THE EFFECT OF DELAYED SERUM SEPARATION AND STORAGE TEMPERATURE ON THE RATE OF SERUM

GLUCOSE CONCENTRATION DECLINE IN ALPACAS, HORSES, DOGS, AND STURGEON

by

NANCY BROWN COLLICUTT

(Under the Direction of Bridget Garner)

ABSTRACT

The objective was to investigate the effect of storage parameters on serum glucose in blood samples from four species. Blood specimens were divided into 7 no-additive tubes and serum separated from one sample after one hour (reference sample). Remaining samples were stored at 4°C and 25°C, then centrifuged and serum glucose measured at 2, 4, and 8 hours post-collection. Serum glucose declines for all samples stored at 4°C were not significantly different, except for the 8 hour samples from sturgeon and dogs. At 25°C, serum glucose was comparable to reference values at 2 hours in the horse, sturgeon, and alpaca, but significantly lower at 4 and 8 hours in those species and at all time points in dogs. Storage at 4°C limits serum glucose decline for at least 4 hours in all species tested. At 25°C, serum-clot contact time should not exceed 1 hour in dogs and 2 hours in horses, alpacas, and sturgeon.

INDEX WORDS: metabolism, pre-analytical, erythrocytes, artifact, hypoglycemia

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CHAPTER 1

INTRODUCTION

Purpose of the Study

This study seeks to investigate the extent of serum glucose concentration decline in stored whole

blood samples from three mammalian species (dog, horse, and alpaca) and one non-mammalian species

(sturgeon). It is widely known that serum glucose concentration declines in stored whole blood samples,

especially when stored at room temperature. The primary objective of this study was to determine the

rate of serum glucose decline in whole blood samples from four animal species stored at room

temperature and refrigerated temperature for 2, 4, and 8 hours after blood collection. Erythrocytes in

whole blood samples are thought to contribute to the majority of serum glucose consumption, although

the human literature indicates that leukocytes can also contribute to glucose decline in samples with

elevated leukocyte counts (details in Chapters 2 and 3). The findings of this study offer veterinarians

clinically-useful, species-specific information regarding blood specimen collection and handling, which

will prevent false diagnoses of hypoglycemia, or of normoglycemia in hyperglycemic patients. This

information will also improve understanding of species differences in glucose metabolism, and it will

permit future study investigating effects of illness on serum glucose measurement in stored samples.

Many veterinary textbooks make the blanket statement that serum glucose concentration in

most veterinary species will decrease at a rate of approximately 5-10% per hour when serum is not

promptly separated from the cells in whole blood samples stored at room temperature[1-6]. However,

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species-specific rates of serum glucose consumption have yet to be compared in the four species of interest (dog, horse, alpaca, and sturgeon). Therefore, the goal of this research was to fill these gaps in the literature and to determine species-specific sample storage recommendations. University-owned horses and sturgeon were used. Per University of Georgia requirements, their care and use was conducted under the approval of the University of Georgia Institutional Animal Care and Use Committee. Client-owned dogs and alpacas were used and study design has been approved by the University Of Georgia College Of Veterinary Medicine Clinical Research Committee. Prior to sampling, informed client consent was obtained from the dog and alpaca owners.

<div align="center">Expected Results</div>

The rate of serum glucose concentration decline was determined for each storage condition (4°C and 25°C) and for each species studied. The author hypothesized that storage at 4°C would prevent or limit serum glucose concentration decline in all species. Additionally, it was expected that at room temperature the samples from sturgeon would have the slowest rates of serum glucose decline given presence of mitochondria (and therefore oxidative phosphorylation for energy generation) and suspected low membrane permeability to glucose in sturgeon erythrocytes. It was also expected that, at room temperature storage, the samples from alpacas would have the fastest rates of serum glucose decline given high glycolytic activity and unique features of alpaca erythrocytes. The unusual characteristics of sturgeon and alpaca erythrocytes that might alter glucose metabolism are discussed in detail in Chapter 2. Based on the results of this study, recommendations for sample storage and processing can be formulated and tailored for each species investigated.

CHAPTER 2

DIFFERENCES IN ERYTHROCYTE GLUCOSE UTILIZATION BETWEEN SPECIES

<u>Energy generation by erythrocytes in mammalian species</u>

Spurious hypoglycemia due to delayed separation of serum or plasma from blood cells is predominantly caused by continued uptake and metabolism of glucose by erythrocytes, as studies have found that erythrocytes metabolize approximately twice as much glucose compared to leukocytes[6-8]. Glucose is the primary substrate for generation of energy in erythrocytes in all species previously studied, except for the pig[9]. Additionally, the rate of glucose utilization by erythrocytes is variable across species. Harvey and Kaneko (1976) determined that *in vitro* erythrocyte glucose utilization in increasing order (of species studied) is: horse, cat, dog, and human[10]. This may signify that serum glucose is expected to decline more rapidly in stored canine blood samples when compared to stored samples from horses in our study.

The biochemical mechanisms causing differences in erythrocyte glucose metabolism are complex. In almost all mammalian species studied, erythrocytes fulfill their energy requirements via anaerobic glycolysis, also known as the Embden-Meyerhof pathway (EMP; Figure 1). In anaerobic glycolysis, glucose is the major substrate for consumption. The pig is the exception in that their erythrocytes utilize inosine rather than glucose for anaerobic glycolysis[9]. After glucose enters the cell, the hexokinase enzyme is responsible for phosphorylating glucose into glucose-6-phosphate (G6P). Subsequently, the majority of G6P is metabolized to lactate or pyruvate, resulting in the generation of adenosine triphosphate (ATP). The energy derived in the form of ATP is used for a variety of cellular processes and

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is important for the maintenance of erythrocyte structure, shape, and deformability. In health and the absence of oxidants, a smaller portion of G6P is metabolized via the hexose monophosphate shunt, also known as the pentose-phosphate pathway (PPP, Figure 2). The PPP is critical in preventing formation of damaging oxidized metabolites by serving as the source for reduced nicotinamide adenine dinucleotide (NADPH)[11]. However, efficiency of glucose utilization by each of these pathways is not constant from species to species. Harvey and Kaneko (1976) found that up to 13% of the glucose utilized by erythrocytes in horses was metabolized through the PPP. This was compared to approximately 6% of glucose utilized for the PPP in cats, dogs, and humans[10]. The remainder of the glucose gets metabolized through the EMP. The significance of this finding is uncertain, especially given the overall slower rate of glucose metabolism in horses. It is unknown whether such variations in glucose metabolism among mammalian species impact rates of glucose decline in stored whole blood samples.

<u>Energy generation by erythrocytes in South American Camelids</u>

A study conducted by Reynafare *et al.*[12] investigated the blood characteristics of alpacas and llamas in order to elucidate the mechanisms by which camelids living in high altitudes can adapt to hypoxia. The results of this study found that the activity of glucose-6-phosphate dehydrogenase, a glycolytic enzyme, was twice as high in camelids as the activity of this enzyme in human erythrocytes. Additionally, the activity of lactic dehydrogenase (another enzyme that plays an important role in anaerobic glycolysis) was approximately 6 times higher than in humans. These results indicated that the glycolytic activity in erythrocytes of camelids is greater than in red blood cells from human beings. The authors of that study theorized that these biochemical differences between human and camelid red blood cells were necessary due to a faster rate of erythrocyte renewal in alpacas, as alpaca erythrocytes have a shorter lifespan than human erythrocytes (60 days and 130 days, respectively)[13]. Such biochemical differences also may act as mechanisms to maintain adaptive features of their erythrocytes

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that allow camelids to survive low oxygen supply to the tissues in high altitudes and unavailability of

water[12]. For example, camelid erythrocytes are mechanically more resistant to osmotic lysis and have

decreased deformability when compared to other mammalian species (discussed in more detail

below)[14,15]. Whether these higher erythrocyte concentrations of glycolytic enzymes play a role in the

rate of serum glucose concentration decline in clinical settings is uncertain.

<u>Energy generation by erythrocytes in teleosts (bony fish)</u>

In contrast to mammalian species, erythrocytes in non-mammalian species, such as fish, gain

most of their energy aerobically via the Krebs cycle (also known as the tricarboxylic acid cycle) and

oxidative phosphorylation (Figure 3)[16]. Retention of organelles such as mitochondria within teleost

erythrocytes allows nucleated erythrocytes to utilize aerobic metabolism. In contrast to anaerobic

glycolysis which primarily utilizes glucose for energy generation, the Krebs cycle utilizes various

substrates such as monocarboxylic acids, glucose, glycolytic intermediates, substrates of the pentose

phosphate pathway and amino acids to oxidize acetate, ultimately leading to the production of ATP,

FADH, H^+ and NADH[17]. The Krebs cycle and subsequent oxidative phosphorylation forms approximately

35 molecules of ATP per molecule of glucose oxidized, compared to only 2 ATP formed by anaerobic

glycolysis alone[18]. Because the Krebs cycle produces relatively more energy per molecule of glucose due

to the availability of more substrates and oxygen, ultimately less glucose is metabolized.

The literature concerning energy generation by erythrocytes in teleosts is limited, and the

metabolic pathways are highly complex and species specific. One study found that rainbow trout

(*Oncorhynchus mykiss*) utilize 28% of erythrocyte glucose for anaerobic glycolysis, leading to the

production of lactate, even with normal oxygen levels[19]. Additionally, glucose remained the dominant

substrate that was oxidized for energy production via the Krebs cycle[19]. In a different species of trout

(brown trout, *Salmo trutta*), glucose also appeared to be the major fuel for red blood cells, but there was a slightly different pattern of glucose metabolism from rainbow trout red blood cells[20].

Cell aging also has an effect on energy production via aerobic and anaerobic metabolism in nucleated erythrocytes from rainbow trout. Ultrastructural studies have shown that there is erythrocyte mitochondrial loss with erythrocyte aging in rainbow trout, which suggests that there would also be a reduction in the capacity to produce ATP aerobically[21]. Another study found that the rate of oxygen consumption for aerobic metabolism in aging erythrocytes decreases; this suggests that the role of anaerobic glycolysis becomes more significant with cell age in rainbow trout erythrocytes[22]. Yellow perch (*Perca flavescens* mitchill) erythrocytes have a low ratio of lactate:pyruvate concentration. This low lactate:pyruvate ratio (much lower when compared to human erythrocytes) correlates with an active Krebs cycle and is characteristic of other cell types that utilize oxidative metabolism for ATP production[23]. In carp (*Cyprinus carpio*) erythrocytes, lactate (rather than glucose) was found to be the predominant energy source, and in the presence of other substrates, glucose utilization was only one-tenth of lactate utilization and oxidation[24]. This finding was in contrast to the previously discussed study concerning glucose utilization by trout erythrocytes[19]. In summary, production of ATP by nucleated RBCs of most teleosts involves both aerobic and anaerobic processes, and the extent of substrate utilization by these pathways is complex, species-specific, and individual cell-specific given that erythrocyte aging is a big factor. Unfortunately, studies of energy production in sturgeon erythrocytes (the species evaluated in the present study) are not available.

<div align="center">Glucose transport across erythrocyte membranes</div>

While there are discernible species differences in the utilization of substrates for energy production by erythrocytes, one must also consider the ability of these substrates to enter into erythrocytes prior to consumption. There are species differences in relative permeability of erythrocyte membranes to

substrates that are utilized for energy production. Erythrocyte permeability to glucose is facilitated by

glucose transporters (i.e. GLUT1 and GLUT4) that propagate passive diffusion of glucose across

erythrocyte membranes. The passive diffusion of glucose into erythrocytes is unaltered by insulin and

independent of energy requirements[25]. Human erythrocytes appear to be the most permeable to

glucose due to the high relative concentration of the glucose transporter GLUT1[26]. Teleost erythrocytes

generally have low glucose permeability across erythrocyte membranes, supporting the theory that

substrates of the Krebs cycle, rather than glucose, play a central role in teleost erythrocyte energy

production[16]. The low glucose penetration rate into erythrocytes of yellow perch, brown trout, and

rainbow trout is considered a rate limiting step in erythrocyte energy generation[16,23]. Similarly, low

glucose permeability may limit the rate of glucose decline when there is prolonged serum-clot contact

time in stored blood samples from many species. However, there is a known exception to this generality

as hagfish erythrocytes have high membrane permeability to glucose that compares to human

erythrocytes[16,26]. Pig erythrocytes appear to be the least permeable to glucose due to the lack of a

functional glucose transporter (except in neonatal pigs and pig reticulocytes)[27]. Erythrocyte membrane

permeability to glucose in species other than humans, fish, and pigs fall between these extremes[28].

Because there is low erythrocyte membrane permeability to glucose in many fish species, the sturgeon

samples in our study may have slower rates of glucose consumption. However, sturgeon-specific studies

are not available to support this hypothesis.

Transport of glucose into cells also can be altered by diseases associated with abnormal glucose

metabolism, such as diabetes mellitus and hyperthyroidism. These disorders are discussed in chapter 5.

<div align="center">Species differences in erythrocyte structure</div>

Aside from the differences in substrates and energy production pathways, an additional

consideration is the alternative erythrocyte structure in certain species such as fish, birds, reptiles, and

camelids. The morphologic differences in erythrocytes from these species, such as nucleated red blood cells in teleosts and elliptical erythrocytes in camelids, may lead to faster or slower glycolytic rates[29]. In mammals, erythrocytes lack a nucleus and therefore cannot synthesize nucleic acids or proteins[11]. The presence of a nucleus in fish, birds, and reptiles enables erythrocytes to produce these substrates that are otherwise not produced in mammalian erythrocytes, contributing to the more active erythrocyte metabolism in these species[30]. In contrast to other mammals, camelid erythrocytes are small in size and elliptical in shape. They are also mechanically more resistant to osmotic lysis, have decreased deformability, and higher affinity for oxygen[14,15]. The resistance to deformability and shape change may be related to the high concentration of band 3 within camelid erythrocytes (the concentration of band 3 in llama was about two and a half to three times that in the humans)[15]. These are believed to be adaptive features which allow these species to thrive in areas with low water or oxygen availability. The high activity of glycolytic enzymes within camelid erythrocytes (described previously) may be necessary to maintain those adaptive features[12,13]. It is uncertain whether these structural differences in fish and camelids will alter the rate of serum glucose concentration decline in stored whole blood samples.

<u>The effect of erythrocytosis on the rate of glucose utilization</u>

With high quantities of erythrocytes in the blood, consumption of glucose is accelerated when there is prolonged serum-clot contact time. In humans, disorders associated with erythrocytosis are known to lead to spurious hypoglycemia due to the accelerated utilization of glucose with prolonged storage prior to processing[31-33]. In one report, a patient with Eisenmenger syndrome (ventricular septal defect) and secondary erythrocytosis (HCT 62.5%) had spurious hypoglycemia (37mg/dL) due to delayed sample processing (storage time and temperature not reported). In another report, spurious hypoglycemia developed in samples from a patient with chronic hemolytic anemia and a subsequent marked metarubricytosis. Lastly, another case report described development of spurious hypoglycemia

(as low as 8 mg/dL) in a patient with polycythemia rubra vera. To the author's knowledge, no similar

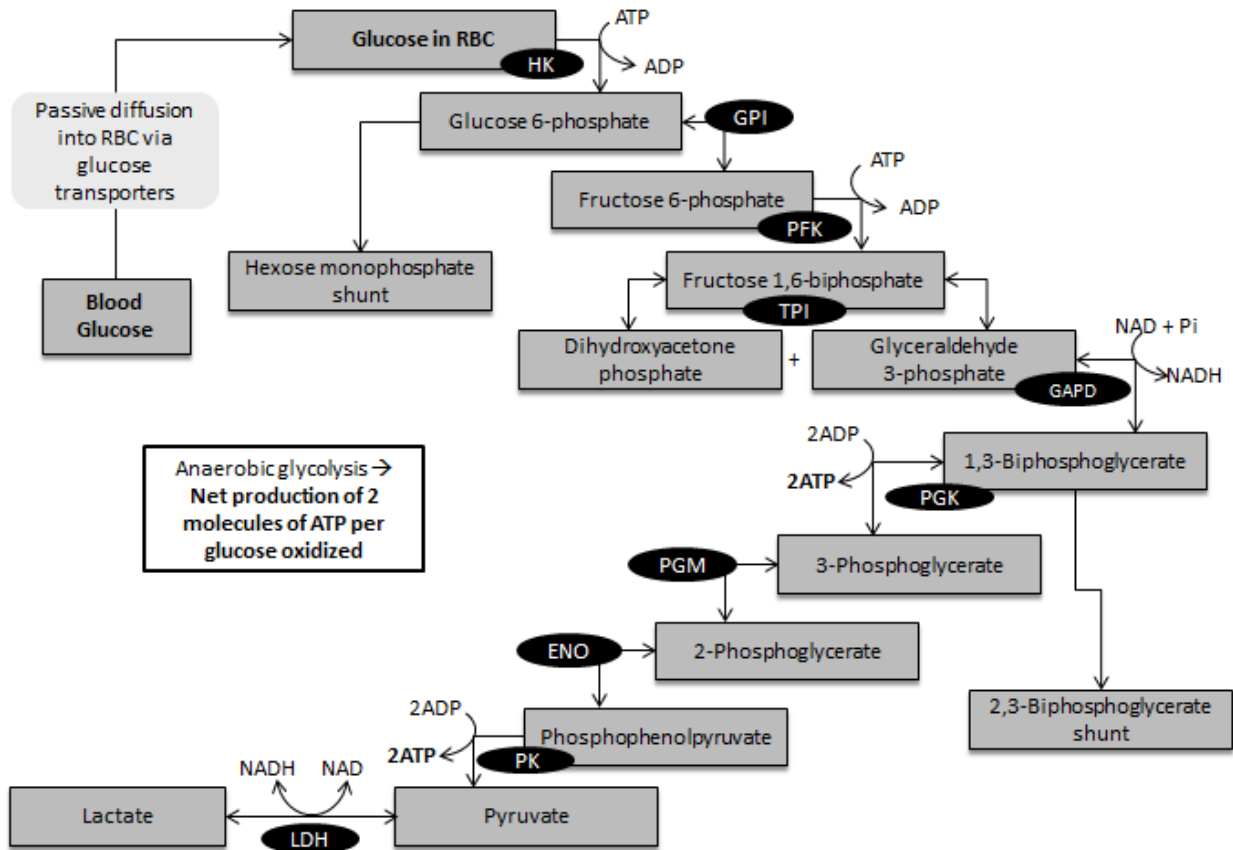veterinary reports are present in the literature.

Figure 1. Diagram of anaerobic glycolysis (also known as the Embden-Meyerhof pathway), the

metabolic pathway of mature mammalian RBCs. Two ATP molecules are consumed in the early steps of

glycolysis, which produces glyceraldehyde 3-phosphate. In the later steps, four ATP molecules and two

pyruvate molecules are produced. In cells that lack mitochondria such as mature mammalian RBCs,

pyruvate cannot enter the Krebs cycle and lactate is ultimately produced instead. The net reaction

produces two ATP molecules per molecule of glucose. HK, Hexokinase; GPI, Glucose phosphate

isomerase; PFK, Phosphofructokinase; TPI, Triosephosphate isomerase; GAPD, Glyceraldehyde 3-

phosphate dehydrogenase; Pi, Inorganic phosphate; NAD, Nicotinamide adenine dinucleotide; NADH,

Reduced adenine dinucleotide phosphate; PGK, Phosphoglycerate kinase; PGM, Phosphoglycerate

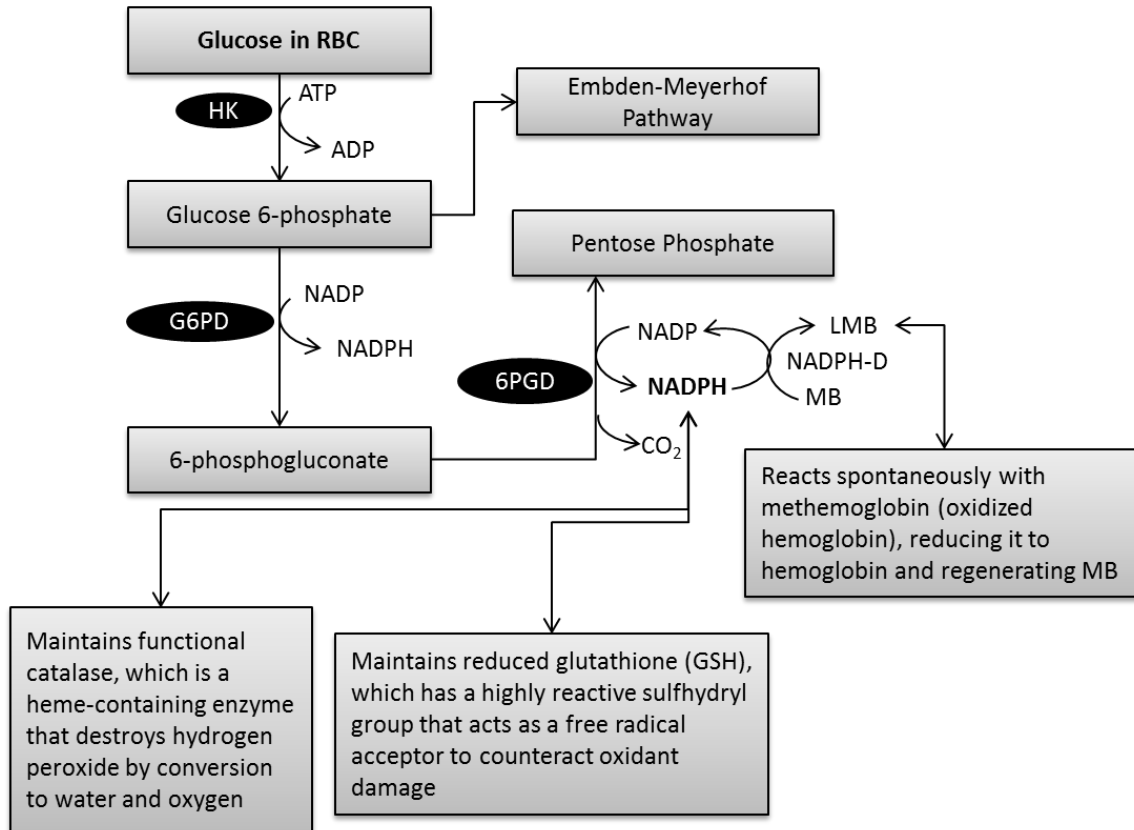mutase; ENO, Enolase; PK, Pyruvate kinase; LDH, Lactate Dehydrogenase.

Figure 2. Diagram of the pentose phosphate pathway (PPP), by which 5-13% of erythrocyte glucose is

metabolized[11]. The PPP is the major source of NADPH, which provides reducing molecules to protect the

erythrocyte against oxidative damage. G6PD, glucose-6-phosphate dehydrogenase (rate controlling

enzyme); NADP, nicotinamide adenine dinucleotide phosphate; NADPH, reduced nicotinamide adenine

dinucleotide phosphate; 6PGD, 6-phosphogluconate dehydrogenase; LMB, leukomethylene blue; MB,

methylene blue; NADPH-D, reduced nicotinamide adenine dinucleotide phosphate diaphorase.
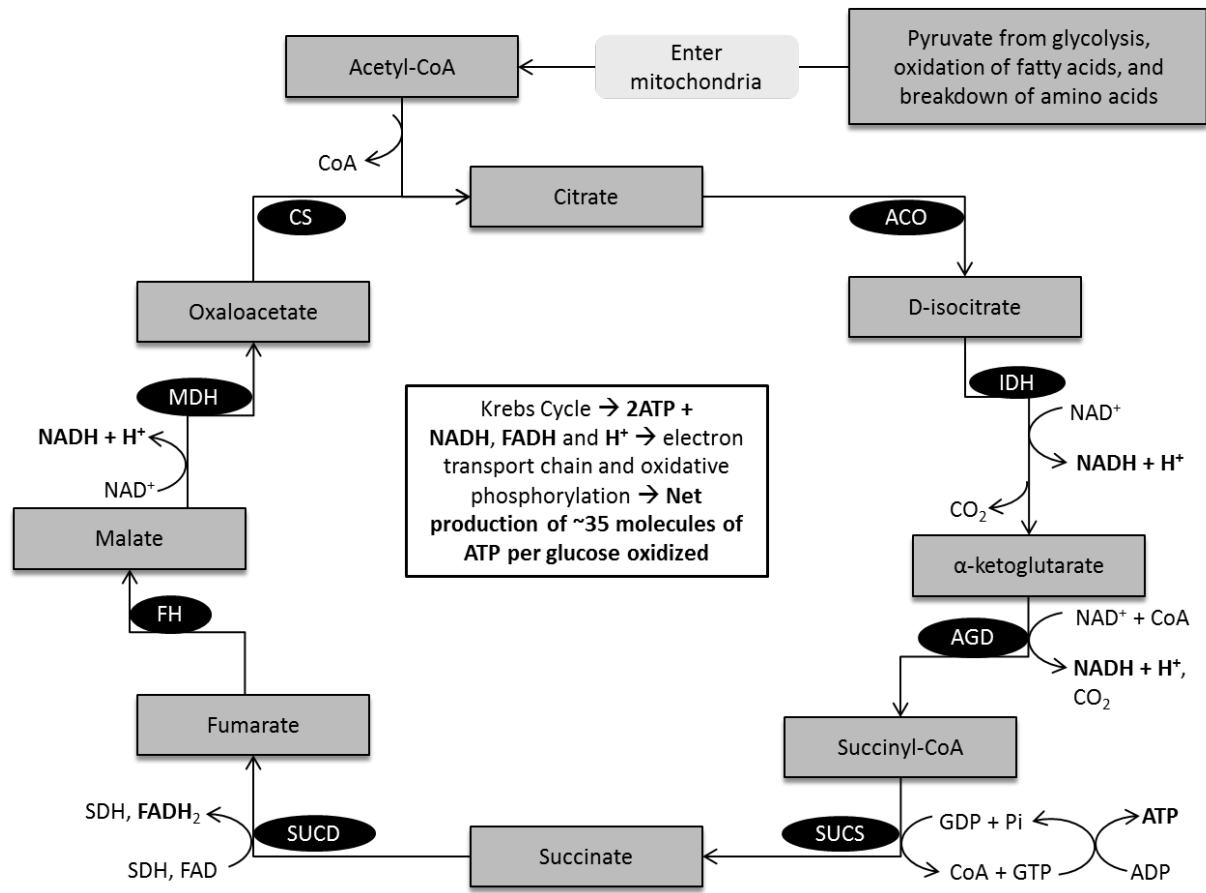
Figure 3. Diagram of the tricarboxylic acid (TCA) cycle (also known as the Krebs cycle), which occurs in cells that have mitochondria, including nucleated erythrocytes of fish species. Pyruvate produced in glycolysis, free fatty acids, and products of amino acid breakdown lead to the formation of acetyl-CoA. Carbon enters the cycle as acetyl-CoA and exits as CO_2, with the production of 2 ATP molecules. Electrons liberated in the TCA cycle will then flow through the electron transport chain, and NADH and FADH will undergo oxidative phosphorylation to produce a total of 30-38 molecules of ATP per glucose oxidized. NAD, Nicotinamide adenine dinucleotide; FAD, Flavin adenine dinucleotide; NADH, Reduced adenine dinucleotide phosphate; FADH, Reduced flavin adenine dinucleotide; ACO, Aconitase; IDH, Isocitrate dehydrogenase; AGD, α-ketoglutarate dehydrogenase; SUCS, Succinyl-CoA synthetase; GDP, Guanosine diphosphate; GTP, Guanosine triphosphate; Pi, Inorganic phosphate; SUCD, Succinic dehydrogenase; FH, Fumarase; MDH, Malate dehydrogenase; CS, Citrate synthase.

CHAPTER 3

UTILIZATION OF SERUM GLUCOSE BY LEUKOCYTES

Energy generation by leukocytes

Metabolism of neutrophils and other leukocytes is dependent on available glucose stores, and because they have organelles (i.e. mitochondria), they generate energy via anaerobic and aerobic processes[34-37]. Due to the combination of metabolic processes, leukocytes metabolize approximately half the amount of glucose that erythrocytes metabolize[7]. Therefore, it is suspected that leukocytes play a lesser role in glucose decline in stored samples than erythrocytes, although unfortunately experimental confirmation of this theory is limited. Most of the literature on this topic is found within human transfusion medicine journals and is focused on the effect of blood storage on neutrophil function. One study found that pyruvate kinase activity within neutrophils increased with storage, while 13 other glycolytic enzymes showed no major changes during storage[35]. However, the effect of increased pyruvate kinase activity within neutrophils on the concentration of glucose in the serum was not evaluated.

The effect of leukocytosis on rate of glucose utilization

While leukocytes are believed to play a lesser role in glucose metabolism when compared to the consumption of glucose by erythrocytes[7], human blood samples with marked leukocytosis, such as from leukemic patients, have been associated with accelerated serum glucose consumption and spurious hypoglycemia[38-43]. In these case reports, the human disorders and diseases associated with spurious

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hypoglycemia included a marked paraneoplastic neutrophilia (79.5 neutrophils G/L) secondary to lung

cancer[42], leukemias with varying intensities of leukocytosis and cell type of origin[41], leukocytosis (33.2 X

10^9 WBC/L) secondary to polycythemia vera (which also contributed to the spurious hypoglycemia)[40],

myeloproliferative disease (WBC count of 109,200/µL; 74% segmented neutrophils and 12% band

neutrophils)[39], eosinophilic leukemoid reaction (96,000 WBC/µL; >50% mature eosinophils)[39], and

paraneoplastic leukemoid responses secondary to hematopoietic cytokines (G-CSF and IL-6) from a

variety of neoplasias[43]. In the latter case series, hematopoietic cytokines are thought to cause spurious

hypoglycemia by increasing the absolute peripheral granulocyte concentration and by inducing a variety

of energy-consuming processes by the granulocytes (such as protein synthesis[44], phosphorylation[45],

superoxide ion generation[46], and prostaglandin synthesis[47])[43]. One of these reports states that not until

the WBC count exceeds 60,000/cu mm do the leukemic blood samples consume more glucose than

normal controls, and the accelerated glucose consumption is due to high quantities of leukocytes rather

than cell immaturity as in acute leukemias or left-shifted neutrophilias[39]. Additionally, they concluded

that spurious hypoglycemia can occur with either lymphocytic or granulocytic leukemia, but glucose

consumption typically occurs more rapidly in granulocytic leukemias[39]. Finally, treatment with drugs that

are used during chemotherapy protocols to increase leukocyte counts (i.e. human G-CSF/filgrastim) can

also lead to spurious hypoglycemia due to the development of leukocytosis[48].

The subjects included in the present study did not have any known leukemias or evidence for

leukocytosis. However, complete blood counts were not performed in species other than dogs due to

financial constraints. Despite this, it is suspected that a widened buffy coat would have been observed in

if there was a significant enough leukocytosis to alter the rate of serum glucose decline in this study. The

effects of the described human disorders and diseases on serum glucose concentration measurement

after delayed serum separation is not described in the veterinary literature, but warrants further study

once species-specific effects of storage time and temperature on glucose decline is understood in

samples from healthy animals.

CHAPTER 4

UTILIZATION OF SERUM GLUCOSE BY PLATELETS

<u>Energy generation by platelets in mammalian species</u>

Much of the literature on this topic is regarding cell culture media and energy sources

necessary for maintenance and survival of cultured platelets. In contrast to mammalian erythrocytes

which primarily utilize anaerobic metabolism for ATP generation, platelets consume glucose for

anaerobic glycolysis, aerobic glycolysis and carbohydrate oxidation, as platelets have mitochondria[49-52].

Niu *et al*. found that when glucose and glycogen are present in low concentrations (compared to

physiologic plasma levels), most of the glucose and glycogen utilized is converted to lactate via

anaerobic metabolism, but lactate is also oxidized during that time[49]. Another similar study by Niu *et al*.

found that platelets use glucose, glycogen, lactate, free fatty acids, and amino acids for production of

ATP, and glucose was the major fuel consumed[52]. There was another study whereby fuel choices by

human platelets were investigated, but in this study an *in vitro* system that more closely approximated

physiologic human plasma was used[51]. In that system, anaerobic glycolysis contributed to 24% of total

ATP turnover while oxidation of a variety of fuels (glucose, 3-hydroxybutyrate, acetate, palmitate,

oleate, and other fuels) contributed to 76% of total ATP production[51]. This suggests that aerobic

metabolism, which utilizes a minimal proportion of glucose, within platelets may play a more significant

role in energy generation in freshly collected whole blood. Finally, glucose transport across plasma

membranes of platelets may also represent a rate-limiting step in its utilization for ATP production[53]. In

sum, these findings suggest that platelets are additional blood cells that likely contribute to serum

glucose consumption in stored blood samples. However, this glucose consumption is most likely to a

lesser degree than erythrocytes given the studies that indicate the propensity of human platelets to

utilize aerobic metabolism to generate ATP.

Energy generation by platelets in non-mammalian species

The literature concerning energy generation by platelets in non-mammalian species is limited[54].

Similar to the studies in mammalian species, the one study available describes cell culture preparations

and fuel choices necessary for maintenance and survival of cultured thrombocytes, artificial conditions

that do differ from the whole-blood storage conditions utilized in the present study. Thrombocytes are

the non-mammalian equivalent to human platelets. They serve the same function as platelets but, in

contrast to mammalian platelets, retain a nucleus. Rainbow trout (*Oncorhynchus mykiss*) thrombocytes

utilize the majority of glucose via glycolysis, with only 0.8% of glucose being consumed via aerobic

metabolism, which accounts for only 9% of total ATP turnover (in contrast to 76% of ATP turnover in

human platelets)[51,54]. Therefore, anaerobic glycolysis may be responsible for the majority of energy

generation within fish thrombocytes while aerobic processes may play more substantial roles in

mammalian platelets. It is uncertain whether these species differences in platelet and thrombocyte fuel

utilization have any effect on the results of the present study. Additionally, in the study described

platelet parameters were only evaluated in canine subjects; therefore, the possibility for a connection

between platelets and serum glucose decline was not investigated in all species.

The effect of thrombocytosis on rate of glucose utilization

Similar to leukocytosis being a known cause for artifactual hypoglycemia in humans, it is

possible that extreme thrombocytosis (i.e. more than 900,000 platelets/μL) may also contribute to the

formation of spurious hypoglycemia. However, to the author's knowledge, there are no studies or case

reports to confirm this suspicion, and no dogs in the present study were known to have an extreme

thrombocytosis, preventing evaluation of this possibility.

CHAPTER 5

ALTERATIONS IN GLUCOSE UTILIZATION

The effects of sepsis on glucose utilization

It may be inappropriate to assume that the rate of glycolysis is constant in ill animals as compared to healthy animals. For example, systemic disorders may alter glycolysis and/or glucose uptake. The first systemic disease considered is sepsis. True hypoglycemia is a well-known manifestation of sepsis due to *in vivo* depletion of glycogen stores, diminished gluconeogenesis, and increased peripheral glucose utilization[55]. Additionally, in fresh whole blood stored at room temperature, the rate of glucose consumption does not increase secondary to the induction of bacteria that is present in the blood of septic patients[55]. Therefore, hypoglycemia associated with sepsis appears to be a true pathologic manifestation and an *in vivo* occurrence rather than an *in vitro* phenomenon due to bacterial consumption of glucose.

Alternatively, one must consider that patients with a focus of infection or sepsis may also have elevated leukocyte counts. If the degree of leukocytosis is severe enough, then there will likely be accelerated glucose consumption and an artifactual hypoglycemia may be detected (especially if there is delayed sample processing and if samples are stored at room temperature)[56].

The effects of hemoparasites on glucose utilization

Sheep that are infected with *Mycoplasma ovis* (previously *Eperythrozoon ovis*) are known to have low blood glucose levels and increased blood lactate levels[57]. The same study remarked that the

hypoglycemia can be attributed to increased glycolytic activity of infected erythrocytes[57]. Alternatively,

another study concerning the same genus of hemoparasite in pigs (*M. suis*) actually found that glucose

consumption decreased proportionally with the decline in the percentage of parasitized erythrocytes in

an *in vitro* culture system, indicating that glucose consumption was due to the glycolytic activity of *M.

suis* and not erythrocytes[58]. Therefore, it is likely that there is enhanced consumption of glucose by both

infected erythrocytes and by the hemoparasite itself. Infected animals may present with clinical signs of

hypoglycemia because the consumption of glucose is an *in vivo* occurrence. However, some

Mycoplasma infected animals with measurable hypoglycemia do not present with clinical signs of

hypoglycemia. In one of these cases, it was documented that *M. ovis* infection in a lamb caused

accelerated *in vitro* consumption of glucose in stored whole blood samples when compared to samples

from a control sheep[59]. Hemoplasmosis in llamas and other species have also been associated with

concurrent hypoglycemia[60,61]. In those cases, it was suspected that the hypoglycemia resulted from

glucose metabolism by the hemoparasites, either *in vitro*, *in vivo*, or both. However, other causes for

hypoglycemia (i.e. bacterial sepsis or anorexia) were also considered[57,60,62]. Because hemoplasmosis is

common in South American camelids in the southeast, and due to the potential for *in vitro* glucose

consumption by hemoplasma organisms, two goals of our research were to 1) identify hemoplasma-

infected alpacas and then 2) to investigate a possible association between positive animals and serum

glucose decline in stored samples.

The effects of altered systemic glucose metabolism on rate of glucose utilization

Several studies have found that glucose uptake into many cell types is significantly increased in

human patients with type I diabetes mellitus (DM), and this increased glucose uptake is often

responsible for the many pathologies of DM, including nephropathy, retinopathy, and neuropathy[63-68].

However, one study found that glucose uptake into human platelets was significantly reduced in

patients with DM[65]. Additionally, studies concerning glucose uptake into erythrocytes of diabetic

patients are contradictory. For example, four studies found that influx of glucose into erythrocytes was

significantly reduced in diabetic patients[25,66,69-71], while two studies described increased glucose uptake

in erythrocytes from diabetic patients[72,73]. It has been shown that hyperglycemia causes lipid

peroxidation of RBC membranes[74], and because of this membrane damage, RBC glucose uptake is

reduced[25]. As previously mentioned, RBC glucose uptake is unaffected by insulin. Therefore, it can be

suspected that treated diabetic patients with normoglycemia would have "normal" RBC glucose uptake

that is comparable to healthy patients. Furthermore, it can also be theorized that glucose uptake would

be reduced in any patient with hyperglycemia, regardless of the etiology. Given the inconsistencies in

the literature on the topic, it would be quite interesting to determine the rate of serum glucose decline

in stored whole blood samples from diabetic patients with normoglycemia, diabetic patients with

hyperglycemia, and healthy patients with hyperglycemia due to other causes. Future studies can be

tailored to include those patients.

Hyperthyroidism is another systemic disease that was found to perpetuate an overall higher

metabolic rate within human erythrocytes[75], and glucose transport activity in hyperthyroid rats was

significantly higher than that from euthyroid controls[76]. These alterations in systemic glucose

metabolism due to endocrine dysfunction may or may not affect the rate of serum glucose decline in

stored samples. The present study did not include any patients with previously diagnosed or clinically

evident endocrine disease, so investigation of this occurrence warrants additional prospective studies.

CHAPTER 6

PRE-ANALYTICAL SAMPLE STORAGE STUDIES

Many veterinary textbooks state that serum glucose concentration in most veterinary species will decrease at a rate of approximately 5-10% per hour when serum is not promptly separated from the cells[1-6]. Additionally, it is suggested that storage of the samples at 4°C or separation of serum from the clot within 15-30 minutes will prevent this decrease in glucose concentration[8]. However, the original literature supporting these claims is limited and it may be inappropriate to generalize the rate of serum glucose decline to all veterinary species. In human studies, the rate of glucose decline has been reported as 6-10mg/dL per hour at 25°C[6,77]. Feline blood samples stored at 25°C exhibited serum glucose decline of 15% in the first hour and 10% each subsequent hour, though storage at 4°C prevented any glucose changes for up to 8 hours[8]. One study found that there was a reduction in serum glucose by 2.6mmol/l after 24 hours and 3.7mmol/l after 72 hours in equine blood samples stored at 25°C[78]. In cattle blood samples stored at 23°C, glucose dramatically declined beginning 2 hours after sampling and at 4°C glucose concentrations were stable for 24 hours[79]. In Loggerhead sea turtles, storage at 4°C prevented a significant decline in glucose for up to 48 hours, but there was a statistically significant 7% decrease in heparinized plasma glucose concentration after 96 hours[80]. It is the goal of this research to elucidate additional species differences in serum glucose decline within stored whole blood samples.

CHAPTER 7

RESEARCH DESIGN AND METHODS

Study Animals

Adult animals from four species (horses, n = 28; alpacas, n = 20; Siberian sturgeon, n = 31; and dogs, n = 26) were used for this prospective study. Prior to inclusion in the study, the animals were deemed healthy on the basis of physical examination and clinical history. The amount of blood drawn from each animal was between 5-15 ml depending on the species. In the horses and alpacas, blood was collected via jugular venipuncture. For the sturgeon, blood was collected from the caudal tail vein. For the dogs, blood was collected either from the jugular or external cephalic vein.

University-owned horses and sturgeon were used for those portions of this study. Per University of Georgia requirements, their care and use was conducted under the approval of the University of Georgia Institutional Animal Care and Use Committee. Client-owned dogs and alpacas were used for those portions of the study, and study design was approved by the University Of Georgia College Of Veterinary Medicine Clinical Research Committee. Prior to sampling of dogs and alpacas, informed client consent was obtained.

Sample handling and serum glucose measurement

Blood from each animal was collected into a single syringe and then immediately aliquoted into seven glass tubes (horses and alpacas) or plastic tubes (sturgeon and dogs) with no additive and one EDTA (dogs, horse, and alpacas) or lithium heparin (sturgeon) anti-coagulant tube. Plastic tubes were used for the sturgeon and canine samples due to the relatively small sample volume size from those

species. Packed cell volumes (PCV) and plasma protein concentrations (PP) were determined from the anti-coagulated samples. For the canine samples, complete blood counts and full biochemical profiles were performed in lieu of PCV and PP to encourage study enrollment. One of the tubes with no additive from each animal was centrifuged at 2000 g for 10 minutes after one hour of storage at room temperature (25°C) and served as the reference sample. Serum was separated, immediately frozen and maintained at -20°C for $\leq$ 30 days prior to glucose measurement. One hour was used as the reference time point to ensure adequate time to clot formation, as average time to clot is 60 minutes in equine blood samples[27]. Three of the remaining no-additive tubes were stored at room temperature (25°C) and the other three were stored in a refrigerator (4°C). One sample from each storage temperature was then centrifuged as previously described at 2, 4, and 8 hours post-collection. Serum was separated from the cells and immediately frozen as previously described. Serum samples were thawed in batches, and serum glucose was immediately measured using a clinical chemistry analyzer (hexokinase test, *P-Modular Analytics System*, Roche Diagnostics, Indianapolis, IN, USA).

<u>Hemoplasmosis screening of alpaca samples</u>

The alpaca EDTA-anticoagulated blood samples were sent to Purdue University for screening for *hemoplasma organismal DNA* by PCR. DNA was extracted from the alpaca EDTA-anticoagulated whole blood samples using Quick-gDNA[TM] Blood MiniPrep and then screened in duplicate for hemoplasma infection using the PCR Syber-Green screening test (qPCR), as described previously.[28] According to the reference paper and the lab that performed the PCR testing, samples that had both duplicates with Ct (threshold cycle value) above 34.4 were considered negative and samples that had both duplicates with values of Ct below 34.4 were positive, with the lower Ct values corresponding to the highest bacteremias. Samples that had one duplicate below and one above 34.4 were considered "suspect." A positive result indicates infection with any species of hemoplasma (it does not indicate infection with a specific species of hemoplasma).

<u>Statistical analyses</u>

Statistical analysis was performed using a commercially available software program (MiniTab, version 15.1.0.0, Minitab Inc., State College, PA, USA). Data was compared within each species using repeated measures ANOVA and Tukey's test for multiple comparisons. Additionally, each species was divided into two binary variable groups: those with a PCV above the median PCV for that species, and those with a PCV below the median PCV for that species. ANOVA was then used to determine if there was an interaction between PCV and serum glucose concentration when comparing the "high" PCV and "low" PCV groups for each species. ANOVA was also used to determine if there was an interaction between hemoplasma qPCR status and serum glucose concentration. Statistical significance was set at $p < 0.05$ for all analyses.

CHAPTER 8

RESULTS

<u>Serum glucose concentration change over time</u>

Average rates of serum glucose concentration decline (expressed as percentages) are

summarized in Table 1. At 4°C, the serum glucose concentration declined significantly after 8 hours of

storage (8H) in the stored sturgeon and canine samples. The decline in serum glucose concentration for

all other samples stored at 4°C was not statistically significant over the 8 hour time period, although in

equine samples there was a minimal downward trend at 4H and 8H.

At 25°C, the serum glucose concentration declined significantly at 4H and 8H ($p < 0.0001$) in the

stored equine, sturgeon, and alpaca samples. For the canine samples stored at 25°C, the decline in

serum glucose concentration was statistically significant at 2H, 4H and 8H. The declines in serum glucose

concentration for all other samples stored at 25°C (horse, sturgeon, and alpaca samples at 2H) were not

statistically significant. However, the serum glucose decline for the equine samples stored at 25°C at 2H

was 3.52% ($p = 0.0500$), approached statistical significance. After 8H, the canine samples had the most

substantial average rate of decline (49%) and the equine samples had the least substantial average rate

of decline (25%).

Figures 4 and 5 graphically display the serum glucose concentrations at each time point in each

species, at 25°C and 4°C, respectively. Figure 6 depicts the average glucose concentration at each time

point for each species at both storage temperatures, with one graph for each species. Dashed lines are

added to Figure 6 (defined in the figure legend) to indicate the lower limit of the reference interval for

serum glucose for that species, as well as the lower limit of total allowable error based on clinical decision limits published by the ASVCP[81]. Additionally, Figures 7 and 8 depict the average serum glucose concentration +/- the standard deviation at each time point in each species, at 25°C and 4°C, respectively.

<div align="center">Hemoplasma qPCR screening results and effect on rate of glucose decline in the alpacas</div>

Table 2 depicts the results of the screening, which revealed 11 positives, 4 suspects, and 5 negatives. The 5 samples with the highest bacteremias (lowest Ct values) were compared with the 5 negative samples using ANOVA to assess for an interaction between qPCR status and serum glucose decline.

There was no statistically significant interaction between hemoplasma qPCR screening status and serum glucose decline when comparing qPCR negative samples to those samples with the highest bacteremias for samples stored at both 25°C (p = 0.985) and 4°C (p = 0.971). Figure 9 graphically displays the average serum glucose concentration as it declines for each time point for the samples from alpacas with negative qPCR status and highest bacteremias.

<div align="center">The effect of PCV status on rate of glucose decline</div>

The median PCV or HCT for each species was as follows: alpaca 24.5%, dog 48.7% (HCT), sturgeon 25%, and horse 34%. At both 25°C and 4°C, there was no statistically significant interaction between PCV and serum glucose when comparing samples grouped by PCV/HCT above or below the median PCV/HCT for each species. Table 2 lists all of the p values calculated from the analysis. Figures 10 and 11 graphically explore the similarities of the slopes of serum glucose decline for all species at each storage temperature when the average glucose of samples with PCV/HCT above and below the median are compared.

Table 1. Average percent change of serum glucose concentration from the reference time point compared to subsequent time points. The p value is given in parentheses. *Statistically significant difference ($p < 0.05$) from reference sample.

| Storage Temperature	Hours post-blood collection	Horse	Alpaca	Fish (sturgeon)	Dog
4°C	2 hours	↓ 0.03% (p = 0.9997)	↑ 1.04% (p = 0.9046)	↑ 0.72% (p = 0.8631)	↑ 2.01% (p = 0.0991)
	4 hours	↓ 1.16% (p = 0.3727)	↑ 0.68% (p = 0.9705)	↓ 0.40% (p = 0.9725)	↑ 1.51% (p = 0.3046)
	8 hours	↓ 0.41% (p = 0.7552)	↑ 1.04% (p = 0.9046)	**↓ 6.45%*** (p < 0.0001)	**↓ 4.17%*** (p = 0.0001)
25°C	2 hours	↓ 3.52% (p = 0.0500)	↓ 1.99% (p = 0.2790)	↓ 1.90% (p = 0.6829)	**↓ 8.34%*** (p < 0.0001)
	4 hours	**↓ 10.88%*** (p < 0.0001)	**↓ 7.67%*** (p < 0.0001)	**↓ 13.94%*** (p < 0.0001)	**↓ 22.91%*** (p < 0.0001)
	8 hours	**↓ 24.85%*** (p < 0.0001)	**↓ 26.23%*** (p < 0.0001)	**↓ 30.21%*** (p < 0.0001)	**↓ 49.22%*** (p < 0.0001)
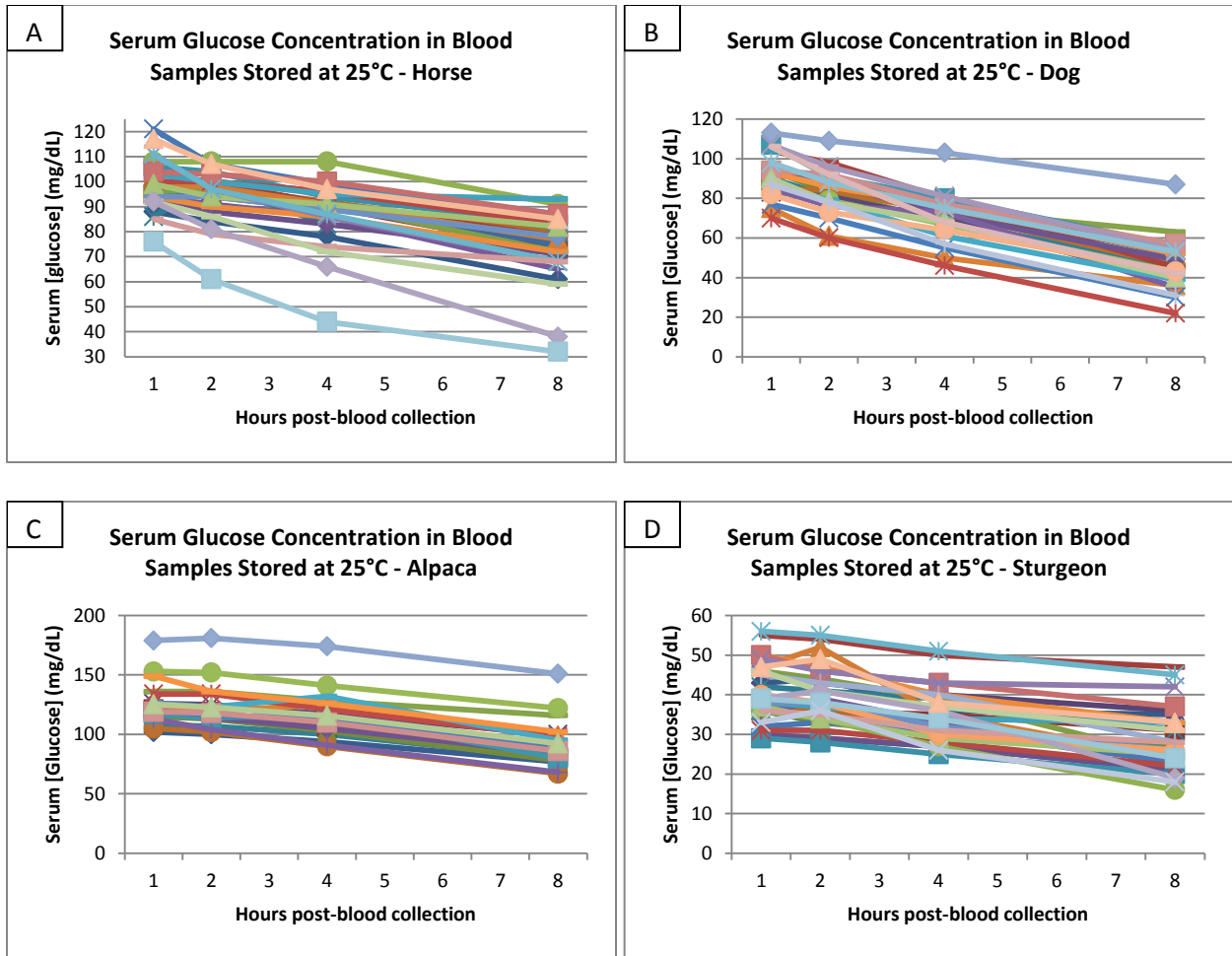
Figure 4. Line plots of serum glucose concentration at each time point for all individual samples stored at 25°C; results from each species are within a separate graph as indicated in the graph titles. **A**, Horse results; **B**, Dog results; **C**, Alpaca results; **D**, Sturgeon results.

Figure 5. Line plots of serum glucose concentration at each time point for all individual samples stored at 4°C; results from each species are within a separate graph as indicated in the graph titles. **A**, Horse results; **B**, Dog results; **C**, Alpaca results; **D**, Sturgeon results.
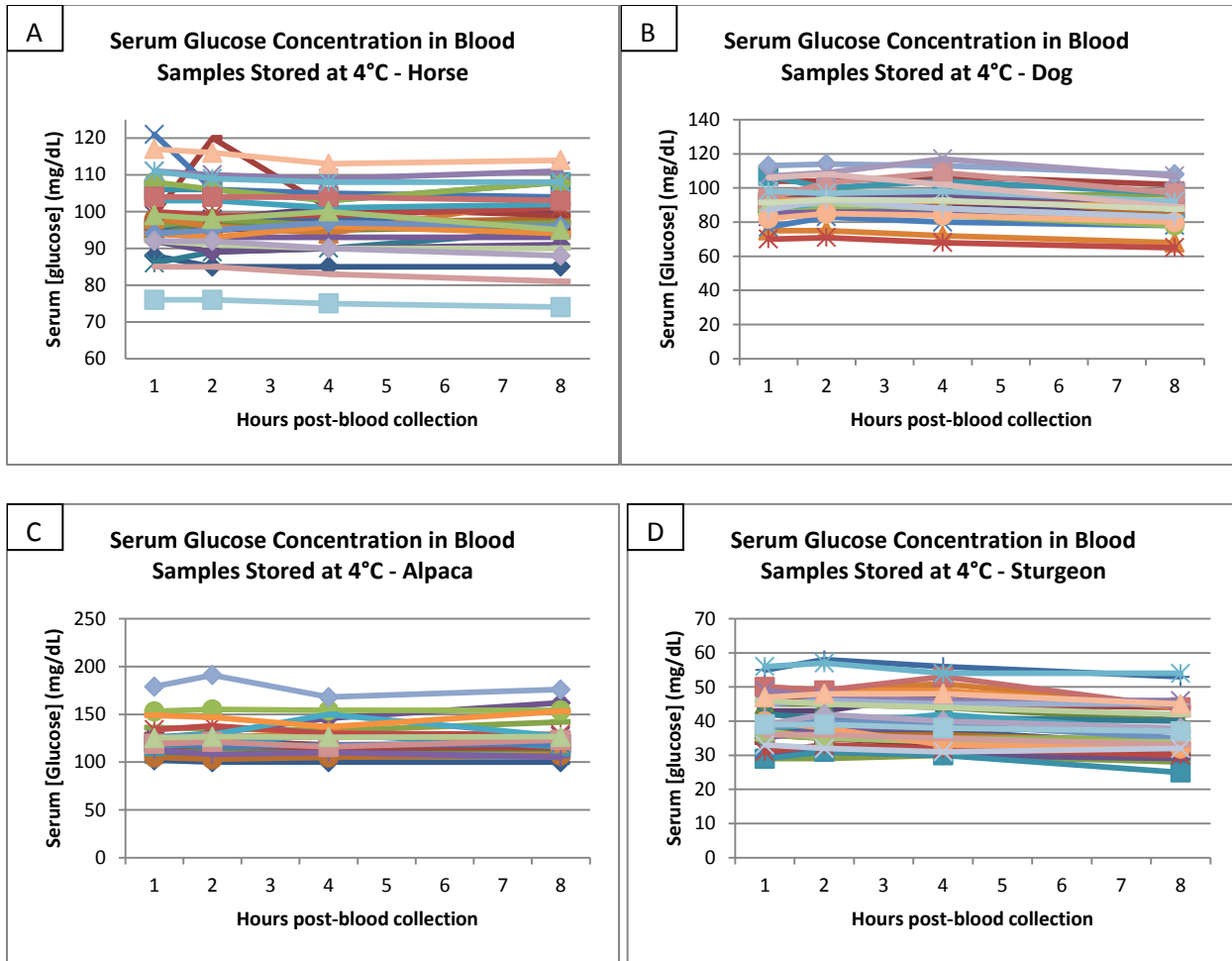
Figure 6. Line plots of average serum glucose concentration over time in whole blood samples stored at 4°C and 25°C from horse (**A**), dog (**B**), alpaca (**C**), and sturgeon (**D**). Percent change from the reference time point is given for each statistically significant difference. Red dashed lines indicate the lower limit of the reference interval for serum glucose for that species (not available for sturgeon). Blue dashed lines indicate a 20% decline from the reference sample (ASVCP published total allowable error for glucose values within reference interval)[81]. *Statistically significant difference (p < 0.05)

Figure 7. Line plots of average serum glucose concentration +/- standard deviation at each time point for all samples stored at 25°C; results from each species are within a separate graph as indicated in the graph titles. **A**, Horse results; **B**, Dog results; **C**, Alpaca results; **D**, Sturgeon results. *Statistically significant difference ($p < 0.05$)
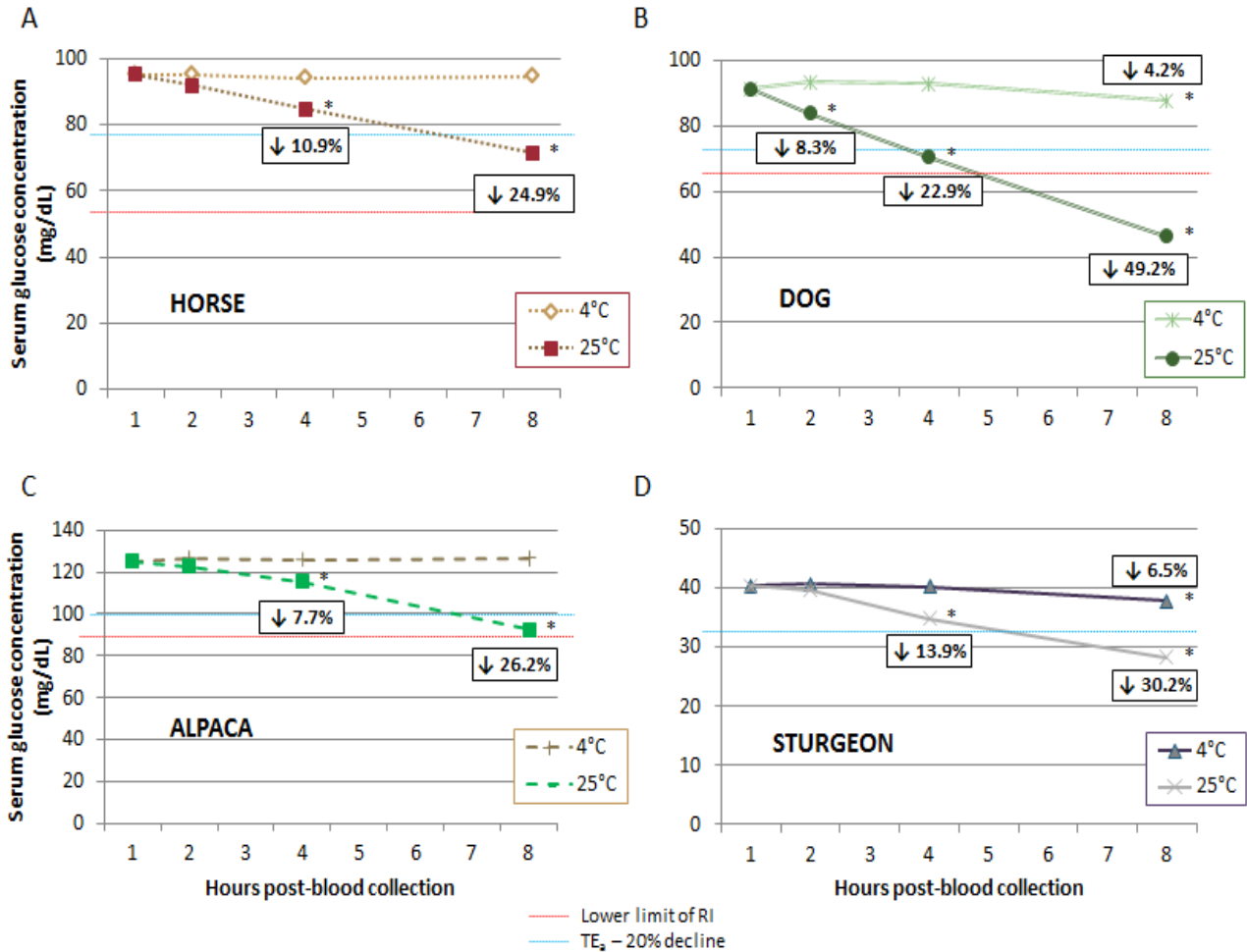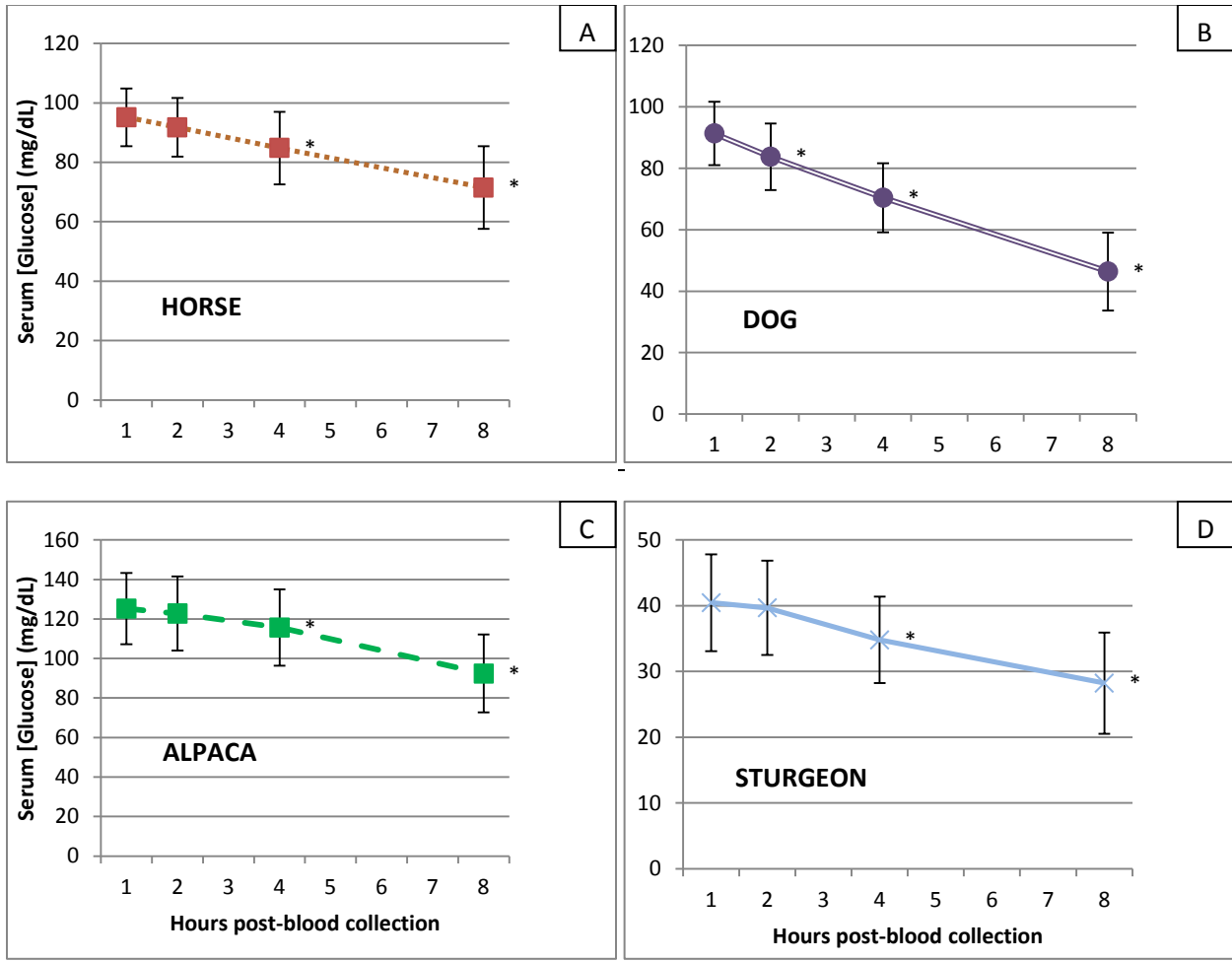
Figure 8. Line plots of average serum glucose concentration +/- standard deviation at each time point for all samples stored at 4°C; results from each species are within a separate graph as indicated in the graph titles. **A**, Horse results; **B**, Dog results; **C**, Alpaca results; **D**, Sturgeon results. *Statistically significant difference ($p < 0.05$)
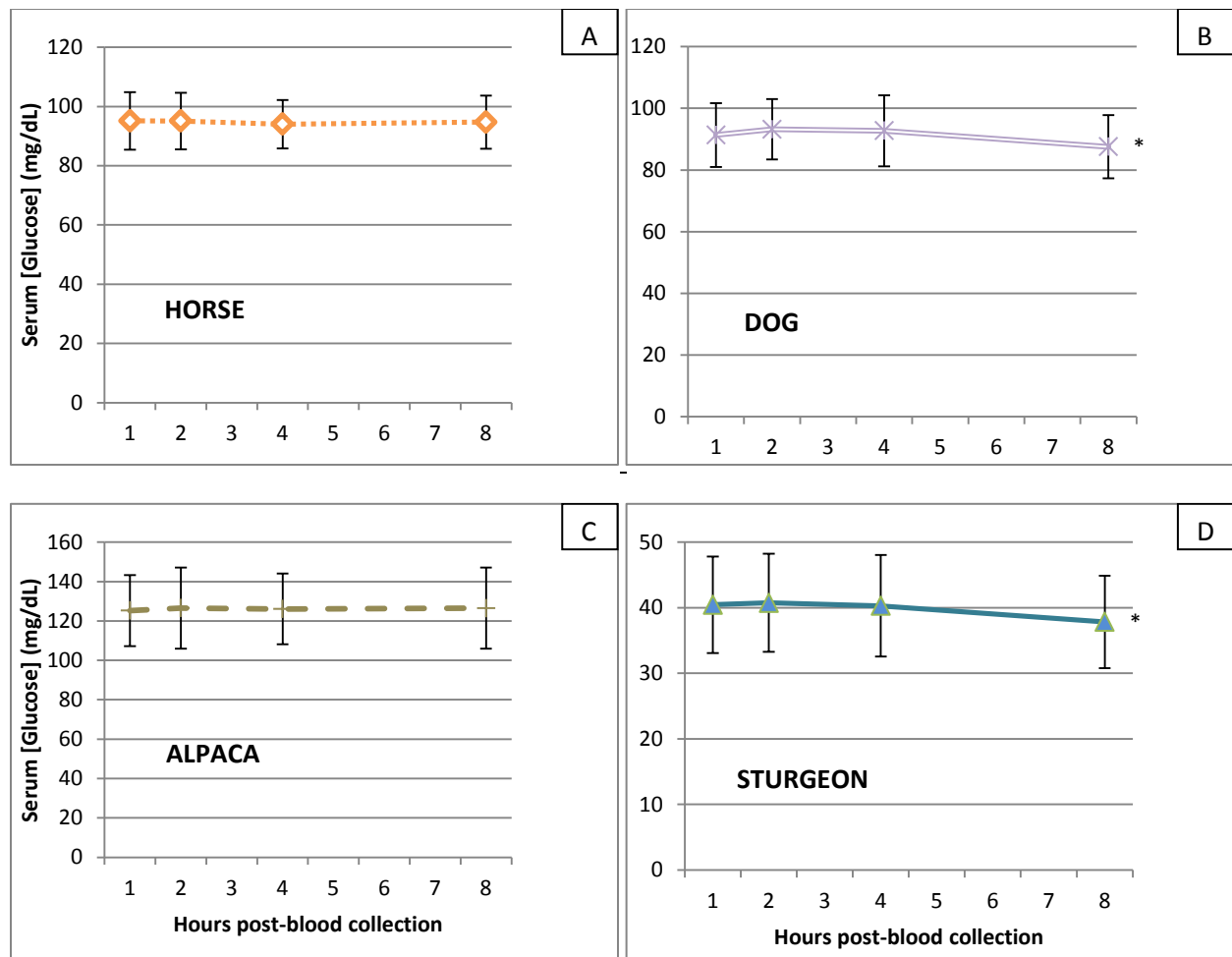
Table 2. Results of qPCR screening for hemoplasma. Negative status; highest bacteremias.

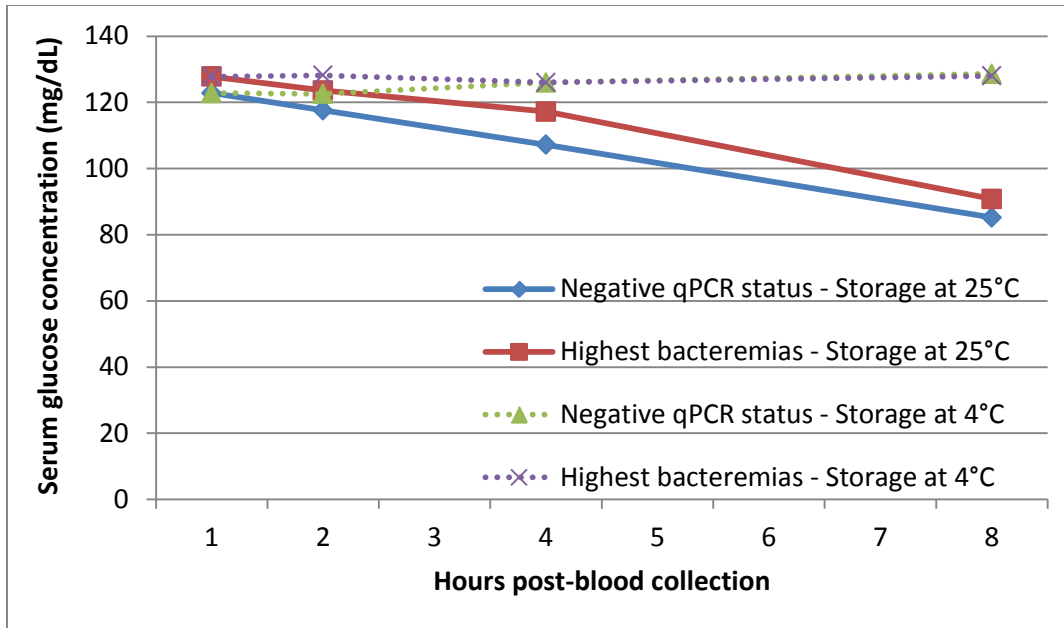| Alpaca ID	Ct (duplicates)	Interpretation
A	27 28	Positive
B	37 32	Suspect
C	34 35	Suspect
D	23 22	Positive
E	30 36	Suspect
F	36 36	Negative
G	24 24	Positive
H	36 37	Negative
I	23 23	Positive
J	35 36	Negative
K	22 22	Positive
L	25 25	Positive
M	21 21	Positive
N	23 22	Positive
O	35 36	Negative
P	37 36	Negative
Q	26 26	Positive
R	23 23	Positive
S	31 35	Suspect
T	19 19	Positive

Figure 9. Line plot of the average serum glucose concentration at each time point for both storage temperatures for the samples from alpacas with negative qPCR status and highest bacteremias.

Table 3. List of P values calculated from ANOVA assessing the relationship between PCV/HCT and serum glucose concentration. All values were statistically insignificant.

Species	Storage Temperature	P value
Horse	25°C	0.972
	4°C	0.967
Dog	25°C	1.000
	4°C	0.992
Alpaca	25°C	0.995
	4°C	0.926
Sturgeon	25°C	0.939
	4°C	0.997

Figure 10. Comparison of the average glucose concentration from samples with PCV/HCT above and

below the median for samples stored at 4°C. **A**, Horse results; **B**, Dog results; **C**, Alpaca results; **D**,
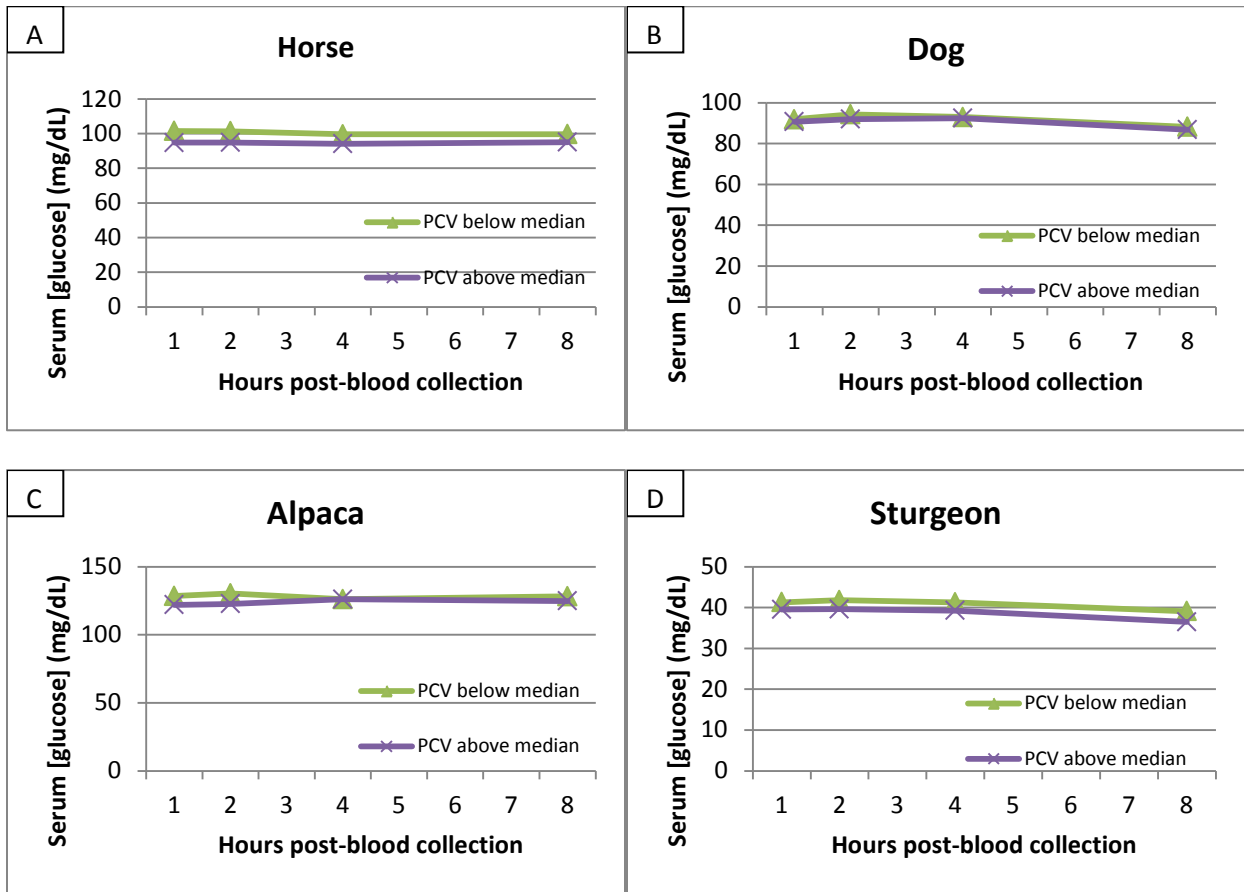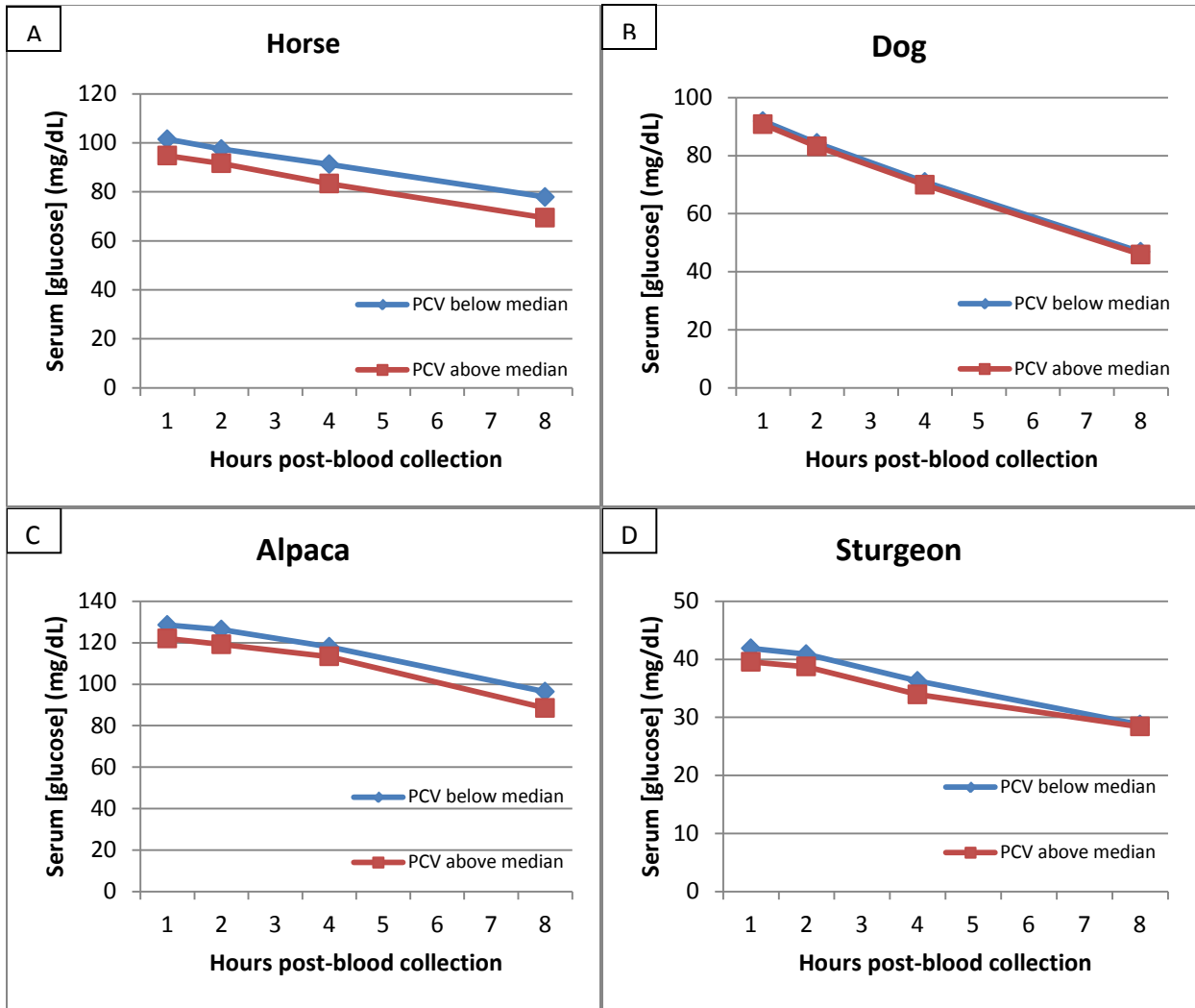
Sturgeon results.

Figure 11. Comparison of the average glucose concentration from samples with PCV/HCT above and below the median for samples stored at 25°C. **A**, Horse results; **B**, Dog results; **C**, Alpaca results; **D**, Sturgeon results.

CHAPTER 9

CONCLUSIONS

Based on the results of this study, species-specific recommendations for sample handling and storage to preserve endogenous serum glucose concentrations can be made for healthy patients. Many veterinary clinical pathology laboratories receive specimens from both clinical patients and research animals. Therefore, to err on the side of caution, conclusions were made based on statistically significant changes rather than total allowable error. The decline in serum glucose concentration for all samples stored at 4°C except for the 8 hour samples from sturgeon and dogs was not significant; therefore it can be concluded that storage at 4°C limits serum glucose decline for at least 4 hours in all species tested and for up to 8 hours in horses and alpacas. This is consistent with previous work showing that storage at 4°C prevents accelerated serum glucose decline with continued serum-clot contact.[5,8,78,79]

It is uncertain why storage at 4°C did not prevent a significant decrease in serum glucose concentration after 8 hours of storage in the dog and sturgeon whole blood samples, as was expected. Perhaps the degree of glucose utilization by canine erythrocytes is substantial enough that storage temperature alone is ineffective at preventing glucose decline if the time of storage is lengthy enough.[10] In sturgeon erythrocytes, it is possible that the efficiency of glucose uptake may rely on an optimal temperature to ensure glucose homeostasis as fish are notoriously affected by their habitat.[82] Regardless, these significant glucose changes after 8 hours of storage in a refrigerator were initially unexpected and are important species differences to note when making sample storage recommendations.

At 25°C, serum glucose concentrations were comparable to reference values at 2 hours post-blood collection in the horse, sturgeon, and alpaca blood samples, but significantly lower at 4 and 8 hours in those species. In canine samples, the decline in serum glucose concentration was significant at all time points. Therefore, at room temperature, serum-clot contact time should not exceed 1 hour in dogs and 2 hours in horses, alpacas, and sturgeon. The degree of serum glucose decline was most substantial in dogs and least sizable in horses after 8 hours of storage at room temperature. Given the unique qualities and characteristics of their erythrocytes as described in the introduction, it was initially suspected that alpacas would have the most substantial degree of serum glucose decline. However, in the study herein, stored canine samples consumed serum glucose at more rapid rates than other species, and this lead to serum glucose concentrations that fell outside of the reference interval for serum glucose after 8 hours of storage at room temperature. This was the only species in which this occurred. The species-specific difference in dogs causing this sensitivity to prolonged serum-clot contact time remains uncertain, but this finding corresponds with the findings of Harvey and Kaneko's study, which also found that canine erythrocytes metabolized glucose faster than cats, horses, and humans.[10]

Given the low glucose permeability in some fish erythrocytes, and the ability of fish to use additional substrates for the Krebs cycle, it was initially suspected that serum glucose in the sturgeon samples would be the least affected by storage parameters. However, this was not the case as sturgeon erythrocytes consumed glucose at the second fastest rate after 8 hours of storage at room temperature. To explain this discrepancy, there may be species-specific disparities unique to glucose uptake and metabolism by sturgeon erythrocytes that have yet to be described, as there is much species-specific variability in glucose use in piscine erythrocytes described in the literature.[19,23,24]

The authors speculated there would be an association between HCT/PCV and serum glucose concentration decline over time because erythrocytes are thought to consume more glucose than other blood cells.[1,6-8,83] However, there were no significant interactions between serum glucose concentration

and red blood cell mass in all species at both storage temperatures. The lack of significant correlation between serum glucose decline and PCV/HCT may signify a true lack of an association, or, more likely, it may be due to the fact that only clinically normal animals were included in this study. If anemic animals or animals with an erythrocytosis were included, a possible association between red blood cell mass and serum glucose concentration in stored samples may have been revealed. It is suspected that hemoconcentrated samples would affect the serum glucose concentration more considerably when compared to anemic samples, as marked erythrocytosis (i.e. due to polycythemia vera) has led to artifactual hypoglycemia in stored human blood samples.[31-33] To the author's knowledge, no similar veterinary reports are present in the literature. Future studies can be tailored to investigate this possibility so that sample storage recommendations can be adapted for anemic and dehydrated patients.

Results of the hemoplasma qPCR screening revealed 11 positive alpacas and 4 suspect positive alpacas. This was not entirely surprising because *Mycoplasma hemolamae* is a very common hemoplasma infection in alpacas and most infections are subclinical with anemia occurring infrequently.[84] In this study, all of the animals sampled were clinically normal and the owner was unaware of the herd's *M. hemolamae* status. Hemoplasmosis has been associated with development of clinical significant hypoglycemia in individual animals of several species, including sheep, llamas, cattle, and swine.[60,61] Sheep that are infected with *Mycoplasma ovis* frequently have low blood glucose concentrations, which one study attributed to increased glycolytic activity of infected erythrocytes.[57] Alternatively, another study concerning the same genus of hemoparasite in pigs (*M. suis*) found that glucose consumption was due to the glycolytic activity of *M. suis* and not erythrocytes.[58] *M. hemofelis* also generates energy via anaerobic glycolysis, similar to erythrocyte energy metabolism.[85] Therefore, it is likely that there is both enhanced *in vivo* and *in vitro* consumption of glucose by infected erythrocytes and by the hemoparasite itself in animals with hemoplasmosis. Other etiologies of hypoglycemia (i.e.

bacterial sepsis or anorexia) can also contribute to development of hypoglycemia in animals with

hemoplasmosis.[57,60,62] *M. ovis* infection in a lamb caused accelerated consumption of glucose in stored

whole blood samples when compared to a control sheep.[59]

Based on these case reports that exposed the association between hemoplasmosis and

hypoglycemia, the author expected the alpacas with the highest bacteremias to elicit a faster rate of

glucose decline in stored blood samples when compared to the negative alpacas. However, serum

glucose rate of decline did not differ between animals with low and high bacterermias. The cause for the

discrepancy between this study and the previous case reports is uncertain. One consideration is that

some alpacas that tested negative for hemoplasmosis may have been chronically infected with very low

bacteremias that fell below the detection limit of the test.[86] The possible presence of false negatives in

our statistical analysis could have precluded revelation of a difference between negative alpacas and

alpacas with highest bacteremias. Another possibility is that the lamb in the referenced case report may

have had a very high bacteremia that exceeded the bacteremias of the alpacas in this study. Whether or

not there is a true association between hemoplasma status and serum glucose concentration in alpacas,

one must consider that it may actually be more clinically relevant to include infected alpacas when

establishing sample storage recommendations because of the high prevalence of *M. hemollamae* in

alpaca herds.

While erythrocytes contribute to the majority of serum glucose consumption in stored blood

samples, leukocytes may also play a role. One limitation of this study is the absence of leukocyte counts

from the sample animals (except for the dogs), as extremely high leukocyte counts could have affected

glucose concentrations after prolonged storage. The veterinary literature is lacking in this field, but in

human blood samples with marked leukocytosis (i.e. due to leukemia), spurious hypoglycemia has

resulted from accelerated serum glucose consumption.[38-43] However, all animals in this study were

clinically normal and samples with abnormally thick buffy coats were not identified, so it is unlikely that samples with extreme leukocytosis were present.

Another limitation of this study was the lack of an immediate blood glucose concentration from each animal, as the reference glucose concentration in this study was measured 1 hour after blood collection to allow for clot formation. Future studies can be tailored to provide an immediate glucose concentration using a glucometer. However, one potential issue with using an immediate glucometer reading is the different specimen that is used (whole blood versus serum), and the different methodology used to measure glucose concentration. Comparisons made between different specimens and different methodologies should be made with caution. Similarly, the reference glucose concentration could have been measured using a sample placed into a sodium fluoride tube, which inhibits glycolysis, but once again there is potential issue with comparing glucose concentrations made on different specimens. Furthermore, a key goal of this research was to provide clinically-relevant suggestions for veterinarians, and, in the "real world," most veterinarians will be collecting blood for chemistry analysis into red top tubes and then waiting for the blood to clot before measuring serum glucose.

An additional limitation of this study was the lack of sick animals, as we only included healthy animals. In particular, knowing the rate of glucose decline in samples from animals with hyperglycemia, which is a very common biochemical abnormality, would be clinically useful. It is the hope of the author that future studies will include animals with hyperglycemia.

In summary, delays between blood sample collection and analysis are common in veterinary medicine, and while most laboratories recommend prompt sample processing, the effect of prolonged serum-clot contact time on serum glucose concentration has previously not been well established in a variety of species. Prolonged serum-clot contact time at room temperature is associated with continued glycolysis by blood cells (predominantly erythrocytes), leading to a decrease in measured serum glucose

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concentration. The findings of the present study suggest that storage of whole blood at 4°C limits serum glucose concentration decline for up to 8 hours of storage in healthy horse and alpaca samples. Storage greater than 4 hours, even at 4°C, should be avoided in samples from healthy dogs and sturgeon in order to prevent significant alterations in serum glucose. At 25°C, serum-clot contact time should not exceed 1 hour in healthy dogs and 2 hours in healthy horses, alpacas, and sturgeon. The degree of serum glucose concentration decline at 25°C after 8 hours of storage at room temperature was fastest in dogs, followed by sturgeon, alpaca, and horses. Red blood cell mass (PCV or HCT) in all species and hemoplasma qPCR screening status in alpacas did not have statistically significant effects on serum glucose concentration over time at both storage temperatures. The results of this study provide veterinarians with clinically-useful information regarding blood sample handling and storage to prevent a false clinical diagnosis of hypoglycemia. It also lays the framework for future studies that may help prevent misdiagnosis of normoglycemia in a hyperglycemic patient.

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