non-Greyhounds to determine whether or not decreased GFR contributed to high serum creatinine in the breed. Greyhounds had significantly higher GFR and serum creatinine concentrations than non-Greyhound dogs (Drost and others 2006). Based on these results, the most likely cause of high serum creatinine concentrations in Greyhounds is the large muscle mass. Pursuing additional evaluation in an otherwise healthy Greyhound with mild increases in creatinine concentration (i.e.; 1.2 to 2.1 mg/dL) is not warranted. These differences in creatinine concentrations were recently confirmed using a large number of Greyhounds, reporting an even narrower reference interval (1.1-2.0 mg/dL), respectively (Dunlop and others 2011).

Liver enzymes

Some liver enzymes are used as markers of hepatocellular injury (i.e.; ALT, AST) or cholestasis (i.e.; ALP, GGT). Reference values for select liver enzymes have been reported in a small group of racing Greyhounds (Egan 1978). The ranges for ALT and ALP activities reported in that study were very wide in Greyhounds; however, mean activities were comparable to those in other breeds (Egan 1978). Egan concluded that the wide range of values for these enzymes
makes their use as indicators of hepatic disease unreliable in Greyhounds (Egan 1978). Recently, Dunlop has reported narrower reference intervals for liver enzymes in Greyhounds, finding higher ALT activities compared to the canine reference intervals (Dunlop and others 2011). As muscular dystrophy (and subsequent necrosis) can increase ALT in dogs with no evidence of liver disease (Valentine and others 1990), we hypothesize that muscle damage due to intense racing could be the cause of this mild ALT elevation in Greyhounds.

**Serum Electrolytes and Acid-Base Balance**

Higher serum concentrations of Na and/or Cl in Greyhounds than in non-Greyhound dogs have been reported (Egan 1977; Lassen and others 1986; Porter and Canaday 1971; Steiss and others 2000). Previously published Greyhound-specific reference ranges for Na and Cl are 149-157 mmol/L and 110-122 mmol/L, respectively (Steiss and others 2000). Greyhounds were also reported to have increased serum total CO₂ concentration compared with that of non-Greyhound dogs. Although increased total CO₂ concentration is typically indicative of metabolic alkalosis, none of the dogs in the study had any findings consistent with alkalosis (Steiss and others 2000).

Lassen et al. found that mean serum calcium concentrations in racing Greyhounds decrease during the racing season, but are never outside the reference interval (Lassen and others 1986). However, a lower calcium reference interval than the standard reference interval has been recently reported using a large population of healthy non-racing Greyhounds (Dunlop and others 2011). In
a study in retired racing Greyhounds using a critical care analyzer (STP CCX Analyzer, Nova Biomedical, Waltham, MA, USA) performed by our group (Zaldívar-López and others 2010) we found that potassium, ionized calcium and ionized magnesium concentrations were significantly lower than in the non-Greyhound group. In contrast, Greyhounds had higher glucose concentration when using this instrument, but a lower glucose concentration than the non-Greyhounds when using a Hitachi 911 analyzer (data not published). As previously reported (Feeman and others 2003; Steiss and others 2000), bicarbonate concentrations were also higher in Greyhounds than in non-Greyhounds (Zaldívar-López and others 2010).

Protein

Recently published Greyhound-specific reference ranges for total protein, albumin, and globulin are 5.2-6.7 g/dL, 2.7-3.7 g/dL, and 2.2-3.3 g/dL, respectively (Dunlop and others 2011). That is the largest but not the only study reporting differences in Greyhounds: lower plasma and serum protein concentrations (Fayos and others 2005; Porter and Canaday 1971; Sullivan and others 1994) and significantly lower globulin concentration (Steiss and others 2000) were also reported by other groups. Hypoglobulinemia in Greyhounds was investigated by analyzing serum proteins using agarose gel protein electrophoresis (Fayos and others 2005). The concentrations of total protein (mean ± SD, 5.56 ± 0.39 g/dL), total globulins (2.23 ± 0.24 g/dL), and \( \alpha_1, \alpha_2, \beta_1, \) and \( \beta_2 \) globulins (0.33 ± 0.05, 0.27 ± 0.10, 0.20 ± 0.06 and 0.21 ± 0.07 g/dL, respectively) were significantly lower and the albumin-to-globulin (A:G) ratio (1.23 ± 0.25 g/dL) was
significantly higher in Greyhounds than in non-Greyhounds, whose respective values for concentrations of total protein, total globulins, and $\alpha_1$, $\alpha_2$, $\beta_1$, and $\beta_2$ globulins and A:G ratios were $6.07\pm0.45$, $2.83\pm0.35$, $0.46\pm0.14$, $0.47\pm0.14$, $0.32\pm0.12$, $0.34\pm0.09$, and $1.17\pm0.18$ g/dL. (Fayos and others 2005). No significant difference was found in albumin or $\gamma$-globulin concentrations. Further studies are necessary to identify the individual proteins associated with low $\alpha$- and $\beta$-globulin concentrations in Greyhounds. Differences in serum concentrations of acute phase proteins may help explain the low $\alpha$-globulin concentration (see below), and lower IgA and IgM concentrations in Greyhounds may contribute to the low $\beta$-globulin concentrations in Greyhounds (Clemente and others 2010). Possible mechanisms of hypoglobulinemia include plasma volume expansion associated with chronic conditioning and training; however, this mechanism does not explain why only some protein fractions are affected or why they persist after Greyhounds retired from racing. Since hyperviscosity has been associated with hyperglobulinemia in humans and dogs with myeloma (Lindsley and others 1973; Ramaiah and others 2002) and in people receiving immunoglobulin therapy (Bentley and others 2011), we hypothesize that opposite phenomenon (i.e. hypoproteinemia, hypoglobulinemia) may happen in Greyhounds, being an adaptive mechanism to decrease serum viscosity, as Greyhounds have higher PCVs and blood viscosity than do other breeds (Bodey and Rampling 1998).

We also evaluated the concentration of acute phase proteins in Greyhounds (Couto and others 2009). Serum concentrations of C-reactive protein (CRP), haptoglobin (Hp), acid-soluble glycoprotein (ASG), ceruloplasmin (CP), and serum amyloid A (SAA) were measured and compared
between a group of healthy RRGs and age- and gender-matched healthy non-Greyhound controls. The concentrations of Hp (by both colorimetric and immunoturbidimetric methods) and ASG were significantly lower in Greyhounds than in non-Greyhounds; CRP and CP concentrations were not significantly different between groups; SAA concentration was below the detection limit in all dogs. The cause for low Hp concentration in Greyhounds is not apparent, and is being investigated (more details in Chapter 3), as findings may provide insight regarding the value of Hp as an acute phase marker in Greyhounds. As ASG constitutes a heterogeneous group of proteins that may vary among animal species, it is unknown which of the individual proteins may be responsible for low ASG values in Greyhounds. Because both Hp and ASG migrate in the \( \alpha \)-globulin fraction, results from this study may explain the previously described low \( \alpha \)-globulin concentrations in Greyhounds (Fayos and others 2005).

**Thyroid hormones**

Greyhounds have basal total T\(_4\) (tT\(_4\)) concentrations below non-breed-specific reference intervals (Beale and others 1992; Ferguson 1997; Gaughan and Bruyette 2001; Hill and others 2001; Lee and others 2004; Nachreiner and Refsal 1992; Panciera and others 2003; Rosychuk and others 1988; van Geffen and others 2006). Free T\(_4\) (fT\(_4\)) concentrations can also be low, although not to the same extent as tT\(_4\), with reported mean values ranging from 6.0 to 11.6 pmol/L (Gaughan and Bruyette 2001; Hill and others 2001). No significant difference
has been described between the concentrations of TSH in Greyhounds and those in other breeds; interestingly, most Greyhounds have TSH concentrations in the lower end of the reference interval (Gaughan and Bruyette 2001; Hill and others 2001). Highly variable T₃ concentrations have been reported (Shiel and others 2007b); however, fT₃ concentrations in Greyhounds are usually below non-breed-specific reference intervals (Cowan and others 1997; Hill and others 2001; Rosychuk and others 1988). A lack of increases in tT₄ concentration after administration of exogenous TSH has also been described in Greyhounds (Gaughan and Bruyette 2001; Rosychuk and others 1988). Hill showed that trained and racing Greyhounds had lower tT₄ concentrations than retired racers, and that tT₄ concentrations were higher five minutes after racing (Hill and others 2001). In 2007, Shiel investigated thyroid hormone concentration in pre-training Greyhounds (Shiel and others 2007b). Results of this study showed that young pre-training Greyhounds had lower tT₄ and fT₄ concentrations and a tendency toward higher total T₃ (fT₃) concentrations when compared to non-breed-specific reference intervals (Shiel and others 2007b). Concentrations of TSH were within the non-breed-specific reference intervals, a finding that is consistent with previous studies investigating TSH in adult Greyhounds (Gaughan and Bruyette 2001; Hill and others 2001).

Pinilla and his group evaluated thyroid scintigraphy as a method of assessing thyroid function in Greyhounds suspected of having primary hypothyroidism based on standard thyroid hormone testing. Thyroidal (99m)TcO₄⁻ uptake values (mean +/- SD, 0.76 +/- 0.26%) were within the
reference intervals published for euthyroid dogs (0.39-1.86%) making hypothyroidism highly unlikely in the study Greyhounds (Pinilla and others 2009).

A recent study by Shiel’s group retrospectively assessed the use of serum thyroid hormone concentrations by veterinarians to diagnose hypothyroidism in 398 sighthounds including Greyhounds (n=347) and other sighthounds such as Borzois (n=22), Salukis (n=11), Irish Wolfhounds (n=14), and Scottish Deerhounds (n=4). A cross-sectional study was also performed to assess serum thyroid hormone concentrations in healthy Salukis. Results of this study revealed that practitioners who had submitted blood samples for thyroid hormone testing had diagnosed hypothyroidism in 286 of 398 (71.9%) sighthounds on the basis of low serum concentrations of tT₄ or tT₃ alone. Seventeen (4.3%) sighthounds also had low fT₄ or fT₃ concentrations, and 30 (7.5%) sighthounds had a diagnosis of hypothyroidism made despite the fact that all measured thyroid hormone concentrations were within their respective reference intervals. Only 65 (16.3%) sighthounds had additional abnormalities suggestive of hypothyroidism (high serum TSH concentration or positive thyroglobulin autoantibody [TGAA]) (Shiel and others 2010). In addition, when compared with standard (non-breed-specific) reference intervals, 154/282 (54.6%) and 67/216 (31%) of the Salukis had tT₄ and fT₄ values (respectively) below reference intervals. These findings support the fact that other sight hound breeds also have low serum tT₄ concentrations (Shiel and others 2010).
Cardiac biomarkers

Previously documented cardiovascular findings in healthy Greyhounds include a higher heart weight-to-body weight ratio, higher left ventricular free wall thickness, functional murmurs with no detectable structural or physiologic abnormalities, and higher vertebral heart scores than non-Greyhounds (Carew and Covell 1978; Fabrizio and others 2006; Gunn 1989; Marin and others 2007; Page and others 1993; Pape and others 1986; Pape and others 1984; Snyder and others 1995). Cardiac troponin I (cTnI) is a polypeptide found specifically in cardiac muscle; serum concentrations of cTnI have been used as a diagnostic and prognostic indicator of heart disease, including cardiac infarction in humans and cardiomyopathy in dogs. In 2009, LaVecchio compared serum cTnI concentrations between RRGs, Boxers with and without arrhythmogenic right ventricular cardiomyopathy (ARVC), and non-Boxer control dogs (LaVecchio and others 2009). Greyhounds had significantly higher serum cTnI concentrations compared to non-Greyhound dogs; there were no significant differences in serum cTnI between Greyhounds and Boxers with and without ARVC. Interestingly, several Greyhounds in this study had cTnI concentrations within or above the range of the Boxers with ARVC. Possible explanations for this high concentration include the Greyhound’s higher heart weight-to-body weight ratio (Gunn 1989) and undetermined underlying myocardial pathology; however, the latter explanation is less likely given that the Greyhounds in LaVecchio’s study were healthy, had no arrhythmias on auscultation, and remained asymptomatic for heart disease months after completing the study.
Greyhounds with a heart murmur, high vertebral heart size (VHS), and high cTnI could be incorrectly diagnosed with myocardial disease; thus, until a more precise reference range is established, caution should be employed when interpreting serum cTnI concentrations in Greyhounds with suspected cardiac disease (LaVecchio and others 2009).

Natriuretic peptides of the B-type (BNP) are neuroendocrine peptides synthesized constitutively in atrial myocytes and released in response to volume or pressure overload. In humans, BNP concentrations increase in association with heart disease and reflect disease severity and prognosis. Furthermore, plasma NT-pro BNP has the potential to distinguish between heart failure and respiratory disease in humans and small animals (Oyama and others 2007; Schober and others 2011). We recently measured NT-proBNP concentration in Greyhounds using a commercially available assay (Cardiopet® proBNP, IDEXX Laboratories, Westbrook, ME, USA), and found that Greyhounds have significantly higher concentrations than non-Greyhound dogs (Greyhounds: mean 1051 pMol/L, SD 439 pMol/L; non-Greyhounds: mean of 684 pMol/L, SD 446 pMol/L). Fourteen of the Greyhounds had results above 900 pMol/L, which is the cutoff for heart disease diagnosis. Therefore, results from this test in this breed should be interpreted cautiously.

**Urine**

The fractional excretion (FE) of an electrolyte is an expression of the amount excreted in the urine in relationship to the amount that is filtered and reabsorbed
by the kidneys. Due to the fact that serum concentrations of Na and Cl are higher in Greyhounds than in non-Greyhounds (Lassen and others 1986; Porter and Canaday 1971; Steiss and others 2000), the use of Greyhound-specific reference limits of urinary FE of electrolytes is recommended when investigating renal tubular disease in the breed (Bennett and others 2006). Bennett established reference intervals for urinary FE of electrolytes from 48 Greyhounds using a bootstrap estimate, and found that although the fractional excretions of K, Cl, Ca, and phosphate were significantly lower in Greyhounds than those published for dogs, the differences were likely not been clinically relevant (Bennett and others 2006). However, in that study non-Greyhound control dogs were not used for comparison. Veterinary practitioners may use these reference limits when investigating renal tubular disease in Greyhound dogs (Bennett and others 2006).
Establishment of Greyhound-specific reference intervals at The Ohio State University

Due to the well-known clinicopathological differences of this breed, the establishment of Greyhound-specific reference intervals has been of great interest for the Greyhound Health and Wellness Program at The Ohio State University Veterinary Medical Center for a long time. Several studies have been done, involving different aspects of the breed’s physiology (hematology, chemistry, coagulation and blood gases), with the purpose of characterizing these differences, and therefore proposing reference intervals that are valid and specific for the breed.

Dogs used for generation of reference intervals over the years have been from 2 different populations: 1) the Greyhound spay/neuter/dental clinic for third or fourth year veterinary medical students, and 2) the Greyhound blood donor population. All studies were under approved Institutional Animal Care and Use Committee protocols (IACUC protocol). All reference intervals were established by using the 5th and 95th percentiles, despite the fact that the sample size was not optimal in some cases and not all parameters were normally distributed. All samples were collected from the jugular vein and placed into different tubes depending on analysis (sodium EDTA for CBCs, tubes with sodium citrate for hemostasis assays, and tubes without anticoagulant for biochemical profiles (Monoject, Sherwood, St Louis, MO); samples were processed and analyzed
within 4 hours of collection. CBCs were performed using a LaserCyte or a ProCyte Dx analyzer with the appropriate software settings (Table 2). If flags were obtained, the dog was not re-evaluated owing to lack of additional blood samples, and the sample was excluded. In a subset of dogs, CBCs were evaluated using a Cell-Dyn 3500 hematology analyzer. Differential WBC counts were performed manually.

For conventional hemostasis assays, OSPT, aPTT, and fibrinogen concentration, 2 analyzers were used: the ACL 200 coagulation analyzer (Instrumentation Laboratory, Lexington, MA) and the Stago Compact analyzer (Diagnostica Stago, Parsippany, NJ) (Table 3). Serum biochemistry profiles (n=100) were performed on a COBAS c501 chemistry analyzer (Roche Diagnostics, Indianapolis, IN, USA) (Table 4). Thrombelastography was done as previously described (Vilar and others 2008). Initially, 20 µl of CaCl₂ were placed in the prewarmed cup of the TEG-5000 (Thrombelastograph, TEG Haemoscope, Niles, IL); 340 µl of citrated blood was then added to make a total volume of 360 µl. No activator was used. Tracings were obtained after 120-180 minutes of running time at 37°C (Table 5).

In summary, Greyhounds have hematologic and serum biochemical values that frequently lie outside the reference intervals established for non-Greyhound dogs, suggesting differences in many aspects of their physiology. For these reasons, the establishment of breed- or group-specific (e.g.; sighthounds) reference intervals is essential for the correct diagnosis and subsequent treatment of medical ailments based on clinicopathologic abnormalities. As the number of
RRG adoptions increases in the United States, veterinarians will be faced with
the challenge of interpreting laboratory parameters in light of the hematologic
differences of the breed. Although the sample size in these studies is not as large
as recommended for the creation of reference intervals, we provide approximate
intervals that will benefit clinicians by providing a better understanding of the
hematologic and biochemical differences between Greyhounds and non-
Greyhound dogs, and helping to avoid misdiagnoses based on incorrect reference
intervals for these sighthound breeds.
Table 2. Greyhound-specific and non-breed-specific hematology reference intervals at The Ohio State University and published reference intervals for Greyhounds.

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<td></td>
<td>Greyhounds LaserCyte†</td>
<td>Greyhounds ProCyte†</td>
</tr>
<tr>
<td></td>
<td>(n = 151)</td>
<td>(n = 48)</td>
</tr>
<tr>
<td>Total WBC (x10^9/L)</td>
<td>4.4-10.8 (5.5-16.9)</td>
<td>3.6-6.9 (5.1-16.7)</td>
</tr>
<tr>
<td>Lymphocytes (x10^3/L)</td>
<td>0.2-2.5 (0.5-4.9)</td>
<td>0.8-2.2 (1.1-5.1)</td>
</tr>
<tr>
<td>Neutrophils (x10^3/L)</td>
<td>2.6-7.4 (2.0-12.0)</td>
<td>2.1-5.2 (2.9-11.6)</td>
</tr>
<tr>
<td>Monocytes (x10^3/L)</td>
<td>0.3-1.1 (0.3-2.0)</td>
<td>0.1-0.3 (0.2-1.1)</td>
</tr>
<tr>
<td>Eosinophils (x10^3/L)</td>
<td>0-0.1 (0-1.5)</td>
<td>0-1.0 (0.2-1.2)</td>
</tr>
<tr>
<td>Basophils (x10^3/L)</td>
<td>(0.00-0.01)</td>
<td>0.0-0.1 (0.0-0.1)</td>
</tr>
<tr>
<td>HCT (%)</td>
<td>42.7-61.5 (37.0-55.0)</td>
<td>51.5-71.0 (37.3-61.7)</td>
</tr>
<tr>
<td>HGB (g/dL)</td>
<td>15.1-20.4 (12.0-18.0)</td>
<td>17.4-24.1 (13.1-20.5)</td>
</tr>
<tr>
<td>RBC (x10^12/L)</td>
<td>6.0-9.4 (5.5-8.0)</td>
<td>7.4-10.2 (5.6-8.8)</td>
</tr>
<tr>
<td>Reticulocytes (x10^6/L)</td>
<td>17.2-45.7 (14.7-17.9)</td>
<td>10.0-97.7 (6.6-100.7)</td>
</tr>
<tr>
<td>MCV (fl)</td>
<td>66-79 ND</td>
<td>63-76 (62-74)</td>
</tr>
<tr>
<td>MCHC (g/dL)</td>
<td>29.4-38.2 (30.0-37.5)</td>
<td>33.1-35.1 (32.0-37.9)</td>
</tr>
<tr>
<td>MCH (pg)</td>
<td>20.9-28.6 (18.5-30.0)</td>
<td>21.5-26.2 (21.2-25.9)</td>
</tr>
<tr>
<td>RDW (%)</td>
<td>14.7-15.9 (14.7-17.9)</td>
<td>16.0-22.2 (13.6-21.7)</td>
</tr>
<tr>
<td>Platelet count (x10^9/L)</td>
<td>117-295 (175-500)</td>
<td>112-205 (148-484)</td>
</tr>
<tr>
<td>MPV (fl)</td>
<td>6.9-11.8 (NA)</td>
<td>8.6-11.9 (8.7-13.2)</td>
</tr>
</tbody>
</table>

*Intervals in parentheses are reference intervals for dogs (all breeds) provided by the manufacturer.
†LaserCyte and ProCyte, IDEXX Laboratories, Westbrook, ME; Cell-Dyn 3500, Abbott Diagnostics, Abbott Park, IL; Advia 120 and 2120, Siemens Healthcare Diagnostics, Deerfield, IL.
ND, not done; NA, not available.
Greyhound-specific reference intervals (Campora and others 2011); non-breed canine specific reference intervals (Moritz and others 2004).
### Table 3. Greyhound-specific and non-breed-specific reference intervals for hemostasis profiles at The Ohio State University.

<table>
<thead>
<tr>
<th>Assay</th>
<th>Greyhounds ACL 200* (n = 88)</th>
<th>Greyhounds Stago Compact† (n = 62)</th>
<th>Non-breed-specific ACL 200*</th>
</tr>
</thead>
<tbody>
<tr>
<td>OSPT (seconds)</td>
<td>6.2-7.6</td>
<td>6.9-8.3</td>
<td>6-7.5</td>
</tr>
<tr>
<td>aPTT (seconds)</td>
<td>11.2-18.1</td>
<td>9.7-12.1</td>
<td>9-21</td>
</tr>
<tr>
<td>Fibrinogen (mg/dL)</td>
<td>83-190</td>
<td>89-180</td>
<td>100-384</td>
</tr>
</tbody>
</table>

*Samples collected in 3.8% sodium citrate or in 3.2% sodium citrate; ACL 200 (Instrumentation Laboratory, Lexington, MA); STA Compact CT (Diagnostica Stago, Parsippany, NJ).

OSPT, one-stage prothrombin time; aPTT, activated partial thromboplastin time.

### Table 4. Greyhound-specific and non-breed-specific reference intervals for serum biochemical profiles and protein fractions at The Ohio State University

<table>
<thead>
<tr>
<th>Serum Biochemistry*</th>
<th>Greyhounds (n=100)</th>
<th>Non-breed-specific</th>
</tr>
</thead>
<tbody>
<tr>
<td>BUN (mg/dL)</td>
<td>11-21</td>
<td>5-20</td>
</tr>
<tr>
<td>Creatinine (mg/dL)</td>
<td>1.0-1.7</td>
<td>0.6-1.6</td>
</tr>
<tr>
<td>Phosphate (mg/dL)</td>
<td>2.3-5.3</td>
<td>3.2-8.1</td>
</tr>
<tr>
<td>Calcium, total (mg/dL)</td>
<td>9.4-11.4</td>
<td>9.3-11.6</td>
</tr>
<tr>
<td>Sodium (mmol/L)</td>
<td>144-156</td>
<td>143-153</td>
</tr>
<tr>
<td>Potassium (mmol/L)</td>
<td>3.5-4.4</td>
<td>4.2-5.4</td>
</tr>
<tr>
<td>Chloride (mmol/L)</td>
<td>108-119</td>
<td>109-120</td>
</tr>
<tr>
<td>Anion gap</td>
<td>9.0-19.9</td>
<td>15-25</td>
</tr>
<tr>
<td>Osmolality, calculated (mmol/L)†</td>
<td>285-310</td>
<td>285-304</td>
</tr>
<tr>
<td>Bicarbonate (mmol/L)</td>
<td>20-31</td>
<td>16-25</td>
</tr>
<tr>
<td>Alanine aminotransferase (U/L)</td>
<td>28-82</td>
<td>10-55</td>
</tr>
<tr>
<td>Aspartate aminotransferase (U/L)</td>
<td>24-57</td>
<td>12-40</td>
</tr>
<tr>
<td>Alkaline phosphatase (U/L)</td>
<td>19-90</td>
<td>15-120</td>
</tr>
<tr>
<td>C-ALP (U/L)‡</td>
<td>0-31</td>
<td>0-6</td>
</tr>
<tr>
<td>Creatine kinase (U/L)</td>
<td>76-254</td>
<td>50-400</td>
</tr>
<tr>
<td>Cholesterol (mg/dL)</td>
<td>91-210</td>
<td>80-315</td>
</tr>
<tr>
<td>Bilirubin (mg/dL)</td>
<td>0.1-0.3</td>
<td>0.1-0.4</td>
</tr>
<tr>
<td>Glucose (mg/dL)</td>
<td>77-121</td>
<td>77-126</td>
</tr>
<tr>
<td>Total protein (g/dL)</td>
<td>4.8-6.3</td>
<td>5.1-7.1</td>
</tr>
<tr>
<td>Albumin (g/dL)</td>
<td>2.9-3.9</td>
<td>2.9-4.2</td>
</tr>
<tr>
<td>Globulin (g/dL)</td>
<td>1.7-3.0</td>
<td>2.2-2.9</td>
</tr>
<tr>
<td>Albumin:globulin ratio</td>
<td>1.0-2.2</td>
<td>0.8-2.2</td>
</tr>
</tbody>
</table>

**Agarose Gel Electrophoresis**

<table>
<thead>
<tr>
<th>Protein</th>
<th>Greyhounds (n=100)</th>
<th>Non-breed-specific</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-1 globulin (g/dL)</td>
<td>0.3-0.4</td>
<td>0.3-0.9</td>
</tr>
<tr>
<td>α-2 globulin (g/dL)</td>
<td>0.1-0.5</td>
<td>0.3-0.7</td>
</tr>
<tr>
<td>β-1 globulin (g/dL)</td>
<td>0.1-0.3</td>
<td>0.2-0.7</td>
</tr>
<tr>
<td>β-2 globulin (g/dL)</td>
<td>0.1-0.3</td>
<td>0.2-1.4</td>
</tr>
<tr>
<td>γ-globulin (g/dL)</td>
<td>0.8-1.7</td>
<td>1.0-1.9</td>
</tr>
</tbody>
</table>

*All analytes were measured on the COBAS c501 chemistry analyzer (Roche Diagnostics, Indianapolis, IN).
†Osmolality was calculated using the following formula: 1.86 x Na + (Glucose/18) + (UN/2.8) + 9.
‡Corticosteroid-induced alkaline phosphatase.
Table 5. Reference intervals for thromboelastographic (TEG) measurements in Greyhounds

<table>
<thead>
<tr>
<th>Measurement</th>
<th>Reference Interval Greyhounds</th>
<th>Reference Interval Non-breed-specific\textsuperscript{31}</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>((n = 129))</td>
<td>((n = 15))</td>
</tr>
<tr>
<td>R-time (min)</td>
<td>2.5-8.8</td>
<td>2.0-8.2</td>
</tr>
<tr>
<td>K-time (min)</td>
<td>1.9-6.4</td>
<td>0.8-3.4</td>
</tr>
<tr>
<td>angle (degrees)</td>
<td>34.5-62.9</td>
<td>49.4-80.5</td>
</tr>
<tr>
<td>MA (mm)</td>
<td>38.6-61.2</td>
<td>43.8-74.2</td>
</tr>
<tr>
<td>G (dyn/cm\textsuperscript{2})</td>
<td>3148-7900</td>
<td>3843-7810</td>
</tr>
<tr>
<td>LY60 (%)</td>
<td>0-8.9</td>
<td>0-8.8</td>
</tr>
</tbody>
</table>

Abbreviations: MA, maximum amplitude; LY60, percent lysis at 60 minutes
II. Iron

Most functional iron in humans is located in the oxygen-carrier heme proteins Hb (~70%) and myoglobin (Mb~5%); most of the non-heme iron is in form of storage iron such as ferritin or hemosiderin in hepatocytes or macrophages (~25%), and minor amounts (<1%) are bound to the transferrin plasma carrier or incorporated into enzymes (e.g. peroxidase, catalase, cytochromes) (Coleman 2007). Although the majority of the iron is used for Hb production, iron is also necessary as a neurotransmitter and for myelin production, collagen formation, immune system function, energy metabolism, and DNA and RNA synthesis (Andrews and Smith 2000; Lieu and others 2001). Therefore, there are three different pools of iron: functional, storage and transport. Iron plays an important role as an electron carrier and in metabolic oxidation and reduction (redox) reactions, which are essential, but it can also produce reactive oxygen species (ROS) causing significant tissue damage (Lieu and others 2001). Disturbances of the iron balance (intake, absorption, regulation) can lead to pathological states of different/varying severity.

Diet is the main source of iron, and it can be intestinally absorbed as heme or non-heme forms. The heme form is more effectively absorbed than the non-heme form (Coleman 2007). The duodenum and upper jejunum are the sites of maximal absorption. Iron must be in the ferrous form (Fe$^{2+}$) for oxygen
transport in Hb, but also for intestinal absorption. Heme iron binds to the enterocyte, is internalized (through the membrane protein heme carrier protein 1) and the enzyme heme oxygenase degrades it into Fe\(^{2+}\) iron, carbon monoxide and bilirubin IXa (Coleman 2007). Non-heme iron is usually in the form Fe\(^{3+}\) and is reduced by the enzyme cytochrome reductase b to Fe\(^{2+}\) iron. (McCown and Specht 2011) This Fe\(^{2+}\) is then transported into cells by the divalent metal transporter (DMT1) and exported to the portal circulation through ferroportin (with help of hephaestin) (Coleman 2007). Oxidized iron must be bound to transferrin to be transported, but some remains in the enterocytes stored as ferritin (McCown and Specht 2011). Transferrin-bound iron allows the circulation of nonreactive iron while delivering it to cells with transferrin receptors: rapidly dividing cells (e.g. erythroid precursors, rapidly dividing cells, activated lymphocytes, hepatocytes) (McCown and Specht 2011).

Although there is little information about iron metabolism and iron requirements in dogs, most commercial pet foods are supplemented with iron (McCown and Specht 2011; National Research Council (U.S.). Ad Hoc Committee on Dog and Cat Nutrition. 2006). Recommended allowance is 0.36-0.5 mg/kg/day, depending on bioavailability (McCown and Specht 2011). Excess iron is stored as ferritin or hemosiderin. Storage as an iron oxide in the central cavity of ferritin in the cytoplasm of hepatocytes is the primary site (95%). The remaining 5% is in Kupffer cells (liver macrophages) as hemosiderin (McCown and Specht 2011) Excretion of iron is not an effective mechanism, so it has to be
regulated by absorption (iron absorbed is inversely related to the amount of iron stores and erythropoiesis).

Regulation and maintenance of iron balance (intake/excretion) is critical for the organism because in its free form or in elevated amounts are toxic (Coleman 2007). Therefore, a tight regulation between intestinal absorption, erythropoiesis, recycling from senescent RBC and storage is needed (McCown and Specht 2011). Bioavailability depends on the chemical form, and can also be modified by other components of the diet that can act as enhancers or inhibitors of the absorption (Coleman 2007). The amount of iron consumed influences the absorption; in one study in growing dogs, enterocytes became resistant to iron absorption after the administration of an iron bolus (Andrews 1999). Iron regulation is complex and carefully controlled in order to avoid iron deficiency or overload. Hepcidin is a negative regulator of the intestinal iron absorption and release from macrophages, which binds to the ferroportin receptor causing degradation of ferroportin; therefore iron remains in the enterocyte, decreasing absorption and mobilization of iron stores. There is increased synthesis of hepcidin when transferrin saturation is high, and viceversa (decreased synthesis when transferrin saturation is low) (Coleman 2007). Hepcidin also has antimicrobial effects by itself, but at a much higher dose than for impairment of iron homeostasis (Nemeth and Ganz 2006).
Clinical manifestations of iron imbalance

- Iron deficiency

Clinical effects of iron deficiency have been described since the Middle Ages, when it was called “chlorosis”, which affected young menstruating women. Years later it was recognized that the disease responded to iron treatment (Andrews 2000; Guggenheim 1995). Today, iron deficiency still exists, most commonly in children (due to the rapid growth) and premenopausal women, but it also occurs in patients with chronic parasitosis, tumors, inflammation, infections or due to familial causes (Andrews 2000).

Iron deficiency due to inadequate intake of iron is rare in veterinary medicine, but it has been reported in nursing animals because the iron content in milk is low. In animals, the major cause of iron deficiency is chronic blood loss from the following: gastrointestinal tumors, gastric ulcers, inflammatory bowel disease, or parasites (Giger 2005). Clinical manifestations of iron deficiency anemia include pallor, weakness, lethargy, tachypnea, tachycardia and abnormal behavior such as pica, craving to eat, chew or lick non-nutritive objects (e.g. rocks, dirt, metal). Iron deficiency is characterized by the absence of iron stores, low serum iron concentration, low transferrin saturation, and low HCT and Hb values, and is usually regenerative. Although rarely seen, other clinical manifestations can be secondary to impaired proliferation, growth and function
on non-erythroid tissues, reduced muscle activity, abnormal behavior and skin or nail changes (Giger 2005).

Anemia of inflammatory disease occurs secondary to changes in iron homeostasis, altered proliferation of erythroid cells, altered production of erythropoietin (EPO), and decreased RBC lifespan (Weiss and Goodnough 2005). Activation of T cells and monocytes results in the production of cytokines, which alter iron homeostasis. Lipopolysaccharide (LPS) and IL-6 induce production of hepcidin, causing inactivation of ferroportin and downregulation of DMT1 as previously mentioned, impairing intestinal absorption and decreasing iron release from stores in macrophages and hepatocytes. This impairment limits the availability of iron for hematopoiesis. The anemia of inflammatory disease is non-regenerative; the potential mechanisms of this inhibition of proliferation and differentiation of erythroid precursors are: induction of apoptosis, down-regulation of EPO receptor expression, and reduced expression of other prohematopoietic factors (e.g. stem cell factor) (Means 2003; Taniguchi and others 1997; Wang and others 1995). RBC lifespan can be also shortened, with an inadequate EPO response for the degree of anemia. Inflammatory cytokines can alter binding affinity of EPO-inducing transcription factors and also cause damage to EPO-producing cells, interfere with EPO signal transduction and down-regulation of EPO receptors (Jelkmann 1998).

The safest and cheapest treatment of iron deficiency is oral iron supplementation, and among the choices ferrous sulfate and ferrous gluconate
are the most appropriate due to their high bioavailability and low cost. Iron supplementation can color the feces and give false positives in the guaiac test for fecal occult blood, but it does not usually affect the benzidine test for occult blood. Treatment can also be parenteral as iron dextran, iron gluconate, or iron sucrose (McCown and Specht 2011).

- **Iron overload**

Tissue storage happens in two different manners: a) when plasma iron exceeds the iron binding capacity of transferrin, parenchymal liver cells absorb the iron, and, when they are saturated, other parenchymal cells start accumulating iron too (e.g. cardiac myocytes, pancreatic acinar cells); b) when excess of iron comes from increased catabolism of RBC (e.g. repeated blood transfusions) the accumulation occurs in the reticuloendothelial macrophages first, and later in parenchymal cells. The storage in parenchymal cells can lead to tissue damage and fibrosis, so iron accumulation in the reticuloendothelial cells is safer (Andrews 2000).

Iron disorders are classified as hemosiderosis (asymptomatic increase in iron accumulation in tissues) or hemochromatosis (organ dysfunction secondary to iron-induced injury). Intravenous (IV) administration has the highest potential of toxicity, followed by intramuscular and oral administration. In overload situations, the organism tries to regulate the metabolism through a decrease in intestinal absorption and saturation of iron-binding proteins (McCown and Specht 2011). It has been shown that in these cases, iron can be absorbed in all
parts of the gastrointestinal tract, not just in the duodenum and upper jejunum (Albretsen 2006).

In dogs, ingestion of <20 mg/kg does not result in clinical signs; problems can develop ingesting 20-60 mg/kg of elemental iron, and serious illness can occur in dogs ingesting >60 mg/kg of elemental iron; oral ingestion of 100-200 mg/kg is lethal in animals (Albretsen 2006; McCown and Specht 2011). Acute iron intoxication signs start as gastrointestinal irritation and distress, followed by peripheral vascular collapse (e.g. depression, weak pulse, hypotension, cyanosis, ataxia, coma), followed by pulmonary edema, vasomotor collapse, hepatic failure, coma and death (Albretsen 2006; McCown and Specht 2011; Plumb 2008).

Besides acute intoxication, tissue damage (e.g. liver) resembling hemochromatosis has been reported in dogs after repeated transfusions (Sprague and others 2003) and in Basenjis with pyruvate kinase deficiency (Giger and Noble 1991); canine hemochromatosis has also been experimentally induced by IV iron administration (Lisboa 1971). The treatment of choice in these situations is an iron chelator such as deferoxamine, administered at a constant rate infusion of 15 mg/kg/h (Dean and Krenzelok 1987).
**Diagnostic tests**

- **Serum Iron Concentration**

  Serum iron concentration refers to the Fe$^{3+}$ bound to serum transferrin, and does not include iron in serum as free Hb. It can be measured by chromogen-spectophotometric methods. It has diurnal variation (highest in the morning and lowest in the evening). Serum iron can be decreased by other pathologies (not only in iron deficiency anemia) such as inflammatory disorders, acute infection, certain immunizations or myocardial infarction.

- **Iron-binding capacity**

  Although total transferrin can be measured, it is not commonly determined. Only 1/3 of the iron binding sites of transferrin are occupied by Fe$^{3+}$, so 2/3 of the iron binding sites do not carry iron: this is known as serum unsaturated iron-binding capacity (UIBC). The total number of available sites is the total iron-binding capacity (TIBC), which reflects the iron content when transferrin is saturated with iron. UIBC can be measured by chromogen-spectophotometric methods; TIBC can be measured indirectly by chemical means, and directly by immunologic methods (Coleman 2007). TIBC is usually low in anemia of inflammatory disease, but normal in dogs with chronic iron deficiency anemia (Meyer and Harvey 1998). Percent transferrin saturation is the % of available sites that are carrying iron. It is the ratio of serum iron to TIBC, and the normal
range is 20-60%. It is decreased in iron deficiency, but clinical utility is affected by the diurnal variations (Meyer and Harvey 1998).

- **Ferritin**

  Although present in plasma in low amounts, ferritin concentration correlates well with tissue iron stores in people, where it is a good method of measuring total body iron; it is usually measured by immunologic methods. It is present in plasma in low concentrations in equilibrium with iron stores (changes in iron stores is reflected by plasma ferritin concentration). Plasma ferritin declines early in the development of iron deficiency. Ferritin is also a positive acute phase reactant protein, so it can be increased in animals with inflammation (Coleman 2007; McCown and Specht 2011). Ferritin concentrations can also be high in patients with liver disease, hemolytic disorders and some neoplasms (Feldman 2005).

- **Hematological parameters**

  Serum iron, transferrin, and ferritin are considered direct measurements of iron. There are also indirect ways to measure the iron status such as MCV, MCHC, RDW and reticulocyte indices. Iron deficiency anemia is initially strongly regenerative, and becomes non-regenerative when it is more severe, but hematological changes do not occur until late in iron deficiency. In these cases, RBCs become microcytic (low MCV) and hypochromic (low MCHC) due to the increased synthesis of RBCs, delayed cell maturation, and extra mitotic
divisions. MCHC occurs after the decrease in MCV, and it is common in severely affected dogs (but does not occur in adult cats with iron deficiency anemia). Red cell distribution width (RDW) is often increased. In contrast to these findings, anemia of inflammatory disease is usually non-regenerative, normocytic and normochromic, and not that severe. Reticulocytes circulate 1-2 days before maturing into RBCs, so they are useful in evaluating and monitoring iron deficiency and response to therapy (Coleman 2007; McCown and Specht 2011).

- **Iron staining**

Tissue iron concentration can be assessed in dogs by obtaining a biopsy sample and staining it with Prussian blue; the amount of iron (hemosiderin) present in macrophages, nucleated RBCs and reticulocytes can be then estimated visually. This technique also can be used in peripheral blood and bone marrow aspirate smears or preparations (Coleman 2007; McCown and Specht 2011).

Measuring only serum iron gives little information regarding the actual iron status of the animal, so the combination of various parameters will give more clinical information and diagnostic capability.

**Iron in exercise physiology**

Iron is the functional component (i.e. oxygen carrier) of hemoglobin and myoglobin, but also plays an important role in the electron transport chain
because cytochromes and mitochondrial enzymes are also heme-containing proteins that promote oxidative phosphorylation in the mitochondria (Williams 2005). Severe iron deficiency results in anemia, and athletic performance can be affected due to the decreased oxygenation at the tissue level (Peeling and others 2008).

Iron deficiency is common in athletes, and more common in those engaged in endurance sports (Beard and Tobin 2000); in addition, low iron stores can decrease athletic performance (Schumacher and others 2002). Iron is in delicate balance in the organism; proposed mechanisms of iron deficiency in athletes are: hemolysis, hematuria, sweating, and gastrointestinal bleeding (Babic and others 2001; DeRuisseau and others 2002; McInnis and others 1998; Peeling and others 2008; Zoller and Vogel 2004) and more recent studies have also demonstrated the role of the inflammatory driven hormone hepcidin (iron regulator) and cytokines (Peeling 2010; Roecker and others 2005). It is hypothesized that hepcidin activity in exercise is similar to its activity in chronic inflammation, since inflammatory symptoms are similar, but more studies are needed to establish this association (Peeling and others 2008; Roecker and others 2005).

As mentioned earlier in this section, hepcidin is a hormone produced by the liver, and is thought to be the master regulator of iron homeostasis (Kemna and others 2005). Hepcidin is expressed in response to inflammation, hypoxia and elevated iron levels, but its primary mediator is interleukin 6 (IL-6). Peak hepcidin levels occur 3 h after the peak of IL-6 activity, and such increases are
reflected as a rapid decrease of plasma iron, which can end in a decrease in Hb synthesis and subsequent anemia (Nemeth and others 2004).

Running results in “plantar hemolysis”, that increases post exercise levels of serum iron and IL-6 (Janakiraman and others 2011a, b; Telford and others 2003); therefore, as previously mentioned, hepcidin levels increase 3h after exercise. As a result of this hemolysis, rupture of RBCs releases all their contents to the blood stream (including iron) and serum iron levels increase. Macrophages “eat” all debris, avoiding oxidative damage in tissues. However, the increase of hepcidin results in entrapment of iron (hemosiderin) inside the macrophages, which impacts the recycling mechanism of iron in order to form new RBCs. Also, the increase in hepcidin levels 3h post exercise may impair the iron absorption from the diet in that time frame (Peeling 2010).

Exercise-induced physiological changes in dogs have been studied particularly in two dog populations: racing sled dogs and racing Greyhounds. Based on these studies, two types of exercise physiology can be assessed, endurance (sled dogs) and sprint (Greyhounds). There is little information regarding iron metabolism in exercising dogs. Recently, a prospective study evaluated iron status and acute phase proteins in racing sled dogs before and after a long distance race. Mean serum iron was low after the race; ferritin was higher after the race, but only in the dogs that did not finish the race and only 3.5% of the dogs had low ferritin levels (suggestive of iron deficiency) after the race. Ceruloplasmin and C-reactive protein were significantly increased after the race, suggesting inflammation. They concluded that iron deficiency is
uncommon in racing sled dogs after racing, or that inflammatory changes can be masking such iron depletion (Kenyon and others 2011). However, more studies are needed to understand the influence of the number of races, training intensity, hepcidin levels and inflammatory markers (e.g. IL-6) on iron homeostasis.

**Iron in blood donors**

Maintenance and monitoring of iron status is very important in blood donors, especially in people that donate blood regularly. Studies related to this topic have been done in many populations in different parts of the world. As an example of the importance, in a literature search in a scientific database (e.g. PubMed) using the words “iron”, “blood” and “donor”, generates over 700 articles, most of them on iron deficiency.

People can donate whole blood every 56 days (i.e. 8 weeks) without harmful effects for the individual: 200-250 mg of iron are lost in each blood donation (Simon 2002). This repeated blood loss is associated with a progressive depletion of the body’s iron stores; this is more pronounced in premenopausal women, but can also occur in men (Mast and others 2008; Simon and others 1981). A decrease in serum ferritin (8% in males, 23% in females) has been associated with blood donation during the prior 4-5 years (Finch and others 1977; Simon and others 1981).

Genetic factors such as polymorphism in the transferrin gene and genes for hemochromatosis have been described, and can define people as “at risk” or
“protected” for iron deficiency. Racial and socioeconomic factors have been recently assessed through a large study from the National Heart, Lung, and Blood Institute’s Retrovirus Epidemiology Donor Study-II (REDS-II). This study, named RISE (REDS-II Donor Iron Status Evaluation), was designed to evaluate the effects of blood donation intensity on iron and Hb concentrations, and to provide information about needs and blood donation frequency guidelines. In that study, more than 2,000 blood donors were evaluated for 2 years. Researchers concluded that there is a high prevalence of iron deficiency among the blood donor population, particularly in young women. There was a strong association between iron depletion and prior donation intensity and time since the most recent donation. Many first-time donors developed iron deficiency within 15 to 24 months, but iron status changes little among longtime donors. Donors that donated blood every 14 weeks had a higher risk of iron depletion than people who donated every 14-20 weeks. The main cause of deferral from the blood donor program was iron deficiency (77%). Higher weight and smoking were found to be protective factors for a decrease in iron stores. Ethnicity or dietary factors did not have a major impact, except iron supplementation decreased the prevalence of iron deficiency (Bahrami and others 2011; Cable and others 2011a; Cable and others 2011b).

The term high-intensity blood donors (“superdonors”) defines a selected population of individuals that/who donate blood 13 times in 2 years with less prevalence of deferral due to low HCT. Evaluation of iron metabolism in this population indicates that iron deficiency is present in 60%. The most important
finding in superdonors was that they have greatly decreased serum hepcidin levels, consistent with a higher ability to absorb iron in order to replace iron losses. After genetic analysis, superdonors that were heterozygous for the H63D mutation in *HFE* (hemochromatosis) also had significantly decreased hepcidin (Mast and others 2008).

**Iron in Greyhounds**

Due to previously reported low Hp, high HCT, high Hb concentration and high oxygen affinity (Zaldivar-Lopez and others 2011b), and the high prevalence of osteosarcoma (OSA) in the breed (Rosenberger and others 2007), we recently investigated the iron status of Greyhounds (G) with and without OSA. Dogs were divided into four groups: healthy Greyhounds (n=25), OSA Greyhounds (n=28), healthy non-Greyhounds (n=30) and OSA non-Greyhounds (n=32). TIBC was lower [and consequently transferrin saturation (%SAT) was higher] in healthy Greyhounds than in healthy non-Greyhounds. Both OSA Greyhounds and OSA non-Greyhounds had lower serum iron and %SAT than their healthy counterparts. We concluded that low TIBC and high %SAT may be another Greyhound idiosyncrasy, and that all OSA dogs had lower serum iron and %SAT than the healthy ones (Couto and others 2011).
III. Hemoglobin

_Hemoglobin physiology_

Hemoglobin (Hb) is the iron-containing oxygen-transport metalloprotein in RBCs of vertebrates, comprising about 97% of the dry matter of RBCs in mammals, and about 35% of the total content including water. Each RBC contains approximately 270 million Hb molecules (Telen and Kaufman 2004). Hb’s main function is to transport oxygen from the lungs (high-affinity) to other tissues and organs such as muscles, where the oxygen is released (low-affinity) at the capillary level for its use in cell metabolism. The relationship between oxygen and Hb is described by the oxyhemoglobin dissociation curve (ODC, figure 2). The sigmoidal shape of the ODC promotes rapid uptake of oxygen in the lungs (high PO₂) and facilitates efficient delivery of oxygen to the tissues (low PO₂) (Clerbaux and others 1993). Under standard conditions (37°C, pH 7.4, PCO₂ 40 mmHg), species variations in the ODC and oxygen affinity are determined mainly by the primary structure of the Hb molecule (i.e. genetic features) and the chemical composition of RBCs (i.e. 2,3-DPG concentration). Among domestic animals, dogs have higher 2,3-DPG concentrations than cattle and humans (Clerbaux and others 1993). Oxygen affinity is measured through the P₅₀, which is the partial pressure of oxygen when the Hb molecule is 50% saturated with oxygen. Oxygen affinity in dogs varies among breeds (mean P₅₀: 30.0 mmHg, SD 1.3), ranging from 25.9 mmHg (Boxer) to 35.8 mmHg.
(hounds), with no correlation found with 2,3-DPG (Cambier and others 2004; Clerbaux and others 1993).

Figure 2. Diagram of the oxyhemoglobin dissociation curve (ODC) and factors that affect the position of the curve.
The Hb molecule has been widely studied during the last 60 years, primarily with the purpose of clarifying the pathophysiology of Hb disorders in humans. This molecule is expressed at very high concentrations in the highly specialized mammalian anucleated erythrocyte, resulting in an efficient oxygen transport mechanism from lungs to tissues and vice versa. At the molecular level, Hb is formed by symmetrical pairing of two dimers of polypeptide chains, resulting in a tetrameric structural and functional unit (Provan and Gribben 2010; Schechter 2008). These subunits (globins) are composed of a protein chain tightly associated with a non-protein heme group (composed of an iron ion held in a porphyrin ring). The iron ion is the site of oxygen binding.

In humans, the maturation process of erythroid cells starts in the embryo (i.e. yolk sac), where a set of early embryonic genes is expressed, forming the embryonic hemoglobins (Hbs). Approximately 6-8 weeks post-conception, erythropoiesis switches to the fetal liver, where embryonic Hbs are silenced and fetal Hb gamma- (HBG) genes are up-regulated, forming the fetal Hb (HbF). HbF has slightly higher oxygen affinity than adult Hbs because it binds 2,3-DPG less strongly. At birth, alpha-genes remain active (Hb alpha), but gamma- genes are downregulated, and the beta-like [delta (HBD) and beta- (HBB)] genes are activated. Adult Hbs are predominantly HbA (α_{2}β_{2}) and HbA_{2} (α_{2}δ_{2}). In erythrocytes of normal human adults, HbA accounts for about 97% of the protein molecules, HbA_{2} for 2%, and HbF for 1% (Provan and Gribben 2010; Schechter 2008).
The mechanisms by which the globin genes are expressed at such high levels and the switching between gene types have long been of interest due to the relatively high prevalence of hemoglobinopathies, such as sickle cell anemia and thalassemias in certain ethnic human groups. There are two different thalassemia syndromes, alpha- and beta-, depending on which globin gene carries the mutation. The alpha-thalassemia syndrome is caused by mutations in the alpha-globin gene (HBA). Both the beta-thalassemia and sickle cell anemia are caused by mutations in the HBB gene, and have been reported to respond to a therapeutic increase in HbF (Provan and Gribben 2010; Schechter 2008). Although the basic globin gene structure and protein folds are conserved in evolution among all mammals, hemoglobinopathies like sickle cell disease and thalassemia have not been recognized in other species.

Blood gases and acid-base balance have been of special interest in racing Greyhounds, due to the importance in exercise physiology. Numerous studies demonstrate that racing Greyhounds experience more extreme changes in acid base status during a race (Ilkiw and others 1989; Lassen and others 1986; Nold and others 1991; Rose and Bloomberg 1989) than other sporting species, such as horses and humans (Nold and others 1991). These changes return to baseline values within 1-3 hours. Selective breeding in Greyhounds could contribute to altered Hb function, due to the need for oxygen delivery at the tissue level during a race (Sullivan and others 1994; Zaldivar-Lopez and others 2011a). Sullivan’s study reported that Greyhounds had lower hemoglobin P_{50} values than non-Greyhound dogs (Sullivan and others 1994), which means that the ODC is left-
shifted, implying that Hb has a higher affinity for oxygen in Greyhounds than that in non-Greyhound dogs. Interestingly, RBC 2,3–DPG concentration, which influences the position of the ODC, was not different between Greyhounds and non-Greyhounds. In the same study, the high Hb and PCV in Greyhounds were suggested to be a compensatory change secondary to decreased oxygen delivery to the tissues (low P$_{50}$), as seen in humans with high-affinity hemoglobinopathies (Sullivan and others 1994).

Recently we conducted additional studies on the high-affinity Hb in RRGs using a different method (which will be explained and discussed in detail in Chapter 2), and confirmed a low P$_{50}$, as well as higher oxygen content and oxygen capacity of the Hb molecule in Greyhounds (Zaldivar-Lopez and others 2011a). Although previous studies in exercise physiology had demonstrated that a decrease in oxygen affinity is beneficial for athletic performance (Biolo and others 2009; Cambier and others 2004), because oxygen diffuses to the tissues more readily. The Greyhound adaptation is similar to recent studies on hemoglobin-based oxygen carriers (HBOCs) that demonstrate a beneficial effect of higher oxygen affinity due to the later release of oxygen to deeper oxygen-deprived tissues (Dimino and Palmer 2007).
Crystal structure of Greyhound hemoglobin

Based on previously mentioned physiological properties of Greyhound Hb (GrHb), we further analyzed the crystal structure (GrHb) at 1.9 Å was further analyzed (Bhatt and others 2011). The objective of this study was to correlate the high affinity of GrHb with its structure. First, the ODC of GrHb was determined, and high-affinity in GrHb and GrRBCs was confirmed (Figure 3).

Figure 3. Greyhound oxyhemoglobin dissociation curve

Abbreviations: GrHb, Greyhound hemoglobin; GrRBCs, Greyhound red blood cells
GrHb crystallized in the R2 state, despite high salt conditions used for crystallization. Cooperative binding has been traditionally explained by the two-state model (Monod and others 1965): T-state (tense, unligated) and R-state (relaxed, ligated). Years later, another quaternary structure was described, the R2-state, which was first thought to be intermediate between R- and T-states (Silva and others 1992), but later was shown as the endpoint transition state of the Hb quaternary form (Schumacher and others 1997).

The GrHb sequence was identical to the generic dog Hb. Structural analysis of GrHb and its comparison/superposition with the R2-state of human Hb revealed several regions different between these two structures, which could potentially explain the additional stability of the R2-state of GrHb compared to R2 state of human Hb (HuHb):

1) GrHb is in the R2 state, as shown in figure 4. Structural alignment and superposition of the α1β1 subunits of the tetramer of GrHb (gray) with the R2 state of HuHb (R2-HuHb, purple), the R state of HuHb (R-HuHb, yellow) and the R2 state of guinea pig Hb (R2-PiHb, pink) shows that GrHb belongs to the R2 state, since the GrHb structure is better superimposed to R2-HuHb and R2-PiHb. The curved arrow in the figure highlights the relative rotation of α2β2 subunits, and the inset magnifies the relative orientation of the residues αThr38, αThr41 and βHis97 in the structural alignment.

2) Restricted conformation of the α C-termini of GrHb relative to HuHb. A variation from αHis89 in HuHb (purple) to Tyr in GrHb results in the formation
of van der Waals stacking interactions as well as a hydrogen bond network at the C-termini of GrHb. This in turn could possibly restrict the transition from the liganded (R or R2) to the unliganded (T) states. Dotted green lines represent hydrogen bonds.

3) A water-mediated hydrogen-bond network is uniquely present in GrHb. A group of three variations, αAsp30 in GrHb replacing Glu in HuHb, αGln34 in GrHb replacing Leu in HuHb and βGln125 in GrHb replacing Pro in HuHb, creates a water-mediated hydrogen-bond network at the α1β1 interface of GrHb (gray), hence lowering its free energy relative to the R2 state of HuHb (purple).

4) A previously well-studied hydrophobic cluster of bar-headed goose Hb (BaHb) near α119 was incorporated in the comparison between GrHb and human Hb. A variation from βVal33 in HuHb (purple) to Ile in GrHb (gray) and BaHb (green) leads to a stronger hydrophobic interaction at the α1β1 interface of GrHb compared with HuHb.
Figure 4. Crystal structure of Greyhound Hb tetramer, which belongs to the R2 state

GrHb: gray; R2-HuHb: purple; R2-PiHb: pink; R-HuHb: yellow. Relative rotation of $\alpha_2\beta_2$ shown with arrow, and relative rotation of residues $\alpha$Thr38, $\alpha$Thr41 and $\beta$His97 are magnified.
Finally, a structural comparison with generic dog Hb and maned wolf Hb was conducted. This revealed that in contrast to GrHb these structures belong to the R state of Hb, raising the intriguing possibility of an additional allosteric factor copurifying with GrHb that can modulate its quaternary structure. This structure, in addition to previous studies where high affinity Hb was found (Zaldivar-López 2010; Zaldivar-Lopez and others 2011a), indicates that GrHb can serve as a model for high-oxygen affinity hemoglobin (Hb) in higher mammals, especially humans.
IV. Haptoglobin

• *The role of haptoglobin in hemolysis*

Hb is the most abundant protein in blood and a high number of pathological conditions (e.g., porphyria, jaundice, and anemia) are associated with abnormalities in its synthesis and/or metabolism. Hb in RBCs is preferentially cleared from the blood by extravascular hemolysis, which is the process of red blood cell (RBC) phagocytosis by macrophages in the spleen and bone marrow. The other clearance route occurs during intravascular hemolysis, during which Hb released from ruptured RBCs is immediately bound to circulating haptoglobin (Hp) (see below). Other mechanisms of Hb clearance occur if iron in Hb becomes oxidized, forming methemoglobin. The heme molecule then binds to hemopexin, a plasma protein produced by the liver. These mechanisms prevent iron from urinary loss, and renal tubular oxidation. Hemopexin-metheme binds to hepatocyte receptors and is internalized, recycling iron, protoporphyrin and hemopexin. Metheme can also bind Hb, but it is a temporary holding state (before binding to hemopexin, which has higher binding affinity) (Rodak and others 2007).

When free in plasma Hb has harmful effects, such as oxidative damage or nitric oxide scavenging and subsequent hypertension. To avoid such damage, Hb binds to Hp, a plasma protein produced by the liver. By this strong binding, Hb avoids filtration by the glomerulus and can be recycled. The Hp-Hb complex is carried to the liver and is recognized by the CD163 receptor on monocytes and macrophages, which internalize and degrade the complex, recycling the receptor (Figure 4) (Nielsen and Moestrup
In the macrophage, the iron is recycled, and protoporphyrin is converted to bilirubin. Hp is depleted, but the amount produced by the liver is sufficient for normal intravascular hemolysis. There is no compensatory mechanism to produce more Hp secondary to hemolysis (Rodak and others 2007), (Figure 5). The removal of Hb from plasma prevents oxidative stress and, furthermore, the downstream metabolic pathway of Hb downregulates the inflammatory response of macrophages (Nielsen and Moestrup 2009). The interaction of Hp-Hb seems to have several purposes: one is to promote clearance of Hb and another is to prevent glomerular filtration of Hb and heme intoxication of the kidney (Nielsen and Moestrup 2009). This Hb-Hp binding also has a bactericidal effect in infected wounds by limiting the availability of iron for bacterial growth (Ceron and others 2005).

If previous mechanisms are inadequate, iron can be retained in the kidney; some Hb or (Met)heme is reabsorbed. Inside the renal tubular cell, iron is separated from protoporphyrin, and stored as ferritin or hemosiderin.
Figure 5. Diagram summarizing the haptoglobin physiology cycle.
• *The role of haptoglobin in inflammation*

Acute phase proteins (APP) are a group of blood proteins that change in concentration shortly after tissue injury due to infectious, neoplastic, immunologic or traumatic diseases; the purpose of the inflammatory response is to remove the noxious stimuli and restore homeostasis. Besides systemic effects (i.e. fever, leukocytosis, etc), the APP response includes changes in the concentrations of APPs, which are classified as negative (or downregulated) APPs (albumin, transferrin) and positive (or upregulated) APPs [C-reactive protein (CRP), serum amyloid A (SAA), Hp, alpha-1-acid glycoprotein (AGP), ceruloplasmin (Cp), and fibrinogen].

Hp is a positive APP whose concentration increases in plasma shortly after inflammation or tissue injury (Martinez-Subiela and others 2002). Hp is involved in the host defense response to infection and inflammation, and acts as a natural antagonist for receptor–ligand activation of the immune system. Hp also inhibits granulocyte chemotaxis and phagocytosis (Ceron and others 2005).

Whereas classical activation of macrophages by interferon-gamma and tumor necrosis factor (TNF) leads to a decreased expression of CD163, the expression of CD163 is highly induced by glucocorticoids, IL-6, and the anti-inflammatory IL-10. These findings support the fact that CD163 is a marker of ‘alternative macrophage activation’ (Nielsen and Moestrup 2009). In addition to the membrane-inserted variants of CD163 a soluble variant (sCD163) is present in plasma and other tissue fluids (Nielsen and Moestrup 2009). Rather than being
an alternative splice variant, sCD163 is a shedding product (i.e. CD163 receptors that do not bind Hp-Hb complexes are cleaved from the macrophage surface). Interestingly, neither Hp knockout mice nor humans with anhaptoglobinemia display compromised plasma Hb clearance, and the cellular distribution of Hb uptake in liver and spleen is not changed during hemolysis, providing additional evidence of such a pathway (Schaer and Alayash 2010). Schaer et al recently demonstrated a biphasic model of macrophage-mediated Hb clearance. In this model, small amounts of free Hb are removed by the well-characterized, high-affinity interaction of CD163 with the Hb-Hp complex. However, once plasma Hp is depleted, CD163-mediated uptake of free Hb is maintained through an apparently lower-affinity, Hp-independent interaction between free Hb and the macrophage scavenger receptor CD163 (Schaer and Alayash 2010).

- **Haptoglobin in dogs**

In dogs, an acute phase response for CRP, Hp, SAA, and AGP has been described in different diseases (Ceron and others 2005). Hp is a moderate APP in this species (Conner and others 1988), and changes in serum Hp concentration can be used as diagnostic and prognostic markers in various inflammatory disorders (Martinez-Subiela and others 2002). The Hp concentration in healthy dogs is 0-3 g/L (Eckersall and others 1999); the magnitude of response to stimuli is a 2- to 10-fold increase in plasma concentration occurring in 24 hours, and peaking at 3-4 days (Ceron and others 2005). In dogs with intravascular hemolysis (i.e. immune-mediated hemolytic anemia), Hp concentrations are
lower because it binds strongly to Hb, and the Hb-Hp complexes are removed from the circulation by macrophages via CD163 (Harvey and West 1987).

A significant increase in serum Hp concentration has been demonstrated after administration of glucocorticoids, anthelmintics, or phenobarbital (Martinez-Subiela and others 2004; Ndung'u and others 1991). Also, significant positive correlation has been established between Hp and WBC and neutrophil counts (Solter and others 1991). Hp is more stable than the cellular components of blood, thus assays can be performed on frozen serum or plasma samples. However, a decrease in Hp concentration in canine serum stored at -20ºC has been described; thus, -70ºC has been suggested for prolonged storage (Weidmeyer and Solter 1996). Serum Hp concentration can be measured by spectrophotometry and by immunoassays. It is important to note that canine serum specimens often must be diluted when Hp assays developed for other species are used, as the concentrations of Hp in health and disease are significantly higher in dogs than in other species (Ceron and others 2005).

Former racing Greyhounds have low serum protein concentration (Steiss and others 2000) due to a lower globulin concentration; primarily, serum α- and β-globulin concentrations (Fayos and others 2005). In 2009, our group measured serum Hp concentrations in Greyhounds (n=15) and non-Greyhound (n=11) dogs using colorimetric and immunoturbidimetric methods (previously validated in the laboratory of one of the co-authors). The Hp concentration was significantly lower in Greyhounds (p=0.0009 using the colorimetric method; p=0.019 using the immunoturbidimetric method), and later confirmed by protein
electrophoresis, in which negligible bands were seen in the Greyhound samples (Couto and others 2009). The low Hp concentration seems to be breed-specific, and could partially account for the low α- globulin concentrations in the breed.
Chapter 1: IRON METABOLISM IN GREYHOUNDS

INTRODUCTION

Iron is necessary in the body for multiple biological functions (e.g. RBC production), but its metabolism (intake/excretion) has to be tightly regulated because its free form and excess are toxic (Coleman 2007). Functional iron can be found as Hb, myoglobin, storage iron (ferritin or hemosiderin), bound to the transferrin plasma carrier or forming enzymes (Coleman 2007). There are no effective iron excretion mechanisms; therefore, the maintenance of an appropriate iron balance is regulated via intestinal absorption, erythropoiesis, recycling from senescent RBCs and storage (McCown and Specht 2011).

Iron deficiency due to inadequate intake is rare in veterinary medicine because most commercial foods are supplemented with iron. The major cause of iron deficiency in veterinary medicine is chronic blood loss (e.g. gastrointestinal tumors, gastric ulcers, inflammatory bowel disease, and parasites). Anemia associated with inflammatory disease can occur secondary to changes in iron homeostasis (Weiss and Goodnough 2005). Iron can also be in excess (overload), which is toxic. Besides acute iron intoxication (Brutlag and others 2012), tissue damage (e.g. liver) resembling hemochromatosis has been reported in dogs after repeated transfusions.
(Sprague and others 2003). It has also been experimentally induced by IV administration (Lisboa 1971), and it has been reported in Basenjis with pyruvate kinase deficiency (Giger and Noble 1991).

Iron status and safety of blood donation frequency has been assessed in people through a large study called RISE (REDS-II Donor Iron Status Evaluation). This study concluded that there is a high prevalence of iron deficiency among the blood donor population, particularly in young women, and that there is a strong association between iron depletion and prior donation intensity/time since last donation. Many first time donors developed iron deficiency within 15 to 24 months but iron status changes little among long time donors. People that donated blood every 14 weeks had higher risk of iron depletion than people who donated every 14-20 weeks. (Bahrami and others 2011; Cable and others 2011a; Cable and others 2011b).

Iron is the functional component (i.e. oxygen carrier) of hemoglobin and myoglobin; severe iron deficiency results in anemia, which can affect athletic performance due to the decreased oxygenation at the tissue level (Peeling and others 2008). Iron deficiency is common in human athletes, and more frequent in those engaged in endurance sports (Beard and Tobin 2000). Exercise-induced physiological changes in dogs have been studied particularly in two dog populations: racing sled dogs and racing Greyhounds (endurance and sprint runners, respectively). Recently, iron metabolism in racing sled dogs has been reported. Dogs had lower mean serum iron and higher ferritin concentration after the race, but levels were not indicative of iron deficiency (Kenyon and others 2011). We recently investigated the iron status of Greyhounds with and without osteosarcoma (OSA) (Couto and others 2011). We
found that TIBC was lower in Greyhounds than in non-Greyhound controls, consequently increasing transferrin saturation (%SAT) was in the Greyhound group. We hypothesized that this could be a breed-associated difference.

Although there is abundant information about other physiological parameters in Greyhounds (Zaldivar-Lopez and others 2011b), there is little information about iron homeostasis in this breed, and in the canine species in general. Additionally, to the author’s knowledge, there are no prior reports on iron status in blood donor dogs. Therefore, this study had the following two objectives:

a) To investigate iron metabolism in Greyhounds compared to other dog breeds, and to evaluate whether there are breed-specific differences;

b) To assess iron status in the canine blood donor population.

MATERIALS AND METHODS
We studied a total of 130 healthy dogs: 88 Greyhounds (G) and 42 non-Greyhound breeds (NG); 75 were blood donors (BD) and 55 were not (controls, C). Blood donors were part of the blood donor program (Animal Blood Bank, OSUABB) at The Ohio State University Veterinary Medical Center (OSU-VMC). Control dogs were patients admitted to OSU-VMC for either wellness checks or prior to elective surgeries (i.e. spay/neuter). In BD, all blood samples were collected before blood donation. Also, 16 of the blood donor dogs had two samples taken: the first when they were enrolled in the blood donor program (T0), and a second sample at some point later (T1), in order to assess the effects of recurring donation on iron homeostasis; the number of
donations between the two sampling times were recorded. The Animal Blood Bank has a current animal use protocol approved by the Institutional Animal Care and Use Committee (IACUC) for collection of blood to establish reference intervals. Blood samples in non-donor dogs were collected after signed owner’s consent. All dogs were healthy based on normal physical examination, PCV/total solids, temperature, heart and respiratory rate; in addition, all blood donor dogs get annual CBC and chemistry panels to ensure health. Blood samples were drawn (Figure 6) from the jugular vein, placed into tubes without anticoagulant, and allowed to clot before being centrifuged at 1380g for 10 minutes. Sera were frozen at -30°C and batched for analysis. Serum iron concentration and unsaturated iron-binding capacity (UIBC) were analyzed in the OSU-VMC chemistry laboratory (COBAS Analyzer, Hitachi). Total iron-binding capacity (TIBC) was calculated by adding UIBC and serum iron concentration, and percentage transferrin saturation (%SAT) was calculated using the formula: serum iron/TIBC x 100.

Descriptive statistics and normality test (D’Agostino & Pearson omnibus test) were performed. Two-way analysis of variance (ANOVA) was done to compare G and NG and the blood donor status factor (BD/C), with Sidak post-hoc test comparing main effects and interactions. A paired t-test was performed to compare the blood donor

Figure 6. Blood sample collection in a Greyhound
dogs that had two samples (in different time points) taken, in order to assess the
effects of blood donation. Statistical significance was set at P<0.05. Statistical
software used for the analysis were SPSS 19 (descriptive and 2-way ANOVA) and
GraphPad Prism® 5.0 (paired t-test).

RESULTS

Iron metabolism in Greyhounds

Results from the descriptive statistics of the serum iron panel are shown in table 6.
Serum iron concentrations were not different between G and NG, but they were
significantly higher (p=0.004) in BD (183.74 ± 55.31 µg/dL) when compared to C
(153.90 ± 51.73 µg/dL); no interaction between the two factors was detected (figure
7). The UIBC was lower (p<0.001) in G (141.22 ± 68.12 µg/dL) than in NG (270.09
± 107.09 µg/dL), and it was lower (p=0.002) in BD (152.6 ± 73.35 µg/dL) than in C
(224.12 ± 120.61 µg/dL); interaction between the two factors (p<0.001) was found
(figure 7). TIBC was lower (p<0.001) in G (315.96 ± 52.94 µg/dL) than in NG
(433.98 ± 87.28 µg/dL) but there was no statistical difference between BD and C, and
there was interaction between the two factors (p<0.001) (figure 7). The %SAT was
higher (p<0.001) in G (56.04% ± 17.12 µg/dL) than in NG (39.38 ± 16.24 %). The
BD group had higher (p=0.007) %SAT (55.69 ± 17.44 %) than C (43.80 ± 17.85 %),
and interaction between the two factors was found (p=0.024) (figure 7).
Effects of blood donation over time

All dogs in this part of the study were Greyhounds. The number of donations between T0 and T1 was 8.68 (range 1 – 21, SD 6.09). Using the dates of each sample (before and after) and the number of donations in between, we calculated that the average time between donations of our canine blood donor population was 60.74 days (8.67 weeks). Average time between T0 and T1 was 75.6 weeks. For this part of the study, we included packed cell volume (PCV) and total protein (TP) in addition to the iron panel (iron, UIBC, TIBC and %SAT), for a better assessment of the overall health of our blood donor population. Results from the comparison between the first (T0) and the second (T1) samples showed statistically significant differences in PCV and in all iron parameters (figure 8). PCV decreased (p=0.0328) from 61 ± 4 % to 58 ± 6 %. Serum iron decreased over time (p=0.029) from 194.9 ± 57.2 µg/dL to 156.1 ± 59.8 µg/dL; consequently, %SAT also decreased (p=0.009) over time from 58.70 ± 19.34 % to 41.69 ± 18.51 %). In contrast, UIBC and TIBC increased (p=0.0016 and p=0.0009, respectively) from 142.9 ± 73.2 µg/dL to 228.4 ± 83.2 µg/dL (UIBC), and from 337.8 ± 41.6 µg/dL to 384.4 ± 51.7 µg/dL (TIBC).
Table 6. Descriptive statistics for iron parameters in Greyhounds, non-Greyhounds, blood donors and control dogs.

A) Dependent Variable: IRON

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<th>N</th>
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B) Dependent Variable: UIBC

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<td>42</td>
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</tbody>
</table>

C) Dependent Variable: TIBC

<table>
<thead>
<tr>
<th>BREED</th>
<th>BLOOD DONOR</th>
<th>Mean</th>
<th>SD</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>Greyhound</td>
<td>Blood Donor</td>
<td>328.69</td>
<td>49.85</td>
<td>63</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>283.88</td>
<td>47.36</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>315.96</td>
<td>52.94</td>
<td>88</td>
</tr>
<tr>
<td>Non-Greyhound</td>
<td>Blood Donor</td>
<td>376.50</td>
<td>50.97</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>456.50</td>
<td>88.84</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>433.64</td>
<td>87.28</td>
<td>42</td>
</tr>
</tbody>
</table>

D) Dependent Variable: %SAT

<table>
<thead>
<tr>
<th>BREED</th>
<th>BLOOD DONOR</th>
<th>Mean</th>
<th>SD</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>Greyhound</td>
<td>Blood Donor</td>
<td>56.48</td>
<td>18.02</td>
<td>63</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>54.95</td>
<td>14.90</td>
<td>25</td>
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<tr>
<td></td>
<td>Total</td>
<td>56.04</td>
<td>17.12</td>
<td>88</td>
</tr>
<tr>
<td>Non-Greyhound</td>
<td>Blood Donor</td>
<td>51.56</td>
<td>13.91</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>34.51</td>
<td>14.61</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>39.38</td>
<td>16.24</td>
<td>42</td>
</tr>
</tbody>
</table>

Abbreviations: IRON, serum iron; UIBC, unsaturated iron-binding capacity; TIBC, total iron-binding capacity; %SAT, transferrin saturation; SD, standard deviation; N, number of animals. Units: IRON, UIBC and TIBC expressed in µg/dL; %SAT is expressed as %.
Figure 7. Graphs showing iron parameters in Greyhounds and non-Greyhounds (X-axis), blood donors and control dogs (maroon and green, respectively)

IRON: serum iron; UIBC: unsaturated iron-binding capacity; TIBC: total iron-binding capacity; SATURATION: transferrin saturation. Blood donors: maroon; control dogs: green. Segments represent the 95% confidence interval of the mean.
Figure 8. PCV, total protein and iron parameters in blood donor dogs (Greyhounds) at two different time points.

PCV, packed cell volume; IRON: serum iron; UIBC: unsaturated iron-binding capacity; TIBC: total iron-binding capacity.
DISCUSSION

a) Iron metabolism in Greyhounds

For the two measured parameters (serum iron and UIBC) in G and NG, serum iron concentration was within the previously published reference interval (Kenyon and others 2011) and no statistical difference was found between the G and NG groups; however, UIBC was lower (p<0.001) in G. Consequently, G have lower (p<0.001) TIBC and higher (p<0.001) %SAT that NG.

TIBC, which is calculated by adding the serum iron concentration to the UIBC, reflects the total number of available iron binding sites, and is therefore an indirect calculation of the total serum iron content of the individual. Percentage transferrin saturation (%SAT) represents the % of available sites bound to iron, which in a healthy individual should be approximately 33% of the total binding sites (range 20-50%); therefore, this parameter is more clinically useful than serum iron concentration alone. Percentage transferrin saturation (%SAT) was higher in G than in NG; most of the values in G were on the high end or outside the reference interval.

Decreases in UIBC/TIBC occur in acute phase reactions (e.g. inflammation, neoplasia, chronic disease), or they can occur due to decreased hepatic production or liver disease. Transferrin can also be decreased due to protein-losing conditions. Decreased TIBC along with high serum iron and %SAT are common in pathological states such as iron overload and hemolytic anemia (McCullen and others 2002). The low UIBC/TIBC found in G could be a breed-specific feature (as many others) or be caused by chronic inflammation (e.g. gastrointestinal disorders, arthritis), both of
which are common in the breed (Lord and others 2007). However, all these dogs were clinically healthy, and the remaining clinicopathologic findings were not consistent with inflammation.

Iron-related genetic variation has been selected through evolution. For example, for oxygen carrier properties of hemoglobin, iron excess is associated with different types of cancer, possibly due to the formation of reactive oxygen species (ROS) (Toyokuni 2009). Increases in transferrin saturation are associated with cancer (Mainous and others 2005; Stevens and others 1988), and a %SAT higher than 60% increases risk of certain types of cancer in people, such as colorectal and lung cancer (Knekt and others 1994). The better studied examples of iron carcinogenic properties due to previous tissue damage are hepatocellular carcinoma in hemochromatosis (usually after cirrhosis) (Hsing and others 1995), lung carcinoma and malignant mesothelioma from exposure to asbestos (Kannerstein and Churg 1972), and ovarian carcinoma in women with history of endometriosis (endometriotic cysts are rich in iron) (Brinton and others 1997). Increases in ferritin are associated with increased risk of breast cancer in atomic bomb survivors (Stevens RG 2011). Numerous animal models of iron-induced cancer have been developed, demonstrating its carcinogenic potential (Campbell 1940; Li and others 1987; Richmond 1959). OSA is the most common cancer and cause of death in Greyhounds (Lord and others 2007; Rosenberger and others 2007). We recently reported that Greyhounds with OSA have lower serum iron and %SAT than healthy Greyhounds (Couto and others 2011). Further studies are needed in veterinary medicine (and in Greyhounds) in order to correlate body iron with prevalence of cancer.
There are controversial studies linking iron stores and stroke risk. In general, epidemiologic studies have associated serum transferrin saturation, stroke incidence and mortality in people (Gillum and others 1996). Ferritin levels have been correlated with mortality in individuals with peripheral arterial disease (Depalma and others 2010). In another study, iron stores and HFE genotype were not related to increased risk of ischemic stroke (Ekblom and others 2007). In postmenopausal women, neither serum iron nor transferrin saturation are associated with stroke risk, but higher serum ferritin concentrations increase the risk of ischemic stroke (van der A and others 2005). Although there is not conclusive evidence, some reports have shown a decreased cancer risk in people with peripheral arterial disease undergoing iron reduction through controlled phlebotomies (Zacharski and others 2008).

Cerebrovascular accidents (i.e. strokes) have been reported in Greyhounds (Garosi and others 2005; McConnell and others 2005), so it would be interesting to investigate iron metabolism in affected dogs to see if there is association between iron biomarkers and strokes.

Platelet counts are inversely correlated with iron stores: decreased iron saturation is associated with increased platelet counts in women with iron deficiency anemia, and it has been suggested that low %SAT stimulates megakaryopoiesis, or that iron has an inhibitory effect on PLT counts (Kadikoylu and others 2006). Greyhounds have low PLT counts (Shiel and others 2007a; Sullivan and others 1994) and high transferrin saturation (Couto and others 2011), which supports the generality of this association, but this inhibitory mechanism of iron is yet to be determined.
b) Iron metabolism in blood donor dogs

In our first part of the study (n=130) we incidentally found that iron is higher and UIBC is lower in BD when compared to C, which makes TIBC (serum iron + UIBC) no different between groups. Also, percentage transferrin saturation (%SAT) is higher in BD. The increased serum iron concentration was unexpected based on the human scientific literature, since the most common adverse effect of frequent blood donation is iron depletion. From the second part of our study, where we evaluated the effects over time of being part of the canine blood donor program on iron status, we found a decrease in PCV, serum iron and transferrin saturation; despite this decrease, none of the dogs developed iron deficiency anemia during the 75.6 weeks (~1.5 years) of duration of the study. Our canine population donates blood approximately every 2 months average time between each donation in our study: 8.67 weeks), meaning that each dog gives a standard unit of blood (~450 ml, same as humans) 6 times per year with no decrease of HCT. This is comparable to human high intensity blood donors (or superdonors), blood donors who, despite donating blood 6.5 times a year do not develop iron deficiency anemia. The fact that same amount of blood (450 ml) is drawn from an animal weighing half as much makes this physiology more striking. Contrary to what it was expected, we find that serum iron and transferrin saturation are higher, and UIBC is lower in BD dogs. With this study we demonstrate that blood donation of ~450ml every 2 months does not impair iron homeostasis of canine donors. Identification of the biology underlying this – possibly a trait selected for in Greyhounds – has important implications for human medicine.
Chapter 2: HEMOGLOBIN IN GREYHOUNDS

2.1. Hemoglobin function in Greyhounds

INTRODUCTION

As previously mentioned, Greyhounds have a high mean hematocrit (Hct), hemoglobin (Hb) concentration and red blood cell (RBC) counts (Shiel and others 2007a); lower white blood cell, neutrophil, and platelet counts; and atypical eosinophil morphology, when compared to dogs of other breeds (Couto and Iazbik 2005; Neuhaus and others 1992; Shiel and others 2007a). Greyhounds may have the highest resting Hct of any mammalian species, which increases significantly during a race due to splenic contraction, RBC release from the bone marrow (Neuhaus and others 1992; Rose and Bloomberg 1989; Snow and others 1988), or by translocation of water out of the vascular space and subsequent hemoconcentration (Neuhaus and others 1992; Nold and others 1991; Shiel and others 2007a; Snow and others 1988; Sullivan and others 1994). Some of these features have been hypothesized to be physiologic adaptations to racing in order to increase oxygen delivery to tissues. However, little is known about Hb function, including oxygen affinity, in Greyhounds compared to non-Greyhound breeds. In a previous study (Sullivan and others 1994), Hb function in Greyhounds was evaluated by
determining the partial pressure of oxygen at which hemoglobin is 50% saturated (P$_{50}$), using oxyhemoglobin dissociation curves (ODC). The P$_{50}$ values were lower in Greyhounds than in other breeds, representing a left shift in the ODC, and therefore a higher affinity for oxygen (Sullivan and others 1994). The RBC 2,3-diphosphoglycerate (2,3-DPG) content was not significantly different between the Greyhounds and the non-Greyhounds in that study. Based on those results, the authors proposed that decreased oxygen release to the tissues could cause increases in erythropoietin production and increased RBC production. They also proposed this higher oxygen affinity, rather than breeding selection, as the likely cause of the high Hb and Hct in this breed (Sullivan and others 1994).

Gas exchange and Hb function can be assessed using blood gas analyzers and cooximetry, respectively. Cooximeters are instruments that measure percentages of the four hemoglobin moieties spectrophotometrically: oxyhemoglobin (O$_2$Hb), deoxyhemoglobin (HHb), carboxyhemoglobin (COHb), and methemoglobin (MetHb) (Myers and Browne 2007). The objectives of this study were to evaluate the oxygen affinity of hemoglobin in healthy retired racing Greyhounds using a critical care blood gas analyzer with a cooximeter, and to establish reference intervals for blood gases and cooximetry in this breed.

MATERIALS AND METHODS

Venous blood samples were obtained from 57 healthy, adult, retired racing Greyhound dogs and 30 healthy, adult dogs of other breeds. Blood samples were analyzed using a
Stat Profile® Critical Care Xpress Analyzer with a cooximeter (STP CCX Analyzer, Nova Biomedical, Waltham, MA, USA), following the manufacturer’s instructions (Nova Biomedical STP CCX Analyzer User Manual, 2003). The analyzer directly measures pH, partial pressure of carbon dioxide (Pco₂), partial pressure of oxygen (Po₂), oxygen saturation (So₂%), hematocrit (Hct) and hemoglobin (Hb). The methods used to measure these parameters were specific electrode (pH), Severinghaus method (Pco₂), amperometric (Po₂), optical reflectance (So₂%), conductivity/Na correction (Hct), and multiple wavelength/conductivity correction (Hb). The cooximeter directly measures by multiple wavelength spectrophotometry the percentages of oxyhemoglobin (O₂Hb%), deoxyhemoglobin (HHb%), carboxyhemoglobin (COHb%), and methemoglobin (MetHb%). The instrument’s software automatically calculates other parameters such as P₅₀, oxygen content (O₂Ct) and oxygen capacity (O₂Cap). Dogs were divided into two groups, Greyhounds (G) and non-Greyhounds (NG), and data were analyzed using GraphPad Prism® statistical software (GraphPad Software, Inc., San Diego, CA, USA). Descriptive statistics and normality testing (D’Agostino & Pearson omnibus normality test) were performed. Unpaired two-tailed Student’s T-tests were used to compare values between both groups when data were normally distributed, and a Mann-Whitney test was used when data did not have Gaussian distribution. Statistical significance was set at P<0.05. Reference intervals for G and NG were established using the central 95% of values (mean ±2SD) when data were normally distributed. For variables that did not follow Gaussian distribution, observed ranges are listed (method used based on the small number of data points, and non-normal distribution (Thrall 2004)).
RESULTS

The Greyhound group had 30 males (53%) and 27 females (47%), with a mean age of 5.7 years (SD 1.65 years), and a mean weight of 32 kg (SD 4.49 kg). The non-Greyhound group had 20 males (67%) and 10 females (33%), with a mean age of 4.7 years (SD 2.51 years), and a mean weight of 31.72 kg (SD 12.30 kg). All the dogs included in the study were spayed/neutered. The other breeds included a wide range of weights and muscle masses. All dogs included in this study were pets with similar activities. The non-Greyhound group included a variety of large and small breeds. All data was normally distributed with the exception of MetHb in both groups, and pH and P50 in the G group.

As has been previously reported (Porter and Canaday 1971; Shiel and others 2007a), we found a significantly higher Hct in Greyhounds (P<0.0001) compared to non-Greyhounds. Values from the blood gas analyzer and cooximeter are shown in Table 7. Greyhounds had significantly higher pH, P02, SO2%, O2Hb%, tHb, O2Ct, O2Cap, and lower HHb% and P50 compared to non-Greyhounds. The remaining parameters (PCO2, COHb%, MetHb%) were not statistically different between Greyhounds and non-Greyhounds. As shown in Figure 9, the distribution of the P50 values was much narrower in Greyhounds (range 26.00-28.40 mm Hg; SD=0.40) than in the non-Greyhounds (range 25.90-38.50; SD=4.28).

Both SO2% and O2Hb were significantly higher (p<0.0001) in Greyhounds (SO2 mean, 89.18%; O2Hb mean, 86.51%) than in non-Greyhounds (SO2 mean, 77.05%; O2Hb mean, 75.41%). This result contrasts with the lower percentage of deoxyhemoglobin in Greyhounds compared to the non-Greyhound group (10.77% versus 21.36%; p<0.0001). Greyhounds also had higher total tHb (mean, 21.53 g/dL; p<0.0001) than non-
Greyhounds (mean, 18.16 g/dL). Greyhound-specific reference intervals for parameters measured in this study are shown in Table 8, compared to reference intervals for non-Greyhounds.

Table 7. Venous cooximetry and blood gas mean values ± SD of Greyhounds and non-Greyhounds exhibiting significant difference

<table>
<thead>
<tr>
<th>Value</th>
<th>Greyhounds (n=57)</th>
<th>Non-Greyhounds (n=30)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td>7.41±0.03</td>
<td>7.40±0.03</td>
<td>P=0.034</td>
</tr>
<tr>
<td>Hematocrit (Hct, %)</td>
<td>51.7±3.9</td>
<td>46.1±2.8</td>
<td>P&lt;0.0001</td>
</tr>
<tr>
<td>Partial Pressure of Oxygen (PO₂, mmHg)</td>
<td>60.3±12.0</td>
<td>52.1±8.7</td>
<td>P=0.0014</td>
</tr>
<tr>
<td>Oxygen Saturation (SO₂, %)</td>
<td>89.2±5.3</td>
<td>77.0±11.3</td>
<td>P&lt;0.0001</td>
</tr>
<tr>
<td>Oxyhemoglobin (O₂Hb, %)</td>
<td>86.5±5.5</td>
<td>75.4±10.3</td>
<td>P&lt;0.0001</td>
</tr>
<tr>
<td>Deoxyhemoglobin (HHb, %)</td>
<td>10.8±5.2</td>
<td>21.4±9.3</td>
<td>P&lt;0.0001</td>
</tr>
<tr>
<td>Hemoglobin (tHb, g/dL)</td>
<td>21.5±1.7</td>
<td>18.2±1.6</td>
<td>P&lt;0.0001</td>
</tr>
<tr>
<td>P₅₀ (mmHg)</td>
<td>26.5±0.3*</td>
<td>29.9±4.3</td>
<td>P&lt;0.0001</td>
</tr>
<tr>
<td>Oxygen Content (O₂Ct, mL/dL)</td>
<td>25.9±3.1</td>
<td>19.0±2.8</td>
<td>P&lt;0.0001</td>
</tr>
<tr>
<td>Oxygen Capacity of Hb (O₂Cap, mL/dL)</td>
<td>29.0±2.6</td>
<td>24.3±2.1</td>
<td>P&lt;0.0001</td>
</tr>
</tbody>
</table>

Data non normally distributed, presented as median and inter quartile range
Table 8. Greyhound-specific and non-breed-specific reference intervals for venous cooximetry and blood gas analysis

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Reference Interval Greyhounds (n = 57)</th>
<th>Reference Interval Non-breed-specific (n = 30)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pO₂ (mmHg)</td>
<td>36.3–84.3</td>
<td>34.6 – 69.6</td>
</tr>
<tr>
<td>pCO₂ (mmHg)</td>
<td>25.6–39.9</td>
<td>24.7 – 44.4</td>
</tr>
<tr>
<td>SO₂ (%)</td>
<td>78.6–99.8</td>
<td>54.4 – 99.8</td>
</tr>
<tr>
<td>tHb (g/dL)</td>
<td>18.1–25.0</td>
<td>15.0 – 21.3</td>
</tr>
<tr>
<td>O₂Hb (%)</td>
<td>75.6–97.4</td>
<td>54.7 – 96.1</td>
</tr>
<tr>
<td>COHb (%)</td>
<td>0.9–3.9</td>
<td>0.4 – 4.5</td>
</tr>
<tr>
<td>MetHb (%)</td>
<td>0.0–2.2</td>
<td>0.1 – 2.8</td>
</tr>
<tr>
<td>HHb (%)</td>
<td>0.4–21.2</td>
<td>2.7 – 40.0</td>
</tr>
<tr>
<td>P50 (mmHg)</td>
<td>26.0–28.4</td>
<td>21.4 – 38.4</td>
</tr>
<tr>
<td>O₂Ct (mL/dL)</td>
<td>19.7–32.0</td>
<td>13.3 – 24.6</td>
</tr>
<tr>
<td>O₂Cap (mL/dL)</td>
<td>23.8–34.1</td>
<td>20.2 – 28.5</td>
</tr>
</tbody>
</table>

pO₂, partial pressure of O₂; pCO₂, partial pressure of O₂; SO₂, oxygen saturation; tHb, total hemoglobin; O₂Hb, oxyhemoglobin; COHb, carboxyhemoglobin; MetHb, methemoglobin; HHb, deoxyhemoglobin; O₂Ct, oxygen content; O₂Cap, oxygen capacity.

Figure 9. Hemoglobin P50 values (partial pressure oxygen at which hemoglobin is 50% oxygenated) of healthy Greyhounds and non-Greyhounds
DISCUSSION

In summary, Greyhounds have higher $P_{O_2}$, $SO_2$, $O_2Hb$, tHb, $O_2Ct$, and $O_2Cap$ than non-Greyhounds. These parameters assess the oxygenation and function of the Hb molecule, and higher values support the fact that Greyhounds are able to carry a higher concentration of total oxygen in the blood. However, Greyhounds also have lower $P_{50}$ than non-Greyhounds, which could be due to higher oxygen affinity. Previous studies have proposed that a decrease in oxygen affinity (higher $P_{50}$) is beneficial for athletic performance (Biolo and others 2009; Cambier and others 2004; Fenger and others 2000; Henderson and others 2000; Richardson and others 1998; Schumacker and others 1985; Watanabe and others 2008), since the oxygen is more easily released from the Hb to tissues. In Greyhounds, the apparent high oxygen affinity hemoglobin seems to contradict this theory.

In people, hemoglobinopathies are the most frequently found monogenic disorders worldwide (Gonzalez Fernandez and others 2009). Over 1500 Hb structural variations have been described, including single mutations, deletions, or insertions in the genes that encode either the $\alpha$ or $\beta$ globin chain. In many of these structural variations, there is a single amino acid mutation that leads to changes in stability, solubility, and function. To date, there are 93 reported hemoglobinopathies associated with high oxygen affinity according to the HbVar database (database of hemoglobin variants), and they are associated with tissue hypoxia (Wajcman and Galacteros 2005). This hypoxia triggers production of erythropoietin by hypoxia-inducible factors (HIF), leading to secondary
erythrocytosis (Semenza 2009). Patients with high-affinity Hb have low $P_{50}$, as is the case in Greyhounds.

As previously mentioned, the ODC in dogs is strongly influenced by breed, with $P_{50}$ values widely dispersed among breeds, ranging from 25.8 mmHg in spaniels to 35.8 mmHg in hounds (Clerbaux and others 1993). In the present study, the unusual minimal dispersion as well as the low $P_{50}$ in Greyhounds suggests that unknown factors have selected for a very specific Hb oxygen affinity this breed. Since previous reports show that 2,3-DPG concentrations in Greyhounds are not different that in other breeds (Sullivan and others 1994), one of our hypothesis is that Greyhounds may be more sensitive to pH changes (Ilkiw and others 1989), causing the ODC left shift.

Although arterial samples are more traditionally used for the assessment of oxygenation, venous samples were used in this study based on the guidelines for routine measurement of blood Hb oxygen affinity (Wimberley and others 1991) because $P_{50}$ should not vary among them, and venous samples are more commonly obtained, stable and easy to run in the clinical setting. The $O_2$Ct is the total amount of oxygen in the blood (dissolved oxygen and oxygen bound to Hb) and is calculated by the cooximeter using the equation $1.39*Hb*SO_2\% + 0.003*P_{O_2}$. $O_2$Cap is the total amount of oxygen that Hb can carry and is calculated by the cooximeter using the equation $1.39*(O_2Hb\%+HHb\%)/100*[tHb]$. In the present study both $O_2$Ct and $O_2$Cap were significantly higher in Greyhounds, which suggests that it could be a consequence of the high affinity hemoglobin and stronger binding between Hb and $O_2$.

It is still unclear how an athletic breed like Greyhounds can benefit from a low $P_{50}$. Researchers working on hemoglobin-based oxygen carriers (HBOCs) have
demonstrated that a high-affinity oxygen carrier is beneficial, suppressing vasoconstriction elicited by early off-loading and over-oxygenating tissues at the level of the pre-capillary sphincter (Dimino and Palmer 2007). In normal individuals, oxygen is released at the arteriolar level before it reaches the capillaries. When the Hb has higher affinity, the oxygen remains bound longer (i.e. through arteriolar circulation), and can be released at a deeper tissue level (i.e. capillaries), where oxygen tension is lower (Vandegriff and Winslow 2009). In the case of racing Greyhounds, this mechanism may allow delivery of oxygen to the most hypoxic tissues. Therefore, although counterintuitive to traditional exercise physiology theories, benefits of having a high-affinity Hb in an athletic breed like Greyhounds could be explained based on that.

Results from this study, in combination with the previous findings of decreased cooperative binding of Hb (Sullivan and others 1994), strongly indicate that Greyhounds may have a unique structural variation in the Hb molecule. Alternatively, given the high Hct (mean, 51.70±3.92%), the high viscosity in Greyhounds could have impeded a constant flow rate through the channel in the instrument, thus altering the cooximeter values. In order to test this hypothesis, we run a small number of additional samples in duplicate: one raw sample, and the other 20% diluted with PBS solution, reaching a non-Greyhound Hct (~40%) without changing the pH (data not shown) and the results were similar (no statistical difference between non-diluted and diluted samples). Preliminary data from electrophoresis of Hb from retired racing Greyhounds (data not shown) did not reveal any different mobility patterns compared to non-Greyhounds. However, this technique may not be useful in the evaluation of high affinity Hb because many of the mutations are electrophoretically silent (Jones and others 1990). Crystallography is
helpful in characterizing this high affinity Hb (Balasubramanian and others 2009; Sundaresan and others 2009), and results of Greyhound Hb crystal structure will be discussed in the next section.

This study had some potential limitations: 1) as in most clinical studies, sample handling could have influenced venous SO$_2$%, P$_{CO_2}$ and P$_{O_2}$ (Rezende and others 2007), but this potential influence was minimized since all samples were handled by the same operator (G and NG), and SO$_2$% and P$_{O_2}$ in Greyhounds are still significantly higher than in non-Greyhounds; such high SO$_2$% may be because the Greyhound high oxygen affinity makes their Hb more likely to remain bound to oxygen, giving therefore higher SO$_2$%. 2) Also, although this instrument is widely used in hospitals and emergency practices (Grosenbaugh and others 1998; McMillan and others 2009; Scott and others 2005), no validation for its use in dogs has been reported. The only parameter that has been validated in vitro is oxygen saturation (SO$_2$%) (Jahr and others 2000).
2.2. Greyhound hemoglobin genetics

INTRODUCTION

Hemoglobin’s (Hb) main function is to transport oxygen from the lungs (high affinity) to other tissues and organs such as muscles (low affinity), where the oxygen is released at the capillary level for its use for cell metabolism. As shown by crystallographic studies, Hb is formed by symmetric pairing of alpha-like (i.e. alpha, zeta) and beta-like (i.e. epsilon, gamma, delta, beta) globin polypeptide chains, into a tetrameric structural and functional unit (Perutz 1963). Each of the subunits (globins) has an iron ion (oxygen-binding site) held in a porphyrin ring, and the binding of the oxygen molecules will lead to conformational changes (T, R or R2 states). Under standard conditions (37°C, pH 7.4, PCO₂ 40 mmHg), variation in Hb’s affinity for oxygen is determined mainly by the primary structure of the Hb (i.e. point mutations, nucleotide variations) and the chemical composition of RBCs (i.e. 2,3-DPG levels).

At the molecular level, alpha- and beta-like chains are synthetized from two different gene clusters, located in different chromosomes (in human chromosomes 16 - chr16- and 11 -chr11-, respectively); they are arranged in developmental order, being turned on and off sequentially (developmental switches): embryonic → fetal → adult (beta), and embryonic → adult (alpha) (Tang and others 2008). Both beta-globin and alpha-globin gene clusters’ expression patterns are regulated by promoters of the individual genes, but also by a large sequence located upstream of both clusters. This
sequence is called the locus control region (βLCR) in the beta cluster, and upstream regulatory element (αURE) in the alpha cluster (Bulger and Groudine 2011; Fromm and Bulger 2009; Tang and others 2008). In humans, βLCR starts 2 Kb upstream from HBE1, extending approximately 20 to 30 Kb, and αURE is located upstream from the first alpha-globin gene (HBZ), extending approximately 10 to 50 Kb (Tang and others 2008). These regulatory elements are marked by erythroid-specific DNase I hypersensitive sites, and have a high density of transcription factor binding sites (Fromm and Bulger 2009). Such protein complexes (transcription factors) can be activators or repressors, regulating expression and developmental switches (Chen and others 2010; Fromm and Bulger 2009). Physiologically, fetal Hb (HbF, 2 alpha and 2 gamma chains, α2γ2) has slightly higher oxygen affinity than adult Hbs because it binds 2,3-DPG less strongly. Adult human Hbs are predominantly HbA (2 alpha and 2 beta chains, α2β2) and HbA2 (2 alpha and 2 delta chains, α2δ2); HbA accounts for about 97% of the erythrocyte protein molecules, HbA2 for 2% and HbF for 1% in normal adults. The basic globin gene structure and protein folds are similarly conserved in evolution among all placental mammals. A recent phylogenetic study of the beta-globin cluster in placental mammals has shown that: the embryonic (ε) gene is present in all species; fetal globins branched in two, with Euarchontoglires (e.g. primates, rodents) retaining the γ-globin gene, and Laurasiatheria (e.g. carnivores, ungulates, bats) retaining the η-globin gene; β-globin is present in all species studied except for the order Eulipothyphia (e.g. shrew, hedgehog); and δ globin gene is not present in orders Rodentia (e.g. mouse, rat), Lagomorpha (e.g. rabbit) or Afrotheria (e.g. lesser hedgehog tenrec) (Opazo and others 2008).
The dog’s Hb oxygen affinity has been shown to differ among breeds (mean P$_{50}$: 30.0 mmHg, SD 1.3), with no correlation found with 2,3-DPG (Cambier and others 2004; Clerbaux and others 1993). As previously explained, we recently studied blood gases and Hb function in RRGs, showing that this sporting breed’s Hb molecule is able to carry more oxygen in the blood, and has higher oxygen affinity (lower P$_{50}$) than that in other breeds (Zaldivar-Lopez and others 2011a). We have also reported the crystal structure of Greyhound Hb at 1.9 Å, which was found to crystallize in the R2 state; this fact, in addition to the low P$_{50}$, supports the high affinity of Greyhound Hb (Bhatt and others 2011; Pairet and Jaenicke 2010). This finding is counterintuitive since previous studies in exercise physiology had demonstrated that a decrease in oxygen affinity is beneficial for athletic performance (Biolo and others 2009; Cambier and others 2004). However, current studies on hemoglobin-based oxygen carriers (HBOCs) have demonstrated that a higher oxygen affinity could be beneficial, by suppressing the vasoconstriction elicited by early off-loading and over-oxygenating tissues at the level of the pre-capillary sphincter, and thus delivering more oxygen to the tissues (Dimino and Palmer 2007). This allows for delivery to the tissues that are in immediate need of oxygen, which should confer a benefit during strenuous exercise (i.e. Greyhounds) (Sullivan and others 1994; Zaldivar-Lopez and others 2011a).

Due to the high prevalence of hemoglobinopathies in people, the alpha- and beta-globin genes of humans, mice, and chickens, are well characterized (Fromm and Bulger 2009; Kiefer and others 2008; Schechter 2008). However, despite the increasing importance of dog as models for human diseases (Rowell and others 2011), almost nothing is known about dog Hb genetics, and it is reported they only have one beta globin
gene, and that they lack fetal Hb (Brimhall and others 1977; LeCrone 1970; Seal 1969). Evidence of high affinity Hb in Greyhounds suggests that Hb genes might be mutated, affecting the physiological properties of this breed’s Hb.

Based on our previous work and preliminary computational analysis, we hypothesized that dogs have at least six active globin genes (not only one, as previously reported), and that at least one of them is mutated in Greyhounds. The specific aims of the study are:

1. To define the gene structure of canine alpha- and beta-globins, and established that they are expressed in vivo
2. To investigate if there are differences in the globin genes in Greyhounds compared with non-Greyhound dogs.

MATERIALS AND METHODS

Animals

A total of 13 dogs were used for this study. Genomic DNA was isolated from venous blood drawn from 10 retired racing Greyhounds (G), and one non-Greyhound dog (NG). Two additional dogs were used to isolate RNA and synthetize cDNA: one RRG and one NG (German Shepherd Dog), both of which were healthy.

All retired racing Greyhounds and non-Greyhound dogs used for this study were recruited at The Ohio State University Veterinary Medical Center, and samples were collected after signed owner consent. All dogs from this study were purebred, and all samples were verified for registration and pedigree information: for racing Greyhounds,
NGA (National Greyhound Association) registration; and for non-Greyhounds, AKC (American Kennel Club) registration paperwork. This study was approved by the Institutional Animal Care and Use Committee (IACUC, protocol number 2010A0025-AM1) and hospital Clinical Research Advisory Committee (CRAC).

**Blood processing**

Venous blood was collected from the dogs and placed into tubes containing EDTA anticoagulant or into RNA tubes. DNA was isolated using the Gentra Puregene genomic DNA purification kit (QIAGEN Inc., Valencia, CA, USA), adding an ethanol precipitation step to the original protocol; RNA was isolated using the PAXgene blood RNA kit (QIAGEN Inc., Valencia, CA, USA). RNA isolation was followed by cDNA synthesis using Superscript II Reverse Transcriptase (Life Technologies Corp., Grand Island, NY, USA).

**Computational analysis**

Computational analysis was done using the Basic Local Alignment Search Tool database and tool ([http://blast.ncbi.nlm.nih.gov/Blast.cgi](http://blast.ncbi.nlm.nih.gov/Blast.cgi)) and UCSC Genome browser ([http://genome.ucsc.edu/](http://genome.ucsc.edu/)). The dot plot images of the regulatory region analysis were obtained from BLAST. Protein alignments were done using ClustalW2 ([http://www.ebi.ac.uk/Tools/msa/clustalw2/](http://www.ebi.ac.uk/Tools/msa/clustalw2/)) and consensus sequences were obtained using Weblogo ([http://weblogo.berkeley.edu/](http://weblogo.berkeley.edu/)).

**DNA sequencing**
Primers were designed in order to PCR amplify and sequence all the globin genes, including a minimum of all exons and immediate flanking intron sequence, and promoter regions (500 base pairs upstream each gene) from genomic DNA and cDNA. PCR forward and reverse primers were custom designed to amplify products of ~800 bp. PCR amplification was performed using JumpTaq polymerase (JumpTaq Hot Start Polymerase, Sigma); Tm, annealing time and number of cycles was adjusted to primer sets in order to optimize amplification with similar conditions for all PCRs. Products were purified (PCR purification/gel extraction kits; QIAGEN Inc., Valencia, CA, USA) and sequenced (Eurofins MWG Operon, Huntsville, AL, USA), and results obtained were analyzed using the computer software DNASTAR Lasergene Core Suite (DNASTAR Inc., Madison, WI, USA), comparing the sample data to the corresponding gene of the canine reference sequence (Boxer breed; canFam2). Variant nomenclature used was according to recommendations of the Human Genome Variation Society (HGVS).

RESULTS AND DISCUSSION

a) Mapping and gene structure of canine alpha/beta globin genes

Computational analysis (BLAST) of the dog genome (canFam2) was performed in order to map and characterize canine globin genes. Canine alpha- and beta- globin gene clusters are located in different chromosomes (CFA6 and CFA21, respectively), as in humans. Five genes constitute each one of the clusters, and all of them have the same basic structure as the human globin genes (3 exons with 2 introns), arranged in developmental order.
We were unable to identify the alpha-globin genes in the available dog genome assembly (canFam2), since the human alpha-globin region mapped to the Unknown or Unmapped canine chromosome (chrUn or CFAUn) and was incomplete. We then selected human alpha-globin cluster flanking sequences/genes, which mapped to canine chromosome 6 (CFA6), and a large sequence gap between them (where the alpha-globin gene cluster should be located) could be observed. Fortunately, the canFam3.1 dog genome was published during the execution of this study with that gap resolved, and a total of 5 alpha-like genes were identified in dog chr6: three embryonic-like (HBZ1, HBZ2 and HBM) and two adult-like (HBA1 and HBA2). As in humans, the genes are arranged in order of developmental expression (embryonic to adult): HBZ1-HBZ2-HBM-HBA1-HBA2 (Figure 10A). There are duplicated genes in this cluster, with the same coding sequence, but different introns: HBZ1 and HBZ2 (which have identical protein sequence), and HBA1 and HBA2 (same protein sequence with one amino acid change [Ala/Thr] in position 131).

In the beta-globin computational analysis, we identified the entire gene cluster in the canine chromosome 21 (CFA21). Due to the lack of specific annotation in the canine genome (canFam2), we compared protein and DNA sequence from multiple mammalian species and canine examined expressed sequence tags (ESTs). We thus determined that the Genscan gene predictions appeared to be correct, and that this genomic region has five beta globin genes in a single locus: two embryonic/fetal-like genes and three adult-like genes. Based on previous phylogenetic analyses of the globin genes in different mammals by Opazo and others (Opazo and others 2008), we named the embryonic/fetal-like genes HBE1 and HBE2 and the adult-like genes HBD1, HBD2 and HBB. As in the
alpha cluster, beta-globin genes are arranged in developmental order (embryonic to adult): HBE1-HBE2-HBD1-HBD2-HBB (Figure 10B). There is a partial duplication in this cluster: HBD1 and HBD2 have the same protein sequence, but different intronic sequences. We aligned the alpha- and beta-globin genes of different placental mammals in order to see the conservation of the different parts of the genes (Figures 11 and 12). The species selected and their classification and scientific information are shown in table 9. Computational analysis of the adult beta-like globin gene (HBD1, HBD2 and HBB) ESTs demonstrated that both HBD1 and HBD2 are abundantly expressed in dogs whereas HBB is rarely expressed. This finding is counterintuitive and contrary to humans, where HBB is the most commonly expressed gene and protein (accounting for 97% of the adult beta globin chains). Since we had previously published the crystal structure of Greyhound Hb (Bhatt and others 2011), we compared the sequence of the beta chain crystallized to the adult beta-globin protein sequences (HBD1, HBD2 and HBB) and we found that the reported protein sequence corresponded to HBD (protein product from either HBD1 or HBD2) or δ-globin chain. Therefore, contrary to humans, HBD-like globin genes (HBD1 and HBD2) are the highly expressed beta-globins in dogs, meaning that the predominant Hb tetramer combination is likely α2δ2 (equivalent to HbA2 in humans).

As previously mentioned, globin genes expression is regulated by the αURE (alpha-globin gene cluster) and the βLCR (beta-globin gene cluster). Given the homology found between humans and dogs in the alpha- and beta-globin gene clusters, we further analyzed upstream regions in order to investigate similarities between dog and human regulatory elements. Based on the human literature, we selected the genomic region
extending 60 Kb upstream $HBZ$ and $HBZ1$ genes and aligned them (using BLAST tool) in order to assess the similarities between human and canine $\alpha$URE (Figure 13A). We repeated the same analysis for the $\beta$LCR, selecting 30 Kb upstream the $HBE1$ and $HBE1$ and aligning both sequences (Figure 13B). Results demonstrate that the canine and human genomic sequences upstream alpha- and beta-globin clusters are well conserved (Figure 13), suggesting a similar regulatory role of canine upstream globin loci sequences. In order to demonstrate this theory, further studies are needed.

Figure 10. Diagram of alpha- (A) and beta-globin (B) gene clusters in human (above) and dog (below) genomes, including upstream regulatory regions (URE, upstream regulatory element; LCR, locus control region).
Table 9. Phylogenic information of the species used for the alignments of the globin genes

<table>
<thead>
<tr>
<th>KINGDOM</th>
<th>PHYLUM</th>
<th>CLASS</th>
<th>INFRACLASS</th>
<th>CLADE</th>
<th>SUPERORDER</th>
<th>ORDER</th>
<th>SPECIES NAME</th>
<th>COMMON NAME</th>
</tr>
</thead>
<tbody>
<tr>
<td>Animalia</td>
<td>Chordata</td>
<td>Mammalia</td>
<td>Eutheria</td>
<td>Boreoeutheria</td>
<td>Euarchontoglires</td>
<td>Primates</td>
<td>Homo sapiens</td>
<td>Human</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Macaca mulatta</td>
<td>Rhesus macaque</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Otolemur garnettii</td>
<td>Small-eared Galago</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Rodents</td>
<td>Mus musculus</td>
<td>Mouse</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Laurasiatheria</td>
<td>Perissodactyla</td>
<td>Equus caballus</td>
<td>Horse</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Chiroptera</td>
<td>Rhinolophus ferrumequinum</td>
<td>Greater horseshoe bat</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Eulipotyphla</td>
<td>Sorex araneus</td>
<td>Eurasian shrew</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Carnivora</td>
<td>Canis familiaris</td>
<td>Dog</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Atlantogenata</td>
<td>Xerathra</td>
<td>Dasypus novemcinctus</td>
<td>Nine-banded armadillo</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Afrotheria</td>
<td>Loxodonta africana</td>
<td>Elephant</td>
</tr>
</tbody>
</table>
Figure 11. Alignment (A) and consensus sequence (B) of the alpha-globin genes in placental mammals

A

B
Figure 12. Alignment (A) and consensus sequence (B) of the beta-globin genes in placental mammals
Figure 13. Graphical representation of the alignment between alpha- (A) and beta-globin (B) regulatory regions in humans (X axis) and dogs (Y axis).
b) **Discovery of genetic variation in Greyhound alpha/beta globin loci**

As mentioned earlier in the chapter, G Hb has higher affinity for the oxygen than other dog breeds (Bhatt and others 2011; Zaldivar-Lopez and others 2011a). Since intra-species’ variation in hemoglobin affinity is determined by the primary structure of Hb, we investigated if G peculiarities are due to sequence structure modifications. This investigation of potential genetic variation in the G breed was structured in two parts:

1. Alpha and beta-globin genes from one G and one NG dogs were amplified, sequenced and analyzed, comparing them to the reference dog sequence, in order to identify variations.

2. Where nucleotide changes were found in G, 8 or more additional individuals were analyzed, with the purpose of assessing the variation frequency in the breed.

These two clusters have a total of ten genes and all can be detected in adult blood cDNA. Our physiological findings suggest that all or almost all Greyhounds have the atypical oxygen carrying properties and this is consistent with reduced variation pertaining to an ancestral sighthound haplotype. Given the variation of interest is extremely common, we next sequenced all ten alpha/beta globin genes in a small number of Greyhounds (Table 10) and compared to a non-Greyhound and the reference sequences of a Boxer and a Poodle (NCBI Genbank). In the alpha-globin gene cluster, only one polymorphism was found in the G *HBZ2* promoter (g.-155C>T), but such variation was not present in the additional 9 G sequenced. Therefore, no G-specific nucleotide changes were identified in the canine alpha-globin cluster. In the beta cluster,
no changes were found in coding sequences from G embryonic genes (*HBE1* and *HBE2*), but a single nucleotide polymorphism (SNP) was detected in the G *HBE1* promoter region (g.-156C>T), and this SNP was present in all the additional G studied (100%). No polymorphisms were found in G *HBD1* and *HBD2* coding sequence. Five novel SNPs were detected in the G *HBD1* genomic sequence: in the promoter (1), first (1) and second (3) introns. Our results suggest there are two haplotypes in this region, given that either the dogs had no SNPs, or all 5 SNPs.

Multiple SNPs were found in Greyhound *HBB* gene. From all the variations found in the promoter region and first exon (n=13), 2 different patterns of variations were observed (table 11): 2/8 dogs share one of the patterns, meanwhile 4 other dogs (4/8) share a different one, with only three of the thirteen possible SNPs being present in both potential haplotypes. Of the SNPs found in this region, the best candidate to explain unusual oxygen carrying properties is Ile12Val, which is found in all Greyhounds. Strikingly for a gene that is so highly conserved within species, four of the SNPs (first exon) lead to amino acid substitutions (Figure 14), and one in the first intron. All amino acid substitutions in G *HBB* were located at the beginning of the first exon. Interestingly, all of them were replaced by the amino acid that is located in that position in *HBD* (i.e. positions 12, 14, 15, 17 and 22), therefore transforming that region of the *HBB* in *HBD*-like in Greyhounds (Figure 15). Two different patterns of variation were also observed in this *HBB* region, with all the variants being in one *HBD-like* haplotype. Since we had recorded all dogs’ information and racing tattoo numbers, we performed a pedigree analysis (http://www.greyhound-data.com/) and found that the dogs that shared SNP patterns also shared a common ancestor, both of them are in the Greyhound Racing Hall.
of Fame (Kunta Kinte and Dutch Bahama). Both haplotypes are common and we speculate that one or both were under selection for racing performance at one time or another (Figure 16).

The mammalian beta-globin locus is considered a model of gene regulation, where gene expression can be modulated by a large sequence located upstream of the cluster, by the promoters of each gene, or other conserved distal enhancers (Bulger and Groudine 2011; Fromm and Bulger 2009). In humans, several reports have demonstrated that differences in expression of globin genes can be caused by mutations or polymorphisms in gene promoters, coding sequences or introns (Giardine and others 2011; Jouini and others 2012). Human high affinity Hb variants are often associated with erythrocytosis and thrombocytopenia (Wajcman and Galacteros 2005) and caused by amino acid mutations that cause an increase in the affinity for oxygen, leading to hypoxia; this decrease in oxygenation is compensated by an increase in erythropoiesis (Kauppi and others 2012). All human Hb variants and thalassemias are registered in the HbVar database (http://globin.bx.psu.edu/hbvar/menu.html). There is evidence showing that the human embryonic (epsilon) globin gene expression is controlled by the βLCR (Raich and others 1990) and the gene promoter (Alhashem and others 2011). In the present study, we did not sequence the βLCR, but we found a SNP present in the HBE1 promoter of all the Greyhounds studied; since an increase in embryonic and fetal globin expression increases the Hb oxygen affinity, we suggest that this Greyhound-specific variation could be the cause of the difference in affinity of this breed. However, further work is needed in order to test this hypothesis.
Despite being the minor adult globin in humans (~2%), we conclude based on Opazo and others’ work (Opazo and others 2008) and our own analysis that δ-globin (encoded by the \textit{HBD} gene) is the main globin in adult dogs, and likely in the other members of the superorder Laurasiatheria (e.g. bat, hedgehog, cat, horse). Canine δ-globin binds to α-globin in order to form the functional Hb tetramer, which will transport oxygen from the lung to the different body tissues and organs. Mutations in the \textit{HBD} gene and protein have been described in humans (Bouva and others 2006; Papadakis and others 1997). HbA$_2$ ($\alpha_2\delta_2$) has similar properties to fetal Hb (HbF): higher oxygen affinity and avoids HbS polymerization in thalassemias (Inagaki and others 2000). We have discovered 6 SNPs in the promoter and introns of the \textit{HBD1} gene in Greyhounds; one of them (g.191_192insG) is present in all the Greyhounds, and the other 5 in almost half of the population. These nucleotide polymorphisms could change \textit{HBD} expression and overall Hb affinity, but further studies are needed.

HbA ($\alpha_2\beta_2$) is the main Hb type in people (i.e. \textit{HBB} is the most expressed adult beta globin gene), but β has evolved as the minor adult beta globin in dogs (i.e. \textit{HBB} is the least expressed adult beta globin gene). Polymorphisms in the βLCR and promoter of \textit{HBB} can vary such expression (Giardine and others 2011; Kukreti and others 2010). In Greyhounds we detected several SNPs in the promoter and first exon, which could cause a change in \textit{HBB} expression. From all the SNPs detected, 4 of them lead to an amino acid change; surprisingly, 3 out of 4 are substituted by the amino acid that is in that position in \textit{HBD}, transforming Greyhound \textit{HBB} in \textit{HBD-like} in the proximal region. This finding is even more important knowing that canine \textit{HBB} and \textit{HBD} protein sequences differ in only 7 amino acids (Figure 15), therefore Greyhound \textit{HBB} is more similar to \textit{HBD}. We
conclude that Greyhound $HBB$ Ile12Val, possibly in combination with regulatory variants, is the best candidate to explain the unusual Hb properties of Greyhound Hb.
Table 10. Summary of genetic variants found in Greyhound alpha- and beta-globin genes.

<table>
<thead>
<tr>
<th>GENE</th>
<th>VARIANT</th>
<th>LOCATION</th>
<th>BREED</th>
<th>FREQUENCY</th>
</tr>
</thead>
<tbody>
<tr>
<td>HBZ2 (LOC100855600)</td>
<td></td>
<td></td>
<td></td>
<td>Not present in additional G</td>
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<tr>
<td></td>
<td>g.-155C&gt;T</td>
<td>Promoter</td>
<td>G</td>
<td></td>
</tr>
<tr>
<td></td>
<td>p.Phe34Phe</td>
<td>Exon 1</td>
<td>NG</td>
<td></td>
</tr>
<tr>
<td>HBE1 (LOC100855634)</td>
<td></td>
<td></td>
<td></td>
<td>10/10 (100%)</td>
</tr>
<tr>
<td></td>
<td>g.-156C&gt;T</td>
<td>Promoter</td>
<td>G</td>
<td></td>
</tr>
<tr>
<td>HBE2 (HBG)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>g.790A&gt;G</td>
<td>Intron 2</td>
<td>NG</td>
<td></td>
</tr>
<tr>
<td></td>
<td>g.1205G&gt;A</td>
<td>Intron 2</td>
<td>NG</td>
<td></td>
</tr>
<tr>
<td></td>
<td>g.1217&gt;A</td>
<td>Intron 2</td>
<td>NG</td>
<td></td>
</tr>
<tr>
<td>HBD1 (LOC609402)</td>
<td></td>
<td></td>
<td></td>
<td>4/9 (44.4%)</td>
</tr>
<tr>
<td></td>
<td>g.-61A&gt;C</td>
<td>Promoter</td>
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<td>g.122T&gt;C</td>
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<td>g.812C&gt;A</td>
<td>Intron 2</td>
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<td>g.825G&gt;A</td>
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<td>G</td>
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<td></td>
<td>g.914T&gt;G</td>
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<td>G</td>
<td></td>
</tr>
<tr>
<td>HBB (HBD)</td>
<td></td>
<td></td>
<td></td>
<td>3/8 (37.5%)</td>
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<tr>
<td></td>
<td>g.-357A&gt;T</td>
<td>Promoter</td>
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<td>g.-351C&gt;T</td>
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<td>g.-292A&gt;T</td>
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<td>g.-256C&gt;T</td>
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<td>g.-210A&gt;G</td>
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<td>g.-197T&gt;A</td>
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<td>g.-141G&gt;A</td>
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<td>g.-125C&gt;T</td>
<td>Promoter</td>
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<td></td>
</tr>
<tr>
<td></td>
<td>p.Ile12Val</td>
<td>Exon 1</td>
<td>G</td>
<td>8/8 (100%)</td>
</tr>
<tr>
<td></td>
<td>p.Ser14Gly</td>
<td>Exon 1</td>
<td>G</td>
<td>5/8 (62.5%)</td>
</tr>
<tr>
<td></td>
<td>p.Met15Leu</td>
<td>Exon 1</td>
<td>G</td>
<td>5/8 (62.5%)</td>
</tr>
<tr>
<td></td>
<td>p.Gly17Asp</td>
<td>Exon 1</td>
<td>G</td>
<td>2/8 (25%)</td>
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<tr>
<td></td>
<td>p.Asp22Asp</td>
<td>Exon 1</td>
<td>G</td>
<td>7/8 (87.5%)</td>
</tr>
<tr>
<td></td>
<td>g.130G&gt;A</td>
<td>Intron 1</td>
<td>G</td>
<td>2/11 (18.1%)</td>
</tr>
</tbody>
</table>

Gene names in parentheses correspond to the names in the NCBI gene database. Breed: G (Greyhounds), NG (non-Greyhounds). Variant: g. (genomic) or p. (protein) followed by position (position 1 is the A from the starting methionine) and followed by the nucleotide or amino acid change. Transcription start sites are not yet mapped; I refer to the sequence upstream of the initiating ATG (5' untranslated region and promoter) as Promoter.
Table 11. Description of variants in each dog following two distinct patterns (green and orange) and potential combinations between them. Dogs are named with a G and consecutive numbers; X represents the presence of the nucleotide change; blue means heterozygous and red represents homozygousity

<table>
<thead>
<tr>
<th>DOG</th>
<th>VARIANTS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>g.357A&gt;T</td>
</tr>
<tr>
<td>G1</td>
<td>X</td>
</tr>
<tr>
<td>G2</td>
<td>X</td>
</tr>
<tr>
<td>G3</td>
<td>X</td>
</tr>
<tr>
<td>G4</td>
<td>X</td>
</tr>
<tr>
<td>G5</td>
<td>X</td>
</tr>
<tr>
<td>G6</td>
<td>X</td>
</tr>
<tr>
<td>G7</td>
<td>X</td>
</tr>
<tr>
<td>G8</td>
<td>X</td>
</tr>
</tbody>
</table>
Figure 14. 3D model of dog β-globin chain (light blue) superimposed to 3pel Greyhound globin dimer ($\alpha_1\delta_1$; brown); red area corresponds to the Greyhound-specific amino acid substitutions ($\beta$-to-$\delta$ changes)
Figure 15. Comparative alignment of Greyhound HBB gene with canine HBB and HBD genes (A); also shown the consensus sequence of the adult beta globins (β and δ) in placental mammals.
Figure 16. Common ancestors of the Greyhounds studied that could be the founders of the two different SNP patterns observed.
2.3. Hemoglobin function in Galgos (Spanish Greyhounds)

INTRODUCTION

The Galgo Español (Spanish Greyhound, GE) is a dog breed closely related to the Greyhound (Figure 17). According to The Fédération Cynologique Internationale (FCI, http://www.fci.be/nomenclature.aspx) classification, both Greyhounds (G) and GE belong to group 10 (sighthounds), section 3 (short-haired sighthounds). Similarly to G, the GE is also a sporting breed, although Galgos Españoles (GEs) are mainly used for hunting hares and lure coursing instead of sprint racing. Based on their phenotypic similarities and common origins we hypothesized that, similar to G, blood gas parameters and oxygen carrying properties of Hb in Galgos would be different from those in other canine breeds. The specific objective of this study was to compare venous blood gas analysis in a group of Galgos Españoles (GEs) to a group of mixed-breed dogs (M), using a widely available portable point-of-care blood gas analyzer (VetStat Electrolyte and Blood Gas Analyzer, IDEXX Laboratories, S.L., Barcelona, Spain).
Figure 17. Phenotypic similarities between Galgos (A) and Greyhounds (B), both sighthounds.

MATERIALS AND METHODS

Venous whole blood samples from 20 GEs and 17 M dogs were collected. All GEs, and 7 of the M dogs were at a shelter at the time of the study (Scooby Medina, Medina del Campo, Valladolid, Spain); the remaining 10 mixed-breed dogs were patients from a private veterinary practice located in Madrid (Spain), geographically close to the shelter (~100 miles). All dogs were considered healthy based on history and normal physical examination. Ages ranged from 1 to 7 years, and males and females were evenly distributed. At the time of sampling, all GEs were spayed or neutered, but all the mixed-breed dogs were intact. Signed consent was obtained from the director of the shelter and from dog owners. Samples were obtained by venipuncture of the cephalic vein, using a 2 ml plastic syringe and a 23 gauge needle, and blood was immediately placed into a 1 ml heparinized tube. All samples were analyzed immediately after collection, using the VetStat® Electrolyte and Blood Gas Analyzer with respiratory/blood gases cassettes.
(Idexx Laboratories, Inc.); all parameters measured are listed in Table 12. The P_{50} value was calculated in order to assess the Hb oxygen affinity, using the formula used by another instrument (Stat Profile® Critical Care Xpress Analyzer, Nova Biomedical, Waltham, MA, USA; (Nova Biomedical STP CCX Analyzer User Manual, 2003.)). The data obtained were analyzed using the statistical software GraphPad Prism® 5.0 (Graphpad Software, Inc., San Diego, CA, USA). Descriptive statistics and normality testing (Kolmogorov-Smirnov test) were performed, and then both groups (GE and M) were compared using t-test or Mann-Whitney test, depending on the distribution of the data. Statistical differences were set at p<0.05.

RESULTS
From the 17 parameters measured by the analyzer, 11 showed statistically significant differences between groups. All the results are expressed as mean ± standard deviation (SD) and the p value in those parameters statistically different between GE and M are shown in Table 12. GEs had higher bicarbonate concentration (HCO_3, p<0.0001), partial pressure of carbon dioxide (PCO_2, p=0.0002), and total carbon dioxide (tCO_2, p=0.0002) than M. GEs also had higher total Hb content (tHb, p<0.0001) and oxygen content (O_2Ct, p=0.0005). In contrast, GEs had lower pH (p=0.0386), chloride concentration (Cl, p=0.0123), and P_{50} (p=0.0013) than the M group. Graphical representations of the parameters statistically different between the two groups studied are shown in Figure 15.
DISCUSSION

The most relevant findings were the higher PCO$_2$ and tHb (since HCO$_3$ and tCO$_2$ are calculated using PCO$_2$, and O$_2$Ct using tHb) found in GEs. High Hb and oxygen capacity are consistent with the results of our previous study in RRGs (Zaldivar-Lopez and others 2011a), providing a more efficient oxygen transport. The P$_{50}$, also previously reported to be lower in RRG than in non-Greyhound breeds (as previously mentioned), was also lower in the Galgo group, indicating that Galgos have higher Hb oxygen affinity, feature that could be class-specific (sighthounds).
Table 12. Blood gas values in Galgos and mixed-breed dogs expressed as mean ± standard deviation (SD). P values shown when statistical difference between groups was detected.

<table>
<thead>
<tr>
<th></th>
<th>GALGO</th>
<th>MIXED-BREED</th>
<th>p value &lt;0.05</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean ± SD</td>
<td>Mean ± SD</td>
<td></td>
</tr>
<tr>
<td>pH</td>
<td>7.390 ± 0.0334</td>
<td>7.426 ± 0.0668</td>
<td>p=0.0386</td>
</tr>
<tr>
<td>HCO₃ (mmol/L)</td>
<td>26.61 ± 2.251</td>
<td>23.06 ± 2.237</td>
<td>p&lt;0.0001</td>
</tr>
<tr>
<td>PCO₂ (mmHg)</td>
<td>47.85 ± 6.515</td>
<td>38.29 ± 7.218</td>
<td>p=0.0002</td>
</tr>
<tr>
<td>AnGap (mmol/L)</td>
<td>22.47 ± 2.232</td>
<td>23.01 ± 2.528</td>
<td></td>
</tr>
<tr>
<td>BE (mmol/L)</td>
<td>0.885 ± 1.217</td>
<td>-0.094 ± 2.31</td>
<td></td>
</tr>
<tr>
<td>BE ecf (mmol/L)</td>
<td>1,930 ± 1.947</td>
<td>-0.8471 ± 2.238</td>
<td>p=0.0003</td>
</tr>
<tr>
<td>BE act (mmol/L)</td>
<td>0.47 ± 1.495</td>
<td>-0.9471 ± 2.412</td>
<td>p=0.0359</td>
</tr>
<tr>
<td>BB (mmol/L)</td>
<td>50.9 ± 1.573</td>
<td>39.7 – 51.0 *</td>
<td>p=0.0005</td>
</tr>
<tr>
<td>tCO₂ (mmol/L)</td>
<td>28.08 ± 2.424</td>
<td>24.22 ± 2.363</td>
<td>p&lt;0.0001</td>
</tr>
<tr>
<td>cH⁺ (mmol/L)</td>
<td>40.81 ± 3.157</td>
<td>32.5 – 60.5 *</td>
<td>p=0.01</td>
</tr>
<tr>
<td>PO₂ (mmHg)</td>
<td>49.74 ± 18.5</td>
<td>47.53 ± 10.13</td>
<td></td>
</tr>
<tr>
<td>tHb (g/dL)</td>
<td>20.05 ± 1.885</td>
<td>16.02 ± 2.286</td>
<td>p&lt;0.0001</td>
</tr>
<tr>
<td>SO₂ (%)</td>
<td>77.05 ± 11.65</td>
<td>78.06 ± 8.407</td>
<td></td>
</tr>
<tr>
<td>O₂Ct (vol%)</td>
<td>21.5 ± 3.233</td>
<td>17.59 ± 2.91</td>
<td>p=0.0005</td>
</tr>
<tr>
<td>Na (mmol/L)</td>
<td>157 ± 2.282</td>
<td>155.9 ± 2.315</td>
<td></td>
</tr>
<tr>
<td>K (mmol/L)</td>
<td>4.400 ± 0.2428</td>
<td>4.629 ± 0.4398</td>
<td></td>
</tr>
<tr>
<td>Cl (mmol/L)</td>
<td>112.4 ± 2.088</td>
<td>114.5 ± 2.683</td>
<td>p=0.0123</td>
</tr>
<tr>
<td>P₅₀ (mmHg)</td>
<td>27.78 ± 1.193</td>
<td>29.14 ± 0.965</td>
<td>p=0.0013</td>
</tr>
</tbody>
</table>
Figure 18. Box and whiskers plot graphs of blood gas parameters showing differences between Galgos (red) and mixed-breed (blue) dogs. Whiskers represent range, and boxes the central 50% of the data.
Chapter 3: HAPTOGLOBIN IN GREYHOUNDS

As previously stated, Hp is a moderate APP in dogs (Conner and others 1988), and changes in serum Hp concentration can be used as diagnostic and prognostic markers in various inflammatory disorders (Martinez-Subiela and others 2002). The Hp concentration in healthy dogs is 0-3 g/L (Eckersall and others 1999). Decreased concentrations are described in dogs with intravascular hemolysis (i.e. Hp binds to Hb and the complex is removed from the circulation by macrophages via CD163). On the other hand, increases in serum Hp concentration occur after administration of different drugs (e.g. glucocorticoids, anthelmintics, or phenobarbital). Former racing Greyhounds have low serum protein concentration (Steiss and others 2000) due to a lower globulin concentration; primarily, serum α- and β-globulin concentrations (Fayos and others 2005). We reported that serum Hp concentrations in Greyhounds (n=15) are lower than in non-Greyhounds (n=11) dogs using colorimetric and immunoturbidimetric methods (previously validated in the laboratory of one of the co-authors) and later confirmed by protein electrophoresis (Couto and others 2009). This low Hp concentration was hypothesized to be breed-specific, which could explain the low α- globulin concentrations in the breed.
3.1. Investigation of the haptoglobin gene in Greyhounds

INTRODUCTION

RBC destruction (due to natural or pathologic causes) can be achieved by two different mechanisms: intravascular and extravascular hemolysis. When RBCs are destroyed within the circulation (intravascular hemolysis, 10-20% of normal RBC destruction) hemoglobin (Hb) is released into the bloodstream, with the potential of causing oxidative damage to the tissues (e.g. hypertension, kidney damage, thrombosis). The haptoglobin mechanism is the most efficient mechanism to avoid these complications: the Hb molecule (tetramer) breaks down into dimers, and haptoglobin (Hp) binds very strongly to the free Hb dimers; this Hp-Hb complex is recognized by the CD163 receptor in macrophages and monocytes (blood cells) and binds to it, internalizing and degrading its components (Levy and others 2010). The half-life of the complex is 9 minutes (Levy and others 2010).

In humans, the HP gene has two allelic forms (Hp1 and Hp2), with Hp2 being a partial duplication of Hp1; these alleles lead to three different genotypes: Hp1-1, Hp2-1, Hp2-2. Its primary site of expression is the liver (mRNA and protein), although it has been detected in other organs. Hp circulates in plasma, and concentration increases during the acute phase response (due to enhanced synthesis). Hp is clinical marker of acute phase response (increased) and intravascular hemolysis (decreased) (Nielsen and Moestrup 2009). The protein is composed of α (complement control protein domain) and
β (serine protease domain) subunits, which are linked by disulfide binding. Despite having a protease domain, no proteolytic effect has been assigned to Hp (Levy and others 2010). Hp1 is present in all mammals (Hp2 only in humans), and it is composed of 5 exons. A multimerization domain is located in exon 3. The Hb-binding site in Hp is located in the beta chain. Hp1-1 genotype is superior in avoiding oxidative damage. Hp stabilizes heme within the heme pocket of Hb, preventing release of oxidizing radicals (Levy and others 2010).

In dogs there is only one subtype, which is homologous to human Hp 1-1 (Shim and others 1971). As in humans, two αβ dimers form a tetramer (βααβ), and the Hb-binding site is located in the β chain (Shim and others 1971). Two differences are recognized compared to human Hp: 1) The two αβ chains are joined by a non-covalent interaction; 2) The α chain is glycosylated and has an oligosaccharide-binding sequence. The glycosylation pattern may vary depending on the disease (Ceron and others 2005).

Differences in physiological values may occur among dog breeds. As commented in the literature review section, Greyhounds have many physiological differences compared to other dog breeds. Former racing Greyhounds have low Hp concentration (Couto and others 2009) that could partially account for the low α-globulin concentrations in the breed. Therefore, we hypothesize that the low Hp concentrations found in Greyhounds could be due to a decrease of HP gene expression. The objective of this study was to investigate the HP gene in Greyhounds.
MATERIALS AND METHODS

Animals

A total of 4 dogs were used for this study (2 Greyhounds (G) and 2 non-Greyhounds (NG)). Genomic DNA was isolated from venous blood drawn from 1 G and 1 NG. Two additional dogs (1 G, 1 NG) were used to isolate RNA and synthetize cDNA. All dogs used were healthy based on clinical history and a normal physical exam. All dogs used for this study were recruited at The Ohio State University Veterinary Medical Center, and samples were collected after signed owner consent. All dogs from this study were purebred, and pedigree was registered at the time of sampling. This study has IACUC (Institutional Animal Care and Use Committee) and hospital CRAC (Clinical Research Advisory Committee) approval.

Blood processing

Venous blood was collected from the jugular vein by direct venipuncture, and placed into tubes containing EDTA anticoagulant or into RNA tubes. DNA was isolated from the EDTA samples using the Gentra Puregene genomic DNA purification kit (QIAGEN Inc., Valencia, CA, USA), adding an ethanol precipitation step to the original protocol; RNA was isolated from the RNA tubes using the PAXgene blood RNA kit (QIAGEN Inc., Valencia, CA, USA). RNA isolation was followed by cDNA synthesis using Superscript II Reverse Transcriptase (Life Technologies Corp., Grand Island, NY, USA).

DNA sequencing
Exon and intron specific primers were designed in order to PCR amplify and sequence DNA and cDNA of the *HP* gene, including exons, introns and splice sites from genomic DNA and cDNA. PCR forward and reverse primers were custom designed to amplify products of ~800 bp. PCR amplification was performed using JumpTaq polymerase (JumpTaq Hot Start Polymerase, Sigma); Tm, annealing time and number of cycles was adjusted to primer sets in order to optimize amplification. Products were purified (PCR purification/gel extraction kits; QIAGEN Inc., Valencia, CA, USA) and sequenced (Eurofins MWG Operon, Huntsville, AL, USA).

**Computational analysis**

The canine Hp gene was identified using BLAT with other Mammalian Hp protein sequences on the UCSC Genome browser (http://genome.ucsc.edu/). Sequencing results obtained were analyzed using the computer software DNASTAR Lasergene Core Suite (DNASTAR Inc., Madison, WI, USA), comparing the obtained data to the canine reference sequence (canFam2). Using the protein sequence, a predicted 3D model of canine haptoglobin was created using the PHYRE2 software (Kelley and Sternberg 2009). The obtained model was visualized using the UCSF Chimera software (Pettersen and others 2004).

**Quantitative real-time PCR (RT-qPCR)**

Using RNA from G and NG blood, cDNA was synthesized. *HP* and *B2M* (used as housekeeping gene) were PCR amplified using exon-specific primers designed for the RT-qPCR and SYBR Green real time PCR Master Mix using a real time PCR system
Instrument. Gene-specific quantitative data were normalized by B2M (considered a housekeeping gene appropriate for RNA expression normalization) RNA abundance in the same sample and will be expressed as fold-expression relative to the housekeeping gene.

RESULTS

Based on computational analysis, the canine HP gene is located in canine chromosome 5 (CFA5), and it is composed of 5 exons that span 4.6 kilobases (Kb). Due to the lack of annotation in the canine reference sequence (UCSC), we compared multiple gene prediction calling programs and determined that Genscan predictions were likely to be correct. That was predominantly based on expressed sequence tags (EST) library data and manual analysis of cross-species conservation at the DNA and protein levels (Figure 19).

Figure 19. Caption of the HP gene predictions based on computational analysis
Sequencing results of *HP* in both DNA and whole blood cDNA revealed a homozygous nucleotide change (C>T) in the Greyhound. It is located in the first intron, in a non-conserved region. There were also two homozygous SNPs in exon 5 in both G and NG, leading to synonymous changes (CGG>AGG → Arg.Arg; CAC>CAT → His>His).

Using the PHYRE2 software, we a predicted 3D model of canine haptoglobin with a 100% confidence and a 91% coverage. The obtained model was visualized and analyzed using the UCSF Chimera software (Figure 20.A). We added the Greyhound Hb crystal structure (3pel, from the protein database -PDB-) to the Hp model (Figure 20.B) forming the Hb-Hp complex. As shown in Figure 21, the Hp binds to the canine β-globin chain, embracing the heme unit. Hp and Hb structures create inter-model hydrogen bonds, shown as orange lines in Figure 21. We also included the canine CD163 (i.e. the macrophage receptor where the Hp-Hb complex binds in order to be remove from the circulation, avoiding free Hb oxidative damage) in the 3D prediction model (Figure 20.C).

The expression of the *HP* gene in Greyhounds is confirmed by the amplification of the transcript from peripheral blood cDNA. Contamination by genomic DNA was ruled out because the PCR amplicon was designed to span an intron. Real-time quantitative PCR analysis shows that, although expressed in the Greyhound and in the non-Greyhound, the Greyhound has lower *HP* expression (p=0.029) in blood (figure 22).
Figure 20. 3D prediction model of dog Hp (A, brown) forming a strong complex with the Greyhound Hb $\alpha\beta$ dimer (B, blue); the Hp-Hb complex is bound to the CD163 macrophage receptor (C, pink) to be cleared from the circulation.
Figure 21. Hp (brown) binds to the β-globin chain (blue) at the heme site. Orange lines represent hydrogen bonds.
DISCUSSION

Nucleotide polymorphisms (SNPs) can cause changes in expression patterns of genes. Of the three nucleotide changes found in the DNA sequence of the HP gene, only one is present uniquely in the Greyhound. The location of this SNP (in an intronic region not conserved across mammals) suggests that is not regulatory, because a potential indication of the presence of regulatory elements is the conservation across species. The other two SNPs are present in both G and NG, and lead to synonymous amino acid changes therefore they have no effect on protein sequence. However, it is possible that they affect the mRNA structure, which could in turn have diverse effects on gene regulation and function (Wan and others 2011).
Despite having low or undetectable serum concentrations of HP (Couto and others 2009), we find that Greyhound HP mRNA is expressed in blood and lacks amino acid variation. It will be important to also test for Hp mRNA expression in liver, the major site of expression. Should that be normal, it would suggest that the clinical absence of the HP is attributable to post-transcriptional HP effects or to an unknown physiological interaction.
3.2. Haptoglobin in Galgos (Spanish Greyhounds)

INTRODUCTION

As previously mentioned, Galgos (GE, Spanish Greyhounds) share common origins and phenotypic characteristics with Greyhounds. Although there are only a few peer-reviewed articles about Galgos, they also seem to share some physiological peculiarities with Greyhounds. We hypothesize that low Hp concentration is a common feature of the sighthound group, and thus GEs will have similar Hp concentration than Gs. The objective of this study was to measure Hp in GE, and determine if they are similar to previously reported Hp values in Gs.

MATERIALS AND METHODS

Venous samples were collected from the jugular vein of 21 healthy adult GE at the Clinical Veterinary Hospital at the University of Córdoba (Spain). After collection, the blood was immediately placed into tubes with EDTA anticoagulant, and centrifuged at 1300g for 10 minutes. Plasma was aliquoted into Eppendorf tubes and immediately frozen at -80°C, until all the samples were sent as a batch to the Veterinary Clinical Pathology Laboratory at the Veterinary Hospital at the University of Murcia for analysis. Plasma Hp was measured by the same personnel and using the same colorimetric hemoglobin-binding method (Tridelta Phase, Tridelta Development Ltd., Bray, Ireland) as in previous study in Greyhounds (Couto and others 2009). Crossreactivity between the polyclonal goat antihuman Hp antiserum and canine Hp was previously demonstrated by
radial immunodiffusion and ELISA tests (Tecles and others 2007). The analysis was performed using a biochemistry autoanalyzer (Cobas Mira Plus, ABX Diagnostics, Montpellier, France), and results were reported in gram per liter. The same samples were analyzed again the following day in order to evaluate the intra-individual variability through coefficient of variation (CV). For statistical purposes, we used the raw data (Greyhounds (G, n=15) and Non-Greyhounds (NG, n=11)) from previous publication (Couto and others 2009). All three groups were analyzed with descriptive statistics and tested for normality using the D’Agostino & Pearson omnibus normality test; the groups were compared using non parametric Kruskal-Wallis test with post-test (Dunn’s multiple comparison test). Statistical significance was set at p<0.05. GraphPad Prism was the software used for statistical analysis.

RESULTS

Since there were two Hp measurements (one day apart), mean values for each dog were calculated, and that was the value used for descriptive statistics. Data in GEs were normally distributed, with a mean of 1.78 g/L (SD, 1.41 g/L), and ranging from 0.13 to 4.52 g/L. Intra-individual coefficient of variation over the two analyses was very low (8.85%). Raw data (Greyhounds and Non-Greyhounds) from previous publication (Couto and others 2009) was used for the graphical representation (figure 18). Results in the G group were not normally distributed (median 0 g/L, inter quartile range -IQR- 0.15) and ranged from 0 to 0.61 g/L. Data in the NG group had Gaussian distribution, with a mean of 0.96 g/L (SD, 0.85 g/L), and ranged from 0.15 to 2.64 g/L. When the three groups
were compared, the Hp in the GE group was higher than G (p<0.0001), but not
statistically different from the NG group (figure 23). The figure shows differences in
haptoglobin concentration between the three groups: Galgos (mean 1.78 g/L; SD 1.41
g/L), Greyhounds (median 0 g/L; IQR 0.15), and non-Greyhounds (mean 0.96 g/L; SD
0.85 g/L); lines indicate the mean/median and SD/IQR.

Figure 23. Differences in haptoglobin concentration between Galgos, Greyhounds, and
non-Greyhounds; Galgos’ Hp concentration (a) is different than in Greyhounds (b), but not different
from non-Greyhounds (a)
DISCUSSION

Galgos Españoles (GE) have plasma Hp concentrations similar to those in other dogs (Ceron and others 2005), in contrast to their closely related sighthound group mates (Greyhounds), who have very low or undetectable Hp levels (Couto and others 2009). Surprisingly, GEs have slightly higher Hp than other dog breeds (from the 21 GEs, only 4 were outside the upper limit of the canine reference interval). This elevation could be breed-specific, or due to a subclinical not previously detected inflammation or infection in some dogs (although all dogs were clinically healthy). Low serum protein concentration in former racing Greyhounds (Steiss and others 2000) has been shown to be due to a lower globulin concentration (primarily, serum α- and β-globulin concentrations) (Fayos and others 2005), and the lower Hp concentration likely contributes to the low α-globulin concentrations in the breed (Couto and others 2009). Further studies are warranted in order to investigate the relationship between serum proteins and Hp in GEs.

Hp is more stable than the cellular components of blood, thus assays can be performed on frozen serum or plasma samples. However, a decrease in Hp concentration in canine serum stored at -20°C has been described, thus -70°C has been suggested for prolonged storage (Solter and others 1991). In this study, samples were processed immediately, and they were kept at -80°C until analyzed to avoid storage changes. Little variation was found between the two measurements of each sample (duplicate measurements, one day apart), as reflected by the low CVs. In dogs with intravascular hemolysis (i.e. immune hemolytic anemia), Hp concentrations are lower because it binds strongly to Hb, and the Hb-Hp complexes are removed from the circulation by
macrophages via CD163 (Harvey and West 1987). Considering the previously reported short half-life of Greyhound RBCs (Novinger and others 1996), the low Hp concentration could be attributed to chronic hemolysis; however, other indicators of hemolysis (i.e.; reticulocytosis, hyperbilirubinemia, increased RDW, etc) are absent in Gs (Zaldivar-Lopez and others 2011b), and more recent reports have shown no differences between Gs and other breeds’ RBC half-life (Garon and others 2010).

Although there is little information in the scientific literature regarding GE (Weidmeyer and Solter 1996), given the phenotypic similarities between these two sighthound breeds (virtually indistinguishable to the untrained eye), and the difference in Hp concentration, we believe that the comparison between Gs and GE could be an interesting natural biological model system to study the physiology of Hp in the canine species. Further studies including more animals from a variety of origins and lifestyles (Gs were all retired racers) could help to better understand the Hp-Hb physiology between Gs and GE, potentially giving new insights and encouraging research on treatments for common Hb-scavenging dependent diseases in dogs (i.e. hypertension, thrombosis or infectious diseases). Characterization and understanding of these mechanisms in Greyhounds will be especially beneficial, since many of their differences or common diseases (i.e. high creatinine concentration, thromboembolism, hypertension) could be linked to increased free-Hb oxidative damage to tissues (i.e. blood vessels, kidney). Differences between Greyhounds and Galgos found in this study are of great importance when interpreting laboratory results, since GE’ labwork is often interpreted as if they were Gs (due to their similarities). As an example, Hp values within the normal range for a Galgo (or any other breed) would be elevated for a Greyhound.
SUMMARY

**Objective 1:** To investigate iron metabolism in Greyhounds compared to other dog breeds, evaluating whether there are breed-specific differences.

From the two measured parameters (serum iron and UIBC), serum iron concentration was within the previously published reference interval and no statistical difference was found between the Greyhound and non-Greyhound groups; however, UIBC was lower in Greyhounds, causing a lower TIBC and higher %SAT. The low UIBC/TIBC found in G could be a breed-specific feature caused by chronic inflammation (unlikely in this study). Elevations in transferrin saturation are associated with cancer, and a %SAT higher than 60% increases the risk. These carcinogenic properties have been demonstrated in animals too.

**Objective 2:** To assess iron status in the canine blood donor population.

Iron is higher and UIBC is lower in blood donors when compared to controls, which makes TIBC (serum iron + UIBC) no different between groups. Also, percentage %SAT is higher in blood donors. The increased serum iron concentration was unexpected based on the human scientific literature, since the most common adverse effect of frequent blood donation is iron depletion. Our canine population donation
intensity is comparable to human “superdonors”; however, the fact that same amount of blood is drawn from an animal weighing half as much makes this physiology more striking. Contrary to what it was expected, we find that serum iron and transferrin saturation are higher, and UIBC is lower in blood donor dogs, which indicates that blood donation of ~450ml every 2 months does not impair iron homeostasis of canine donors. Identification of the biology underlying this – possibly a trait selected for in Greyhounds – has important implications for human medicine.

**Objective 3: To evaluate hemoglobin function and venous blood gases in healthy retired racing Greyhounds.**

Greyhounds have higher partial pressure of oxygen (PO$_2$), oxygen saturation (SO$_2$), oxyhemoglobin percentage (O$_2$Hb), total Hb concentration (tHb), oxygen content (O$_2$Ct), and oxygen capacity (O$_2$Cap) than other dog breeds. These parameters assess the oxygenation and function of the Hb molecule, and higher values support the fact that Greyhounds are able to carry a higher concentration of total oxygen in the blood. On the other hand, they have lower P$_{50}$ than non-Greyhounds, consistent with previous studies in the breed. The unusual minimal dispersion of the P$_{50}$ results in Greyhounds suggests that unknown factors have selected for a very specific Hb oxygen affinity this breed. Although at first glance seems counterintuitive that an athletic breed like Greyhounds have low P$_{50}$, researchers working on hemoglobin-based oxygen carriers (HBOCs) have demonstrated that a high-affinity oxygen carrier is beneficial, suppressing vasoconstriction elicited by early off-loading and over-oxygenating tissues at the level of
the pre-capillary sphincter. In the case of racing Greyhounds, this mechanism may allow delivery of oxygen to the most hypoxic tissues.

**Objective 4:** *To establish reference intervals for blood gases and cooximetry in Greyhounds*

Due to the differences in venous blood gases and cooximetry between Greyhounds and other dog breeds, breed-specific reference intervals were calculated. These reference intervals should be used in the clinical practice to correctly interpret laboratory results and avoid misdiagnoses.

**Objective 5:** *To define the genetics of canine alpha- and beta-globins.*

Using computational analysis of the dog genome, we identified the canine globin genes. Canine alpha- and beta- globin gene clusters are located in different chromosomes, as in humans. Five genes constitute each one of the clusters, and all of them have the same basic structure than human globin genes, arranged in developmental order: 5 alpha-like genes (*HBZ1-HBZ2-HBM-HBA1-HBA2*) and 5 beta-like genes (*HBE1-HBE2-HBD1-HBD2-HBB*). In people, *HBB* is the most commonly expressed gene and protein, and constitutes the most abundant Hb protein HbA (97% in adults). However, the HBD-like globin genes (*HBD1* and *HBD2*) are the highly expressed beta-globins in dogs, meaning that the predominant Hb tetramer combination is \(\alpha_2\delta_2\) (equivalent to HbA\(_2\) in humans). The two large regulatory regions located upstream the globin clusters were identified and compared to their human counterparts, where a good positive correlation was found.
Objective 6: To investigate if there are differences in the globin genes in Greyhounds compared with non-Greyhound dogs.

Since intra-species’ variation in hemoglobin affinity is mainly determined by the primary structure of Hb, we investigated if Greyhound oxygen carrier properties are due to sequence structure modifications. We found that adult dogs express 10 globin genes, contrary to old reports. No Greyhound-specific nucleotide changes were identified in the canine alpha-globin cluster. Since an increase in embryonic and fetal globin expression increases the Hb oxygen affinity, we suggest that the Greyhound-specific variation in HBE1 could be the cause of the difference in affinity of this breed. Despite being the minor adult globin in humans (~2%), we found that δ-globin (encoded by the HBD gene) is the main globin in adult dogs, and likely in the other members of the superorder Laurasiatheria (e.g. bat, hedgehog, cat, horse). HbA₂ (α₂δ₂) has similar properties to fetal Hb (HbF): higher oxygen affinity and avoids HbS polymerization in thalassemias. We have discovered 6 SNPs in the promoter and introns of the HBD1 gene in Greyhounds that could change HBD expression and overall Hb affinity. HbA (α₂β₂) is the main Hb type in people (i.e. HBB is the most expressed adult beta globin gene), but β has evolved as the minor adult beta globin in dogs (i.e. HBB is the least expressed adult beta globin gene). We found several SNPs in Greyhounds’ HBB (in the promoter and first exon), which could cause a change in expression. Three out of four amino acids affected are substituted by the amino acid that is in that position in HBD, transforming Greyhound HBB into HBD-like in the proximal region. This finding is even more important knowing that canine HBB and HBD protein sequences differ in only 7 amino acids. We conclude
that Greyhound \textit{HBB} resemblance to \textit{HBD} (main canine Hb) may explain better oxygen-carrying properties that Greyhound Hb has.

\textbf{Objective 7: To evaluate hemoglobin function and venous blood gases in Galgos Españoles (Spanish Greyhounds).}

Galgos Españoles have higher bicarbonate concentration, partial pressure of carbon dioxide, total carbon dioxide, Hb content, and oxygen content than the mixed-breed group. In contrast, they have lower pH, chloride concentration, and \(P_{50}\) than the mixed-breed group. Since \(\text{HCO}_3\) and \(\text{tCO}_2\) are calculated using \(\text{PCO}_2\), and \(\text{O}_2\text{Ct}\) using \(\text{tHb}\), the most relevant findings were the higher \(\text{PCO}_2\) and \(\text{tHb}\) found in Galgos Españoles. High Hb and oxygen capacity are consistent with the results of our previous study in Greyhounds, which denotes a more efficient oxygen transport. The \(P_{50}\), low in Greyhounds, was also lower in the Galgos Españoles than in the mixed-breed dogs, indicating that Galgos have higher Hb oxygen affinity. We conclude that this feature that could be class-specific (sighthounds).

\textbf{Objective 8: To investigate if the cause of low haptoglobin in Greyhounds is due to variations in the HP gene.}

Only one (out of three) nucleotide change found in the DNA sequence of the \textit{HP} gene is present uniquely in the Greyhound. It is located in a non-conserved intronic region, which suggests that is not regulatory. The other two SNPs are present in both Greyhounds and non-Greyhounds, and cause synonymous amino acid changes (no effect on protein sequence). Despite having low or undetectable serum concentrations of Hp, we
find that Greyhound *HP* mRNA is expressed in this breed, and it does not have sequence variations that can explain this anhaptoglobinemia. This question remains unanswered, suggesting that the clinical absence of the *HP* is attributable to post-transcriptional *HP* effects or to an unknown physiological interaction.

**Objective 9: To measure haptoglobin in Galgos Españoles in order to determine if they have low haptoglobin, similarly to Greyhounds.**

We found that plasma Hp concentrations in Galgos Españoles are similar to those in other dogs, in contrast to their closely related sighthound groupmates (Greyhounds), who have very low or undetectable Hp levels. Surprisingly, Galgos Españoles have slightly higher Hp than other dog breeds; this elevation could be breed-specific, or due to a subclinical not previously detected inflammation or infection in some dogs (although all dogs were clinically healthy).

Although there is little information in the scientific literature regarding Galgos Españoles, given the phenotypic similarities between these two sighthound breeds, and the remarkable difference in Hp concentration, we believe that the comparison between Gs and GEs could be an interesting natural biological model system to study the physiology of Hp in the canine species. Differences between Greyhounds and Galgos found in this study are also of great importance when interpreting laboratory results, since Galgos Españoles’ labwork is often interpreted as if they were Greyhounds.
CONCLUSIONS

1. Greyhounds have lower UIBC and TIBC, and higher %SAT than non-Greyhound dogs.

2. Iron and %SAT are higher, and UIBC is lower in blood donor dogs (when compared to controls). The increased serum iron concentration is totally unexpected because same-intensity human blood donors usually have iron depletion, and the same amount of blood is drawn from dogs.

3. Greyhounds have better oxygen-carrying properties and higher Hb oxygen affinity than other dog breeds, suggesting evolutionary selection for better exercise performance.

4. Canine alpha- and beta- globin gene clusters are located in different chromosomes and arranged in developmental order. Although in people HBB is the most commonly expressed globin gene, HBD is the highly expressed beta-globin in dogs.
5. Adult dogs express all 10 globin genes (embryonic and adult). The Greyhound-specific variations in \textit{HBEI}, \textit{HBD} and the transformation of Greyhound \textit{HBB} into \textit{HBD-like} could be the cause of the better oxygen carrying properties and higher affinity in this breed.

6. Galgos Españoles also have a more efficient oxygen transport and lower P\textsubscript{50} than mixed-breed dogs, but not as prominent as Greyhounds.

7. Despite having low or undetectable serum concentrations of Hp, we find that Greyhound \textit{HP} mRNA is expressed in this breed, and it does not have sequence variations that can explain the anhaptoglobinemia found in the breed.

8. Plasma Hp concentrations in Galgos Españoles are within normal levels for dogs; differences with Greyhounds could make them an interesting natural biological model system to study the physiology of Hp in the canine species.


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DEPALMA, R. G., HAYES, V. W., CHOW, B. K., SHAMAYEVA, G., MAY, P. E. & ZACHARSKI, L. R. (2010) Ferritin levels, inflammatory biomarkers, and mortality in peripheral arterial disease: a substudy of the Iron (Fe) and Atherosclerosis Study (FeAST) Trial. J Vasc Surg 51, 1498-1503


risk of ischemic stroke. A prospective nested case-referent study. Cerebrovasc Dis 24, 405-411


HENDERSON, K. K., MCCANSE, W., URANO, T., KUWAHIRA, I., CLANCY, R.
& GONZALEZ, N. C. (2000) Acute vs. chronic effects of elevated hemoglobin O(2)
affinity on O(2) transport in maximal exercise. J Appl Physiol 89, 265-272

HILL, R. C., FOX, L. E., LEWIS, D. D., BEALE, K. M., NACHREINER, R. F.,
Effects of racing and training on serum thyroid hormone concentrations in racing

HILPPO, M. (1986) Some haematological and clinical-chemical parameters of sight
hounds (Afghan hound, saluki and whippet). Nord Vet Med 38, 148-155


HSING, A. W., MCLAUGHLIN, J. K., OLSAN, J. H., MELLEMKJAR, L.,
hemochromatosis: a population-based cohort study in Denmark. Int J Cancer 60, 160-162

granules in Greyhound eosinophils. Vet Clin Pathol 34, 140-143

IAZBIK, M. C., O’DONNELL, M., MARIN, L., ZALDIVAR, S., HUDSON, D. &

blood-gas, and acid-base values in greyhounds before and after exercise. Am J Vet Res
50, 583-586


polymorphisms to raise fetal hemoglobin levels in normal adults. Mol Biol Rep 39, 4619-4625


LEVY, A. P., ASLEH, R., BLUM, S., LEVY, N. S., MILLER-LOTAN, R., KALET-LITMAN, S., ANBINDER, Y., LACHE, O., NAKHOU, F. M., ASAIF, R., FARBSTEIN, D., POLLAK, M., SOLOVEICHIK, Y. Z., STRAUSS, M., ALSHIEK, J.,


MARTINEZ-SUBIELA, S., TECLES, F., ECKERSALL, P. D. & CERON, J. J.
(2002) Serum concentrations of acute phase proteins in dogs with leishmaniasis. Vet Rec 150, 241-244

MAST, A. E., FOSTER, T. M., PINDER, H. L., BECKKIEWICZ, C. A.,
BELLISSIMO, D. B., MURPHY, A. T., KOVACEVIC, S., WROBLEWSKI, V. J. &
metabolism in high-intensity blood donors. Transfusion 48, 2197-2204

imaging findings of presumed cerebellar cerebrovascular accident in twelve dogs. Vet Radiol Ultrasound 46, 1-10


MCINNIS, M. D., NEWHOUSE, I. J., VON DUVILLARD, S. P. & THAYER, R.

MCMILLAN, M. W., WHITAKER, K. E., HUGHES, D., BRODBELT, D. C. &
and carbon dioxide in spontaneously breathing, conscious dogs in an intensive care unit.
Journal of veterinary emergency and critical care (San Antonio) 19, 564-570

Curr Hematol Rep 2, 116-121


inducing the synthesis of the iron regulatory hormone hepcidin. J Clin Invest 113, 1271-1276


164
PERUTZ, M. F. (1963) X-ray analysis of hemoglobin. Science 140, 863-869

PETTERSEN, E. F., GODDARD, T. D., HUANG, C. C., COUCH, G. S.,


Ames, Iowa, PharmaVet ;

Distributed by Blackwell Pub. pp 382-384


3,5,3'-triiodothyronine in dogs before and after administration of freshly reconstituted or previously frozen thyrotropin-releasing hormone. Am J Vet Res 49, 1722-1725


hypothyroidism in sighthounds and investigation of serum thyroid hormone concentrations in healthy Salukis. J Am Vet Med Assoc 236, 302-308


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