(SLIDE 1: *Title*) Narrative Text for:
"An Historical Perspective: Iron Overload Disease in Browser Rhinoceroses"
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(SLIDE 2: *Sir Winston Churchill*) Given the venue of our meetings, it seems appropriate to begin with a trenchant comment from Sir Winston Churchill. Long before he was *Sir* Winston, he presented a speech in the House of Commons in which he said, *"When the situation was manageable, it was neglected, and now that it is thoroughly out of hand we apply too late the remedies which then might have effected a cure."*

At the time, he was talking about violations of the Treaty of Versailles, but his comment applies equally to the topic of this presentation, ***iron storage disease (ISD)***. My primary purpose today is to dispel persistent misconceptions about the nature and significance of ISD, a disorder that develops in browser rhinoceroses, tapirs and many other wildlife species when they are displaced from their natural habitats and constrained under captive conditions. Sir Winston's words are especially pertinent for those of you in this audience who represent our next generation of rhino keepers. Your knowledge of mistakes made in the past, *(and there have been many!)* will allow you to help prevent or correct such shortcomings in the future.

(SLIDE 3: *Clinical Problems*) We are all aware of the multiple problems that plague various rhino species in captivity. As keepers, you deal with these every day. It's important to emphasize that virtually none of these occur in rhinoceroses that are free-ranging in their native habitats. Some, such as hoof problems, are clearly caused by specific conditions of captivity, like hard artificial ground surfaces. Others, however, more likely result from *deprivation* of nutritional, environmental, social, or other essential factors present in the natural ecosystems where they evolved over millions of years. For the moment, let's focus on the last of these, anemia and *iron storage disease (ISD)*, also known as *iron overload disorder* or *syndrome*. These two *deadly* conditions are also historically important because the frequent occurrence of one (*hemolytic anemia*) has long obscured the true nature of the other (ISD).

(SLIDE 4: *RBC "Abnormalities"*) Several decades ago, the most common cause of black rhino deaths in captivity was sudden onset of anemia caused by *hemolysis*, the massive disruption of circulating red blood cells. These *acute hemolytic episodes* were often initiated by exposure to drugs, chemicals, infections or other forms of stress. In 1984, the Hematology Research Laboratory at UCLA's School of Medicine was recruited to investigate possible causes of these hemolytic events because of our experience with similar conditions in humans. Our initial studies of rhinoceros erythrocytes revealed numerous, radically unique, enzymatic and biochemical features that would be considered metabolic "deficiencies" if they were found in any other animal.

One of the most remarkable characteristics of rhino red cells was their minimal (2-5%) reserves of adenosine triphosphate (ATP), the essential fuel for initiating important biochemical reactions and for pumping ions against cell-membrane gradients. Additionally, we discovered significant impairments in their capacity to neutralize ambient oxidants. The latter was reflected by presence of Heinz bodies (aggregates of denatured hemoglobin) in 10-15% of rhino red cells. Either of these features alone would be expected to be pathological and predispose them to hemolytic episodes, but these "abnormalities" (when compared to other mammals) were present in *all* rhinoceros species, so they eventually had to be considered "normal" for *Rhinocerotidae.*

African black rhinos were the most severely "deficient" among the four species we studied, consistent with their tendency to experience hemolytic episodes, which are rare or unreported in other rhino species. Since metabolic characteristics of erythrocytes often reflect those in other tissues, the ominous probability of impaired anti-oxidant capacities in other organs remains very real and consistent with observations, but as yet unproven.

Unfortunately, a 2007 *Journal of Zoo & Wildlife Medicine* review of health issues in captive black rhinoceroses completely misinterpreted these studies and incorrectly concluded, "earlier studies of black rhino red blood cells (RBCs) found no metabolic abnormalities to explain the hemolysis." In fact, precisely the opposite was true, as confirmed and quantified by two other laboratories with internationally recognized expertise in blood cell metabolism.

(SLIDE 5: *Acute Hemolytic Anemia*) Fortunately, extensive experience with a nearly identical syndrome in humans provided highly effective preventative and therapeutic strategies. One was based on inducing high plasma phosphate concentrations, which are known to enhance red cell ATP levels. Since these preventative measures were instituted two decades ago, there have been only two or three other primary hemolytic episodes with no mortalities.

Historically, hemolytic anemia in black rhinos was also important because its initially high frequency obscured the true nature of iron storage disease. When hemoglobin is released from ruptured red cells, it is deposited in organs throughout the body. There it degrades into an iron-protein complex called *hemosiderin*, which also happens to be a natural pigment that accumulates in ISD.

Given the prominence of hemolytic anemia as a highly lethal (~75%) disorder of black rhinos, it's not surprising that hemosiderin seen at necropsy was routinely misinterpreted as residua of past hemolytic events rather than a hallmark of possibly deranged iron metabolism.

(SLIDE 6: *Necropsy Reports*) Over the past seven decades, virtually all necropsy reports of both African black and Sumatran rhinos dying in prolonged (>3 years) captivity note the presence of substantial hemosiderin deposits (*"hemosiderosis"*), and most of these reports erroneously ascribe them to hemolysis rather than ISD.

(SLIDE 7: *Joe Smith*) The first evidence that there might be an underlying disorder involving iron came from my long-time friend and colleague from a rival laboratory

also studying red cell metabolism, Dr. Joseph Smith. Joe was the pathologist who headed Kansas State University's Veterinary Diagnostic Laboratory and an acknowledged expert in iron metabolism. Tragically, his untimely death in a sports accident prevented him from completing his seminal work in this field.

In 1993, at the First International Workshop on Diseases of Black Rhinos at White Oak, he presented data indicating that black rhinos progressively load iron in proportion to their time in captivity, whereas white rhinos do not. This received little attention at the time because of overriding concerns about hemolytic anemia and other clinically prominent disorders, such as fungal pneumonias and ulcerations of skin and mucous membranes. Perhaps more importantly, the problem remained unappreciated because chronic iron toxicity produces no overt clinical signs or distinguishing symptoms until it has caused dysfunction or failure of some critical organ system, and by then it is often too late.

(SLIDE 8: *"Silent" Disorders*) In that sense, ISD resembles several other so-called "silent" disorders, conditions that can be completely devoid of clinical signs or symptoms until they reach often irreversible, terminal stages. For example, you wouldn't know you had hypertension or diabetes if your blood pressure or blood sugar was never measured. Similarly, because there are no unique or obvious indicators, the only way to detect iron overload in susceptible species is by specific analyses of blood or tissues. We'll examine those tests momentarily, but first let me start with a few basics regarding iron metabolism. This will give us a common point of departure for the remainder of this presentation and for appreciating the importance of data that Roxanne Losey will be presenting to you next.

(SLIDE 9: *Iron Cycling*) Vertebrates have no physiological mechanism to excrete iron. Iron balance is therefore almost entirely dependent on modulating its dietary uptake. When our bodies have enough iron, hormones signal intestinal lining cells to stop absorbing iron. When we need more, the signal is reversed to reinitiate uptake. Similar signals govern the storage pool of iron sequestered primarily within liver cells and macrophages.

Free iron is extremely reactive chemically, so its absorption, transport and storage require combining it with various protective proteins that neutralize its toxicity. The two that are most pertinent to our discussion are *transferrin* and *ferritin*. *Transferrin* is the plasma protein that carries iron from the intestinal tract to the bone marrow for hemoglobin production and to the liver and other organs for enzyme production and intracellular storage in *ferritin* protein complexes.

Senile erythrocytes are removed from the circulation by specialized macrophages, and their degradation products, including iron, are almost completely recycled. Humans have 2.5-3.5 g of total-body iron and, because of efficient recycling, we only require a milligram or two daily to stay in balance.

(SLIDE 10: *"Goldilocks"*) Iron is one of several elements that are essential to biological systems. I like to refer to it as the "Goldilocks metal" because its concentration has to be "just right" or major problems occur. Iron's unique chemical properties result from its dexterity in either donating or accepting electrons in

oxidation-reduction reactions that are fundamental to the biochemistry of oxygen-breathing animals. These properties, for example, allow hemoglobin to transport and exchange oxygen and carbon dioxide with absolutely no expenditure of energy, a most remarkable molecular feat.

Iron deficiency is the primary cause of *anemia* in this country and affects more than a billion people worldwide. When iron is present in excess, as it is in the Western World's most common genetic disorder, *hereditary hemochromatosis*, those same chemical properties make it extremely hazardous. In hemochromatosis, mutations in a gene called *HFE* cause subjects to increase slightly their uptake of dietary iron, leading inexorably to ISD unless interdicted by preventative measures. Approximately 10% of the people in this room are carriers of these mutations, and one in two-to-three-hundred are likely to have the full homozygous disorder. Fortunately, this provides us with a huge base of experience from which we have developed preventative strategies applicable to rhinos and other species that are similarly affected in captivity. (A chance for us to partially repay some of the benefits we have been accorded from research on animals.)

(SLIDE 11: *Iron Storage Disease)* With that background, let me present several statements to summarize the scope and severity of captivity-induced ISD. Within weeks of birth or capture, browser rhinos will have detectable elevations in iron levels that continue to increase with time in captivity. To re-emphasize, this occurs in *all* African black and Sumatran rhinoceroses so far studied, so it represents a species-wide susceptibility, *not an inherited "disease"*. Tenfold elevations in total body burdens develop in as little as 3-5 years. Our eldest rhinos have literally kilograms of excess iron in their bodies. Despite lack of overt symptoms, untreated chronic iron toxicity of this magnitude reduces normal life expectancy by 20-30% or more and contributes to deteriorating quality of life as various affected organs begin to falter.

(SLIDE 12: *Evidence for ISD*) Now let me try to justify the dogmatic statements I made earlier. More importantly, let me present the data and ask you to judge for yourselves. These data are derived from our studies of over 250 blood specimens from all four captive species submitted over the last 31 years to the UCLA Hematology Research Laboratory, and on my personal performance of, participation in, or review of over 60 necropsies of all five extant species of rhinos. Necropsies, of course, provide the definitive data. (That's the advantage of being a pathologist, you get the final word!)

(SLIDE 13: *Serum Iron Analytes*) But first, let's focus on blood studies, not only because they're relatively noninvasive, but more importantly, because they can alert us to intercede *before* the situation becomes deleterious and irreversible. In ISD, *serum iron concentration* is elevated, but this is a nonspecific effect since other conditions can also cause increases or decreases. The two most useful analyses are *transferrin saturation* and *serum ferritin concentration*.

Because free iron is so highly reactive, we all depend on a variety of proteins to transport and store it in nontoxic complexes. As previously noted (**SLIDE 9: *Iron***

Cycling), *transferrin* is the plasma protein that allows transport of dietary iron to various sites for utilization or storage. It's normally present in great excess, so *transferrin saturation* (the ratio of *serum iron* to *total iron binding capacity*) is usually around 25-35%. Values above 45-50% are considered pathological, and therapeutic intervention in humans is the standard of practice for elevations above 60%. Values in the 80-100% range are not uncommon in susceptible animals held in prolonged captivity.

Ferritin is a large iron-protein complex that is the principal storage form of intracellular iron. *Serum ferritin concentrations provide the most valuable indirect metric of ISD because they correlate closely with total body burdens of iron in humans and other species.* Current ferritin assays require species-specific reagents, and the system devised by Joe Smith at the Kansas State University Veterinary Diagnostic Laboratory remains the gold standard for rhinoceroses, tapirs and other species. Unfortunately, ferritin values are also influenced by a variety of other conditions, so it's important not to place too much emphasis on individual values. *Serum iron and ferritin concentrations should be assayed in multiple specimens taken over periods of time to identify trends rather than relying on isolated tests.* Current criticisms of this assay by some colleagues are probably due to failure to take these caveats into account. This is particularly important because extremely high ferritin concentrations in captive rhinos require *serial dilutions* of sera to bring them into standard ranges, compounding the probability of amplifying small dilution errors into large.

(SLIDE 14: *Ferritin vs. Captivity Time, Indian Rhinos*) Now let's concentrate on just one of these protein complexes, *ferritin*, but keep in mind that trends in *transferrin saturation* are almost equally reliable. As with serum iron assays, it is important to obtain multiple blood specimens over periods of time, as individual measurements can be affected by other conditions.

In the next few slides, we are going to compare serum ferritin concentration with time in captivity. Note that the ferritin scale is logarithmic with tenfold increments. Humans and horses generally have about 100-300 ng/ml as shown by brackets on the left. This is the same range of values that we have measured in both black and white rhinos free-ranging in Southern Africa. On the right side, we have values for greater one-horned rhinos in U.S. zoos. These are connected by lines only for ease of following them on subsequent slides. As you can see, there is no statistically significant difference among these groups regardless of how long they have been in captivity.

(SLIDE 15: *Ferritin, White Rhinos*) On the next slide, we add data for African white rhinos in captivity up to nearly four decades, and again there is no significant difference.

(SLIDE 16: *Ferritin, Sumatran Rhinos*) When we add Sumatran rhinos to the graph, however, that pattern no longer holds. Blue triangles are those in U.S. captivity, and while they show no elevations initially, they rapidly and progressively increase with time in captivity. Again, let me emphasize that this is a log scale, so

each increment represents a tenfold increase. For the moment, let's ignore the Sumatran rhinos in Indonesia and Malaysia and skip to the even more dramatic data for captive black rhinos on the next slide.

(SLIDE 17: *Ferritin, Black Rhinos*) Here again we see that, early in captivity, there's no significant difference from their free-ranging counterparts, but this rapidly changes during prolonged captivity. Within the first few years, ferritin values elevate markedly and progressively, reflecting accumulation of *kilogram quantities of excess iron*. Because it takes time to synthesize the protein component of ferritin, values that reach into the thousands and tens of thousands provide *evidence of active organ damage* releasing intracellular ferritin directly into the blood stream.

(SLIDE 18: *Ferritin, Black Rhino Calves*) The rapidity of iron loading is even more dramatically demonstrated by charting ferritin concentration as a function of age in black rhino calves born in captivity. On day of birth, serum ferritins are even higher than shown here, averaging about 20,000 ng/ml and most likely reflecting high values present in their mothers, as well as the stress of birthing, since ferritin is an acute-phase reactant that elevates under inflammatory or other stressful states. Within the first few days of life, however, ferritin drops precipitously to normal levels but then begins to increase progressively over the first year, reaching levels more than tenfold above normal within the first two to three years of life.

(SLIDE 19: *Lung Histopathology*) Necropsies provide definitive evidence of organ damage. The next three slides compare organ histopathology of white and black rhinos of comparable ages and times in captivity. These are stained with Prussian blue, a stain that is specific for oxidized (ferric) iron. You don't need to be a pathologist; you can judge for yourselves. If cells stain blue, they contain iron: the bluer they are, the more iron is present. It's that simple and unequivocal. In the first slide, sections of lung show only a few iron-positive macrophages in the walls of the white rhino air sacs, while the alveolar walls of the black rhino are thickened and packed with iron (hemosiderin) laden macrophages.

(SLIDE 20: *Bone Marrow*) The next slide shows broad sheets of blood-forming elements in vertebral bone marrow. Only a few clusters of macrophages stain positively for iron in the white rhino's marrow. By comparison, marrow in this black rhino has been compressed into a small residual island (next to a bone spicule on the far right) by broad confluent sheets of iron-laden macrophages.

(SLIDE 21: *Liver Histopathology*) In the liver, African white and Indian rhinos usually exhibit sparse storage-iron deposits in scattered macrophages, including those lining the portal sinusoids. African black and Sumatran rhinos show dense deposits not only in the macrophages, but also in the liver cord cells themselves, where there is additional morphologic evidence of cell damage and death.

(SLIDE 22: *Liver Cancer*) If affected animals live long enough, this degree of damage can progress to complete disruption of liver architecture and induce scarring (cirrhosis) and malignant transformations as shown in this elderly black rhino where every cell contains enormous amounts of iron.

(SLIDE 23: *Tissue Iron Assays*) An unequivocal indicator of iron overload is provided by quantitative assays of necropsy tissues for elemental iron. This graph shows the huge amounts of iron that accumulate progressively in black rhino liver and spleen over a captivity period of nearly thirty years. Interestingly, the heart does not appear to be a major target organ in rhinos in contrast to its marked vulnerability in human ISD (hemochromatosis).

(SLIDE 24: *Biochemical Effects*) If the precise cause of ISD in captive browser rhinos remains uncertain, there is no doubt about its consequences. Elemental (non-protein-bound) iron actively catalyzes generation of hydroxyl free radicals and so-called "reactive oxygen species" that are extremely reactive chemically and highly destructive biologically. Most creatures have evolved enzyme systems to rapidly neutralize these free radicals that are normal byproducts of physiologic processes. Our studies at the UCLA Hematology Research Lab, however, have shown that the entire family of *Rhinocerotidae* lacks many of these metabolic countermeasures, making them all highly vulnerable to oxidative damage, especially the African black and Sumatran species. (See **SLIDE 4: *RBC "Abnormalities"*.**) Resultant peroxidative injury to cell and organelle membranes, to nucleoproteins, and to structural, functional and enzymatic proteins eventually becomes cumulative and irreparable.

(SLIDE 25: *Clinical Consequences*) Subcellular and cellular damage inevitably lead to dysfunction and failure of multiple organs throughout the body. Liver, bone marrow, heart, spleen, endocrine and reproductive systems are among those most commonly affected in rhinos. Liver cell damage and destruction lead to progressive scarring and end-stage cirrhosis. One-third to one-half of hemochromatosis patients with untreated cirrhosis will develop liver-cell malignancies, an outcome we have observed in several of our eldest captive black rhinos.

At a clinical level, one of the most important consequences of excess iron is highly increased susceptibility to infections, not only those caused by pathogens but also from organisms that are normally innocuous.

(SLIDE 26: *Role in Infectious Diseases*) Unusual infections (for example, fungal pneumonias and aggressive tuberculosis) have long been serious problems among both African black and Sumatran rhinos in captivity. Since microorganisms cannot proliferate and survive without access to their host's iron stores, one of our best natural defenses against infection is so-called "nutritional immunity" by which we sequester essential elements in protein complexes. This deprives invaders of their use, reducing their infectivity. Conversely, availability of free iron greatly enhances the virulence of invading microbes. Excess iron also impairs the ability of white blood cells and tissue macrophages to phagocytize and kill bacteria and other pathogens. The culmination of these effects is a host with compromised immunity,

highly susceptible to microorganisms that are not particularly threatening under normal circumstances.

(SLIDE 27: *Captivity-Induced ISD*) Now that you've seen these data, I hope you're equally convinced that ISD acquired under captive conditions represents a clear and present danger to susceptible species. Indeed, many of us consider ISD *the singular, most important challenge to long-term viability of African black and Sumatran rhinoceroses and their tapir cousins in captivity*. I would be remiss, however, if I didn't point out that not everyone agrees. A few still consider ISD an "epiphenomenon", perhaps related to one or more of the other pathologic conditions affecting these species in captivity.

ISD has also been hypothesized to be a consequence of undefined and conceptually obscure "metabolic disturbances." The 2007 *JZWM* article mentioned previously (**SLIDE 4: *RBC "Abnormalities"***) is but one example. The authors of this review skeptically dismissed iron overload in regard to its possible relation to other black rhino disorders but, more importantly, they failed to consider it an inherently deleterious condition in and of itself, an egregious error of omission.

Equally disturbing, the 2014 revision of the *Rhino Husbandry Manual*, sanctioned by the Association of Zoos and Aquariums' Rhino Advisory Group and the International Rhino Foundation, also treats the problem in a dismissive and perfunctory manner. This is not meant as an indictment of individuals or institutions, but rather an apologetic recognition of my own ineffectiveness as a professional educator unable to convince colleagues of the lethal consequences and ready preventability of captivity-induced ISD.

While our focus has been on rhinos, it's important to recognize that multiple other species of exotic wildlife also develop ISD when displaced from natural habitats into captivity. These include not only tapirs, but lemurs, marmosets, fruit bats, and a huge number of tropical avian, simian, feline and marine species. As with so many problems affecting the world today, from habitat destruction to global warming, human intrusion is the common denominator, so an anthropogenic label seems appropriate.

(SLIDE 28: *Etiologic Possibilities-Nutritional Factors*) At the 1993 White Oak Conference, when Joe Smith first reported disparities in iron loading between black and white rhinos, he suggested that nutritional factors might be responsible. This was consistent with a then current observation, "White rhinos are grazers and when they're brought into captivity and fed grass, they do fine; but black rhinos are browsers, and when they're brought into captivity and fed grass, they *don't* do well."

Compared to grasses, natural browse contains many components that form insoluble complexes with iron, allowing passage through the intestinal tract without absorption. These include tannins, fiber, phytates, phenolics, and phosphates, among others, that are abundant in twigs, leaves and bark. In the wild, black rhinos forage on over two-hundred different species of plants, shrubs, grasses and trees, a menu that cannot be duplicated in domestic captivity. Some of these, like *Mimosa* and *Leucania,* are highly toxic for other animal species, but contain compounds such as L-mimosine and dihydroxybenzoic acid that have extremely high iron-binding

coefficients. Theoretically, small non-toxic amounts of these could retard enteric uptake sufficiently to prevent iron overloading in the wild. (These might also have potential value as additives to reduce bioavailability of iron in captive diets.)

The most plausible theory is that browsers instinctively modulate their iron stores by such dietary choices. In the absence of appropriate research, this hypothesis remains unproven but supported by another notable study presented at the White Oak Conference by veterinary pathologist, Nancy Kock and her colleagues. In necropsies of seven black rhinos free-ranging in Zimbabwe, they found no evidence of iron-pigment deposition in those captured less than two weeks previously. (In keeping with the prevailing opinions, this was interpreted as evidence that hemolytic episodes did not occur in the wild.) However, they found abundant hemosiderin in multiple organs in 14 of 18 other black rhinos that died between two weeks and two years of capture, and the amounts of pigment deposition appeared to increase with time in captivity.

These rhinos were corralled in the field and fed copious amounts of local browse, but browse was selected by rangers, *not ad libitum* by the animals. Although it was unrecognized at the time, this study confirmed Joe Smith's observations and provided the first documentary evidence that ISD could be induced rapidly in free-ranging rhinos by captive restraint. Without access to natural forage of their own selection, it's not surprising that metabolic imbalances such as ISD are inevitable consequences in susceptible species.

(Etiologic Possibilities-Genetic Predispositions) Since *all* animals so far studied are affected, ISD cannot be considered an "inherited disease" as inaccurately claimed in recent press releases regarding Sumatran rhino deaths at the Cincinnati Zoo. Rather, variations in iron homeostasis reflect genetic predispositions that are the *norm* for these species. Our understanding of iron metabolism has been enormously expanded over the past decade by studies in the laboratory of my UCLA colleagues, Tomas Ganz, M.D., Ph.D., and Elizabeta Nemeth, Ph.D. Their research teams discovered the principal hormones (*hepcidin* and *erythroferrone*) that maintain iron balance in vertebrates, and elucidated the molecular mechanisms involved.

(SLIDE 29: *Molecular Regulation of Iron Balance*) *Ferroportin* is the membrane channel through which iron passes between cells and plasma. As the key iron regulator, *hepcidin* acts by blocking iron passage through ferroportin channels, preventing absorption of iron from the diet or its mobilization from the storage pool. Interactions between hepcidin and ferroportin are additionally influenced by other regulatory molecules, such as hemojuvelin, *HFE*-protein, and transferrin receptor-2.

(SLIDE 30: *Hepcidin Deficiency*) Hepcidin *deficiencies* (or defects in its interactions with modulators) are now known to be responsible for ISD syndromes in humans and other animals. Specific deficiencies have not yet been identified in rhinos or tapirs despite extensive studies. This reinforces the concept that iron overloads in affected species develop from *normal* genetic predispositions when these animals are deprived of access to essential components of their native environments.

(SLIDE 31: *Evolutionary Aspects*) How could this happen? What evolutionary pressures could have caused so many species to become vulnerable to this deadly disorder? To understand possible underlying mechanisms, it's again useful to review history, in this case, the geologic and fossil records.

Earth's ancient atmosphere contained very little free oxygen until aquatic microorganisms developed photosynthetic capacity, using energy from the sun to split water and carbon dioxide and recombine their products into organic compounds and molecular oxygen. About a quarter of a billion years ago, plants did this so effectively that atmospheric oxygen rose to almost twice the concentration that exists today. This intensely oxidizing atmosphere converted most of Earth's iron into its highly insoluble *ferric* form, basically iron oxide (rust).

Since iron is an essential trace element, animal life depended on evolving mechanisms to solubilize and assimilate dietary iron rather than excreting it (see **SLIDE 9: *Iron Cycling***). This included the first browsing rhinoceroses that began to appear in the Eocene Epoch about 50-60 million years ago. During the subsequent Miocene, 25-million years ago, savannah grasslands dominated the Earth, and grazers evolved with their specialized mouths, teeth and digestive abilities. Because they consumed large amounts of soil along with grasses, grazers also had to develop feedback-control mechanisms to limit dietary iron uptake, a physiologic capacity that eluded browsers which continued to rely on selective forage to balance their iron requirements. The precise genetic and molecular mechanisms imparting ISD susceptibility remain to be identified, but intensive investigations continue.

(SLIDE 32: *Prevention & Therapy*) By now I hope you, too, are convinced that ISD is a potentially lethal disorder affecting not only browser rhinos, but their tapir cousins and a broad range of other species as well. So what can we (you) do about it? Again, I return to the extensive experience provided by studies and treatment of ISD in humans. The two general approaches are obvious, prevention and therapy. Therapeutic drugs exist that chemically chelate iron allowing it to be excreted in the urine, but these are prohibitively expensive for use in rhinos. Prevention, on the other hand, is inexpensive and straightforward: simply return to the ancient (but anachronistic) art of blood-letting.

(SLIDE 33: *Phlebotomy Rationale*) Periodic removal of small aliquots of venous blood will eventually induce a slight anemia. Consequent decreases in blood-oxygen levels activate erythropoietin, the hormone responsible for initiating new red cell production which mobilizes stored iron for hemoglobin synthesis.

When you remove and discard a liter of blood, the body burden is reduced by approximately 0.5 g of hemoglobin iron. This procedure may seem primitive and simplistic, but it's relatively noninvasive and highly effective, and it is performed daily on thousands of patients with hereditary hemochromatosis. It carries the added advantage of allowing precise calculation of the quantities of iron removed by measuring the volumes and hemoglobin content of extracted blood.

Repetitive phlebotomy is a well-established and effective technique to *prevent* damage due to iron toxicity. It's *not* therapy for damage that has already occurred.

It is far, far easier to train young or newly captured animals to tolerate the procedure, before they build up significant overloads, than it is to reduce huge iron stores present in older, long-term captives. In fact, phlebotomy is *contraindicated* in rhinos that have overt clinical problems because it would add the hazards of anemia to any organ dysfunction already extant.

(SLIDE 34: *Rhino ISD Historical Chronology*) None of what I have presented to you here is new information: As shown in this chronology, ISD has been reported extensively in the literature and at multiple national meetings. Two international symposia have been convened specifically to review these data and recommend remedial actions. Phlebotomy protocols developed for rhinos and tapirs have been available for a decade and a half. Nonetheless, despite repeated recommendations from Taxon Advisory Groups and Research Councils, very few institutions have undertaken phlebotomy programs. Further, the responsibility for administering captive breeding programs resides with the AZA, which apparently has no mechanism to ensure that TAG recommendations are implemented, something that I (as an outsider) view as a major flaw in their organizational matrix.

(SLIDE 35: *Sumatran Breeding Program*) One last historical synopsis should dispel any lingering doubts about the enormous impact of ISD on captive breeding programs. Between 1984 and 1996, some 44 Sumatran rhinoceroses were transferred from the wild into worldwide institutions to attempt *ex situ* breeding. Captures were eventually discontinued because of high mortality (>50%) and an absence of newly conceived offspring.

A consortium of zoos was then formed to congregate survivors at the Cincinnati Zoo. Research breakthroughs at their Center for Reproduction of Endangered Species eventually allowed matriarch *"Emi"* to deliver the first captive births of this species in over a century. Unfortunately, in the absence of phlebotomies, this monumental achievement was overshadowed when deaths due to ISD progressively reduced this group to one last 8-year-old survivor, "*Harapan"*, whose name ironically means "hope" in his homeland of Indonesia. Phlebotomies were not initiated until two years ago when *"Suci",* older sibling of *"Harapan"*, also began to deteriorate clinically, *(a condition that should have <u>contra</u>indicated the procedure, since it induces the additional burden of anemia.)*

In my view, this complete collapse of the Sumatran rhino captive breeding program is directly attributable to ISD that would have been prevented by a serial phlebotomy program, (in Sir Winston's words, the *"remedy which then might have effected a cure"*.)

(SLIDE 36: *Phlebotomies: Cost/Benefit Analysis*) So, given the strength of these data and the efficacy of a ready remedy, why haven't preventative phlebotomies become standard of practice for ISD-susceptible animals, as they are for so many humans? The simple answer is probably "cost/benefit analysis." Administrators, confronted with the challenges, complexities and competing priorities that exist in zoological institutions are strongly influenced by cost/benefit analyses. They have an understandable reluctance to commit staff personnel and other valuable

resources to train and attend animals that exhibit no outward signs of any underlying disease. When chronic iron toxicity causes sufficient organ dysfunction to become overt, as it apparently did in *"Emi"* and *"Suci",* it's generally irreversible. At that stage, trying to convert *preventative* phlebotomies into *therapeutic* measures becomes a futile act of desperation, and euthanasia remains the only alternative.

Again, much can be learned from a parallel situation in human medicine. Two decades ago when molecular tests to detect *HFE* mutations cost thousands of dollars, a major Health Maintenance Organization determined that it was cost-effective to screen *all* of their patients (tens-of-thousands) to detect the one in two- or three-hundred who would develop pathologically significant ISD from hereditary hemochromatosis. Detection of this gene allows early interdiction by preventative phlebotomies, avoiding the enormous expense of treating terminal multi-system disorders that are the inevitable consequence of prolonged iron overburden, (since euthanasia is *not* an option.)

(SLIDE 37: *"Tipping Points"*) If you need further convincing, you might find the following argument even more compelling. Current threats of overpopulation, global warming, pollution, criminal poaching, etc., have prompted development of computer models to predict when and if certain species might cross over into extinction. Obviously, sustainability of any population in peril depends on a favorable balance between births and deaths, which in turn depends on its "tipping point," when one outweighs the other. At our current rate of three rhino poaching deaths per day, the *International Union for Conservation of Nature* (IUCN) and *Save the Rhino* predict that the tipping point in South Africa will occur between 2016 and 2018. An alteration in the current birth/death ratio of as little as 2-6 %, however, can reverse that imbalance. Is it possible to achieve this? Again, we can learn from our extensive experience with the common human form of ISD, hereditary hemochromatosis.

(SLIDE 38: *Human ISD - Hereditary Hemochromatosis*) In humans with *HFE*-gene defects, nonspecific symptoms of iron overload typically begin to appear in their 30s or 40s, even later in women, who benefit from menstrual blood loss. If untreated, liver damage eventually terminates in cirrhosis. One-third to one-half of cirrhotic patients develop liver cell malignancies by their 50s or 60s, *decreasing their life spans by 20-30%.* On the other hand, if excess iron is depleted by phlebotomies *before* cirrhotic changes occur, *life expectancy is entirely normal.*

(SLIDE 39: *Phlebotomies: Potential Benefits – Long-Term Captives*) Even though long-term captives may have huge iron burdens, tangible benefits from repetitive phlebotomies are attainable. Any reduction in the toxicity of excess iron is beneficial, enhancing quality of life, possibly extending life spans, and delaying lethal progression of organ deteriorations. Fortunately, our colleagues at Disney Animal Kingdom have refined our proposed protocols into sophisticated, highly efficient procedures for rapid, large-volume phlebotomies with the outstanding results that Roxanne Losey will be presenting to you in the next session.

(SLIDE 40: *Phlebotomies: Potential Benefits - Juveniles*) Two target populations would benefit most from serial phlebotomies: newly captured animals and captive-born calves and juveniles. Body iron stores are at their lowest in both groups, and younger animals are more receptive to operant conditioning to tolerate repetitive phlebotomies without hazards of sedation. If human response to early intervention is any indicator, phlebotomy programs in rhinos could *add five to ten or more years of good health to captive life spans and at least one or two more reproductive cycles. This could radically shift the tipping point away from extinction, toward sustainability.*

Demise of the Sumatran captive breeding program was not without a note of encouragement. *"Andalas"*, born to *"Emi"* in 2001, was repatriated to his ancestors' Indonesian homeland after 5½ years residency in U.S. zoos. By that time, his body burden of iron had increased twenty- to forty-fold as estimated by serum iron analytes. He quickly adapted to the Sumatran Rhino Sanctuary (SRS) in Way Kambas National Park where a wide variety of native rainforest browse is available. Animals in this sanctuary have the lowest iron burdens we have yet measured among worldwide populations of Sumatran rhinos, and iron metrics closely correlate with their foraging tendencies and preferences. This adds strong support to our hypothesis that browser rhinos, tapirs, and perhaps other ISD-susceptible species, rely on selective natural forage to reduce bioavailability of dietary iron and avoid ISD that has been the inevitable consequence of confinement in artificial environments.

"Andalas" continues to thrive and he sired his first offspring three years ago. A number of us, including my friend, colleague, and Sumatran rhino expert, Dr. Robin Radcliffe of Cornell's Veterinary Conservation Medicine Program, have been lobbying to have his brother *"Harapan"* join him, confident that he would benefit equally from the more natural conditions of this protected environment.

(SLIDE 41: *Summary*) So there you have it. If we can condense three decades of extensive research into three sentences, I would leave you with these. *Iron storage disease* is a devastating disorder affecting multitudes of exotic wildlife species when they are confined under captive conditions that deprive them of essential components of natural ecosystems in which they evolved over eons. The causes and consequences of ISD are well established for comparable syndromes in humans and other animal species, providing highly effective strategies for prevention and therapy. Despite its simplicity and efficacy, preventative phlebotomy programs continue to be resisted but, if adopted, could extend captive life spans 20-30% and add reproductive cycles that might radically shift "tipping points" away from extinction and toward sustainability of highly endangered species.

If the tragic demise of the Sumatran-rhino captive-breeding program is to provide anything positive, it might serve as a wake-up call to responsible administrators to avoid similar fates for other ISD-susceptible species, African black rhinoceroses and their tapir cousins foremost among them.

(SLIDES 42, 43: *Max Planck*) I would like to conclude with another pertinent (or impertinent) observation, this by Nobel Laureate physicist Max Planck: *"A new scientific truth does not triumph by convincing its opponents and making them see the*

light, but rather its opponents eventually die, and a new generation grows up that is familiar with it." *You* represent that new generation. I leave it in your hands. Thank you and good luck….!

(SLIDE 44: *Acknowledgements)*