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Research article

Haematological and serum biochemistry reference intervals for the Amur leopard (*Panthera pardus orientalis*)

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Abstract

We report on the first investigation into subspecies-specific haematological and serum biochemistry reference intervals for the Amur leopard (*Panthera pardus orientalis*). A total of 62 samples were included in this retrospective study. All animals involved were part of the European Endangered Species Breeding Programme. The samples were collected between December 1995 and May 2015. In broad terms, the reference intervals reported were consistent with existing available Species 360 data for the species, albeit with narrower ranges for some parameters. Fourteen of the 34 amylase results included in the study were found to be unusually high when compared to the Species 360 range. The clinical significance of these values remains unclear, however evidence from the studbook suggests this apparent elevation in amylase levels may be a heritable trait and therefore warrants further investigation. It is hoped the reported reference intervals will not only prove a valuable tool for clinicians dealing with captive animals, but will also provide baseline data for any future re-introduction of Amur leopards in the Russian Far East.

Introduction

Of all the cat species, leopards (*Panthera pardus*) have the widest geographic distribution. They can be found in a wide variety of habitats ranging from sub-Saharan plains, through dense tropical rainforests to the lower reaches of the Himalayas. Local adaptation and habitat fragmentation have given rise to nine genetically distinct subspecies (Uphyrkina et al. 2001). Of these, the Amur leopard (*Panthera pardus orientalis*) is one of the most endangered, with around 70 individuals now thought to exist in the wild. The majority of these are located in a small area of the Russian Far East (Jackson and Nowel 2008) with the remainder in the adjacent region of north east China (WWF 2015). Intensive in-situ and ex-situ conservation efforts are underway to preserve the subspecies, including a European Endangered Species Breeding Programme (EEP), under the auspices of the European Association of Zoos and Aquaria (EAZA), and a proposed re-introduction programme. The aim of the latter would be to re-establish a second Russian population within the subspecies' former range using offspring of leopards sourced from the EEP (Spitzen et al. 2012).

Such intensive population management cannot be achieved without suitable veterinary support (Kelly et al. 2013). Interpretation of haematological and biochemical parameters remains one of the mainstays of veterinary investigation, and its importance cannot be overestimated in non-domestic species where clinical observation may be unrewarding. Blood sampling requires only very basic facilities, and with appropriate training may even be possible without the need for immobilisation (Temple 1995, Reamer et al. 2014). A small sample can be used for multiple tests, enabling the practitioner not only to screen for specific diseases of interest, but also to obtain quantitative data on a wide variety of haematological and biochemical parameters, which in turn may give indications of the animal's health.

The interpretation of blood results from non-domestic species is often confounded by a lack of reference data and further exacerbated in species which are particularly rare. Species 360 (formerly The International Species Information Service, 7900 International Drive Suite 1040 Bloomington, MN 55425) aims to tackle this issue by providing a forum through which blood results from captive individuals in zoos around the world may be pooled and shared. Whilst undoubtedly valuable, it is worth noting that although these databases are often referred to as 'reference ranges', information provided is limited to mean, standard deviation, minimum value, maximum value, sample size and number of animals included. They do not include formally calculated reference intervals. Furthermore, this service is currently only provided at a species, rather than a subspecies, level. While in many cases this may be sufficient (Sabapara et al. 2008), variation between subspecies does occur, particularly where genetic bottlenecks

apply (Foster and Cunningham 2009). Recent work suggests that even within the domestic cat there are breed variations which could prove clinically significant (Reynolds et al. 2010, Paltrinieri et al. 2014).

Morphologically, Amur leopards are the most divergent of the nine subspecies (Kelly et al. 2013). Genomic investigation has so far revealed the wild population to be not only genetically distinct, but impoverished, with only the Florida panther (*Puma concolor coryi*) and the Asiatic lion (*Panthera leo persica*) comparable in terms of their homogeneity (Uphyrkina et al. 2001, Uphyrkina et al. 2002). The current EEP captive population is represented by only nine founders. Despite retaining comparatively good genetic variation (Uphyrkina et al. 2002), the potential effects of such close breeding, however well managed, cannot be ignored.

This retrospective study investigates subspecies-specific reference intervals for commonly used haematological and biochemical parameters in the Amur leopard. It is hoped the results will aid zoo clinicians in their management of these animals and provide a baseline for the rigorous health screening required in the proposed re-introduction project (Kelly et al. 2013).

Methods

Blood samples were collected during routine health screening of captive Amur leopards identified by the EEP as potential breeding stock for the proposed re-introduction programme and held in various zoological collections within Europe. Each animal was identified by its unique International Studbook (ISB) number, which not only aided tracking of animal movements between institutions but also provided ready access to parentage and breeding lines.

Samples were collected between 15th December 1995 and 1st May 2015, and only those from reportedly healthy animals were included. Health status at the time of sampling was determined by observation, clinical history and physical examination under anaesthesia. All subjects were anaesthetised during sampling, obviating the need for physical restraint. As per standard pre-anaesthetic practice, all subjects were fasted prior to sampling. Unfortunately, due to the retrospective nature of the study, detailed information on sampling technique, including the vein accessed and the size of the needle and syringe used, was not available. Similarly, information was not available for all cases regarding sample handling or storage prior to testing.

A total of 62 samples, obtained from 56 animals, were included in this study. In the majority of cases, samples were collected and submitted for biochemistry and haematology simultaneously, although in some instances samples were submitted for one or the other separately. Similarly, for both the biochemistry and haematology submissions, the parameters reported for each sample varied; consequently, the number of results available for each parameter also varied. All parameters were recorded in Standard International units.

Fifty-eight serum samples were submitted for biochemistry. These were taken from 51 animals housed at 18 zoological collections in Belgium (1 sample), the Czech Republic (5 samples), Denmark (2 samples), France (8 samples), Germany (2 samples), the Netherlands (1 sample), Russia (13 samples), Switzerland (1 sample) and the UK (25 samples). Samples were processed at 13 different external commercial or university laboratories local to the zoos of origin (53 samples), and three in-house zoo laboratories (5 samples).

Forty-eight EDTA whole blood samples were submitted for haematology. These were taken from 43 animals housed at 16 zoological collections in Belgium (1 sample), the Czech Republic (1 sample), Denmark (2 samples), France (8 samples), Germany (2 samples), Russia (9 samples), Switzerland (1 sample) and the UK (24 samples). Samples were processed at 13 different external commercial or university laboratories local to the zoos of origin (43 samples) and three in-house zoo laboratories (5 samples). In addition to in-house analysis, blood smears from the three samples from Moscow Zoo were also submitted to Idexx Laboratories in Wetherby, UK.

Results were analysed in accordance with the American Society for Veterinary Clinical Pathology (ASVCP) guidelines for the generation of reference intervals in veterinary species (ASVCP 2011). Initially, given the small numbers involved, data were not partitioned by age or sex. Results for each parameter were assessed visually via a histogram, suspicious outliers were identified and source data were re-checked for potential errors. Any clearly erroneous results were discounted from the study. The distribution was then further assessed for normality using a Shapiro-Wilks test, and any non-Gaussian distributions were transformed. Positively-skewed data were transformed logarithmically, and a box-cox transformation applied if this failed to achieve normality. Negatively-skewed data were first reflected and then transformed as for positively-skewed data. Where normality could not be achieved, the authors selected the transformation which gave the lowest degree of skew, as the robust approach utilised for non-Gaussian data favours symmetry. Once normality had been established, Reed's test for outliers was applied (Reed et al. 1971) and any outliers identified removed from the dataset and the test re-run. Review of the original dataset and consideration given to the potential causes of outlying results were carried out whenever outliers were identified. Due to the small sample size and the need to preserve this where possible, identification of a single outlying result within a sample did not result in the exclusion of the sample. However, if a sample had multiple anomalies or changes consistent with pathology, it was excluded.

Summary data for each parameter were calculated and maximum and minimum values, arithmetic mean, median and standard deviations were recorded. Reference intervals were calculated for all parameters for which 20 or more samples were available. The method of calculation, either parametric or robust, was determined according to the sample size and distribution in line with the ASVCP guidelines.

To assess the need for partitioning, data for each parameter were subdivided into four age and sex classes: females two days to two years old; males two days to two years old; females two years old and over; males two years old and over. The initial choice of age categorisation was adopted from the existing Species 360 data (I.S.I.S 2002). The distributions of each class were compared using a two-tailed Kolmogorov-Smirnov test. Where statistical differences in the distributions were identified, the need for partitioning was considered based on the following criteria:

i) Means of the two classes: A difference in means was considered significant if it was greater than 25% of the 95% range of the two classes combined.

ii) Standard deviation of the two classes: A difference was considered significant if the ratio of standard deviations (larger:smaller) was greater than 1.5.

iii) Percentage of data points of any one class falling outside the combined reference interval: Partitioning was recommended in classes which have either greater than 4.1% or less than 0.9% outside the combined reference interval; partitioning was not recommended if 1.8% to 3.2% fell outside the reference interval. For values between these bounds other factors must be considered (ASVCP 2011).

A minimum of 20 individuals in each subclass is required to calculate partitioned reference intervals. If there are fewer than 40 individuals in each subclass, as was the case with our dataset, the ASVCP only recommends partitioning if there are clear clinical

reasons to do so (ASVCP 2011). Therefore, the final decision was made based on clinical judgement and an assessment of whether the differences in reference intervals for the two classes would be clinically significant. If the decision was made to partition, the data was reviewed graphically to assess whether it supported the initial age boundaries adopted from the Species 360 data. If this was not the case, the boundary was amended accordingly.

Finally, summary data generated for the Amur leopards were compared to those available from Species 360 for all leopard species (*Panthera pardus*). Given the limited data available within the Species 360 and far less stringent inclusion criteria, it was not considered appropriate to compare these results statistically. However, despite its limitations, the Species 360 database remains a valuable resource to many zoological veterinarians and, prior to this study, was the best available source of data for the interpretation of Amur leopard haematology and biochemistry. Therefore, the authors feel some consideration and comparison is appropriate, albeit qualitative rather than quantitative.

Comparison of classes using a two-tailed Kolmogorov-Smirnov test was carried out in R (R CoreTeam 2015), all other analyses were carried out using MedCalc for Windows, version 16.4.3 (MedCalc Software, Ostend, Belgium).

Results

The biochemistry and haematology reference intervals generated and the methods employed to do so are summarised in Tables 1 and 2, respectively. Meanwhile, Figures 1 and 2 provide a graphical comparison to existing Species 360 leopard data.

Of the 38 parameters reported, only four could not be transformed to a normal distribution (see Tables 1 and 2). Two results were identified and excluded as outliers prior to statistical evaluation: the first was a magnesium value of 93, which was so exceptionally high that the authors presumed some error of data entry (the final mean value of magnesium was 0.98). The second was a very low amylase value of 4.5, which on further investigation of the original laboratory report also resulted from data entry error.

A further 16 outliers were identified statistically. These included a sodium result of 170 and a cholesterol result of 9.4, with the remainder being amylase results. In total, 14 of 34 reported amylase results were identified as outliers, all of which fell to the right of the distribution, with values ranging between 835 and 2200 (see Figure 3). These values are dramatically higher than the range reported by Species 360 for leopards, which further supported their status as outliers or abnormal results. The high

Table 1. Calculated biochemistry reference intervals for Amur leopards. N=Number of samples, M=Arithmetic mean, SD=Standard deviation, Med=Arithmetic median, Min=Minimum value, Max=Maximum value, LRIL=Lower reference interval limit, URIL=Upper reference interval limit, for both LRIL and URIL bracketed 90% confidence intervals are given below, D=Distribution, G=Gaussian, NG=non-Gaussian, SA=Statistical approach used in calculating reference intervals, R=Robust, P=Parametric, IS=Insufficient samples, (B)=Box-cox transformation, (FL)=Reflected and logarithmic transformation, (L)=Logarithmic transformation. ALP=Alkaline phosphatase, AST=Aspartate aminotransferase, ALT=Alanine aminotransferase, GGT=Gamma glutamyltransferase, LDH=Lactate dehydrogenase, CPK=Creatinine phosphokinase. *Unable to calculate confidence intervals due to large number of replicate results, in this case 0.

	N	M	SD	Med	Min	Max	LRIL	URIL	D	SA
Sodium (mMol/l)	31	152	5.46	153	133	158	141(138-146)	161 (159-163)	NG	R (FL)
Potassium (mMol/l)	32	3.97	0.310	4.01	3.4	4.8	3.36 (3.20-3.52)	4.58 (4.42-4.74)	G	P
Chloride (mMol/l)	26	118	4.96	119	102	127	106 (100-111)	126 (124-127)	NG	P (FB)
Phosphate (mMol/l)	35	2.02	0.702	1.75	1.23	4.40	1.31 (1.23-1.40)	4.14 (2.99-8.80)	NG	P (B)
Calcium (mMol/l)	37	2.48	0.249	2.50	1.68	2.85	1.99 (1.87-2.11)	2.97 (2.85-3.09)	G	P
Magnesium (mMol/l)	15	0.987	0.185	0.9	0.78	1.3	-	-	NG	IS
Total protein (g/l)	58	72.6	6.12	72.1	59	91	60.6 (58.3-62.8)	84.6 (82.3-86.9)	G	P
Albumin (g/l)	57	37.7	3.03	37.4	29.7	48	32.2 (31.2-33.2)	44.0 (42.7-45.3)	NG	P (L)
Globulin (g/l)	57	35.2	6.21	35.0	24	51	23.0 (20.6-25.3)	47.3 (45.0-49.7)	G	P
Urea (mMol/l)	57	12.3	2.70	11.9	6.78	18.9	7.03 (6.01-8.05)	17.6 (16.6-18.6)	G	P
Creatinine (µMol/l)	58	179	45.3	183.4	82	274	89.8 (72.7-107)	267 (250-284)	G	P
Glucose (mMol/l)	17	9.37	4.68	8.4	4.51	22.3	-	-	NG	IS
Cholesterol (mMol/l)	33	4.14	0.838	4.27	2.80	5.92	2.50 (2.08-2.91)	5.78 (5.36-6.20)	G	P
Triglyceride (mMol/l)	25	0.269	0.159	0.240	0.08	0.88	0.0864 (0.0642-0.116)	0.647 (0.481-0.870)	NG	P (L)
Juvenile ALP (U/l)	23	153	146	139	18.1	549	8.84 (3.32-20.8)	630 (380-1008)	NG	P (B)
Adult ALP (U/l)	35	20.1	11.7	16.8	0.1	43	0 (0-2.96)	43.0 (37.3-48.6)	G	P
AST (U/l)	30	32.2	14.2	28	17.6	79	17.2 (15.5-19.2)	76.1 (52.5-136)	NG	P (B)
ALT (U/l)	57	51.1	22.3	44.0	28	173	28.8 (26.9-31.0)	109 (86.0-148)	NG	P (B)
GGT U/l)	51	1.53	2.34	0.80	0	9	0*	32.6*	NG	R (B)
CPK (U/l)	19	404	488	252	104	1959	-	-	NG	IS
LDH (U/l)	11	157	190	122	1	702	-	-	NG	IS
Amylase (U/l)	20	248	103	209	128	434	123 (106-145)	603 (394-1120)	NG	P (B)

Table 2. Calculated haematology reference intervals for Amur leopards. N=Number of samples, M=Arithmetic mean, SD=Standard deviation, Med=Arithmetic median, Min=Minimum value, Max=Maximum value, LRIL=Lower reference interval limit, URIL=Upper reference interval limit, for both LRIL and URIL bracketed 90% confidence intervals are given below, D=Distribution, G=Gaussian, NG=non-Gaussian, SA=Statistical approach used in calculating reference intervals, R=Robust, P=Parametric, IS=Insufficient samples, (B)=Box-cox transformation. RBC=Red Blood Cell Count, Hb=Haemoglobin, HCT=Haematocrit, MCV=Mean Cell Volume, MCH=Mean Cell Haemoglobin, MCHC=Mean Cell Haemoglobin Concentration, WBC=Blood Cell Count. **Unable to calculate RI as unable to transform to normal and unable to apply Robust methods due to large number of replicate results, in this case 0.

	N	M	SD	Med	Min	Max	LRIL	URIL	D	SA
RBC (×10e12/l)	42	8.35	1.12	8.33	6.01	10.7	6.17 (5.67-6.66)	10.5 (10.0-11.0)	G	P
Hb (g/dl)	45	13.3	2.07	13.6	8.1	18.9	9.26 (8.37-10.1)	17.4 (16.5-18.3)	G	P
HCT (%)	48	41.3	5.37	41.4	30	51.7	30.7 (28.5-33.0)	51.8 (49.6-54.0)	G	P
MCV (fl)	36	48.9	4.47	48.4	40.8	59.6	40.2 (38.0-42.3)	57.7 (55.6-59.8)	G	P
MCH (pg)	32	15.8	1.22	15.8	11.5	18.6	13.2 (12.3-14.0)	17.8 (17.4-18.3)	NG	P (B)
MCHC (g/dl)	36	32.1	2.78	32.4	22.5	36.2	25.0 (18.1-27.9)	36.0 (35.3-36.7)	NG	P (B)
WBC (×10e9/l)	45	11.8	3.05	11.6	6.98	19.7	5.80 (4.50-7.11)	17.8 (16.5-19.1)	G	P
Neutrophils (x10e9/l)	42	9.16	2.70	8.96	5.04	15.5	3.86 (2.65-5.06)	14.5 (13.3-15.7)	G	P
% Neutrophils	46	77.7	7.46	77.5	54	92	63.1 (60.0-66.3)	92.4 (89.2-95.5)	G	P
Lymphocytes (×10e9/l)	42	1.58	0.749	1.28	0.535	3.58	0.591 (0.484-0.722)	3.46 (2.83-4.22)	NG	P (L)
% Lymphocytes	46	13.9	6.08	13.0	3	33	1.94 (0–4.52)	25.8 (23.2-28.4)	G	P
Eosinophils (×10e9/l)	40	0.671	0.481	0.55	0	1.97	0.443 (0-0.110)	1.84 (1.48-2.25)	NG	P (B)
% Eosinophils	45	5.72	4.00	5	0	18	0.143 (0-0.620)	16.1 (13.1-19.3)	NG	P (B)
Basophils (×10e9/l)	38	0.0171	0.0390	0	0	0.12	-**	-**	NG	-
% Basophils	37	0.138	0.346	0	0	1	-**	-**	NG	-
Platelets (×10e9/l)	31	430	88.2	420	254	658	258 (212-303)	603 (558-649)	G	P

Table 3. Assessment of partitioning criteria. Columns 1–4 show the 6 parameters investigated further for partitioning, along with overall and partitioned ranges. Results from the 3 assessment criteria (as described in methodology) are given in columns 5–7. ✓ indicates the result favoured partitioning, × indicates it did not. The last column indicates whether the differences in ranges was felt to be clinically significant, the deciding factor in determining whether to partition.

	Overall Range	Juvenile Range	Adult Range	Criteria (i)	Criteria (ii)	Criteria (iii)	Clinical Sig.
Total Protein	60.7-84.4	59.6–78.3	64.9–88.3	✓	×	✓	×
Albumin	31.8–43.7	34.6–42.9	31.6-44.5	×	×	✓	×
Globulin	22.8–47.3	21.5–38.9	28.9–48.1	✓	×	×	×
Urea	6.98–17.6	5.43-18.3	8.2–17.0	×	×	×	×
Creatinine	88.8-268	61.3-255	118-266	✓	×	✓	×
ALP	0.937-345	8.84-630	0–43.4	✓	✓	✓	✓

	N	Min	Max	Mean	SD
Sodium (mMol/l)	31	133	158	152	5.46
	161	140	162	152	3.99
Potassium (mMol/l)	32	3.4	4.8	3.97	0.310
	170	2.9	5.2	3.96	0.409
Chloride (mMol/l)	26	102	127	118	5.96
	159	105	135	119	5.01
Phosphate (mMol/l)	35	1.23	4.40	2.03	0.702
	167	0.97	2.78	1.78	0.35
Calcium (mMol/l)	37	1.68	2.85	2.48	0.249
	171	2.08	3.08	2.52	0.169
Magnesium (mMol/l)	15	0.780	1.3	0.987	0.185
	19	0.251	1.3	0.709	0.219
Total protein (g/l)	58	59	91	72.6	6.12
	164	53	96	73.0	6.62
Albumin (g/l)	57	29.7	48	37.7	3.03
	150	23	46	34.3	4.35
Globulin (g/l)	57	24	51	35.2	6.21
	151	19	64	34	5.12
Urea (mMol/l)	57	6.78	18.9	12.3	2.70
	187	5.71	28.2	12.0	3.39
Creatinine (µMol/l)	58	82	274	179	45.3
	178	71	522	206	64.1
Glucose (mMol/l)	17	4.51	22.3	9.37	4.68
	183	2.89	17.2	7.11	2.44
Cholesterol (mMol/l)	33	2.80	5.92	4.14	0.838
	180	1.89	11.2	4.60	1.52
Triglyceride (mMol/l)	25	0.08	0.88	0.269	0.159
	107	0	1.28	0.328	0.194
Juvenile ALP (U/l)	23	18.1	549	153	146
	52	17	295	100	64
Adult ALP (U/l)	35	0.1	43	20.1	11.7
	124	0	54	19	10
AST (U/l)	30	17.6	79	32.2	14.2
	181	5	242	35.5	24.4
ALT (U/l)	57	28	173	51.3	22.3
	177	8	226	48.0	31.9
GGT (U/l)	51	0	9	1.53	2.34
	104	0	13	2.72	2.79
CPK (U/l)	19	104	1959	404	488
	119	43	4799	433	559
LDH (U/l)	11	1	702	157	190
	103	20	592	125	99.5
Amylase (U/l)	20	128	434	248	103
	80	40.7	527	170	76.2

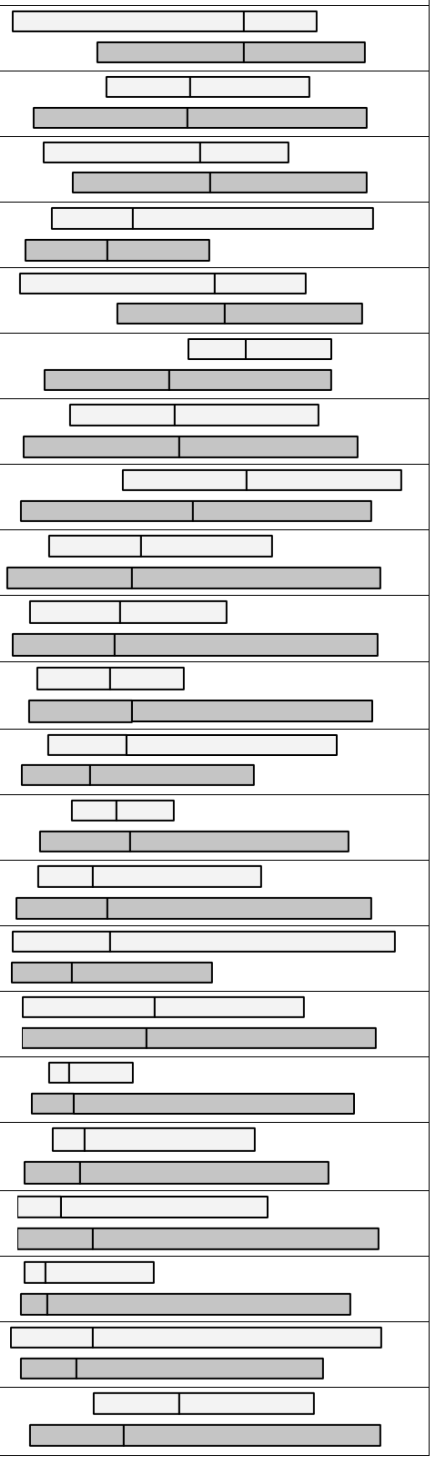

Figure 1. Graphical comparison of Amur leopard biochemistry data with Species 360 leopard biochemistry data. Amur leopard results are shown on top shaded pale grey and Species 360 data below shaded darker grey (I.S.I.S 2002). N=number of samples included, Min=Minimum value, Max=Maximum value, SD=Standard deviation. Bar charts to the right of the table show the data range, with divisions indicating the arithemetic mean. ALP=Alkaline phosphatase, AST=Aspartate aminotransferase, ALT=Alanine aminotransferase, GGT=Gamma glutamyltransferase, LDH=Lactate dehydrogenase, CPK=Creatinine phosphokinase.

	N	Min	Max	Mean	SD
RBC	42	6.01	10.7	8.35	1.12
(×10e12/l)	351	5.05	13.3	8.24	1.35
Hb	45	8.1	18.9	13.3	2.07
(g/dl)	369	8.6	18.0	12.4	1.62
HCT	48	30	51.7	41.3	5.37
(%)	436	23.4	52.0	37.1	5.22
MCV	36	40.8	59.6	48.9	4.47
(fl)	340	29.3	73.2	45.8	5.53
MCH	32	11.5	18.6	15.8	1.22
(pg)	337	10.1	24.6	15.3	1.73
MCHC	36	22.5	36.2	32.1	2.78
(g/dl)	35.8	23.7	42.9	33.4	2.77
WBC	45	6.98	19.7	11.8	3.05
(×10e9/l)	434	5.06	28.9	13.0	3.57
Neutrophils	42	5.04	15.5	9.16	2.74
(×10e9/l)	403	0.806	20.6	9.79	3.34
Lymphocytes	42	0.535	3.58	1.58	0.749
(×10e9/l)	406	0.158	9.51	1.79	1.07
Eosinophils	40	0	1.97	0.671	0.481
(×10e9/l)	348	0	5.65	0.611	0.636
Basophils	38	0	0.12	0.0171	0.0390
(×10e9/l)	60	0	2.30	0.146	0.3567
Platelets	31	254	658	430	88.2
(×10e9/l)	87	96	786	368	130

Figure 2. Graphical comparison of Amur leopard haematology data with Species 360 leopard haematology data. Amur leopard results are shown on top shaded pale grey and Species 360 data below shaded darker grey (I.S.I.S 2002). N=number of samples included, Min=Minimum value, Max=Maximum value, SD=Standard deviation. Bar charts to the right of the table show the data range, with divisions indicating the arithmetic mean. RBC=Red Blood Cell Count, Hb=Haemoglobin, HCT=Haematocrit, MCV=Mean Cell Volume, MCH=Mean Cell Haemoglobin, MCHC=Mean Cell Haemoglobin Concentration, WBC=Blood Cell Count.

proportion of outlying results for amylase prompted further investigation.

Only two animals had repeated amylase results available: ISB 352 and ISB 706. On both samplings, leopard 352 had amylase results within the normal range (214 U/l and 370 U/l), whilst in the case of leopard 706, the amylase result was high on both occasions (835 U/l and 1440 U/l). No relationship was identified with any of the other analytes. Similarly, no relationship was found with any of the laboratories, with high results being reported by three of the 13 external laboratories and a further three in-house processors. Furthermore, no association was found with any holding facility or geographic location.

Analysis of the Amur leopard EEP studbook suggested a degree of heritability in this elevated amylase trait. Unfortunately, biochemistry data were not available for all members of the studbook and, where biochemistry results were available, only 34 out of 58 reports included amylase. However, where both parent and offspring results were available, all (n=7) of the animals for which one or more parents had high amylase results, also had high amylase results (see Tables 4a and 4b). Additionally, two sires and one dam, for which amylase status was not known, had produced multiple offspring with high amylase, and in each of these cases all offspring for which amylase results were available appeared to be affected (see Tables 4a and 4b).

The ASVCP guidelines (ASVCP 2011) recommends a parametric approach to the calculation of reference intervals for normally distributed data where less than 40 results are available. If more than 40 results are available, either a parametric or robust approach can be taken. Seeking consistency, the authors elected to follow a parametric approach wherever possible. There were only two parameters, sodium and GGT, for which the alternative robust approach was necessary, due to the data following a non-Gaussian distribution.

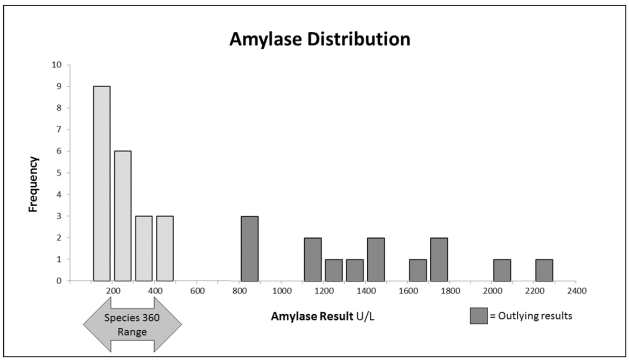

Figure 3. Histogram showing the distribution of Amur leopard amylase results. Results which were found to be outliers are shown in dark grey. The band labelled 'Species 360 Range' indicates the reported range of values given by the Species 360 database.

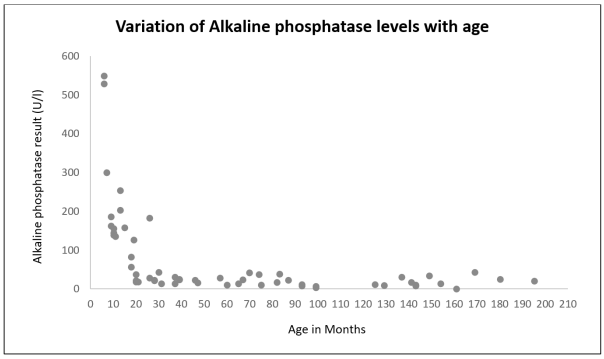

Figure 5. Scatter chart to investigate the relationship between alkaline phosphatase and age and determine appropriate partitioning boundaries.

It was not possible to calculate reference intervals for basophils and %basophils, despite meeting the requisite sample size. These distributions could not be transformed to normal and were unsuitable for a robust approach due to the high number of duplicate values (in this case 0) within the dataset. Four further parameters (magnesium, glucose, CPK and LDH) had insufficient sample numbers to calculate reference intervals, so for these only the summary data are given.

Several parameters showed significant differences between sub-classes using the Kolmogorov-Smirnov test. These were phosphorus, adults vs juveniles ($p=0.81\times10^{-2}$); calcium, adults vs juveniles ($p=0.83\times10^{-3}$); total protein, adults vs juveniles ($p=0.14\times10^{-2}$); albumin, adults vs juveniles ($p=0.038$); globulin, adults vs juveniles ($p=0.35\times10^{-4}$); urea, adults vs juveniles ($p=0.039$); creatinine, adults vs juveniles ($p=0.61\times10^{-2}$); alkaline phosphatase, adults vs juveniles ($p=0.78\times10^{-6}$) and juvenile males vs juvenile females ($p=0.2\times10^{-2}$); creatinine phosphokinase, adults vs juveniles ($p=0.028$); amylase, adults vs juveniles ($p=0.015$); and red blood cell count, males vs females ($p=0.0102$). Of these parameters, only six had sufficient numbers in each comparative sub-class to warrant further investigation into partitioning, namely: total protein, albumin, globulin, urea, creatinine and alkaline phosphatase (see Table 3). In all six cases, the difference investigated was between the adult and juvenile subclasses. Given the small sample sizes available, the need to partition was evaluated quite conservatively. Alkaline phosphatase (ALP) was the only parameter where the differences in the sub-class reference intervals were deemed clinically significant to support partitioning. It was felt that a failure to partition for this parameter could result in potential misdiagnoses. For example, due to high values in juveniles, the upper limit of the non-partitioned overall

Figures 4a and 4b. Investigating potential heritability of high amylase results. 4a: Lists the sires of all of the Amur leopards for which the amylase result is known. 4b: Lists the dams of all of the Amur leopards for which the amylase result is known. In both 4a and 4b: The number of progeny with known amylase results is given, as are the number of progeny with abnormal/high results. The amylase status of the dam or sire is indicated by highlighting the sire/dam ISB number either red (for high), yellow (for normal) or grey (for unknown). In this case, normal is taken to be a result of 600 or less and abnormal to be a result of over 600.

a)

Sire	No. of progeny	No. progeny abnormal	% Abnormal
135	2	1	50
136	1	0	0
164	1	1	100
193	2	0	0
204	2	0	0
211	2	1*	50
216	2	1	50
284	2	0	0
327	1	1	100
344	1	1	100
384	1	0	0
443	1	0	0
533	1	1	100
535	2	2	100
555	4	4	100
561	1	0	0
698	2	2	100
82	1	0	0

b)

Dam	No. of Progeny	No. progeny abnormal	% Abnormal
122	1	0	0
167	2	1	50
183	1	0	0
203	2	1	50
278	1	0	0
299	4	2**	50
356	2	0	0
376	2	0	0
383	1	0	0
422	2	2	100
536	1	1	100
560	1	0	0
562	1	1	100
574	1	1	100
587	3	3	100
615	1	0	0
643	2	2	100

Amylase status not known
Amylase of parent 'normal'
Amylase of parent 'abnormal'

*Dam was 422 which gave rise to 100% abnormal offspring
** Sire was 535 which had abnormal results and offspring

Where normal< 600 and abnormal>600

range was considerably higher than that of the adult range, which could potentially lead to elevated results in adults being interpreted as falsely normal or negative for disease (see Table 3). In the five other parameters, it was felt that the differences in ranges were comparatively small and unlikely to result in false interpretation by a clinician.

The age at which ALP values fall to adult levels is known to vary between species (Fernandez and Kidney 2007). As the Species 360 age partitioning boundary for leopards is neither ALP nor Amur leopard specific, the authors elected to adopt boundaries based on Amur leopard data. ALP levels were plotted against age in months (see Figure 4). As expected, ALP levels fell with increasing age until reaching a plateau. Based on the graphed data, a partitioning boundary of 30 months was selected, classing juvenile animals as those less than 2.5 years old and adults as 2.5 years old and over.

Discussion

In general, the Amur leopard dataset appeared to have narrower maximum–minimum ranges when compared to Species 360 data (Figures 1 and 2). There were however exceptions to this rule, including in the cases of sodium, phosphate, calcium and, most notably, juvenile alkaline phosphatase. Despite this variation in the spread of data, mean values were broadly in agreement, with exceptions being magnesium, albumin, amylase, HCT, basophils and juvenile alkaline phosphatase.

This outcome is not unexpected as Amur leopards represent a sub-set of the leopard species. As already described in the introduction, there are many morphological differences between the various leopard subspecies and therefore it would not be surprising if some variation was also apparent in their clinical biochemistry. As the Species 360 data are only categorised to species level, this dataset is likely to comprise results from several subspecies and possibly cross subspecies hybrids, and therefore greater variation may be expected. Furthermore, the Amur leopard data were subject to screening for outliers prior to this comparison, which may not have been the case for the Species 360 data. Therefore, there may still be anomalous results distorting the distribution of the Species 360 data, expanding the range and shifting the mean. This would certainly seem a likely explanation in the case of basophils, for example, where the mean total count for Amur leopards is severely offset to the left and the range is dramatically decreased as compared to the Species 360 data (see Figure 2). This is consistent with a small number of very high results in the Species 360 dataset, which may well be outliers that should be discounted. Further comparison using the median would aid interpretation, but unfortunately the authors did not have access to the full Species 360 dataset and Species 360 only published the summary data provided in Figures 1 and 2. However, Species 360 has provided partitioned data and it is worth noting that there do seem to be some inconsistencies and variations which would support the above theory. In the case of basophils, for example, the maximum values for the differing subclasses are given as follows: Males over two years = 0.38x109/l, Females over two years = 0.921x109/l, Males eight days to two years = 0.051x109/l, Females eight days to two years = 1.09 x109/l (Amur leopard basophil maximum value = 0.12x109/l). Even more interestingly combined values for males and females over two years were given as 2.30x109/l which is higher than either of the individual sex categories for this age range (I.S.I.S 2002). Eosinophil and lymphocyte total count results are further examples of the potential skewing of Species 360 data. In both cases, although the Amur leopard and Species 360 mean values are in close agreement, the Species 360 mean is significantly offset to the left of it's range, suggesting the Amur leopard data range is being artificially extended to the right by a small number of high-value outliers. When considering the Species 360 biochemistry results in Figure 1, aspartate aminotransferase (AST) and creatinine phosphokinase (CPK) show similar pictures. In the case of CPK, the Species 360 subclass for males over two years (adult males) had a maximum result of 5999 U/L which was more than double that of the next highest sub-class: females over two years (adult females) (2500 U/L) (I.S.I.S 2002).

Within the Amur leopard data there are two parameters worthy of further discussion, namely juvenile alkaline phosphatase and amylase. Alkaline phosphatase levels are known to be higher in young domestic animals, and similar patterns have been reported in other wild felids (Foster and Cunningham 2009, Garcia et al. 2010). In general, this difference is more pronounced in very young animals (Fernandez and Kidney 2007) and this certainly holds true for the Amur leopard data. Whilst the Amur leopard dataset did include the ages of all the animals sampled, the Species 360 dataset does not. It is, therefore, hard to say whether the two juvenile classes are comparable in terms of the distribution of ages of animals sampled. If they are not, this could explain the difference seen between the Amur leopard and Species 360 results. It is also possible that juvenile Amur leopards do have higher alkaline phosphatase levels, but further work would be necessary to confirm it.

The amylase results are perhaps the most interesting. Initial queries as to whether this may be due to differences in laboratory processing were discounted as the majority of samples were processed at the same laboratory. Idexx Laboratories at Wetherby (U.K.) ran 25 of the 36 amylase assays reported and produced both 'abnormal' and 'normal' results. Additionally, multiple other laboratories reported elevated results. Another question is whether these amylase levels are transiently high at the time of sampling or a persistent state in these animals. There are insufficient data to draw conclusions on this point, although the only animal which had high amylase and was sampled twice had high values on both occasions. These two samples were processed by separate laboratories and the animal was resident at different facilities at the two sampling times, suggesting for this animal at least, that it is a consistent finding. The clinical significance of this amylase level remains unclear. At the time of writing, there is no evidence that any of the animals with high readings were clinically affected in any way. One animal known to have elevated amylase levels died post sampling, ISB 678. Unfortunately, as the animal died whilst undergoing a caesarean section, the cause of death was presumed to be anaesthetic-related and no post-mortem results have been reported. It would be useful in future for institutions holding leopards with abnormal/high amylase results to be made aware of the uncertainty regarding the potential implications of this amylase status, and for thorough post-mortem examinations to be carried out upon death, including histopathology of the pancreas, liver and small intestine.

The suggestion of heritability, with regard to amylase levels, could be significant in terms of the ongoing breeding programme, regardless of any direct impact on health. It is possible that this anomaly, and the scale at which it appears to be present, indicates the accumulation of deleterious recessive genes, as was seen in the case of the Florida panther (Roelke et al. 1993), or the over-representation of a few genetic lines. Further investigation is therefore warranted. Initially, this could take the form of simply maximising existing sampling by ensuring all future Amur leopard samples are submitted for amylase assays, ideally at a nominated laboratory.

Many of the limitations of this study stem from its retrospective nature. Sampling methodology was not standard, nor was sample handling necessarily so. Multiple laboratories were used to analyse samples, and even where the same laboratory was used, samples were analysed over such a long period of time that it is likely the

processors within the facilities will have been changed and/or upgraded. With small populations, such as the Amur leopard, it is likely that an extended sampling period will always be necessary to achieve sufficient sample sizes opportunistically. However, as co-operation between zoological institutions increases, it is possible to reduce these limitations in future studies. Organisations, such as EAZA, could facilitate ongoing research and co-operation between zoos by advocating standardised procedures or nominating specific laboratories for analyses. Similarly, within institutions, sampling opportunities should be maximised, not only to contribute more data to the species pool, but also to create a baseline database of results for individuals.

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