PRESENTATIONS
SESSION VI
VETERINARY CARE - VIRUSES
FIRST EVIDENCE OF EEHV INFECTION IN SUMATRAN ELEPHANTS (*Elephas maximus sumatranus*) IN INDONESIA

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1. Case Kristina

- Captive born, mother captive (wild caught), father wild
- Born 17\textsuperscript{th} September 2003, naturally raised by mother
- Kept in a group consisting of 2,2; regular access to wild elephant habitat
- Neither this elephant itself nor any other elephant that it has been in contact with ever had contact with African elephants.
- Never any visible health problems,
- Routine deworming's every 3 - 4 months with ivermectin or albendazol
1. Case, Kristina

In the evening of the 21.2.2006 mild anorexia and some very mild ataxia symptoms disappeared soon without treatment.

In the afternoon of 22.2.2006 again anorexia, drowsiness, and some mild ataxia observed, died few hours later on the same evening.
ORGAN SAMPLES STORED ON ICE AND IN 96% ETHANOL WERE TAKEN.

NO CASE LIKE THIS HAD SO FAR BEEN REPORTED IN SUMATRA.

LIMITED LAB FACILITIES

TESTED AT A LOCAL LIVESTOCK LAB FOR:
HEMORRHAGIC SEPTICEMIA (PASTEURELLA MULTICODA) => NEGATIVE
E. COLI => POSTIVE BUT NO EXAMINATION FOR ENTERO-PATHOGENICITY / TOXICITY => E.COLI ENTEROTOXAEMIA COULD NOT BE DIAGNOSED

NO LAB FACILITIES FOR EEEHV AVAILABLE IN INDONESIA AT THAT TIME.

NO SAMPLES OF THIS CASE WERE STORED.

THE MOTHER GAVE BIRTH AGAIN ON THE 29th JUNE 2008 TO A MALE CALF, NATURALLY RAISED, STILL ALIVE.
WART LIKE LESIONS FOUND IN OTHERWISE HEALTHY, JUVENILE SUMATRAN ELEPHANTS
BLISTERS AND SMALL LESIONS FOUND ON THE MOUTH MUCOSA IN OTHERWISE HEALTHY SUMATRAN ELEPHANTS
2nd AND 3rd CASE, NAMO AND TANGKA

- Both captive born;
- mothers and father of both are captive but wild caught as juveniles from the same family group -> most likely genetically closely related
- Kept in a group consisting of 1,6; access to wild elephant habitat.
- Neither these two elephants themselves, nor any other elephant they had been in contact with, had contact with African elephants
2\textsuperscript{ND} CASE, NAMO

\textit{NAMO} born 6\textsuperscript{th} November 2010, no complication during birth process, naturally raised; never any visible health problems, from an age of 6 months on, regular antiparasitic treatment with ivermectin and albendazole every 4 months, basic training initiated at 6 months of age.
2ND CASE, NAMO

On the 3rd of April 2012 conducting routine deworming (ivermectin 0,15mg 7 kg BW p.o.)

On the morning of the 5th of April 2012 the calf is found dead, no abnormalities had been observed by the mahouts during the previous day and the evening bathing and feeding procedures.
2ND CASE, NAMO  MAJOR GROSS PATHOLOGICAL LESIONS
3rd CASE, TANGKA

TANGKA born 14th September 2010, no complication during birth process, naturally raised; from the age of about 7 months on, a few times developing some intestinal problems such as constipation and mild diarrhea, these problems usually resolved without any treatment after 1 to 3 days, from an age of 6 months on, regular antiparasitic treatment with ivermectin and albendazole every 4 months, basic training initiated at 5 months of age.
3rd CASE, TANGKA

On the 3rd of April 2012 conducted routine deworming (Ivermectin 0,15mg / kg BW p.o.)

No signs of discomfort or illness is observed during the following days. Despite the normal health appearance started prophylactic treatment with (15mg/kg BW) Famciclovir on the 7th of April.

In the evening of the 10th of April 2012 mild discomfort, anorexia and swelling of the lower jaw is observed, the calf is still normally nursing. The calf goes in recumbent position in the early morning and dies 2,5 hours later.
3\textsuperscript{ND} CASE, TANGKA \hspace{1em} MAJOR GROSS PATHOLOGICAL LESIONS
Two sets of tissue samples from all organs with pathological changes were collected and preserved in 96% ethanol and deep frozen at -20 °C.

Due to a lack of diagnostics for EEHV in laboratories in Indonesia the samples could not be tested immediately.

About 6 months after collection of the samples a specific laboratory facility for molecular diagnosis of EEHV was established in Bogor.
DNA WAS EXTRACTED FROM BOTH FROZEN AND ALCOHOL PRESERVED SAMPLES FROM HEART, SPLEEN AND LIVER.

BOTH CASES WERE IDENTIFIED AS **EEHV 1** BY CONVENTIONAL DIAGNOSTIC PCR FOR PAN-EEHV POL AND EEHV1-SPECIFIC POL.

THESE WERE THEN SUBJECTED TO DETAILED GENE SUBTYPE DNA SEQUENCING AT THREE KEY PCR LOCI, U38/POL, U51/VGPCR, U60/TER.

THESE TWO CASES HAVE IDENTICAL **EEHV1A** DNA SEQUENCES TO ONE ANOTHER INDICATING A COMMON EPIDEMIOLOGICAL SOURCE.
SOME CONCLUSIONS AND QUESTIONS FROM THE CASES

- EEHV IS PRESENT IN SUMATRAN ELEPHANTS IN INDONESIA
- POST MORTEM SAMPLES CAN BE STORED AND PRESERVED DEEP FROZEN AND IN 96% ETHANOL FOR OVER & MONTHS
- WHAT HAS TRIGGERED THE OUTBREAK OF THE LETHAL DISEASE???
- COULD THE GENETIC CLOSE RELATION OF THE PARENTS BE A PRE-DISPOSITIONING FACTOR
I thank Elephant Family, Benindi Fund, AES and the US Fish and Wildlife Service for their funding support helping to enable Vesswic’s veterinary works.

I thank the national and provincial Indonesian Nature conservation Agencies (PHKA and BKSDA), the camp managements and mahouts for the good collaboration.

I thank the Pittsburgh Zoo & PPG Aquarium for inviting me to this symposium and present some of our works in Sumatra.
SEVEN SPECIES OF ELEPHANT ENDOTHELIOTROPHIC HERPESVIRUSES (EEHVs) FORM A NOVEL MAMMALIAN SUB-FAMILY DESIGNATED THE DELTAHERPESVIRINAE.

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Nearly 90 suspected cases of lethal EEHV hemorrhagic disease plus ten more survivors of high load symptomatic EEHV viremia have been reported worldwide, primarily in young Asian elephants, including 37 in North America, 28 in Europe and over 30 in Asia [Zong & Hayward, 2011; Hayward, 2012]. At least 57 of these cases have been confirmed and compared by PCR based “DNA fingerprint” gene sequencing. Another 30 EEHV strains have been identified from trunk wash or saliva shedding in asymptomatic zoo elephants, as well from either biopsied skin nodules or necropsied lung nodules from healthy African elephants [Zong J-C et al; Pearson VR et al. MS in preparation]. The results of extensive PCR genotype sequencing analysis of most of these samples have revealed seven distinct EEHV species [Latimer et al, 2011] and several additional chimeric sub-species that have diverged over the entire 100 million year evolutionary history of the Afrotheria, the ancestors of modern elephants [Fig 1]. These and other recent results from samples obtained from Asian hemorrhagic disease cases in range countries [Zachariah et al, 2013] have also now greatly clarified both the origins and epidemiology of this disease, as well as the evolutionary relationship between the Proboscivirus genus and other mammalian herpesviruses.

In particular, the complete 177,136-bp genome of a strain of the most highly pathogenic species EEHV1A(Kimba) has been compiled by next generation sequencing techniques on DNA extracted directly from infected Asian elephant necropsy liver tissue [Ling et al, 2013]. From a total of 22 gigabases of raw Illumina sequence data a subset of sequence runs at a relatively high abundance of between 20 to 35 copies per cell were filtered out and assembled by de novo bioinformatics approaches without the use of a reference sequence. This yielded a pool of 350 unlinked contigs in all, but with one intact block of 83-kb corresponding closely to the core segment that we had already obtained by PCR walking approaches. Another five of the largest contigs were then joined together with this larger segment by filling in gaps across the ends by PCR amplification yielding a new 162-kb super contig. Two further approaches were then employed to attempt to identify any remaining additional smaller viral contigs. The first used a manual elimination screening process to remove all contigs from the pool that appeared to be cellular, based on the presence of homology to high copy number mammalian sequences, the presence of repetitive telomere-and sub-telomere like sequences or the presence of high pyrimidine-purine tract biases. This process left 11 more potential viral contigs adding up to nearly 15-kb in size (including several with small CA-repeat indels of 15-30-bp in length), which all then proved to be present by PCR in six other infected Asian elephant DNA samples tested, but not in any of six uninfected Asian elephant DNA samples. A second approach involved carrying out a long-range PCR amplification experiment in the outwards directions with primers from the two ends of the 162-kb contig, which yielded a single PCR product of 15.8-kb in size representing an apparent join across a closed circular or palindromic intracellular form of the viral genome. Remarkably, when this PCR “end-join” product was
sequeced all 11 of the small remaining “candidate” contigs above proved to be present within it with only small gaps between them. Subsequently, nearly the entire novel segment was also reconfirmed by standard Sanger PCR sequencing to correct about 50 frame-shift and other errors in the Illumina generated data. Sequence data and annotation for the final complete EEHV1A(Kimba) genome is available online at NCBI Genbank Accession No KC618527.

**Figure 1:** Summary diagram illustrating the genetic relationships between individual EEHV species, including the EEHV1B chimeric sub-species (#). The dendrogram on the left-hand side indicates genetic divergence values as average nucleotide differences (%) across sequenced portions of these genomes. GC-rich and AT-rich branches refer to overall G-plus-C content of the DNA. Red boxes indicate natural host *Elephas maximus*. Green boxes indicate natural host *Loxodonta africana*. The number of confirmed lethal cases associated with each virus species compared to the total number of examples identified so far for each species, together with the year of original discovery are listed on the right-hand-side.

Overall, the data revealed that whilst 51 of the total of 115 genes have orthologues in other herpesviruses, the other 64 are novel, including 23 members of the 7xTM rhodopsin-vGPCR family (eight of which resemble chemokine receptors), ten immunoglobulin family (IgFam) genes and two glycosyl tranferase enzymes (vGCNT1, vFUT9) that all represent “captured” or “pirated” cellular genes. The IgFam includes three versions of cellular vOX2(CD200) genes, including a
duplicated spliced pair of “old” very anciently acquired and now highly diverged genes (vOX2-2 and vOX2-3), each with about 25% residual identity to the mammalian versions, as well as another “new” more recently acquired unspliced vOX2-1 gene that encodes a protein with 98% identity over 280 amino acids within the functional domains in exon3 plus exon4 to the host Elephas, Loxodonta and Mammuthus versions of this protein. Nevertheless, since it is also present in the EEHV2, EEHV5 and EEHV6 genomes and has diverged by 20% at the DNA level from the elephantid host versions, the “new” vOX2-1 gene (despite having not changed significantly at the protein level) must have been captured some 20 million years ago and have been under high selective pressure not to diverge throughout that entire time period. The captured vGCNT1 protein has 69% identity to the equine version and the vFUT9 has 52% amino acid identity to all mammalian versions.

Figure 2: Radial DNA level phylogenetic tree for the intact 2,500-bp DNA polymerase (U38/POL) genes from five species of EEHVs (= Deltaherpesviruses) compared to their orthologues in key representative herpesvirus species from all three other mammalian sub-families (Alpha, Beta and Gamma). The dendrogram was generated in Mega5 after clustal alignments with the program Muscle.
Incomplete PCR-based DNA sequence analysis from another two strains each of EEHV1A, EEHV1B and EEHV2 (totaling 60 to 80-kb each), as well as three strains of EEHV5 and two of EEHV6 (totaling 25 to 30-kb each) has shown that all five AT-rich branch EEHV species (which diverge from each other by 15 to 20% at the nucleotide level) have an inversion of a large 40-kb core segment of the genome relative to all mammalian betaherpesviruses. Three other species EEHV3, EEHV4 and EEHV7 (just 4-kb each) are even more highly diverged (35%) and form a distinct GC-rich branch of the Proboscivirus genus [Fig 1]. The AT-rich branch EEHVs also all encode alphaherpesvirus-like thymidine kinase (TK), ribonucleotide B-subunit (RRB) and UL9-like origin-binding protein (OBP) genes plus a dyad symmetry Ori-Lyt domain that are absent.

**Figure 3:** Summary cartoon showing major features across the complete length of the 177-kb EEHV1A and EEHV1B genomes, including indicating the relative positions of most of the 20x family of tandemly repeated copies of an anciently captured 7xTM/vGPCR host chemokine receptor gene, as well as the inverted 40-kb portion of the conserved herpesvirus core gene block (black arrow), the immediate-early-like nuclear protein gene (ORF-L), the predicted lytic origin of replication (Ori-Lyt = ori), and the five major chimeric (I, II, III) or hypervariable (V, II', IV) domains. All six characterized EEHV1B strains have matching linked “B-allele” versions of variable segments I, II and III that are presumed to have been derived by recombination within one sub-type lineage of EEHV1 with some other unknown EEHV species (approx 1 to 2 million years ago), followed by a more recent recombination event back into a partial EEHV1A genome background.
from betaherpesviruses. They also encode orthologues of the conserved protein kinase (CPK) enzyme that could potentially target the anti-viral drugs FCV or GCV. Most dramatically, all EEHV genes and proteins encoded in common with other herpesviruses are at least 50 to 80% diverged from their nearest orthologues. In both DNA and protein based phylogenetic trees the EEHVs fall into a monophyletic clade branching intermediate between the mammalian gammaherpesvirus and betaherpesvirus sub-families [Fig 2]. Because of all these novel features, we propose that the Probosciviruses (= EEHVs) should be designated as the prototypes of a new Deltaherpesvirinae sub-family, which we estimate has evolved separately from the three other mammalian Herpesviridae sub-families within Afrotherian hosts, including the ancestors of modern elephants, for more than 100 million years.

Many EEHV species also exhibit multiple chimeric features and subtype variants [Zachariah, et al 2013]. In particular, all well characterized EEHV1 strains fall into two sub-species clusters called EEHV1A and EEHV1B that differ overall by 4.5% at the nucleotide level. But this divergence is not uniform. Instead they have three major chimeric domains (totalling 10-kb) within the conserved core herpesvirus gene block that are highly diverged [domains I, II and III in Fig 3]. These encompass part or all of 14 genes, including gB-POL (3.0-kb), ORFJ-gN-gO-gH-TK (3.8-kb, 32%) and ORFM-ORFN-UDG-gL-ORFO-ORFP (6.5-kb) that differ between the two sub-species by 17, 32 and 30% at the DNA level. All six evaluated strains of EEHV1B have linked versions of these three chimeric domains in common and show up to tenfold less overall genetic variation amongst them than do the EEHV1A strains.

The remainder of the EEHV1B genome displays additional mosaic features including large pieces that are almost indistinguishable from of a modern EEHV1A genome, combined with an older EEHV1A segment of 25-kb that has diverged by between 2-3%. These are joined to the three chimeric domains that were evidently derived from one or more other related but unknown EEHV viruses that had diverged considerably further than the 15% that EEHV1A has from EEHV6. In addition to all of that, several other EEHV1 genes such as glycoprotein-H(gH) in [domain II], vGPCR1, vGPCR5, vGPCR6, vOX2-2 and vOX2-3 [domain V] all cluster into between three to five hypervariable sub-types each. Finally, a set of between eight and 14 adjacent genes on the far right-hand-side [domain IV in Fig 3], including vFUT9 (with two subtypes that are 44% diverged), as well as vGPCR8 and five vIgFam genes, are also highly variable and form multiple distinct subtypes that are partially unlinked from the 1A versus 1B sub-species patterns. Most unusually, several of the latter have either been deleted in a sub-set of EEHV1 strains or sometimes instead have "fragmented" versions carrying multiple "out-of-frame" mutations. Overall, we conclude that the vast majority of the numerous lethal and non-lethal EEHV1 strains found worldwide last had common ancestors in the time-frame of between many hundreds of thousands of years ago up to several million years ago. These viruses must have been very common and wide-spread infections within wild Asian elephant populations for at least that length of time. Both EEHV1A and EEHV1B strains have been found in lethal hemorrhagic disease cases in America, Europe and Asia, and many elephant housing facilities have had cases of hemorrhagic viremic disease or identified asymptomatic shedding associated with both EEHV1A and EEHV1B [Stanton et al 2011]. Furthermore, five closely monitored surviving healthy Asian elephants in America have been observed to become infected sequentially with either EEHV1A and then EEHV1B or vice versa [Stanton et al, 2013]. A collaborative group in the United Kingdom has also recently published the complete genome sequences of both another EEHV1A strain (Raman) and an EEHV1B strain (Emelia), including defining the physical ends of the virion DNA molecule. [Wilkie et al, 2013].
Similar analyses have also revealed two major chimeric sub-types of EEHV5 in Asian zoo elephants [Atkins et al, 2013; Denk et al, 2012]. So far four examples of EEHV5A and one of EEHV5B have been studied that differ by 10 to 20% in their gB-POL, TK-U49, vGPCR1 and UDG-gL-ORFO domains in a very similar fashion to the EEHV1A versus EEHV1B patterns. Even the very small segments of EEHV3 and EEHV7 evaluated so far from both wild and zoo African elephants show clear evidence for clustering of the multiple examples identified into two distinctive sub-groups each. The differences are recognizable at all five PCR loci available (U38/POL, U66/TERex3, U71-U72/gM), U73/OBP and U76/POR-U77/HEL), but are most pronounced (between 8 and 17% different at the nucleotide level) within U71-U72/gM and U73/OBP.

Other variable genomic features recognized so far include the absence of one copy of an anciently duplicated glycoprotein gene (ORF-Q) from EEHV2 compared to both EEHV1A and EEHV1B, and the deletion of a small vCXCL ligand-like gene (ORF-N) from all EEHV1B strains compared to all EEHV1A, EEHV2, EEHV5 and EEHV6 strains examined. Most noticeably, whereas EEHV1A and EEHV6 have diverged relatively uniformly from each other by about 16% all the way across their genomes and similarly EEHV2 and EEHV5A have done so by about 18%, both EEHV1B and EEHV5B evidently evolved much more recently as mosaic genomes formed by chimeric recombination events that incorporated several small segments acquired from additional related EEHV-type genomes (quite possibly derived from presumed mammoth EEHVs for example).

Therefore, current evidence indicates that the two most highly pathogenic Proboscivirus types, EEHV1A and EEHV1B, which evidently have been the cause of 90% of the fatal cases of acute systemic hemorrhagic disease seen in North America, Europe and Asia, together with EEHV4, EEHV5A and EEHV5B, are likely all natural endogenous viruses of Asian elephants. In contrast, EEHV2, EEHV3A, EEHV3B, EEHV6, EEHV7A and EEHV7B (but not EEHV1) have all been found within benign lung or skin nodules from healthy adult African elephants in Kenya and in North America. Together with five known species of elephant gamma herpesviruses (EGHVs) [Latimer et al, 2011], that fall into three very highly diverged branches (genus level at 40 to 60% different from one another), including three that have separate A and B versions (3.5% different) in African versus Asian elephants, a total of 12 distinct species and at least two sub-species of elephant herpesviruses have now been identified. The EGHVs are commonly found in oral and genital lesions and conjunctival swabs from healthy adult elephants, but none of have yet been associated with any significant disease conditions.


Seven Species of Elephant Endotheliotropic Herpesviruses (EEHVs) Represent a New Deltaherpesvirus Sub-Family.

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<table>
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<tr>
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Total Cases (North America): Deaths = 28 (21); Survivors = 7 (7)

Total Cases (Europe): Deaths = 26 (12); Survivors = 2

Cases (Asia) – anecdotal Deaths = > 40 (17).
Acidophilic Cowdrey-A Nuclear Inclusion Body in Vascular Endothelial Cell In Asian Heart Tissue.
1. Over 60 cases of EEHV hemorrhagic disease in Western Zoos (80% lethal), and between 30-40 deaths in Asia. Extremely high levels of EEHV DNA in blood and necropsy tissue of viremic cases.

2. Eight different species/types of EEHV identified. EEHV1A, EEHV1B, EEHV4 and EEHV5 have all been lethal in Asians (Elephas maximus) but two chimeric versions of EEHV1A and EEHV1B predominate.

3. EEHV2, EEHV3, EEHV6, EEHV7 found in lung and skin nodules in Africans (Loxodontal africana). Only three known deaths.

4. Many monitored zoo Asians occasionally shed EEHV1A, EEHV1B or EEHV5 in trunk washes, and in some animals all three sequentially.

5. >36 strains of EEHV1A and 6x of EEHV1B identified. Many facilities have had cases of both EEHV1A and EEHV1B and multiple examples of the same strain, but strains found at different facilities always different.

6. Cannot yet be grown in cell culture—work with necropsy samples.
EVOLUTIONARY TREE OF MAMMALIAN HERPESVIRUSES (300 million years)

DNA POL (500-bp) (CODEHOPS)

NEW DELTAHERPESVIRUS = PROBOSCIVIRUS GENUS

7x EEHV species
5x EGHV species

Proboscivirus (100 million y)
HHV6A,6B,7
HCMV
KSHV
Elephant Gamma HVs

HSV1,2
VZV

EVOL
TREE OF MAMMALIAN HERPESVIRUSES (300 million years)
DELTAHERPESVIRINAE

PROBOSCIVIRUS GENUS

GC-rich 63%

AT-rich 42%

EEHV1A *KUMARI EM (35/40) 1999
EEHV1B *KIBA EM (4/8) 2001
EEHV5 *NAP28 EM (1/5) 2008
EEHV4 *NAP22 EM (2/2) 2007
EEHV3 *HANSA LA (1/8) 2007
EEHV7 NAP42 LA (0/6) 2010

No of Deaths/Examples

Year of Discovery

35% 18% 8% 4% 17% 35% 23% 14% 10 20 30 MYA

* = lethal acute disease
# = 1B = chimeric genome

NAP35 LA (0/3) 2009
NAP42 LA (0/6) 2010
NAP22 EM (2/2) 2007
KUMARI EM (35/40) 1999
KIBA EM (4/8) 2001
NAP28 EM (1/5) 2008
HANSA LA (1/8) 2007
EEHV6

18%
PCR DNA Sequence Data Available

177-kb

EEHV1A(K)

EEHV1B(K-B, Ehlers)

EEHV1B(H)

EEHV6 (2x)

EEHV2 (2x)

EEHV5A

EEHV5B

EEHV3A,B

EEHV4

EEHV7A,B

Unique-L

Core

Unique-R

60-kb

30-kb

50-kb

30-kb

21-kb

22-kb

4.1-kb

3.9-kb

3.2-kb

50-kb inversion

III - II - I

30-kb

177-kb

PCR DNA Sequence Data Available
Total EEHV1(Kimba) genome = 177,316-bp, 115 genes. Even the most conserved “core” genes of EEHV species differ by > 50% at the protein level from their nearest orthologues in other herpesviruses. Some retain only 25% protein identity to closest relatives the Roseoloviruses or show no viral homologues at all in BLAST searches.

EEHV1s also encode 60 novel genes plus TK, RRB & OBP (and ori-Lyt) typical of alphas, but usually absent in Betas, and have a 40-kb inversion of the core gene block.

Based on intermediate position between Betas and Gammas in phylogenetic trees, plus the unusual overall gene content and organization, the EEHV1s are sufficiently different from all other mammalian herpesvirus types to be classified as a distinct Deltaherpesvirus sub-family.
(a) Protein Gamma

- Alpha
  - Beta
  - Delta
  - U38/POL Protein
Missing in Gammas and Betas except Roseolos

U73/OBP Protein

Alpha

Delta

Roseolo
U48.5/TK Protein

Missing in Betas and Roseolos
EEHV1A vs EEHV1B Chimerism and Hypervariable Subtype Clusters

35 strains of 1A and 6 strains of 1B evaluated by multi-locus PCR sequencing. Overall DNA differs by > 4.3%.

Ancient chimeras but with over half = /< 1% different only.

Three 1A vs 1B-specific core domains (I,II,III) differ by 17% (gH/POL, 3.2-kb), 32% (gN/gO/gH/TK, 3.8-kb), 42% (UDG/gL, 6.5-kb) often = > difference between EEHV1A and EEHV6.

I, II, III are all linked, but other variable genes are scrambled (i.e. unlinked) especially within unique segment, including a multigene block at one end (IV, vFUT, vGPCR7/8, 6x vlgFam).

Additional hypervariability with 4-6 subtype clusters in at least 10 genes incl gH, vGPCR1, 4, 5, 8, vlgFam & vOX2-2/3.
World-wide EEHV1 strain subtype patterns

Figure 3:

vGPCR

Number of Cases

A  B  C  D  E
INDIA  NORTH AMERICA  EUROPE

gH-TK

Number of Cases

A  B  C  D  E  F
INDIA  NORTH AMERICA  EUROPE
EEHV1A/B Genome Hypervariability

Linked 1A/1B Chimeric Patterns

Multiple Unlinked Subtype Clusters
Pseudo-Species, Subtypes and Chimerism (Human herpesviruses)

**HHV6A**
- 30%

**HHV6B**
- 30%

**EBV-A**
- 45%

**EBV-B**
- 15%

**KSHV-P**
- 30%

**KSHV-M**
- 0.5%

**KSHV-N**
- 2%

**HCMV**
20x hypervariable loci = ave 8x subtype clusters each but order completely scrambled by recombination = “swarm”

**Linked diaspora**

**EBNA2**

**EBNA3A,B,C**

**K1/VIP**

**A/C, B, D/E**

**A/C**

**D**

**B, Q, R, N**

**PA/D/B**
- M* = 70%
- N* = 24%

**K15/TMP**

**REP**
EEHV1A/B Subtype Divergence – Region III

EEHV1A
- HEL
- ORF-M
- vCXCL
- UDG
- ORF-O
- ORF-P
- ORF-Q
- Core

EEHV1B
- ORF-L
- Absent in all EEHV1Bs, present in 1A,2,5,6,
- Core

EEHV2
- Absent in EEHV2
- Core

143-kb
- 1.6
- 1.0
- 0.3
- 0.8
- 1.4
- 1.7
- 2.5
- 1.6
- 1.0
- 0.8
- 1.4

S/T
- Dupl
- ORF-Q
- ORF-P
- ORF-O
- ORF-M

IE-like
- 6.3-kb

30%
- 38

23%
- 30%

29/31%
- 65%

25%
- 37%

37%
EEHV1 Subtype Divergence – Region IV

166-kb

vGPCR7

vGPCR8

vIgF1

vIgF2.1

vIgF2.2

vIgF2.3

vIgF2.5

E49.1

E49.2

E49.3

E49.4

EEHV1A (R)

EEHV1A (K)

EEHV1B (E)

vFUT

vOX2-1

Absent in EEHV1A (R)

Absent in EEHV1A (K)

Deleted in K

Number of subtypes

4x

2x

Absence in EEHV1B (E)

42%

25%

40%

18%

15%

10%

30%

3x

2x

4x
EEHV Disease Pathogenesis

Epidemiology zoo cases—all facilities different strains.
- No evidence for spread between zoos

Multiple examples of two calves with same strain.
- Most likely disease is primary infection

Sporadic shedding in Asian herd mate trunk washes.
- Quiescent/latent infections are very common

Multiple sequential infections by EEHV1A, 1B and 5.
- Give limited immunity against other types

Same disease/multiple strains in the wild in India/Asia.
- EEHV1 present in Asians for >>100,000 years

Latency in lung and skin nodules of African elephants.
- Multiple infections common, but very different types

Need to factor in chimeric EEHV1A, 1B, plus 5 and 4.
- Order and early timing of infection critical?

Why are EEHV1A and EEHV1B so much more pathogenic?
ACKNOWLEDGEMENTS:

Funding Sources:

National Elephant Herpesvirus Laboratory, Smithsonian Zoological Park, Washington DC:

International Elephant Foundation
Ringling, Barnum and Bailey Elephant Research Fund
Morris Animal Research Fund
Smithsonian Institute

Johns Hopkins University: School of Medicine, Baltimore, MD
Viral Oncology Program:

NIH NIAID Research Grant R01 AI2457
International Elephant Foundation
Morris Animal Research Fund
FINDING ELEPHANT HERPESVIRUSES

Virginia Riddle Pearson

Guest Researcher, Department of Molecular Biology, Princeton University
Honorary Research Associate, Department of Vertebrate Zoology,
The Academy of Natural Sciences of Philadelphia

EEHV2, EEHV3A, EEHV3B (a new subspecies), EEHV6, EEHV7A, EEHV7B (a new subspecies) and EGHV1A, EGHV1B (a new species), EGHV2, EGHV4 in Tissue Biopsies and Saliva from African Elephants in Kenya and America 2011-2012

in Botswana (August 26, 2013) and South Africa (? 2013)
HERPESVIRUSES

- Co-evolve with their hosts
- Cause primary disease, establish lifelong infection
- Species-specific, cross-species infection can be lethal

Calf of “Babylon”, Samburu, Kenya
credit Virginia Pearson, 2009

“Nisha” d.12.1.07, Dickerson Park Zoo
Zimbabwe “Nautilus 99” in Florida

courtesy Daryl Heard, DVM, University of Florida College of Veterinary Medicine
Nodules exist on wild African elephants

Virginia Pearson, July 2009

Credit: Virginia Pearson, Samburu, Kenya, 2009
NODULES

RNALater RNA Stabilization Reagent@www.qiagen.com
6 mm punch biopsy, iodine popules@www.midwestvet.net
“Enthusiasm” Virtues Family, Samburu. Kenya
credit Virginia Pearson 2011
KENYA 2011 SUMMARY
PCR/Gel Electrophoresis/DNA Sequencing

Skin Nodules and Lung Biopsy

EEHV2
EEHV3A
EEHV3B (new subspecies)
EEHV6
EEHV7A
EEHV7B (new subspecies)
EGHV1B (new species gammaherpesvirus).
Detection of Herpesviruses in Saliva
USA 2012

Non-invasive buccal swabs for first MHC gene characterization

Human herpesviruses detected in saliva: HCMV, HSV, VZV

Jason Holloway,
Six Flags Safari Park
SIX FLAGS 2012 SUMMARY
PCR/Gel Electrophoresis/DNA Sequencing

Saliva

EEHV2
EEHV3A
EEHV3B new subspecies
EEHV6

EGHV1A
EGHV1B new species of gammaherpesvirus
EGHV2
EGHV4

16” swabs@www.puritanmedproducts.com
RNAprotect Saliva Reagent, RNA protect Cell Reagent@www.qiagen.com
## COMBINED SUMMARY

January 28, 2013

<table>
<thead>
<tr>
<th>samples</th>
<th>EEHV</th>
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</table>

Promega PCR Master Mix@www.promega.com  
QIAdnaquick Gel Extraction Kit@qiagen.com  
DNA Sequencing@genewiz.com
Do hemorrhagic deaths occur in wild African elephants?

Does co-infection with elephant gammaherpesviruses affect pathogenesis of this disease?”
Elephants Without Borders
Dr. Michael Chase
Botswana 2013
KALWESI WATER HOLE
Chobe National Park, Botswana
Keep in sight!!
Immobilization dart
RNALater RNA Stabilization Reagent@www.qiagen.com
Before and after biopsy
Antidote
FIRST RESULTS

BOTSWANA
August 26, 2011
EEHV viral gene sequences: U66TER, U77HEL, U71/gM, U73OBP

<table>
<thead>
<tr>
<th>EEHV2</th>
<th>EEHV3A/3B</th>
<th>EEHV6</th>
<th>EEHV7A/7B</th>
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<tr>
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<td>L.a#1, male, 4yrs old with Four Trunk Nodules</td>
<td>L.a#1, male, 4yrs old with Four Trunk Nodules</td>
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<tr>
<td></td>
<td>L.a#2, female, 3yrs old with One Trunk Nodule</td>
<td>L.a#2, female, 3yrs old with One Trunk Nodule</td>
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</tr>
</tbody>
</table>
ELEPHANTS ALIVE
Dr. Michelle Henley
GPS Collarings, APNR, South Africa
RNA Later RNA Stabilization Reagent@www.qiagen.com
RNA Protect Cell Reagent@www.qiagen.com
16” swabs@Puritanmedproducts.com
Ear Lesion
Balule Reserve, South Africa
ACKNOWLEDGEMENTS

Dr. Lynn Enquist, Chairman, Department of Molecular Biology, Princeton University

Dr. Gary Hayward, Professor, Viral Oncology Program, The Sidney Kimmel Comprehensive Cancer Center, Johns Hopkins School of Medicine

1Enquist Laboratory, Department of Molecular Biology, Princeton University, Princeton, NJ, USA; 2Academy of Natural Sciences of Philadelphia, Philadelphia, PA, USA; 3Save The Elephants, Nairobi, KENYA; 4Kenya Wildlife Service Veterinary and Capture Services Department, Nairobi, KENYA; 5Six Flags Wild Safari, Jackson, NJ, USA; 6Six Flags Discovery Kingdom, Vallejo, CA, USA; 7San Diego Zoo Institute for Conservation Research, Escondido, CA, USA; 8Viral Oncology Program, The Sidney Kimmel Comprehensive Cancer Center, The Johns Hopkins School of Medicine, Baltimore, MD, USA
Colleagues: BOTSWANA/SOUTH AFRICA 2013

Elephants Without Borders/Botswana: Dr. Michael Chase, Kelly Landen, Larry Patterson, DVM

Elephants Alive/Save The Elephants South Africa: Dr. Michelle Henley, Cobus Raath, DVM

WESSA South Africa: Chris Galliers

SANPARKS South Africa: Peter Buss, DVM

University of Pretoria Veterinary Genetics Laboratory, South Africa: Dr. Cindy Harper, Susan Miller

Princeton University, USA: Matthew Aardema

University of Washington, USA: Dr. Samuel Wasser
“What is man without the beasts? If all the beasts were gone, men would die from a great loneliness of spirit. For whatever happens to the beasts, soon happens to man. All things are connected.”

Chief Seattle, 
Suquamish Tribe
Elephant Endotheliotropic Herpesvirus (EEHV): Where we are, where we’re going.

Lauren L. Howard, DVM, Dipl. ACZM
Associate Veterinarian
Houston Zoo, Inc.
The Houston Zoo and EEHV

EEHV History at Houston Zoo
• 1977: 6 year old Katu dies @ Houston
• 1988: 4 year old Beau Thai dies @ Canada
• 1991: 3 year old Pearl dies @ Chicago
• 1997: 11 year old Kiba dies @ Germany
• 2000: 7 year old Singgah dies @ Houston
• 2004: 13 year old Kimba dies @ Houston
• 2008: 2 year old Mac dies @ Houston
The Houston Zoo and EEHV

EEHV History at Houston Zoo

- 2009: Partnership between Baylor College of Medicine and the Houston Zoo
The Houston Zoo and EEHV

EEHV History at Houston Zoo

• Goals of HZI/BCM Partnership:
  – Establish local, rapid EEHV PCR testing
  – Better understand elephant immunity to EEHV
  – Better understand epidemiology of EEHV
  – EEHV treatments and possible vaccine development
The Houston Zoo and EEHV

EEHV: The Great Unknown

• Antiviral efficacy against EEHV?
• Immunity in elephants related to EEHV
• Case definition and epidemiology of EEHV
• Cultivation of the virus in the laboratory
EEHV Workshops

9th Annual International EEHV Workshop

• Houston, Texas USA
• January 27-29, 2013
• 70 participants from 6 countries
• 15 scientific abstracts

For copy of proceedings and/or summary:

• lhoward@houstonzoo.org
N. American Asian Elephant Population

- 2011 ZooRisk Population analysis
- Current population not self sustaining
- Declining at 1.6% annually
- Will have a bottleneck in 15-30 years
EEHV Workshop 2013

EEHV and N. American Asian Elephant Collection

• Population Model by Lisa Faust Dec 2012:
  – Evaluated impact of EEHV on population
  – Assume elephants that died of EEHV actually lived

• Compared this result to 2011 ZooRisk analysis
EEHV Workshop 2013

Eliminating EEHV alone:
• Doubles population size in 100 years
• Improves growth rate to be stable (not decline)
Combination of eliminating EEHV and increasing breeding:

- 97% chance to reach target population size:
- Best scenario of all of them considered
EEHV in North America since 1978:

- 109 elephants in susceptible population
- 21 EEHV deaths
- 13 deaths due to non-EEHV causes

EEHV is the leading cause of death in Asian elephants born in North America since 1978.
EEHV in North America since 1978:

• 21 deaths associated with EEHV infection
• 9 survivors of illness associated with EEHV infection since 1978
  – 5 survivors in the past 4 years

• Overall: 70% fatality rate
EEHV Workshop 2013

EEHV in North America since 2009:

• 5 EEHV survivors
• 1 EEHV-associated death

We are making headway on this disease!
Case Definition for EEHV

- N. American captive born Asian elephants between 0.5 and 15 years of age
- clinical signs associated with hemorrhagic disease
- confirmation of EEHV infection via PCR or histopathology.

Figure 1. (A-D) Pathologic changes of EEHV-associated disease found during field necropsy. (A) Asian elephant with cyanosis of the tongue attributed to EEHV disease; (B) Epicardial surface of the heart (apex view) from an Asian elephant showing severe extensive hemorrhage attributed to EEHV disease; (C) Ventricular endocardial surface of the heart from an Asian elephant showing multifocal areas of ecchymotic hemorrhage attributed to EEHV disease; (D) Serosal membrane surface of the liver showing diffusely scattered petechial hemorrhage attributed to EEHV disease; (E,F) Photomicrograph of two capillary endothelial cells containing typical basophilic intranuclear viral inclusion bodies from necropsy liver tissue. Hematoxylin and eosin stain, bar = 25 µm.
EEHV Workshop 2013

Case Definition for EEHV

• clinic signs associated with hemorrhagic disease
  – Which signs, how severe

• confirmation of EEHV infection via PCR or histopathology.
  – What level of viremia is significant?
Multi-year EEHV Epidemiology Study
Dr. Ramiro Isaza, University of Florida

20 Institutions both w/ and w/o EEHV surveyed

• No apparent association between illness or death from EEHV in Asian elephants and exposure to African elephants.
EEHV Workshop 2013

Dr. Gary Hayward/Johns Hopkins
EEHV1A complete genome compiled
• New virus family: Deltaviruses?

11 EEHV Sub-Types and Distribution
• Asian Elephants:
  – EEHV 1, 4, and 5
• African Elephants:
  – EEHV 2, 3, 6 and 7
EEHV Workshop 2013

EEHV and African Elephants

Baylor College of Medicine evaluated trunk secretions in healthy African elephants.

EEHV shedding in 10 African elephants:

- 4/10: EEHV 3/4
- 4/10: EEHV 6
- 1/10: EEHV 1 *
  - *housed previously with Asian elephants
EEHV Workshop 2013

EEHV and African Elephants
Maryland Zoo / Dr. Bronson

• EEHV-3 associated illness in African calf
• Use of whole blood viral loads as measured by real time PCR to guide therapy
• Kudos to Veterinary and Elephant Team!
Dr. Carolyn Cray (U Miami) evaluated acute phase proteins in blood of elephants

- Serum amyloid levels 10X higher in EEHV viremic elephants
- Prognostic value
- Elephant immunity
Immunity and Elephants

Baylor College of Medicine looked at passive transfer of antibodies in Asian elephants

- Majority of maternal-fetal antibody transfer occurred through the placenta, rather than through colostrum.
EEHV Workshop 2013

Immunity and Elephants
Dr. Byron Martina (Erasmus Medical Center)

• Developing a gB based ELISA for evaluation of EEHV antibodies in elephants
• Have run samples from Europe and the US through the ELISA, results under evaluation
Clinical Management of EEHV
Twycross Zoo/ Dr. Sharon Redrobe

- EEHV-5 associated illness and death in an Asian elephant
- Use of methadone improved attitude
- First time pericardiocentesis was performed
Changes at National Elephant Herpesvirus Laboratory (NEHL)

• No longer performing free EEHV testing

• Facilities needing testing may
  – Join the NEHL Consortium
  – Pay individually per test

For more information: Erin Latimer at NEHL (202) 633 4252 or latimer@si.edu
EEHV Workshop 2013

NEHL Consortium (different levels available)

Advantages of Membership

• Unlimited diagnostic testing of elephants
• Routine calf screening****

For more information: Erin Latimer at NEHL (202) 633 4252 or latimer@si.edu
EEHV Workshop 2013

NEHL Consortium (different levels available)

Advantages of Membership

• Testing & Subtyping for all EEHV
• Meeting AZA elephant accreditation standard for Conservation and research activities
• Media support on EEHV issues, if needed
Future Areas of Focus

• Establishing funding to support research
• Further refinement of epidemiology of EEHV
• Better understanding of elephant immune response related to EEHV
  – EEHV Serology test on the horizon
EEHV Workshop 2013

Future Areas of Focus

• Continued attempts to culture virus
• Ongoing work to confirm antiviral efficacy
• Laying groundwork for vaccine development
The Lonely Rhino: Analyzing Anthropomorphism Toward Solitary Animals

By: Selenia Murillo
Introduction
Mission

To inspire conservation leadership by connecting people with wildlife and nature
Top 5 Favorite Animals

1. Lions
2. Giraffes
3. Dolphins
4. Gorillas
5. Bears
6. Elephant
7. Rhinoceros
I prefer to see animals in group settings.
I prefer to see animals in group settings.
Are we effectively fulfilling our mission?
Methods
2. Using the scale below, please indicate how much you agree or disagree with the following statements about the black rhino.

<table>
<thead>
<tr>
<th></th>
<th>1 Strongly disagree</th>
<th>2 Moderately disagree</th>
<th>3 Slightly disagree</th>
<th>4 Neither agree nor disagree</th>
<th>5 Slightly agree</th>
<th>6 Moderately agree</th>
<th>7 Strongly agree</th>
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3. Based on the behaviors you observed, how would you describe:
   a) the black rhino’s mood or emotional state ____________________________
   ____________________________

   b) your mood or emotional state ____________________________
   ____________________________

4. Which best describes the natural social structure of black rhinos?
   □ Solitary  □ Pair Bond  □ Small group  □ Herd  □ Fission-fusion community  □ Other ____________________________
Pachyderm House
Results
Visitor Demographics

Membership
- 54% Members, n=191

Gender
- 56% Female, n=200

Age
- 39% within 30-39 range
- 27% within 40-49 range, n=171

Ethnicity
- 83% Caucasian, n=171

86% completed most or all of the survey
70% of visitors think that black rhinoceros are social.

n=181
Black Rhino Activity

16% Inactive
- Not alert
- Alert

84% Active
- Moving around
- Eating
- Interacting
- Pacing
- Manipulating objects

n=338
<table>
<thead>
<tr>
<th>The black rhino appears lonely.</th>
<th>Agree</th>
<th>Neutral</th>
<th>Disagree</th>
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<td>47%</td>
<td>32%</td>
<td>22%</td>
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<tr>
<th>The black rhino doesn’t need a companion.</th>
<th>Disagree</th>
<th>Neutral</th>
<th>Agree</th>
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<td>63%</td>
<td>27%</td>
<td>10%</td>
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n=146
Animal Concern: Health

The black rhino appears healthy and well-fed.

- 87% Agree
- 6% Neutral
- 6% Disagree

The black rhino appears sickly and malnourished.

- 87% Disagree
- 5% Neutral
- 7% Agree

n=151
### Animal Concern: Stress

#### The black rhino seems stressed by the presence of visitors.

<table>
<thead>
<tr>
<th></th>
<th>Agree</th>
<th>Neutral</th>
<th>Disagree</th>
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<tr>
<td>10%</td>
<td>8%</td>
<td>82%</td>
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</table>

#### The black rhino seems unaffected by the presence of guests.

<table>
<thead>
<tr>
<th></th>
<th>Disagree</th>
<th>Neutral</th>
<th>Agree</th>
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<td>11%</td>
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<td>81%</td>
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</tr>
</tbody>
</table>

n=152
Animal Concern: Space

The black rhino has adequate space.

- 44% Agree
- 15% Neutral
- 41% Disagree

The black rhino needs more room.

- 18% Disagree
- 19% Neutral
- 63% Agree

n=150
Animal Concern: Behavior

The black rhino’s behavior differs within a zoo setting.

- 29% Agree
- 55% Neutral
- 16% Disagree

The black rhino’s behaviors are similar to those of wild rhinos.

- 26% Disagree
- 62% Neutral
- 12% Agree

n=138
Animal Concern: Enrichment

The black rhino has enough toys and interactive features in its exhibit.

- **37%** Agree
- **20%** Neutral
- **43%** Disagree

The black rhino’s environment lacks engaging and enriching stimuli.

- **28%** Disagree
- **17%** Neutral
- **55%** Agree

n=147
Emotional Responses

Rhino’s Mood or Emotional State

<table>
<thead>
<tr>
<th>Negative Descriptors</th>
<th>Bored</th>
<th>Lonely</th>
<th>Sad</th>
</tr>
</thead>
<tbody>
<tr>
<td>20%</td>
<td>54%</td>
<td>32%</td>
<td>19%</td>
</tr>
</tbody>
</table>

n=185

Visitor’s Mood or Emotional State

<table>
<thead>
<tr>
<th>Negative Descriptors</th>
<th>Reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>3%</td>
<td>Sad for the rhino, Concerned about space, Content, but worried he’s sad</td>
</tr>
</tbody>
</table>

n=179
<table>
<thead>
<tr>
<th>Question</th>
<th>Not at all</th>
<th>Somewhat</th>
<th>Very much so</th>
</tr>
</thead>
<tbody>
<tr>
<td>How much did visiting the black rhinos influence your decision to come</td>
<td>73%</td>
<td>17%</td>
<td>10%</td>
</tr>
<tr>
<td>to the zoo today?</td>
<td>n=174</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Did you have an up-close encounter with a black rhino today?</td>
<td>30%</td>
<td>23%</td>
<td>48%</td>
</tr>
<tr>
<td></td>
<td>n=172</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Did you make eye contact with a black rhino today?</td>
<td>39%</td>
<td>16%</td>
<td>46%</td>
</tr>
<tr>
<td></td>
<td>n=176</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Visitor Comments

33% noted animal concerns

- Improve exhibits
  - More indoor space
  - Naturalistic
  - Increase outdoor access
  - Provide hiding places

- More animals

29% mentioned elephants

n=49
Social Structure

The black rhino doesn’t need a companion.
\[ r_s(145) = -0.345, \ p = 0.000^{**} \]

The black rhino appears lonely.
\[ r_s(144) = 0.186, \ p = 0.026^{*} \]

The black rhino seems stressed by the presence of visitors.
\[ r_s(145) = 0.166, \ p = 0.046^{*} \]

* Correlation is significant at the 0.05 level
** Correlation is significant at the 0.01 level
Behavior

The black rhino appears lonely.
\[ r(194) = -0.198, \ p = 0.006^{**} \]

The black rhino appears healthy and well-fed.
\[ r(200) = 0.159, \ p = 0.024^{*} \]

The black rhino is unaffected by the presence of visitors.
\[ r(196) = -0.164, \ p = 0.022^{*} \]

* Correlation is significant at the 0.05 level
** Correlation is significant at the 0.01 level
Up-close Encounter

The black rhino appears healthy and well-fed.

\[ r(126) = 0.232, \ p = 0.009^{**} \]

The black rhino’s environment lacks engaging and enriching stimuli.

\[ r(125) = -0.185, \ p = 0.039^{*} \]

* Correlation is significant at the 0.05 level
** Correlation is significant at the 0.01 level
The black rhino appears healthy and well-fed.

\[ r(125) = 0.237, \ p = 0.008^{**} \]

The black rhino has enough toys and interactive features in its exhibit.

\[ r(122) = 0.272, \ p = 0.002^{**} \]

* Correlation is significant at the 0.05 level

** Correlation is significant at the 0.01 level
Eye Contact

The black rhino’s environment lacks engaging and enriching stimuli.
\[ r(124) = -0.259, p = 0.004^{**} \]

The black rhino doesn’t need a companion.
\[ r(123) = 0.180, p = 0.046^{*} \]

The black rhino has adequate space.
\[ r(122) = 0.179, p = 0.049^{*} \]

* Correlation is significant at the 0.05 level
** Correlation is significant at the 0.01 level
Conclusion
Summary

• Majority of guests are unaware of the black rhino’s solitary social structure
  o More inclined to think black rhino is lonely and stressed
Summary

• Active black rhinos are seen as less lonely, less stressed, and healthier
• Up-close encounters and eye contact improve perception of the black rhino’s welfare from the visitor’s perspective
Next steps for black rhino conservation
Acknowledgements

Animal Programs
• Susan Hoss
• Scott Katzberger
• Rick Lichner
• George Morgan

Conservation Education and Training
• Debra Kutska
• Jerry Luebke
• Jennifer Matiasek
Questions?

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