PhD Thesis

Techniques to assist conservation breeding of the babirusa (*Babyrousa celebensis*)

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DECLARATION

I declare that this thesis has been composed by me and that the work of this thesis is entirely my own, including generation of all raw data and interpretations of that data, with the exception of:

1) the raw studbook data spanning 1972 to the end of February 2008, kindly supplied by Thomas Kauffels of the Opel Zoo, Germany.

2) the raw genetic data for the European (EEP) and Surabaya populations (listed in ***Appendices 2*** and ***3***) and the wild samples referred to in ***Table 28***, which were generated by the Centre for Research and Conservation at the Antwerp Zoo, Belgium, and kindly supplied by Dr Peter Galbusera of the CRC.

3) the raw steroid data for the North American babirusa females ('Greta' and 'Beret', presented in ***Figures 72***, ***73***, ***80*** and ***81***), which were generated by Dr Joan Bauman of the St Louis Zoo, Illinois, USA, and kindly made available by her for inclusion in this thesis.

4) the raw steroid data for the comparison of methods (presented in ***Figures 67***, ***68*** and ***69***) which was generated and kindly made available to this thesis by Prof. Dr Franz Schwarzenberger of the University of Vienna, Austria.

The work of this thesis has not been submitted for any other degree of qualification except for this PhD. However, the following figures were replicated from my MSc thesis, "The Babirusa: A Pig Like No Other", submitted to the University of Edinburgh in 2004 and are not new to this PhD thesis: ***Figure 7***, illustrating the proposed phylogeny of the suidae; ***Figures 50***-***51*** illustrating steroid structures and ***Figure 52*** showing steroid interconversions; ***Figures 61*** and ***66***, showing the major

metabolites of progesterone breakdown, ***Figure 70*** showing conjugation points for creating group-specific or progesterone-specific antibodies; and ***Appendix 5***, a table listing reproductive parameters for various pig species. Each of these is either original to the MSc thesis or was modified from the original source (as cited in the text) then presented in the MSc thesis.

Signed: Sharron Ogle

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ABSTRACT

With the current rate of loss of biological diversity worldwide estimated at 100-1000x the natural background rate, solutions are urgently needed to avoid a catastrophic and irreversible loss of species. Conservation breeding is a tool now widely used to assist in maintaining populations in a safe environment until such time as they can be released back into their natural habitat.

The babirusa (*Babyrousa celebensis*) is a threatened species for which conservation breeding is an integral part of its conservation management. Native to a few Indonesian islands, the babirusa is under threat from habitat destruction and hunting and is estimated to have a wild population of only a few thousand. Conservation breeding was established in 1972 with the translocation of animals from Sulawesi to the Surabaya Zoo in Java, Indonesia. It is thought that 1.2 animals founded what is now a worldwide *ex-situ* population of around 200 individuals. With concerns mounting regarding likely levels of inbreeding and reduced reproductive success, particularly within the European population, this thesis aimed to utilise a number of techniques to assist the conservation breeding programme.

Extensive studbook analysis was used to assess typical reproductive parameters for the babirusa in a captive setting; genetic analysis using microsatellite and mitochondrial DNA markers was used to clarify some aspects of the studbook and to estimate the level of variation within and between populations in different geographical areas; and faecal steroid analysis has been used to shed light on the physiological processes underpinning female reproduction. The combined output from these studies has added to our knowledge of this species and its performance in

an *ex-situ* setting, and has now been used to inform future management of the conservation breeding programme.

GLOSSARY and ABBREVIATIONS

Adaptation	The changes in individuals/populations developed in response to changes in their environment
Adaptation to Captivity	The selection of characteristics most suitable to a captive breeding situation, which may be detrimental to individuals on release
Allozymes	Different versions of the same protein, used to indirectly assess genetic diversity
Anthropogenic	Caused by humans
ARKS	Animal Record Keeping System, a database system developed by ISIS to record information about individuals. Widely used in zoos and aquaria
AZA	Association of Zoos and Aquaria, the professional body advising zoos and aquaria in North America
AI	Artificial Insemination
BIAZA	British and Irish Association of Zoos and Aquaria, the professional body advising zoos and aquaria in the UK and Ireland
Biodiversity	The amount of biological variety present in a habitat, usually measured as numbers and density of species present
Bottleneck	Reduced genetic diversity resulting from a significant reduction in population size, either through demographic effects or the founder effect
CITES	Convention on the International Trade in Endangered Species, a global agreement to limit movement of endangered species in a bid to limit their trade
CL	Corpus Luteum, the progesterone-producing post-ovulatory follicle of the ovary
Conservation Breeding	The breeding of threatened species in an *ex-situ* environment
D-loop	The triple-stranded section of the control region of mitochondrial DNA

Deleterious Alleles	Recessive alleles that reduce individual fitness when expressed as homozygotes. The principal factor in inbreeding depression
EAZA	European Association of Zoos and Aquaria, the professional body advising zoos and aquaria in Europe
EEP	European Endangered Species Programme, the structure used by European zoos and aquaria to manage threatened species *ex-situ*
EIA	Enzyme-immunoassay, a technique that can be used to assay the steroid content of faecal extracts
Electrophoresis	A method to separate DNA strands by size/charge
ESB	European Studbook, the lowest level of population management used for less threatened species within European zoos and aquaria
Estrous cycle	The cyclical changes in the female reproductive system, driven by changing levels of FSH and LH
Estrus	The physical and behavioural signs of a female around the time of ovulation, sometimes referred to as 'heat'
Estrogens	Multiple 18-carbon sterol-based hormones that act at the estrogen receptor
ESUs	Evolutionary Significant Units, populations deemed to be unique enough and to represent significant adaptive variation, regardless of species or sub-species divisions
Effective Population size	The number of individuals available to contribute to breeding in a given population, typically much smaller than census size
***Ex-situ* Conservation**	Any conservation activity that occurs outside the natural range of a species
Extinction vortex	The irreversible decline in population size driven by multiple cumulative factors
f* or *F	Inbreeding coefficient
***F*-statistics**	Used to assess the heterozygosity of a population as a product of its individuals and sub-populations
Fecundity	The lifetime potential of an individual to reproduce

Fitness	The ability of an individual or species to survive
Follicular phase	The estrogen-dominated phase of the estrous cycle, where follicles mature in response to circulating FSH
Founder	An individual used to establish a conservation breeding programme
Founder Assumption	The assumption that founder animals are unrelated to each other
Founder effect	The inevitable loss of alleles when a population is sampled with insufficient founder numbers
FSH	Follicle Stimulating Hormone, acts on the ovary to promote follicle development
GC	Gas Chromatography, a technique used to separate a mixture of molecules. Often used before MS analysis
Gene pool	The combined genetic content of an entire population
Genetic drift	The changes in the gene pool of a population through generations, driven by random allele sampling during meiosis
Genetic rescue	The recovery of a population driven by the immigration and cross-breeding of new unrelated individuals
Genotype	The alleles present at a given locus/multiple loci
GnRH	Gonadotrophin Releasing Hormone, is produced in the hypothalamus and stimulates release of LH and FSH from the Anterior Pituitary
Hardy-Weinberg Equilibrium	A description of expected heterozygosity at a locus based on the number and frequency of alleles present
Heteroplasmy	The existence of more than one mitochondrial haplotype within a single individual
Heterozygote	Where two different alleles are present at a given locus
Homozygote	Where two identical alleles are present at a given locus
HPA	Hypothalamo-Pituitary Axis, the interconnected components related to control of reproductive function

HPLC	High Performance Liquid Chromatography, a technique used to separate a mixture of molecules. Often used before MS analysis
Inbreeding	The breeding of closely related individuals
Inbreeding Coefficient	A measure of how inbred an individual or population is
Inbreeding Depression	The reduced 'fitness' of an individual or population as a result of inbreeding
ISIS	International Species Information System, an organisation who have developed multiple software tools for record-keeping and population analysis
IUCN	International Union for the Conservation of Nature
Large allele Drop-out	Where smaller alleles are preferentially amplified over large ones, resulting in incorrect scoring of homozygotes at that locus
LC	Liquid Chromatography, a technique used to separate a mixture of molecules. Often used before MS analysis
LH	Luteinising Hormone, acts on the ovary to induce ovulation and promote formation of the corpus luteum
Luteal phase	The progesterone-dominated phase of the estrous cycle, where LH stimulates the conversion of ovulated follicles to CLs
mtDNA	Mitochondrial DNA, circular DNA found only in the mitochondria, useful for assessing maternal lineages in pedigrees
MS	Mass Spectrometry, a technique used to measure the size of molecules (often in conjunction with GC, LC or HPLC)
msats	Microsatellites, short sections of non-coding, repeating DNA useful for assessing genetic diversity
Meta-population	A population consisting of multiple discrete fragments with limited connectivity
MK Strategy	Mean Kinship Strategy, the most effective way of limiting loss of genetic diversity from a population, by pairing least related animals for breeding

Mutation	A change in the genetic code
MVP	Minimum Viable Population, the population size required for long-term survival
N_e	Effective population size
N_e / N	Ratio of effective population size to the census size
Natural selection	The persistence of individuals most suited to their environment
Niche	The combined characteristics of a habitat that best suit a population or species
Null alleles	Alleles not amplified as normal due to mutation(s) in the flanking sequence so primers cannot anneal. Results in incorrect scoring of homozygotes
P4	Progesterone
Paternal leakage	The abnormal release and persistence in the zygote of mitochondria derived from the fertilising spermatozoan
PCA	Principle Component Analysis, a statistical technique used to assess the relative importance of multiple variables on an output or effect
PCR	Polymerase Chain Reaction, a technique used to amplify the DNA content of a sample
Pedigree	A representation of all the individuals within a population showing their relationships with each other
PHVA	Population and Habitat Viability Assessment, an integrated approach to assessing the risks to, and plan the conservation of, wild populations
PM2000	Population Management 2000, a software tool from ISIS used to model the effects of various factors on population survival
PMP	Population Management Plan, a tool used in North American zoos and aquaria to assist management of threatened species. Equivalent to the ESB
Primers	Short DNA sequences used amplify a section of target DNA by flanking the region of interest

Progestagens	Multiple 21-carbon sterol-based hormones that act at the progesterone receptor
Recombination	The creation of new allele combinations at meiosis
Red List	Short name for the IUCN Red List of Threatened Species™, the function of which is to assess the extinction risk of all species for which sufficient data is available
RIA	Radio-immunoassay, a technique that can be used to assay the steroid content of faecal extracts
SB#	Studbook number
SEAZA	South-East Asian Association of Zoos and Aquaria, the professional body advising zoos and aquaria in the SE Asian region
SPARKS	Single Population Analysis and Records Keeping System, a database system developed by ISIS, used to combine and analysis data from populations held by zoos or aquaria
SSC	Species Survival Commission, a division of the IUCN
SSP	Species Survival Plan, the North American version of an EEP in which threatened species are managed in zoos/aquaria
Stochasticity	Chance events that can have negative effects on populations, usually categorised as demographic, genetic or catastrophic
Studbook	A list of all individuals within a breeding programme
TAG	Taxon Advisory Group, a group of experts who advise on the management of threatened species within ESBs and EEPs
Threatened Species	A species considered to be at risk of extinction
WAZA	World Association of Zoos and Aquaria, the overarching professional body advising zoos and aquaria worldwide
WZACS	World Zoo and Aquarium Conservation Strategy, the 2005 document of WAZA outlining the role of zoos and aquaria in conservation and the ways is which they can contribute
ZIMS	Zoological Information Management System, the new online database programme developed by ISIS.

Chapter 1: Introduction

SPECIALISATION vs. ADAPTIVE POTENTIAL

Basic processes of evolution have led to innumerable paths of species diversification over millions of years. In many cases, species have become highly specialised to specific environmental conditions and habitats. In doing so, this may leave them vulnerable to change, with insufficient time or genetic capacity to adapt.

For each species, and each population, there is a balance between specialisation to take advantage of a productive niche and retaining the potential for diversification in new directions. This balance is determined by the genetic diversity contained within the species/population of interest, as genetic diversity provides the potential for adaptation to change. The gene pool of a species or isolated population is all that is available (excepting mutation) to allow adaptation to a changing environment and so to avoid extinction.

THE GLOBAL PERSPECTIVE

It is now widely recognised that the earth is experiencing an unprecedented loss of biodiversity, sometimes referred to as a the 'sixth extinction' (Frankham, Ballou et al. 2002). This is in reference to previous mass extinction events such as that which caused the loss of the dinosaurs. Even outside of these large-scale events, individual species extinctions have always been a natural part of the evolutionary process, with some adapting to new environments and others failing to thrive. The difference today is that the loss of numbers of species is far in excess of the origin of new ones, and the contributory factors are not part of the natural cycle of the earth, but are primarily anthropogenic (Lande 1998; Frankham, Ballou et al. 2002).

On March the 17th 2009 at 14.24 and 50 seconds, the world's human population was estimated on the Berkeley Population Clock at 6,735,652,141, i.e. 6.7 billion (http://math.berkeley.edu/~galen/popclk.html). This number is the result of the almost exponential growth in the human population since the Industrial Revolution (Anon 1999), and is projected to continue for decades to come (***Figure 1***).

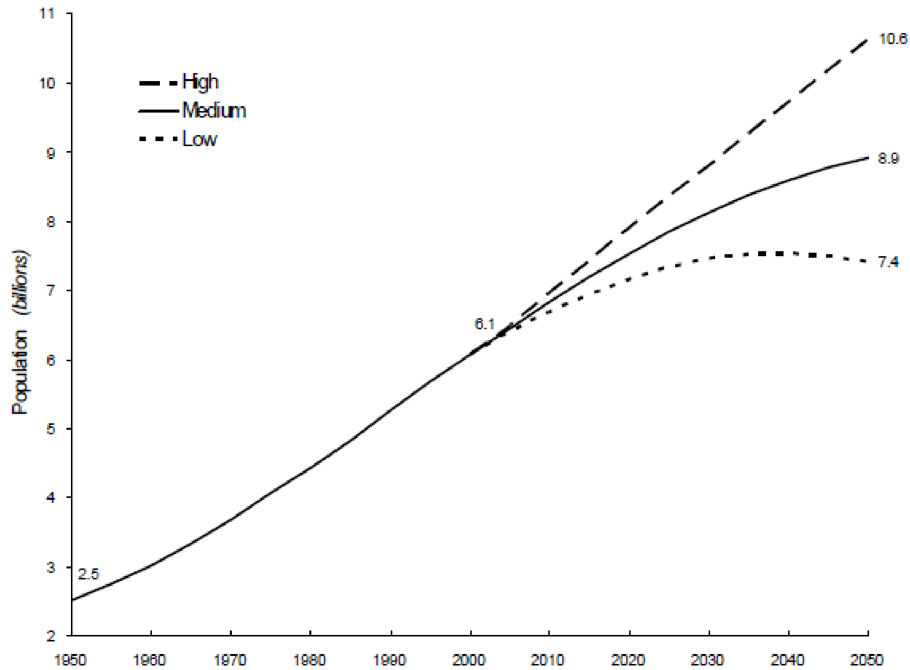


***Figure 1*:** Estimated world population, 1950-2000, and projections based on continued 'Low', 'Medium' or High' population growth: 2000-2050 (Anon 2004).

Anthropogenic effects on non-human species

The effects of this overwhelming human 'mono-culture' on the survival of non-human species act through various mechanisms, from deforestation and land use conversion to pollution, exploitation and over-hunting (Lande 1998). Ultimately, these factors can all result in the decline of non-human species either *directly* by reducing population size or by limiting its potential for growth, or *indirectly* through

habitat fragmentation resulting in meta-populations where contact between sub-populations is limited or impossible. There is now the added impact of climate change to consider, with a proposed increase in global temperatures of at least 2°C by the middle of this century (Kerr 2007). In reality, multiple factors often act together to threaten species survival (Crandall 2009), and they affect small populations the hardest.

SMALL POPULATIONS

There are many reasons why some populations are 'small', not just those mediated by human activities. Perhaps they have adapted to a specialised niche environment and have a limited range, perhaps they are an island species, or perhaps their historically 'large' population has been fragmented through geological processes. Whatever the reason(s), small populations are at greater risk of extinction than equivalent larger ones (Frankham, Ballou et al. 2002; Reed, O'Grady et al. 2003).

The problem with small populations

Although a small population may be very well adapted to its niche habitat, and have survived in that habitat for considerable periods of time, the disadvantage it has over a larger population is it's relative lack of genetic diversity (Frankham, Ballou et al. 2002). The positive correlation between population size and genetic diversity has been illustrated repeatedly in a wide range of taxa and species, as reviewed by Frankham (Frankham 1996).

In general, threatened species are estimated to have only half the genetic diversity of their non-threatened counterparts (Frankham, Ballou et al. 2002) and, as mentioned above, genetic diversity is the key to adaptability in the face of changing habitats.

With limited genetic diversity, a species or population is at greater risk of extinction through multiple inter-related mechanisms. The following illustrates the key parameters thought to impact on the ability of a population to endure in its native range and to ultimately avoid extinction.

Genetic drift

Genetic drift is the inevitable loss of rare alleles during the transition from generation to generation, and has a greater effect on smaller populations (Frankham, Ballou et al. 2002). The random sampling of parental alleles at meiosis means that some alleles will fail to be passed on to offspring (with rare alleles more susceptible to being lost). The result is an overall reduction in total allele numbers and also in genetic diversity, driven by chance 'drift' rather than selection.

Inbreeding

Inbreeding is essentially defined as the breeding of closely related individuals. In more technical terms, it is a measure of the likelihood that individuals share identical alleles by descent, i.e. they share a common ancestor from whom they have both inherited the same genes. The level of inbreeding is usually expressed as the inbreeding coefficient (f), ranging from 0 to 1.0, and reflects the level of relatedness between the parents of an individual. For example, a few benchmark values related to specific inbreeding events, are described in ***Table 1***.

Inbreeding coefficients are routinely used to describe both individuals (f) and populations (mean f). Small populations are at risk of inbreeding as there is a greater likelihood that breeding pairs will be related to each other. Eventually, all individuals in the population will be related to one another (i.e. the mean f will

increase) unless new individuals migrate into the population and successfuly breed. Under 'normal' circumstances, many species employ behavioural mechanisms to avoid inbreeding (Pusey and Wolf 1996) and only do so when there is no alternative (Vila, Sundqvist et al. 2003).

Inbreeding coefficient (*f*)	Parental relationship
0.0625	First cousins
0.125	Half-siblings
0.25	Full-siblings or parent-offspring
0.5	Self-fertilization*

Table 1: Examples of individual inbreeding coefficient (f) values in relation to the specific parental relationship they represent. *Note that this occurs only rarely in animals but is common in many plant species.

Inbreeding depression

Inbreeding depression refers to the reduced ability of individuals or populations to survive, i.e. reduced 'fitness' as a result of inbreeding (Frankham, Ballou et al. 2002), and has greater effects on small, fragmented populations (Dudash and Fenster 2000). There are many proposed mechanisms to explain how inbreeding can lead to inbreeding depression, but it is primarily thought to be the result of the expression of deleterious alleles (Charlesworth and Charlesworth 1999). The effects of inbreeding depression can be expressed in a number of ways, and can be measured either at the individual or population level. Individuals may have reduced fecundity at various life stages (Koeninger-Ryan, Lacy et al. 2003), reduced survivorship or lifespan (Ralls, Brugger et al. 1979; Koeninger-Ryan, Lacy et al. 2003; Charpentier, Williams et al. 2008), reduced reproductive success (Koeninger-Ryan, Lacy et al. 2003; Asa, Miller et al. 2007), reduced immuno-competence (Sanjayan, Crooks et al. 1996; Charpentier, Williams et al. 2008) and/or increased parasitic loads (Charpentier,

Williams et al. 2008). At the population level, inbreeding depression can lead to reduced growth rates (Reed, Nicholas et al. 2007) and so to decreasing population size and also to increased risk of extinction (Frankham 1995; Frankham 1998; O'Grady, Brook et al. 2006), although there is a need to further understand the links between individual effects of inbreeding and how much they impact on populations (Keller, Biebach et al. 2007). There is evidence to suggest that, at least in mice and *Drosophila*, extinction risk is increased with mean inbreeding coefficients beyond 0.25 (Frankham 1995).

Increased susceptibility to stochastic effects

Small populations are more susceptible to stochastic (chance) effects (Frankham, Ballou et al. 2002). In addition to the incidence of genetic drift, inbreeding, inbreeding depression or new deleterious mutations (genetic stochasticity), small populations can be affected by fluctuations in population size (demographic stochasticity), by changes in biotic or abiotic factors (environmental stochasticity), or by the incidence of catastrophes such as extreme environmental events or disease outbreaks. With fewer individuals in a population, small losses can have disproportionately large effects and can be difficult to recover from, especially if multiple effects are combined.

Extinction

It is estimated that the current extinction rate is 100-1000 times that of the historical norm (Pimm, Russell et al. 1995). Although this is undoubtedly driven by multiple anthropogenic effects as previously described, the reduced fitness of genetically impoverished individuals may contribute to the terminal decline in population sizes,

speeding up the process of extinction. Continual erosion of population size, and the resulting increases in genetic drift, inbreeding and inbreeding depression which cause further losses all feed into the 'extinction vortex' (Gilpin and Soulé 1986; Frankham, Ballou et al. 2002), illustrated in ***Figure 2*** from (Frankham, Ballou et al. 2002).

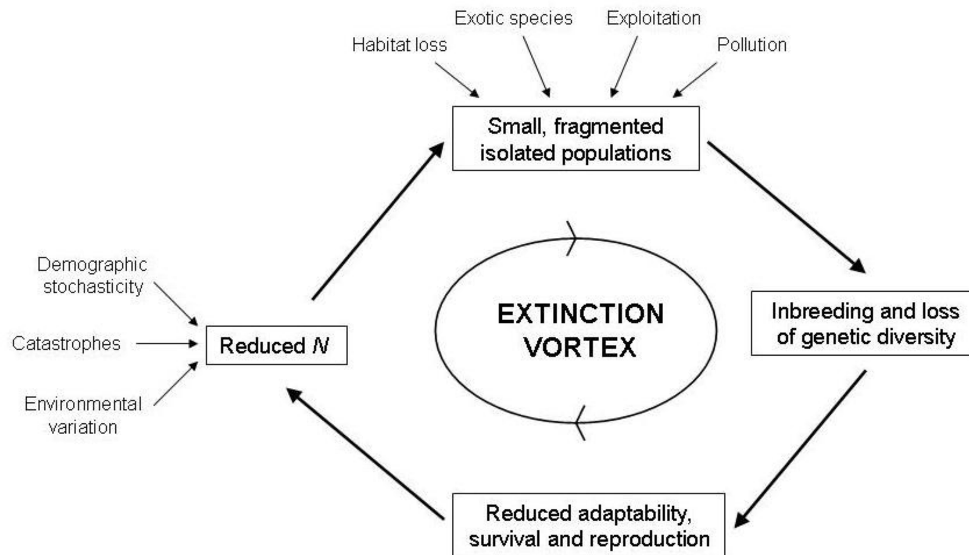


Figure 2: the 'extinction vortex' showing the contributory factors that can contribute to the demise of a population/species and how they are related to one another, reproduced from (Frankham, Ballou et al. 2002).

There is still debate however, over just how important this genetic element is in the big picture of species declines, with some authors arguing that anthropogenic effects will take hold long before genetic effects do (Lande 1988; Caro and Laurenson 1994). Although this is undoubtedly a significant factor, there *are* some extreme examples of populations where their decline to near-extinction has been halted and/or reversed by the addition of new genetic diversity i.e. 'genetic rescue' (Westemeier, Brawn et al. 1998; Vila, Sundqvist et al. 2003; Hogg, Forbes et al. 2006), suggesting that genetic factors must have been important in the decline of these populations.

In reality, of course, there is likely to be some degree of interaction between anthropogenic and genetic effects (Lande 1998; Liao and Reed 2009), so any conservation strategy should include measures related to both. The Population and Habitat Viability Assessment (PHVA) process is designed to do exactly that, where all threats to survival are assessed for a particular species/population and a holistic solution is offered to address the problem of decline in wild populations (Frankham, Ballou et al. 2002).

How small is too small?

The minimum viable population size (MVP) required for long-term survival depends very much on the characteristics of the species. In particular, the level of genetic diversity present in the population, and the ability for the population to grow will determine how likely a population is to survive in its ever-changing niche environment. In terms of how big a population needs to be, it is not so much the census size (N) that is important, but rather the 'effective population size'.

Effective population size (N_e)

Effective population size (N_e) is a measure of the size an idealised population would have to be to lose genetic diversity at the same rate as the actual population (Frankham, Ballou et al. 2002). Put another way, it can be seen as the number of individuals who can contribute genetically to the continuation of the population, i.e. how many are available to breed at any given time. N_e is almost always smaller than census N as not all individuals are available to breed, and that proportion is expressed in the ratio N_e / N. A meta-analysis by Frankham of 47 wild populations calculated a mean N_e / N ratio of only 0.11, and the major factors affecting the ratio

N_e / N are fluctuations in population size, variation in family size, and unequal sex ratio, with decreasing order of importance (Frankham 1995).

CONSERVATION BREEDING

Conservation breeding is the breeding of endangered or threatened species in an *ex-situ* setting (www.biaza.org.uk, accessed Jan 2009). It is the 'endangered' or 'threatened' nature of the species that sets *conservation* breeding apart from any other type of breeding programme, and applies to species at risk of extinction in their wild habitat.

The aim of conservation breeding programmes is to maintain a suitably large and genetically diverse population such that it could be re-introduced to the wild as it becomes safe to do so. For conservation breeding to be successful, it should involve effective sampling of the wild population, propagation of that sample population in a safe *ex-situ* environment until the risk of extinction is reduced, then reintroduction of genetically uncompromised, healthy individuals back to their wild habitat (Ebenhard 1995). For these goals to be achieved, a sufficiently large sample should be taken from across the full range of the species to avoid the founder effect, a strictly managed breeding strategy should be employed to avoid the loss of rare alleles and maintain equal founder representation, and generation numbers in captivity should be minimal to reduce the risk of adaptation to the captive environment before release (Frankham 2008).

Recommended N_e

So, how many individuals are required to achieve the optimal outcome, particularly with respect to retaining genetic variation and adaptive potential? Well, it is

recommended that “to avoid inbreeding depression and retain fitness in the short term, N_e >>50 is required. For threatened species to permanently retain their evolutionary potential N_e of 500-5000 is required.” (Frankham, Ballou et al. 2002). Using a mean N_e / N value of 0.11 it is clear that recommended mean census populations sizes should be >>450 individuals in the short term, and 4500 - 45000 for long-term survival and genetic health.

Why is conservation breeding important?

In cases where population sizes have declined to a point where extinction is a real possibility, efforts have been made to maintain these ‘threatened’ species in protected, *ex-situ* conservation breeding programmes. Early examples of these were for Père David’s deer (*Elaphurus davidianus*) (Zhiganag and Harris 2008) and the Scimitar-horned Oryx (*Oryx dammah*) (IUCN SSC Antelope Specialist Group 2008), both of which now exist only in captivity. Another example is that of the Przewalski’s horse (*Equus ferus przewalskii*), the current living population of which is thought to have been established from thirteen founder animals (Frankham, Ballou et al. 2002). An International Studbook was produced in 1959, followed by the North American Species Survival Plan (SSP) in the 1970s and the European Endangered Species Programme (EEP) in 1986. The population numbered 1,872 in early 2008, and there have been multiple reintroductions back into their native Mongolian range as well as the establishment of large, protected herds elsewhere (van Dierendonck and de Vries 1996; Boyd, Zimmerman et al. 2008). Without conservation breeding, these three species would now be lost entirely.

Conservation breeding is becoming more important as the number of species at risk of extinction in the wild continues to increase (IUCN 2008). Conservation breeding programmes should avoid the need for removing unlimited numbers of animals from the wild by creating sustainable and healthy populations in a captive setting (www.biaza.org.uk, accessed Jan 2009).

What defines a 'threatened' species?

The status of a species in the wild is assessed periodically by the International Union for Conservation of Nature (IUCN), and listed in the 'IUCN Red List of Threatened Species™'. The most recent species assessment was completed and published at the end of 2008 (www.iucnredlist.org). The goal of the Red List is to assess the relative risk of extinction for plant and animal species and to catalogue and highlight those at greatest risk (www.iucnredlist.org/static/introduction, accessed Jan 2009).

According to the 2001 Categories and Criteria (Version 3.1) documentation (www.iucnredlist.org/static/categories_criteria_3_1#categories), species are assessed on the basis of a number of criteria:

A. Declining population (past, present and/or projected)

B. Geographic range size, and fragmentation, decline or fluctuations

C. Small population size and fragmentation, decline, or fluctuations

D. Very small population or very restricted distribution

E. Quantitative analysis of extinction risk (e.g. Population Viability Analysis)

Each criterion is sub-divided into risk categories, defined in each case by specific parameters such as known population size or geographical range. The risk categories are shown in ***Figure 3*** (from: www.iucnredlist.org/static/introduction). You can see

that the ‘Threatened’ category includes the three sub-categories of ‘Vulnerable’, ‘Endangered’, and ‘Critically Endangered’, with increasing risk of extinction.

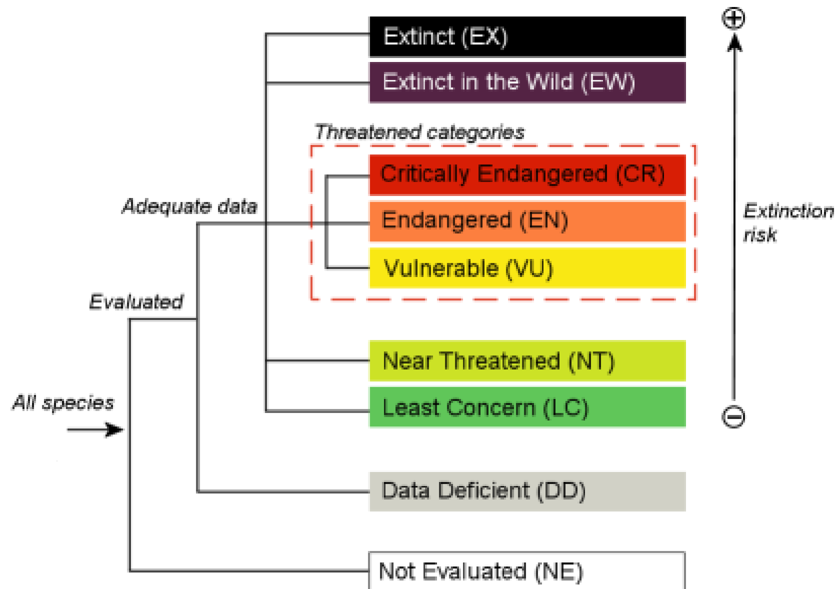


Figure 3: Categories of threat to species survival based on risk of extinction, as described in the IUCN Red List of Threatened Species™.

The overall status of a taxon or species is defined by the highest level of risk as identified in any of the criteria A-E. Full details of the definitions for each risk category for each criterion can be found in ***Appendix 1*** [Table 2.1 in ‘Guidelines for Using the IUCN Red List Categories and Criteria, Version 7.0 (August 2008)].

The role of zoos in conservation breeding

Mission statements for zoos and animal parks often cite their key functions relating to education, conservation and research, as well as their more traditional role as visitor attractions (Hosey, Melfi et al. 2009). Conservation breeding programmes are an obvious and important way zoos can contribute to *ex-situ* conservation and this is

a key recommendation of The World Zoo and Aquarium Conservation Strategy (WZACS) (WAZA 2005). Increasingly, conservation breeding involves the co-ordinated management of a species between large numbers of zoos and/or animal parks. These institutions can provide the space, plus husbandry and veterinary expertise to maximise the success of conservation breeding and through their role as educators can inform the public of the threats faced by species in the wild.

There are of course limitations to what zoos can offer in terms of conservation breeding. The most recent Red List cites almost 6000 vertebrate species classified as 'Threatened', representing 10% of all described species (IUCN 2008). Clearly, with the huge census sizes required to ensure long-term genetic potential of each population (as outlined above), it is beyond the reality of what zoos can offer to maintain all threatened species long-term (Balmford, Mace et al. 1996). Because of this, zoo-based conservation breeding is typically based on "populations of a defined size that are managed to ensure a viable population size, with 90% genetic diversity existing for the next 100 years" (Lees and Wilcken 2009), www.biaza.org.uk, accessed Jan 2009.

How is conservation breeding managed?

Management of conservation breeding can occur at a number of geographical levels, overseen by the relevant zoo association, for example BIAZA (the British and Irish Association of Zoos and Aquaria), EAZA (the European Association of Zoos and Aquaria), AZA (the Association of Zoos and Aquariums, for North America), SEAZA (the South-East Asian Zoos Association) or even WAZA (the World Association of Zoos and Aquariums).

The active management of an *ex-situ* population should aim to build sustainable populations that don't require continuous removal of new stock from the wild (www.biaza.org.uk, accessed Jan 2009). It is recommended by the IUCN that conservation breeding should be instigated before the wild population falls below 1000 individuals (IUCN 1987), although this has rarely been the case (Frankham, Ballou et al. 2002). As mentioned above, an N_e of >>50 (mean census size >>450) is required in the short-term to maintain the genetic health of a population, but many populations at risk have census sizes below this at the time of intervention, making genetic deterioration almost inevitable for many threatened species (Frankham, Ballou et al. 2002).

The level of management of a conservation breeding programme depends heavily on the ultimate aim of the programme (www.waza.org , accessed Jan 2009). In some cases, the aim is to simply maintain good records of individual life-histories and breeding events. In European zoos, this type of management is done through a European Studbook (ESB), and in North America through Population Management Plans (PMP), with some species also under the management of an International Studbook. For species under greater threat in the wild where long-term *ex-situ* breeding is anticipated, or re-introduction is likely the studbook will be actively managed for reproductive success and genetic health of the population. This is usually by through multi-institutional European Endangered species Programmes (EEPs) in Europe or Species Survival Plans (SSPs) in North America on the recommendations of dedicated Taxon Advisory Groups (TAGs) and/or Scientific Advisory Groups.

Record-keeping

With potentially large numbers of animals in any conservation breeding programme, record-keeping is vital to success. Historically, animal records have been kept in inconsistent ways, with the recording method and level of detail dependent to a large extent on the interest and motivation of individual institutions and animal keepers. This has improved with the use of database software such as those created by International Species Information System (ISIS, www.isis.org) e.g. ARKS, SPARKS and most recently ZIMS.

ARKS (Animal Records Keeping System)

This software is used for recording details of individuals in a zoo collection, added to and used by keepers and animal managers in only that institution. Typically it includes behavioural, management and veterinary notes as well as animal identifiers, parentage and significant dates. An example ARKS report for an animal in the Singapore Zoo is shown in ***Figure 4*** below.

G4498 ***Babyrousa babyrussa*** **IZY VulnerableApp.** **Babirusa**

Date in	Acquisition - Vendor/local Id	Holder	Disposition - Recipient/local Id	Date out
5 Dec 1996	Birth	SINGAPORE	Traded to PRETORIA-907881	26 Jan 2000

Sex-Contraception	Female -	Birth type:	Captive Born
Hybrid status	Not a hybrid	Birth Location:	Singapore Zoological Gardens
Enclosure	REMOVED	Birthdate-Age:	5 Dec 1996 - 3Y,1M,21D at transfer
Sire		Dam	G1901 at SINGAPORE
Rearing:	Parent	Transponder ID:	00-0113-BF68
House Name:	Kumai	Global Studbook #:	0305
Studbook Name:	Sin 29		

22 Jan 1997	Sex Modification Log	Sex Code Changed From Unknown to Female
10 Jan 1998	Medical procedure	Dewormed Fenbendazole 10mg.kg bw (Vet-Oh)
29 Oct 1998	Body Temperature	39 Skin 37
22 Jan 2000	Animal management note	Awaiting for export. [[UM]]

Figure 4: An example of an entry in an ARKS report for a babirusa held at Singapore Zoo showing details of the individual and of a few events during her stay.

SPARKS (Single Population Analysis and Records Keeping System)

This software is used to compile information on whole populations of animals held as part of a conservation breeding programme and is the typical tool for Studbook management. SPARKS can be used to produce a wide range of studbook reports by geographical region, institution or individual, analysing anything from census details and whole population parameters to levels of inbreeding for individuals. An example SPARKS report for the babirusa held at Chester Zoo in 2007 is shown in ***Figure 5***.

```
CHESTER - North of England Zoological Society, Upton-by-Chester, England, United Kingdom
   294     M    13 Dec 1996       53        69  LYMPNE      13 Dec 1996 P96048      Birth
                                                BEKESBRNE   29 Oct 1998 H98082      Transfer
                                                MARWELL     17 May 2000    5010      Transfer
                                                CHESTER     18 Jan 2007 C07006      Transfer
                                                            14 Jun 2007             Death
                         [Death by: Euthanasia (medical) □ Unknown □ Unknown (after necropsy)]

   T17     F    23 Aug 2002      170       172  TWYCROSS    23 Aug 2002    3975      Birth
                                                CHESTER      2 Mar 2005   C0558      Transfer

   T34     M     9 Sep 2003      242       440  SO LAKES     9 Sep 2003 BB0003      Birth
                                                CHESTER      7 Sep 2007 C07539      Transfer

Totals: 2.1.0 (3)
```

Figure 5: An example SPARKS report for the babirusa population held at Chester Zoo at the end of 2007.

ZIMS (Zoological Information Management System)

This is the newest studbook management tool from ISIS, and is essentially an online version of the SPARKS concept. It is an improvement on the traditional SPARKS method where only the studbook keeper has free access to the data, as records can be accessed, updated and analysed by member institutions in real-time. It is likely to be available for use sometime in 2010 (www.isis.org).

The key role of the studbook

Whether using SPARKS, ZIMS or some other method, studbooks chart the history of individuals within a breeding programme and are a vital tool in the management of

endangered species in *ex-situ* programmes. They are a record of all individuals within the programme past and present and include information on the parentage and life history of each. As such, they are a mine of information regarding the success of a programme, not just in terms of individuals but also with respect to the species population in a captive environment. This is the basis of **Chapter 2** with respect to the babirusa studbook.

Each studbook is the responsibility of a studbook keeper, usually a zoo animal keeper or registrar. He/she is responsible for the accurate and timely updating of information held in the studbook and is obliged to make management decisions and recommendations to holders of the species to maximise success of the breeding programme.

What if studbooks are unreliable?

Studbooks in the past were not always accurate and information was often missing – this was the biggest downfall of their use to manage breeding programmes. There are currently efforts from ISIS, studbook keepers and zoos around the world to clean up studbook data and make it as accurate as possible, although this cannot fix the problems of unknown parentage and dates of birth/death. In a number of cases, studbook data is simply too fragmented and/or unreliable on which to base sound management decisions. In these cases, where all possible efforts have been made to 'clean' the data, alternative approaches must be found. In recent years, the combining of studbook data with genetic analysis has successfully overcome some of these problems and improved the potential for appropriate population management of species held for conservation breeding.

The role of genetic analyses

Genetic studies have allowed improvement of studbook data in many cases, and these are discussed in more detail in **Chapter 3**. In summary, they are a useful tool to enable clarification of suspect studbook data (such as unknown parentage or sex); to indicate likely relatedness of founder animals; to suggest minimum founder numbers where this is unknown or uncertain; to assess diversity within a population; and to assess the genetic 'value' of individuals.

Ultimately, genetic information can enhance the accuracy and thus the value of a studbook, as well as providing a measure of the genetic potential of a given breeding population. This can help to better address the goals of conserving a given % genetic diversity over a given time period.

What are the limitations?

Despite the potential value in genetic studies, there are of course limitations to their use. First, it can be costly depending on the number of animals to be investigated, and the readiness of the method to be used in the species of interest. Second, it may require access to samples from individuals now dead, especially when clarifying the identity and relationship between founders. Luckily, modern techniques using the Polymerase Chain Reaction (PCR) require very small quantities of DNA which can be obtained from numerous sources, including museum specimens such as skins, bones and hairs. Founder *numbers* can be inferred using only living animals but requires a broad sampling of the populations to reduce the risk of missing genetic lineages, and cannot be considered definitive.

Obstacles to effective conservation breeding

Even with a complete and accurate pedigree, a willing and able zoo community and a sensible breeding plan, effective conservation breeding is difficult to achieve. By its very nature, a conservation breeding programme usually starts with a relatively small founder base, sampling the gene pool available and creating a necessarily 'small' population. The problems inherent in small populations have already been discussed and apply equally to wild or captive populations. It is important to appreciate, however, that the 'migration' of only a few additional unrelated individuals is enough to off-set much of the risk of inbreeding and inbreeding depression. The general rule of 'one-migrant-per-generation' has been widely adopted (Spieth 1974), and although this may be sufficient, it may not be so for many natural populations and up to ten per generation is recommended (Mills and Allendorf 1996; Lees and Wilcken 2009).

As previously mentioned, the gene pool of an ideal founder population would exactly reflect that found in the wild, but this is not practically achievable for a number of reasons: the wild population may be of limited size already so restricting the number that can be removed; it may be difficult to select animals from across its natural range, so excluding some potentially unique lineages; group captures may include related individuals, or the relationship between captured animals may be unknown; animals may not survive the capture and transportation process, or might not breed in the *ex-situ* environment so limiting the growth of particular genetic lineages. Even once the population has been established, it will be limited by the space available to house individuals, the difficulties, costs and risks involved in exchange between institutions, including veterinary restrictions due to disease control (www.waza.org ,

accesses Jan 2009) and the possible effects of 'adaptation to captivity' where individuals most suited to the *ex-situ* environment may be more successful (Lacy 1994). This can change the basic nature of the population which may then be less able to cope if released back into the wild (Frankham 2008).

Successful conservation breeding programmes

Despite all the obstacles, there are examples of successful conservation breeding programmes where the ultimate goal of releasing animals back into their natural environment has been achieved e.g. the return of Przewalski's horse to the Mongolian Steppe, as mentioned previously. Other examples of successful reintroductions include the Californian condor (*Gymnonyps californianus*) back to the Grand Canyon area of the United States (Ralls, Ballou et al. 2000), the black-footed ferret (*Mustela nigripes*) to the American mid-west (Russell, Thorne et al. 1994) and the Golden Lion Tamarin (*Leontopithecus rosalia*) to the forests of Brazil (Kleiman 1989). Each of these cases illustrates important aspects of the reintroduction process. With the Przewalski's Horse, as with many *ex-situ* populations, the founder base was very small as there were few individuals remaining in the wild when the programme started. 5 males and 8 females founded the entire living population, including a domestic mare (only discovered on genetic analysis after the programme was underway) (Frankham, Ballou et al. 2002). Intense genetic management has been employed to try and breed out the contribution of this founder (Frankham, Ballou et al. 2002). For the Californian condor, releases were made far from local human settlements as they were widely persecuted and this had been a major contributor to their initial decline (Ralls, Ballou et al. 2000). The reintroduction of the black-footed ferret has been an example of a somewhat 'ideal'

situation in that animals were bred for limited numbers of generations and the population was increased rapidly then reintroduced before adaptation to captivity could become a significant problem (Frankham 2008). The impact of adaptation to captivity was seen with the return of captive-bred Golden Lion Tamarins back to the Brazilian rainforest. Initial translocations resulted in death of many individuals, due ultimately to lack of food-finding, locomotion skills and orientation (Beck, Kleiman et al. 1991). Further attempts were combined with extensive pre-release 'training' of animals before release, and this seems to have improved survival rates (Frankham, Ballou et al. 2002).

So reintroduction is clearly possible, but it depends on numerous factors, including the removal or modification of initial threats and intensive management, and can be very costly. No doubt these problems contribute to the low rate of success in such programmes. A review by Beck et al concluded that only 11% of 145 species reintroductions of captive-born animals up to that time could be considered successful (Beck, Rapaport et al. 1994). Many of the problems of maintaining a reintroduced population were highlighted with respect to the Przewalski's Horse (van Dierendonck and de Vries 1996), who cited issues of "cooperation, management, habitat choice, insufficient knowledge of the ethology of the species, and current land use within the different project areas." Clearly, reintroduction is only the first step to permanent reinstatement of a species to its native range as further problems may risk their persistence.

THE BABIRUSA (*Babyrousa spp*)

The babirusa is the focus of this thesis, a wild Indonesian pig found only on the island of Sulawesi and a few of its surrounding neighbours. It is thought to have branched off early in the evolution of the suidae, likely between 33.7 and 23.8 million years ago, (Thenius 1970), and has a unique set of characteristics. Its almost hairless body, long slender snout and small ears give it a prehistoric look while still clearly belonging to the pigs (***Figure 6***). The most unusual feature, the upward-pointing tusks that penetrate the snout of the male, adds to its character.

Figure 6: a male (R) and female (L) babirusa (*B. celebensis*) foraging in their grassy enclosure at the Southlakes Wild Animal Park, UK.

Phylogeny

The babirusa is a member of the family Suidae, of the order Arteriodactyla, the even-toed ungulates. Its presumed position within the family is presented in ***Figure 7***.

Class: **Mammalia**
Order: **Arteriodactyla**
Suborder: **Suiformes**
Superfamily: **Suoidea**
Family: **Suidae**

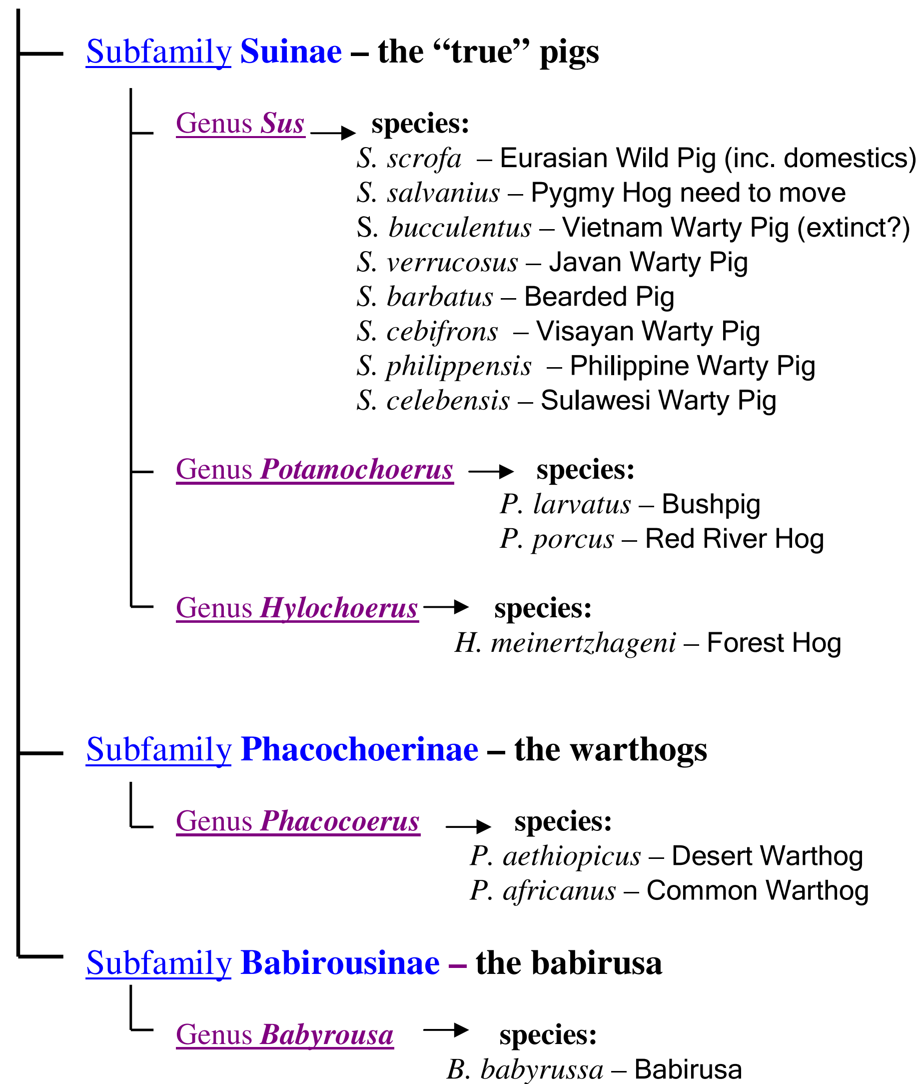


Figure 7: The presumed phylogeny of the Suidae, with the babirusa in its own subfamily and genus, distant to the various *Sus* species, reproduced from (Ogle 2004)

Figure 7 shows the babirusa is the only species in its genus, with the "true pigs" and warthogs in neighbouring subfamilies. Traditionally, these types of species'

divisions have been determined based on anatomical features and/or morphometric studies. The details in ***Figure 7*** are those assumed from such studies (Groves 1980), as presented by Groves and Grubb in 1993 (Groves and Grubb 1993). Since then, genetics have been used to further investigate the phylogenetic relationships within the Suidae, driven mainly by the desire to clarify the position of the pygmy hog, known here as *Sus salvanius*. Mitochondrial DNA has confirmed the basal position of the babirusa, and the uniqueness of the pygmy hog (re-assigning it to its own genus, *Porcula*) but has not yet been able to definitively determine the relationships between the others (Funk, Verma et al. 2007).

Early genetic studies of the babirusa had shown that despite having 36 chromosomes, in common with most other suidae, 5 pairs of autosomes seemingly had no equivalent in the other species (Bosma and de Haan 1981; Bosma, de Haan et al. 1991). A further study, however, concluded that there was in fact correspondence between the five pairs of autosomes in the babirusa and three pairs in the domestic pig (*Sus scrofa*) (Bosma, de Haan et al. 1996).

How many species of babirusa?

Until very recently, it was considered that three living subspecies of babirusa existed: *Babyrousa babyrussa babyrussa*, *B.b. togeanensis* and *B.b. celebensis,* each with their own distinct physical features and geographical range (Macdonald 1993). Meijard and Groves considered these differences to be significant enough to upgrade the three sub-species to full species level (Meijaard and Groves 2002; Meijaard and Groves 2002), and the 2008 IUCN Red List of Threatened Species™ reflects this change, citing three separate species: *Babyrousa babyrussa*, *Babyrousa togeanensis*

and *Babyrousa celebensis*. There is also considered to be a fourth, extinct species, *B. bolabatuensis* as identified from a single sub-fossil skull from central Sulawesi (Groves 1980; Groves and Grubb 1993; Meijaard 2003).

The consequences of upgrading babirusa subspecies' to full species level are varied. The primary advantage is that it should increase the profile of each species in terms of conservation needs, as they will be considered separately on their own merits (Meijaard and Groves 2002). On the other hand, this division into three species is based on morphology, particularly tooth and skull characteristics (Meijaard and Groves 2002) which may prove to be purely artificial. Early results from genetic studies of DNA extracted from skulls collected all over the historical babirusa range and held in museum collections suggest that the story is more complex than that. Mitochondrial DNA analysis has revealed that there are deep divides between groups of individuals found within the proposed geographical ranges for each of the three babirusa species (personal communication, Greger Larson). If this is the case, it may be prudent to protect the wild population in terms of 'evolutionary significant units' (ESUs) (Ryder 1986). This strategy involves preserving 'populations that actually represent significant adaptive variation based on concordance between sets of data derived by different techniques' rather than imposed species or subspecies divisions.

Geographical range

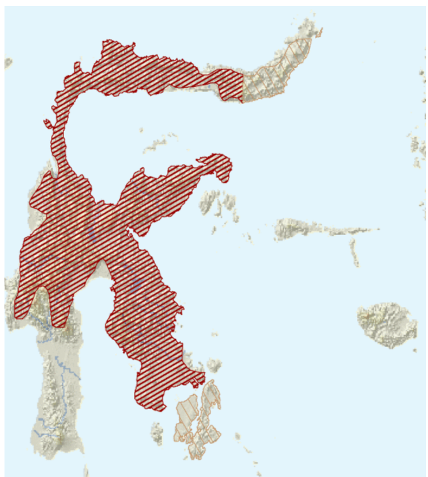
The islands of the Indonesian archipelago (***Figure 8***) have long been isolated from the South-East Asian mainland. Over 200 million years, species have been driven down their own ecologically adaptive routes, making the largest island chain in the world also one of the most biologically diverse. The central Indonesian islands east

of Java, Bali and Borneo are part of the designated Wallacea hotspot for biodiversity (www.biodiversityhotspots.org, visited March 2009); they include Sulawesi, the Moluccas and the Lesser Sunda islands. This area has a high rate of endemism, with an estimated 1500 endemic plant species and 571 endemic mammals, birds, reptiles, amphibians and freshwater fishes, 100 of which are threatened.

Figure 8: Map of South-East Asia showing the Indonesian archipelago (in yellow), including the northern central island of Sulawesi, the home of *B. celebensis*.

The distribution of the three babirusa species is shown in ***Figure 9***, from the 2008 IUCN Red List of Threatened Species™ (www.iucnredlist.org, visited March 2009). It shows the ranges of each species are very discrete and of vastly differing sizes, with *Babirusa babyrussa* existing only on Buru and the Sula Islands of Taliabu and Mangole (Macdonald, Burton et al. 2008), *Babirusa celebensis* on the large island of Sulawesi (Macdonald, Burton et al. 2008), and *Babyrousa togeanensis* on four of the small Togian Islands (Macdonald, Burton et al. 2008).

Babyrousa babyrussa



Babyrousa celebensis

Babyrousa togeanensis

Figure 9: The distribution of the three extant babirusa species in and around the Indonesian island of Sulawesi, as described in the 2008 IUCN Red List of Threatened Species™.

Babirusa status in the wild

As well as being host to so many unique species, Indonesia is the worlds 4th most populous country with an estimated human population of 239.9 million in 2008 and a population density of 126/sq km (Haub and Mederios-Kent 2008). The inevitable conflict between humans and other species for access to resources make this an area in serious need of conservation, but only 1% was protected by Indonesian law in 2006 (Haub, Pollard et al. 2006).

The two most prominent threats to survival of suids in South and South-East Asia are uncontrolled hunting and habitat destruction (Blouch 1995; Lee 2001; Milner-Gulland and Clayton 2002; Lee 2005) with the ever-increasing oil palm industry considered to be a particular threat in this region (Fitzherbert, Struebig et al. 2008; Koh and Wilcove 2008). In addition, there is a lack of financial support for conservation and also cultural difficulties of trying to protect pig species in a predominantly Muslim country (Blouch 1995).

Despite being protected under Indonesian law since 1931, CITES Appendix 1 listed since 1982, and living in several national parks and protected areas (Macdonald 1993) the last 30 years is thought to have seen a rapid decline in babirusa numbers, particularly of *B. celebensis* in the north-eastern peninsula of Sulawesi (Lee 2001; Riley 2002). Accordingly, all three babirusa species are listed in the 2008 IUCN Red List of Threatened Species™ (www.iucnredlist.org, visited March 2009), *B. babyrussa* and *B. celebensis* as 'Vulnerable' and *B. togeanensis* as 'Endangered', the next most serious risk category. The specific justifications given by the IUCN for assigning these threat levels are:

Babyrousa babyrussa (Macdonald, Burton et al. 2008)

"Listed as Vulnerable as the species has a restricted distribution (extent of occurrence less than 20,000 km²), limited to two of the Sula Islands and Buru. Moreover this species has declined in the past largely because of habitat loss through logging and conversion, and to some extent through hunting by non-Muslim communities. At least some level of decline in habitat quality and number of mature individuals can be expected to continue."

Babyrousa celebensis (Macdonald, Burton et al. 2008)

"Listed as Vulnerable because of a population decline, estimated to be more than 30% over the last three generations (approximately 18 years), inferred from over-exploitation, shrinkage in distribution, and habitat destruction and degradation; and because the population size is estimated to number fewer than 10,000 mature individuals, there is a decline of >10% expected over the next three generations. It was once thought to be reasonably common, but the species has largely disappeared from the eastern section of the northern peninsula and is under severe hunting pressure in at least northern and central Sulawesi. Elsewhere on the island there has been, and is ongoing, habitat loss, fragmentation and degradation. The Red Listing of the Sulawesi babirusa is dependent on its taxonomy. ***Should the ongoing genetic and anatomical studies later indicate that central and/or eastern, and/or southeastern babirusa are different from those of the northern peninsula, the red list status of the northern peninsula population will become more severe*****"***

*This is important in the context of the work referred to in the 'How many Species?' section.

Babyrousa togeanensis (Macdonald, Burton et al. 2008)

"Listed as Endangered because its extent of occurrence is less than 5,000 km², its distribution is severely fragmented, and there is continuing decline in the extent and quality of its habitat; and because its population size is estimated to number fewer than 2,500 mature individuals, there is an observed continuing decline in the number of mature individuals, and no subpopulation contains more than 250 mature individuals."

Unique features of the babirusa

Due to its early phylogenetic separation and subsequent geographical isolation, the babirusa has a number of features not seen in other Suidae. Of course, the most unique feature of the babirusa is in the tusks, seen most clearly in the male. ***Figure 10*** shows a series of images illustrating their development.

(a) Only one upper canine showing

(b) Both upper canines showing

(c) Upper and lower canine present and starting to curl backwards

(d) Both sets of canines have lengthened and are very close to touching the skin of the head

Figure 10**:** Images of male babirusa at various ages in the Ragunan Zoo, Jakarta. It is clear that as the animal ages, its upper and lower tusks grow and curl back towards the skull.

The tusks develop by 180° lateral rotation of the entire canine socket into an upright position within the first eight months (personal communication, Alastair Macdonald). The canines then erupt through the snout of the animals, with no apparent inflammation or distress caused. There are museum examples of skulls where the canines have grown so large that they have penetrated the cranial cavity (personal communication, Alastair Macdonald), presumably contributing to the animals demise. It was speculated that the tusks may have been used in combat to establish dominance (MacKinnon 1981) but this has not been observed directly, and is unlikely due to their brittle nature. Instead, males engage in 'boxing' behaviour, where they compete by raising onto their hind legs and try to push each other over. (Macdonald, Bowles et al. 1993; Macdonald and Leus 1995; Patry, Leus et al. 1995).

Other unusual features of the babirusa are its stomach (Leus, Goodall et al. 1999), which has a unique 'honeycomb' epithelial layer (Macdonald 2004) and its relatively long gestation period with small litter sizes compared with the other pigs (Macdonald, Kneepens et al. 1984; Bowles 1986; Macdonald 2000). In terms of behaviours, the babirusa rarely displays the typical 'rooting' behaviour of other pigs (Macdonald 1993), but the male has been seen to scent-mark an area using eye-gland secretions (Leus, Bland et al. 1995), as has the female immediately after birth (Bowles 1986).

CONSERVATION BREEDING OF THE BABIRUSA

Although the babirusa has been held in zoo collections since the early European menageries of the 1800s (Macdonald 1993), conservation breeding of the species did not actively begin until 1972, with the founding of an *ex-situ* population of *B.*

celebensis at Surabaya Zoo, Java, Indonesia. Since then, this population has grown and expanded to establish populations in different world regions, each derived from the original import of animals from Sulawesi.

In response to continued threat to the babirusa in the wild, and as a commitment to creating an *ex-situ* insurance population, 1987 saw the founding of the International Studbook under the umbrella of the World Association of Zoos and Aquaria (www.waza.org , accesses Jan 2009). Subsequently, a Population and Habitat Viability Assessment (PHVA) in 1996 set a target population large enough to maintain 90% heterozygosity i.e. genetic diversity over 100 years (Manansang, Macdonald et al. 1996). Part of this strategy required new animals to be caught from Sulawesi and added to the breeding population in Surabaya, with F1 or later being made available to the rest of the world population. The conservation breeding programme, its successes and limitations, are analysed in detail in **Chapter 2**.

Population growth requires individuals to breed

It is all very well planning population management programmes to best conserve genetic diversity and grow populations, but their success depends on individuals being reproductively successful where required. This is not always the case and may require additional research and support in order to achieve the goals set out. It is a recognised concern that knowledge regarding basis reproductive processes is lacking in the majority of exotic species (Andrabi and Maxwell 2007). One of the most useful techniques commonly used to investigate these is faecal steroid analysis, and this is the topic of **Chapter 4**.

To highlight some of the problems facing individual animals, the following case study presents Muhibah (seen in ***Figure 11***), a female babirusa who was reproductively unsuccessful and whose poor fertility raised concerns about the future of the conservation breeding programme.

Figure 11: Muhibah in her outdoor enclosure at Edinburgh Zoo. Photo courtesy of Darren M^c^Garry, Edinburgh Zoo.

Muhibah's story: A Case Study

As the female babirusa who initiated the work of this thesis, Muhibah and her story illustrate a number of the problems facing the *ex-situ* conservation breeding programme. Her story is presented here and as a footnote to subsequent chapters as an example of the reproductive problems seen in this species in captivity in Europe, and also the investigative approaches available to deal with such problems.

Muhibah (Studbook# 206) was born at Marwell Zoological Park in the UK on 25th December 1993 to dam 160 and sire 75. On 18th January, 1995 she was moved to Edinburgh Zoo and joined by a young male from the Antwerp Zoo, Tonnie (SB# 183), on 7th May that year. There was no pregnancy from this pairing, and Tonnie was vasectomised in December 1998 but remained with Muhibah until his death in December 2005. It wasn't until 2003 that another male, six year old Tolo (SB# 317), was introduced to Muhibah by which time she was already 9 ½ years old. The two males were kept with Muhibah in rotation during the day and separated at night to try and increase her responsiveness (Macdonald and Leus 1995). She is recorded as showing signs of estrous cycling (personal communication, Darren McGarry) and also to have been mated by Tolo on a number of occasions. However, no pregnancy resulted.

In 2005, Muhibah was investigated by ultrasound to visualise her reproductive tract and ovaries. Initial attempts with trans-abdominal ultrasound (see ***Figure 12***) were unsuccessful due to excess fat on the abdomen. A later attempt trans-rectally showed that there were probably corpora lutea on the ovaries but the images were inconclusive (Xavier Donadeu, personal communication).

A couple of questions are raised here. First, obesity in captive animals has been anecdotally associated with poor fertility, as it has in humans (van der Steeg, Steures et al. 2008). For example, a recent study of elephants in North American zoos concluded that excessive body mass index is a significant contributory factor to acyclicity (Freeman, Guagnano et al. 2009). It has been observed that obesity seems to be particularly prevalent in babirusa held in European and North American zoos

more than in Asia (personal observation). Secondly, ultrasonography can be a useful tool in monitoring reproductive activity in large mammals (Hildebrandt, Hermes et al. 2000), and has been used extensively with elephants (Brown, Hildebrandt et al. 1999; Hermes, Olson et al. 2000) and rhinoceros (Hermes, Hildebrandt et al. 2006; Hildebrandt, Hermes et al. 2007). It has been used trans-abdominally to monitor pregnancy in a babirusa (Houston, Hagberg et al. 2001) and was recently verified trans-rectally as an accurate method of counting corpora lutea on the ovaries of domestic sows (Bolarin, Vazquez et al. 2009).

Figure 12: transabdominal ultrasound attempt on Muhibah by veterinary staff at Edinburgh Zoo. Muhibah was awake during this procedure.

Further investigations were carried out: baseline faecal steroid analysis was conducted as part of a study by Berger et al (Berger, Leus et al. 2006); Muhibah was

treated with PG600, a hormonal preparation used in domestic swine, in an attempt to stimulate ovulation; and she was included in genetic analysis work being carried out at the Centre for Conservation and Research (CRC) at the Antwerp Zoo in Belgium. The results of these studies are presented in later chapters as appropriate.

In the end, Muhibah was euthanased aged 14 yrs and 28 days having never been pregnant, the reasons for her infertility unknown. On post mortem examination, she was found to have multiple corpora lutea on her ovaries, suggesting that she was ovulating even at 14 years of age. However, she was also shown to have numerous benign tumors in her uterus – could they have been a cause of her infertility? Alternatively, could they be the *result* of her nulliparous life history, as has been seen in captive rhinoceros (Hermes, Hildebrandt et al. 2006)?

Chapter 2: Studbook & pedigree analysis

INTRODUCTION

As previously mentioned, the studbook is a key tool in the management of a conservation breeding programme. As long as the information within the studbook is accurate and complete, it can be used in the assessment of past trends, current success and future management options of the species under investigation. It can shed light on basic biological processes relevant to the species, including typical reproductive parameters and life histories or can contribute to population planning and management using modelling software such as PM2000 (Pollack, Lacy et al. 2002).

What information can be derived from studbooks?

Basic analyses conducted using SPARKS are often limited to looking at births/deaths over a period of time, census reporting, animal movements and locations, etc. Often these analyses are for management purposes only, but some data has been published for example in the journal Zoo Biology (ISSN: 07333188, published by Wiley-Interscience, accessible at www.interscience.wiley.com). Examples include basic analyses of *ex-situ* populations of Cheetah (*Acinonyx jubatus*) (Marker-Kraus and Grisham 1993), Lion-tailed Macaque (*Macaca silenus*) (Lindburg, Lyles et al. 1989) and Clouded Leopard (*Neofelis nebulosa*) (Yamada and Durrant 1989).

However, the data contained within a studbook can be analysed in much greater detail to reveal more fundamental information on how the species performs in an *ex-situ* setting. Ultimately, if used effectively with full and accurate information, pedigree analysis can minimise overall inbreeding of a managed population and can

inform decision-making to best ensure the healthy growth and survival of the population. This has to be a key consideration if the goal is to eventually re-introduce the species back to its natural habitat. These more detailed analyses have been published for a number of species, for example where reproductive data have been compared for *ex-situ* and wild Black Rhino (Diceros bicornis) (Smith and Read 1992), where reproductive success has been associated with age and inbreeding in captive Red Wolf (*Canis rufus*) (Lockyear, Waddell et al. 2009; Rabon Jr and Waddell 2009) and where effects of inbreeding led to recommendations for the future management on the Red Panda (*Ailurus fulgens*) (Roberts 1982).

Generation levels

Each individual within a pedigree can be said to be part of a certain 'generation'. In simple cases, where each generation gives rise to the next, and breeding only occurs between pairs from the same generation, individuals are assigned to the first, second, third generation and so on. However, in real terms, breeding in captive populations occurs between individuals from different generations. This complicates the assignment of generation levels as they are no longer whole numbers. SPARKS can account for this by calculating generation level based on the entire pedigree history of an individual, resulting in each one being assigned a 'Low', 'High' and 'Average' generation number.

The 'Low' value is the most conservative and is derived from the lower of the parent's 'Low' values, plus one. It is zero for a wild born or unknown birth type specimen. A 'Low' of two, for example, would indicate a full second-generation captive born specimen; meaning both of the specimen's parents were captive born,

and at least one of the specimen's grandparents was captive born. The ‘High’ value is a measure of the longest arm of a specimen’s pedigree and is derived from the higher of the parent’s ‘High’ values, plus one. It is also zero for a wild born or unknown birth type specimen. A ‘High’ of three would indicate at least one grandparent was captive born. The ‘Average’ value takes into account all of the captive births in a specimen's pedigree, and will be greater than or equal to the ‘Low’ value and less than or equal to the ‘High’ value. It is derived from the average of the parent's ‘Average’ values, plus one. Any analyses based on ‘generation level’ presented in the results section use ‘Average’ values as described here.

Inbreeding coefficients

The principle goal of pedigree analysis in modern population management schemes is to minimise mean kinship (MK) within a given *ex-situ* population. MK strategies have been shown to most effectively maintain genetic variation over the long-term in captive populations (Montgomery, Ballou et al. 1997; Rudnick and Lacy 2008). In practical terms, this means avoiding the breeding of closely related individuals, thus avoiding inbreeding and potential inbreeding depression. The SPARKS program assigns an inbreeding coefficient to every individual in the population based on historical pedigree information, and can be used as the basis for planning breeding management. Alternatively, and more recently, PM2000 can be used to estimate in greater detail the impact of breeding plans on the mean kinship of the overall population by allowing multiple theoretical matings and application of different breeding parameters to predict long-term effects.

Conservation breeding supported by studbook analysis

The use of advanced pedigree analysis has been integral to the success of *ex-situ* breeding programmes for a number of high-profile threatened species, including for reintroduction of the European Bison (*Bison bonasus*) to the Carpathians (Olech and Perzanowski 2002) and the previously described programmes for the Black-footed ferret and Golden Lion Tamarin. In each case, intensive studbook management has resulted in the establishment of large genetically healthy populations and in all cases to the successful re-introduction of captive-bred individuals back to their native habitats.

One of the key outcomes of studbook analysis is the measurement of inbreeding parameters for individuals and populations, as described in the previous chapter. Studbooks hold information about the relationships between individuals and can thus be used to create a pedigree. From this, it is possible to follow the lineage of any individual back to the founder animals, and so to calculate its inbreeding coefficient (f). The inbreeding coefficient of an individual is "the probability that both alleles at a locus are identical by descent" (Frankham, Ballou et al. 2002) i.e. they have come from the same common ancestor. Examples of values related to particular parental relationships have already been given in ***Table 1***. Inbreeding values can also be calculated from groups or whole populations, determining the mean f using the values of all contributing individuals.

THE BABIRUSA STUDBOOK

The earliest entries in the babirusa studbook refer to animals held in the large German zoos in the early 1900s, most notably Berlin Zoo where a pair was successfully bred multiple times (Kauffels 2008). Even earlier records exist describing the babirusa being held in Europe since the early 1800s (Quoy and Gaimard 1830; Vrolik 1844). However, the populations kept around the world today are derived from stock animals established in Surabaya Zoo in Java, Indonesia in 1972. Relevant studbook information thus originates from that time.

Regional studbooks

Until very recently, individual studbooks were held in each of the geographical regions with interest in the babirusa: Europe (EEP), North America, Singapore and the Indonesian zoos in Jakarta (Ragunan) and Surabaya. The quality of record-keeping varied widely between these regions and this has made the management process difficult in some cases. In particular, there are still uncertainties regarding the early details of founder animals and how they were bred, and later data from the Indonesian zoos are missing. In addition, each region had different ways of identifying their animals: "Sur" numbers in Surabaya; "G" numbers in Singapore; and regional studbook numbers in North America and Europe. This can make tracing the movements of animals confusing and sometimes inaccurate.

International studbook (ISB)

Since 1987 there has been an 'International' Studbook for the babirusa (Kauffels 2008), and efforts have been made in the last couple of years to resolve unknowns and 'clean up' the data. This has involved substitution of unknown birth and death

dates with estimates, and the inclusion of 'hypothetical' animals to allow the pedigree to be suitably 'founded' (Leus 2004).

Founder uncertainties

As mentioned above, poor record-keeping early in the establishment of the *ex-situ* population has resulted in uncertainty as to the number, identity and relatedness of the founder animals. Anecdotal information (Manansang, Macdonald et al. 1996) suggested that 1.2 animals founded the initial population. In order to make sense of the studbook data, hypothetical animals of assumed non-relatedness (SB#s 9067/8/9) were 'created' to represent these founders (Leus 2004). This allowed the pedigree to be rooted for the first time and gave a foundation for analyses to be carried out. However, there may have been additional founders and/or these three hypothetical founders may have been related to each other. This uncertainty, or 'founder assumption' casts some doubt over the validity of the entire babirusa studbook, the potential impact on subsequent generations depending on where and what any inaccuracies actually are.

Additional wild founders

A Population and Habitat Viability Assessment (PHVA) meeting in Indonesia in 1996 (Manansang, Macdonald et al. 1996) recommended that 20 new wild-caught animals from Sulawesi were incorporated into the breeding stock at Surabaya. Realistically, this probably required the acquisition of up to 40 animals to offset the effect of inevitable losses. F1 and F2 animals resulting from this new stock were to be exported and integrated with the collection outside of Indonesia, enhancing the genetic health and size of the worldwide *ex-situ* babirusa population. In reality, five

males and two females were successfully translocated to Surabaya in 1998 and all but one female has been bred into the population there. Details of how these new animals have contributed to the conservation breeding programme are outlined later in this chapter.

SAMPLES

The analyses presented here are based on the International Studbook for the babirusa (Kauffels 2008) from January 1972 until February 2008. This comprises data for a total of 568 individuals held at some time in the conservation breeding population in the following locations: European zoos (EEP), North American zoos, Singapore Zoo and Night Safari, Indonesian zoos (Surabaya Zoo, Ragunan Zoo, Yogyakarta Zoo, Taman Safari I/II in Java and Jajasan Babirusa in Bali) and the Pretoria Zoo. These locations refer to where each individual was residing at the time the studbook report was produced, or where it was last living before death. Movements between regions or between institutions within regions of course occurred for a number of these animals during their lifetimes, but this is not considered in these analyses.

Exclusions

As with all studbooks, there are a number of instances where full information is not available or is unreliable for an individual. Parentage is considered ‘unknown’ where either one or both parents are not identified by a specific studbook number (often the result of poor record-keeping or uncontrolled breeding) or where they are recorded as hypothetical. It is important to remove these individuals as they give false values for inbreeding and generation level (both recorded automatically as ‘0’), so have been removed from any analyses considering these measures. There are also

knock-on effects for any progeny as their inbreeding and generation levels are calculated at least in part from those of the parents. The implications of this are considered later.

Birth or death dates are still considered 'unknown' in many cases where a date was assigned during the 'clean-up' and are generally recorded as 1st Jan ****. This was deliberately done in order to fill missing information about individuals where actual dates are unknown (Leus 2004). This is particularly the case for wild-caught founder animals from Indonesia. In addition, a number of animals from the Singapore Zoo were culled in November 1998 as the result of disease (swine fever) so their death dates are not true reflections of the potential life span of those individuals. In all of these cases, inaccurate dates result in false values for age at reproduction or death, so have been removed from analyses considering these measures.

Data integrity

The incidence of known parentage, birth and death dates in each of the regions, and the proportion of individuals with complete data is summarised in ***Table 2***.

		No. individuals (% of total)			
Region	**Total n**	**Known parents**	**Known date of birth**	**Known date of death**	**Complete data (%)**
EEP	232	230 (99.1)	224 (96.6)	190/190 (100.0)	218 (94.0)
N. America	89	89 (100.0)	89 (100.0)	45/45 (100.0)	89 (100.0)
Singapore	67	49 (73.1)	67 (100.0)	51/51 (100.0)	33 (49.3)*
Indonesia**	175	70 (40.0)	69 (39.4)	43/77 (62.9)	42 (24.0)
Pretoria	5	4 (80.0)	5 (100.0)	4/4 (100.0)	4 (80.0)
Combined	568	442 (77.8)	454 (79.9)	333/367 (90.7)	386 (68.0)

** and ** are explained below*

Table 2: Summary of data integrity for studbooks by region

There are a couple of considerations when assessing the data presented in ***Table 2***:

*Although the dates of birth and death are known for all possible individuals, and parents are known for 73.1% of animals in Singapore, there are only 49.3% of animals with complete data. This is due to the culling of animals in 1998 i.e. their date of death is known, but it is not considered to be valid as it is not the 'natural' date of death likely if a cull had not occurred. 'Complete' data is that which is valid to be used in the calculation of longevity and the factors contributing to it.

** This is for combined data relating to 'original' and 'new' founder lines. These are described and considered separately below.

'Original' and 'new' founder lines

There were two main founder events in Surabaya, the first around 1972 and the second in 1998. In addition to adding numbers and genetic diversity to the Surabaya population, the new founders stimulated the need to keep complete and accurate records. The marked improvement can be seen in ***Table 3***, where the combined scores from the table above have been split to show the 'original' and 'new' lineages. Note that only animals from the first founding are currently represented outside of Indonesia; the second group of founders and their offspring are considered later.

		No. individuals (% of total)			
Indonesian population	**Total**	**Known parents**	**Known date of birth**	**Known date of death**	**Complete data (%)**
'Original'	132	28 (21.2)	33 (25.0)	34/68 (50.0)	7 (5.3)
'New'	43	42 (97.7)	36 (83.7)	9/9 (100.0)	35 (81.4)

Table 3: Summary of Indonesian studbook data integrity by consideration of data relating to the 'original' and 'new' populations

In cases where parentage or dates are not known or are unreliable, the individual is excluded from analyses dependent on that information. In some cases, particularly

with individuals from Indonesia and Singapore, more than one of these is missing for an individual resulting in their removal from multiple analyses. This should be kept in mind when comparisons are made between regions. In analyses dependent on accurate inbreeding and/or generation level data, the populations from Indonesia and Singapore have been removed completely. This is as a result of the knock-on effects of assigning '0' values to specimens of unknown parentage and the subsequent inaccurate scoring of their offspring. This invalidates the vast majority of data from individuals in these pedigrees and as such, is not useful in drawing conclusions on the possible effects of inbreeding and/or generation level.

METHODS and ANALYSES

The SPARKS program was used to generate numerous studbook reports based on the International Studbook data set for the babirusa from Jan 1972 to Feb 2008. Studbook reports were displayed, 'cleaned' and manipulated using Microsoft Excel 2003 and statistical analyses conducted using the SPSS 14.0 for Windows statistical software package.

The full data set was used to address a wide variety of questions regarding the conservation breeding programme: to give an overview of the institutions who have held the babirusa, the number of animals that have been part of the programme, and major animal movements between geographical regions; to present the founder population pedigree; to assess key reproductive parameters including age distribution for dams and sires, typical birth intervals, and survival rates/lifespans; and to identify any correlation between increasing generation and inbreeding values on survival rates and lifespans. Following this, the living population (at the end Feb 2008) has

been described in a variety of ways: each individual has been followed through female founder lines to the region she is now resident in; each regional population is described in terms of numbers, sex ratios, ages and generation/inbreeding levels; and predictions are made as to the likely impact of various factors on the future potential of each regional population.

Limits to data analysis

There are some important considerations to take into account when drawing conclusions about *ex-situ* breeding of the babirusa from this studbook analysis. Firstly, as has been explained above, exclusion of individuals or groups of individuals has been necessary in a number of cases due to unknown or unreliable data. It has been suggested that this 'missing data' precludes the ability to interpret the data reliably (Nakagawa and Freckleton 2008), particularly when excluding living animals during assessment of mean survival and lifespan (Hutchins 2006).

Secondly, because this data is derived from a pedigree, the individual data points are, by their very nature, dependent on each other. Lack of independence violates a basic assumption in the use of parametric statistical tests, making their use risky at best. Researchers will often turn to non-parametric tests where their data violates basic assumptions for parametric testing. Unfortunately, this not only reduces the power to detect statistical significance if it is there, it still doesn't avoid the requirement for independence of data points (Plowman 2008).

Thirdly, for analyses looking at differences between regional populations i.e. Europe, North America, Indonesia and Singapore, there is an understanding that vast differences exist between and even within them in respect of latitude, climate,

photoperiod and numerous other environmental factors. When the population is then considered as a whole, the data could be considered to be ‘pooled’ with these factors uncontrolled. This is usually advised against (Kuhar 2006; Plowman 2008) but in this analysis, as in many others, it has been considered a necessary compromise.

In all of these situations, and other multi-institute, zoo-based studies it is recommended by BIAZA (Plowman 2008) and the AZA (Kuhar 2006) that data analysis proceeds and parametric statistics are applied where possible. This is on the understanding that not all assumptions can be fully met and results should be viewed with caution, but that this is better than not using the data at all.

DATA PRESENTATION

Animal numbers

The standard way of presenting the numbers of males, females and animals of unknown gender is: males.females.unknown e.g. 2.4.1 represents two males, four females and one of unknown gender. Unknown animals are usually youngsters whose gender has not yet been determined, or those who were stillborn or died peri-/post-partum. Often in these cases, the carcass is eaten or damaged by the mother or others in the enclosure making gender difficult to determine without a genetic test.

Pedigrees

When looking at studbook data for a species such as the babirusa, it is very difficult to present data on parentage and relationships for hundreds of individuals, the output quickly becoming too complex and difficult to interpret. ***Figure 13*** shows how the *ex-situ* babirusa pedigree would look using a typical presentation format (from James Burton, personal communication).

It is clear that only a very general impression of the pedigree can be obtained from this type of diagram, and that generation levels can only be shown as whole numbers (each 'layer' of the diagram). For that reason, pedigrees shown here have been presented only by maternal lineages, and only show those individuals required to lead to currently living animals. The maternal lineage has direct links to the mtDNA data presented later, and allows easy following of separate founder lineages through historical populations to that living at the end Feb 2008.

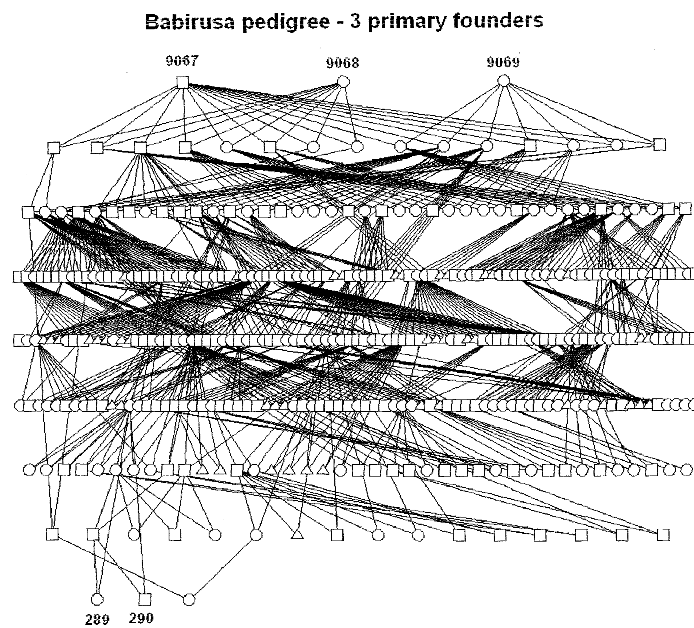


Figure 13: Visual representation of the *ex-situ* babirusa population using a standard pedigree tool, diagram from James Burton.

RESULTS

Overview of the conservation breeding programme, 1972-2007

1) Institution census

In total, 57 different institutions worldwide have held the babirusa in their collection some time between 1972 and 2007. Indonesian zoos have held 175 animals in five institutions since the start of the programme, compared to 232 animals held in 32 European zoos over the same time period. This reflects the differing management approaches to the species, with Indonesian zoos tending to hold large numbers together in a small number of locations compared to Europe where animals are held in small groups, often pairs, in a wide selection of institutions. North American zoos have held 89 animals in 18 institutions since 1982, following the European pattern of dispersing animals between institutions. The Singapore Zoo and Night Safari combined have held a total of 67 animals from 1992 to end 2007, keeping relatively large numbers together at any given time, using single-sex groups to avoid breeding. The Pretoria Zoo has also held five animals between 1993 and 2007.

A summary of institution numbers worldwide holding the babirusa between 1972 and the end of 2007 is shown in ***Figure 14***. It shows a steady increase in numbers to a peak of 51 in 2001 with a gradual decline thereafter.

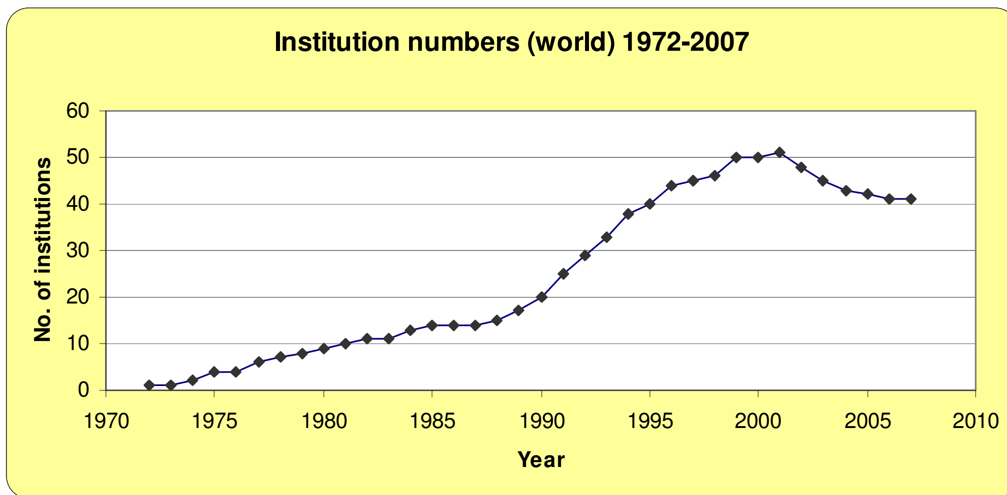
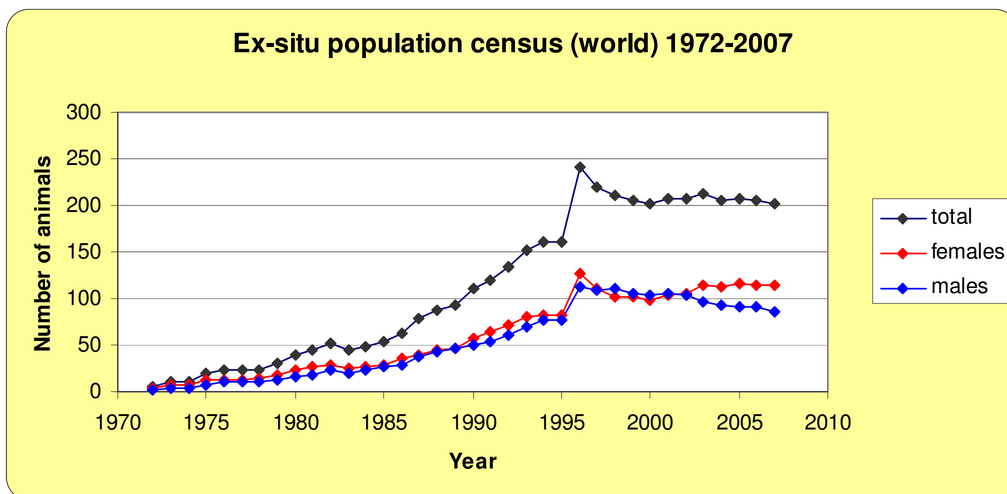


Figure 14: Graph showing the number of institutions that have held the babirusa during the period of the Conservation Breeding Programme, from 1972 to 2007

2) Population census

A summary of the *ex-situ* population census i.e. the total numbers of animals held in the population worldwide during each year of the breeding programme, is shown in ***Figure 15***.



Note: *for the purposes of this illustration, to allow inclusion of all specimens, all dates for animal acquisitions and births given in the studbook are assumed to be correct (despite some being estimates, especially from early in the programme).*

Figure 15: Graph showing the numbers of babirusa held in the worldwide conservation breeding programme each year from 1972-2007

From ***Figure 15***, it is clear that in conjunction with increasing institution numbers, there was an increase in babirusa numbers from the founding of the *ex-situ* population through to a peak in 1996. Between 1995 and 1996 there appears to be a growth rate of 1.497 leading to peak population size of 241 animals (127.112.2) held in 44 institutions at the end of 1996. However, this is an artefact of assigning a birth date of 1st Jan 96 to a large number of animals in Surabaya for whom actual birth date information was not recorded. A more realistic pattern would likely have been a population in 1995 intermediate between that of 1994 and 1996. Subsequent to 1996, numbers levelled off then started to decline from 2003 to the end of 2007.

3) Founder pedigree – the early days

All animals in the babirusa breeding programme were originally founded from wild animals recorded as being caught in North Central Sulawesi (near Poso) and brought to Surabaya Zoo in east Java. Due to incomplete record-keeping in the early stages, there is uncertainty regarding these founder animals and their possible relationships to each other. There are no clear records of when and where each of these animals was captured and dates of birth are, by necessity, simply estimates. Despite this, there was a need to agree the founding of the population to allow analyses to be conducted on the studbook data set. From anecdotal accounts and the assessment of most likely scenarios, the founder pedigree was agreed in 2004 with the addition of hypothetical animals to make best sense of what was known (Leus 2004).

Figure 16 gives an overview of the proposed initial breeding of founder animals within the babirusa breeding programme.

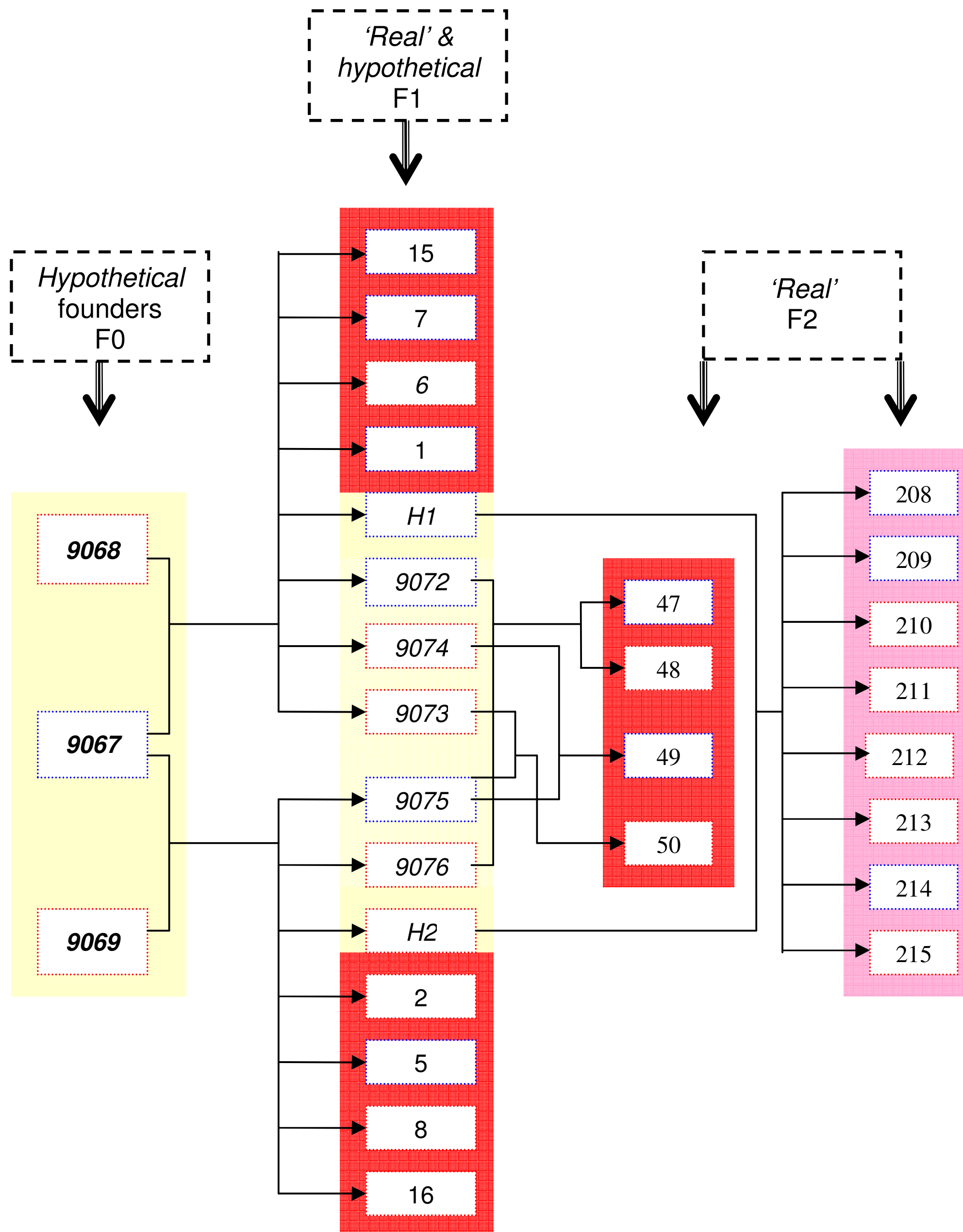


Figure 16: Diagram to illustrate the proposed early generations of the babirusa conservation breeding population, from founder animals to those which moved on to the European and Singapore regions.

Notes: '90 - -' and 'H –' are hypothetical animals
red box = female, blue box = male
yellow area = Indonesia, red area = Europe, pink area = Singapore

4) Major movements of animals (1972 – end 2007)

Once the population was established in Surabaya Zoo, animals were gradually dispersed to other institutions within Indonesia and worldwide. The details of these movements are outlined in ***Figure 17***. More details of the actual animals that were moved between the regions are given on the following page. Numbers shown in brackets after each date are the International Studbook numbers of the animals.

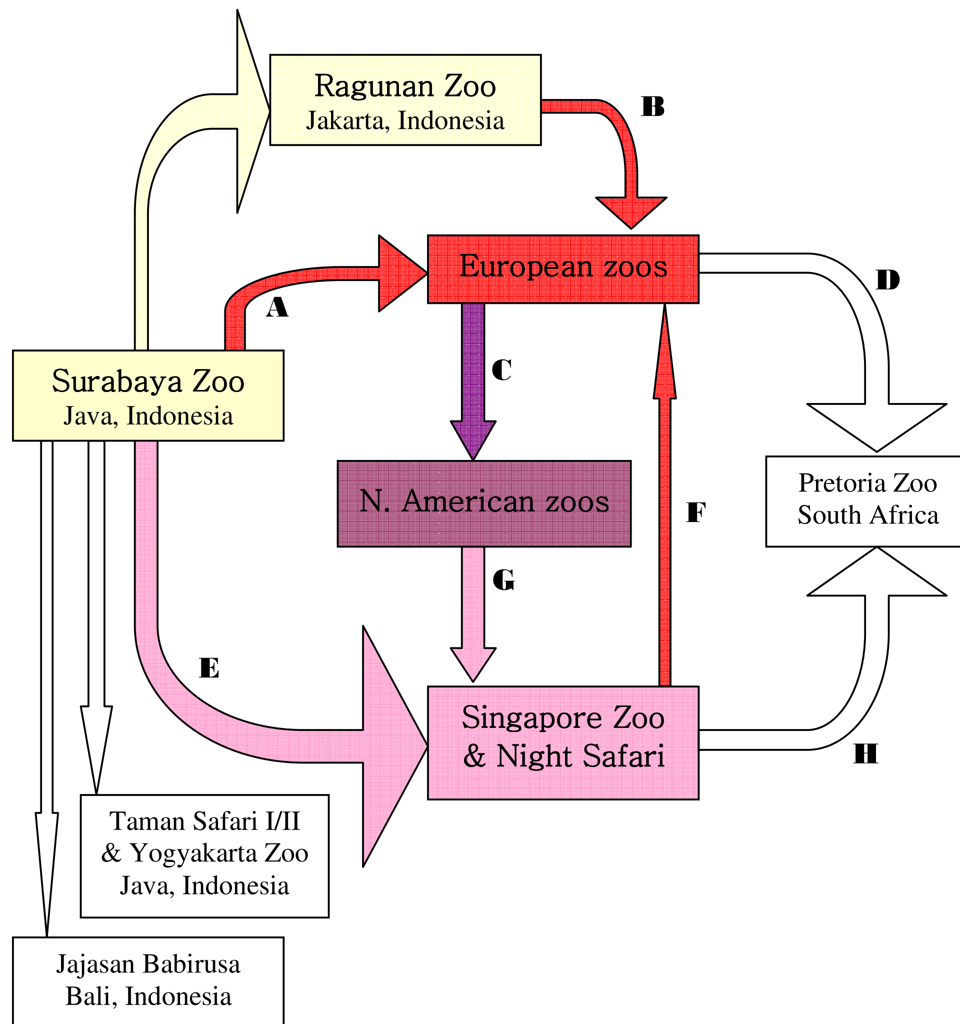


Figure 17: Overview of the movement of animals from the founder population to other geographical regions, and subsequently between the regions.

A Surabaya to Europe (4.4)

1974: 1.1 to Antwerp (1 & 2, proposed ½-sibs)
1975: 1.1 to Stuttgart (5 & 6, proposed ½-sibs)
1976: 1.1 to Stuttgart (7 & 8, proposed ½-sibs)
1977: 1.1 to Rotterdam (15 & 16, proposed ½-sibs)

B Jakarta to Europe (2.2)

1984: 2.2 to Antwerp (47 & 48, proposed sibs; 49 & 50, proposed ½-sibs)

C Europe to North America (8.6)

1984: 1.1 to Los Angeles (37 & 38, full-sib twins)
1985: 1.1 to NY Bronx (36 & 39, 'unrelated'*)
1989: 2.1 to Los Angeles/Cincinnati (60 & 71, ½-sibs; 74, 'unrelated')
1990: 1.2 to Cincinnati/San Diego WAP (63, 'unrelated'; 92 & 93, ½-sibs)
1992: 1.0 to Denver (117)
1994: 1.0 to Oklahoma (168)
1995: 1.1 to Cincinnati (157)

D Europe to Pretoria (1.0)

1993: 1.0 to Pretoria (177)

E Surabaya to Singapore (3.5)

1992: 3.5 to Singapore (208 → 215, proposed full-sibs)

F Singapore to Europe (2.1)

1995: 2.1 to Berlin (217 & 222, ½-sibs; 226, 'unrelated')

G North America to Singapore (1.1)

1997: 1.1 to Singapore (247 & 249, 'unrelated')

H Singapore to Pretoria (1.3)

1997: 1.1 to Pretoria (297 & 299, ½-sibs)
2000: 0.1 to Pretoria (305)
2002: 0.1 to Pretoria (444)

'unrelated' in this context means with different parents

5) Additional wild founders in Surabaya

At the end of 1998, 5.2 illegally-caught babirusas were recovered and successfully transported from North Sulawesi to Surabaya Zoo. 5.1 of these produced a total of 36 offspring, four with two wild parents and the rest through breeding with the original Surabaya stock. As of the end of February 2008, 2.2 of these wild-caught animals still survived (including a non-breeding female), as did 30 of the F1 generation. These founder lines are analysed in more detail later.

Analysis of full studbook data set

Extensive analysis of historical studbook data can allow a description and assessment of various population parameters. Instead of the anecdotal information passed from keeper to keeper and from zoo to zoo, it is possible to apply more rigorous scientific methodology to get a more reliable assessment of how the *in-situ* population 'works'. Analyses 1) to 4) present descriptive accounts of breeding parameters in relation to animals no longer alive in the population. Analysis 5) uses a combination of historical and current information to look at factors affecting survival and lifespan and analysis 6) specifically looks for evidence of inbreeding effects.

1) Litter size

There are 568 individuals listed in the International studbook for the babirusa from 1972 – end February 2008. Each was assigned as a 'single piglet', one of 'twins' or one of 'triplets'. Three was the maximum litter size recorded within the studbook. 115 piglets could not be assigned to a given litter with certainty and these cases have been removed from the following analysis: 88 due to unknown parentage (12 of these had unknown wild parents); and 27 due to hypothetical assignment of parents

(as outlined previously). The remaining 453 individuals were assigned to 311 litters, giving a mean litter size of 1.46. There were 181 single piglets (58.2% of litters), 236 piglets as twins (118 pairs, 37.9% of litters) and 36 piglets as triplets (12 sets, 3.9% of litters).

Interestingly, five of the litters of triplets were born to the same dam, #50, with additional single births at the beginning and end of her reproductive life. She was one of two females brought from Jakarta to Antwerp in 1984, with a hypothetical generation level of two. The other (with different hypothetical parentage but same hypothetical generation level), #48, also produced a set of triplets as well as one set of twins and 2 single piglets. Female #35 produced triplets on two occasions, as well as five sets of twins and four single piglets. With a generation level of 2.5 and inbreeding coefficient (f) of 0.3125, she was born to a father-daughter pairing, and although she conceived on many occasions, only six of her 20 offspring (30%) survived past 30 days. None of the triplets from either litter survived. The remaining four sets of triplets were born to dam #s 71, 141, 172 and 303. Female #71 was the grand-daughter of #50, #s 141 and 172 were third generation from the Surabaya-derived lineage that first came to Europe, and #303 is a 3.5 generation level female from Singapore with an f of 0.4375. One of her triplets survived.

2) Age distribution for dams

The age distribution of dams recorded as giving birth during 1972 – Feb 2008 was analysed. 58 further litters were removed from the 311 analysed above due to unknown dam (12 litters) or unknown age of dam (46 litters), leaving 253 available for analysis. Note that six dams (#s 2, 6, 8, 16, 48 and 50, all sent to Europe from

Indonesia as founder stock), were retained in this data set despite having only estimated dates of birth. These females all produced multiple litters and, combined, contributed 38 litters to the total of 253 (15%). Although their dates of birth were estimated, they were done so as accurately as possible after careful consideration of data from available records (Leus 2004). In addition, they are some of the most interesting females in the data set, and in some cases had very long, successful reproductive lives. As such, they have been deemed suitable for inclusion despite their potential for reducing the accuracy of the analysis.

Ages at conception were calculated by subtracting the recorded 158 day gestation period (Macdonald 1993) from the age (in days) of the mother on the date the litter was born, then dividing by 365 days to express her age in years. The remaining data for the conception of 253 litters from 72 dams is shown in ***Figure 18***. Each age category includes all dams of the age shown up to one day less than the next category.

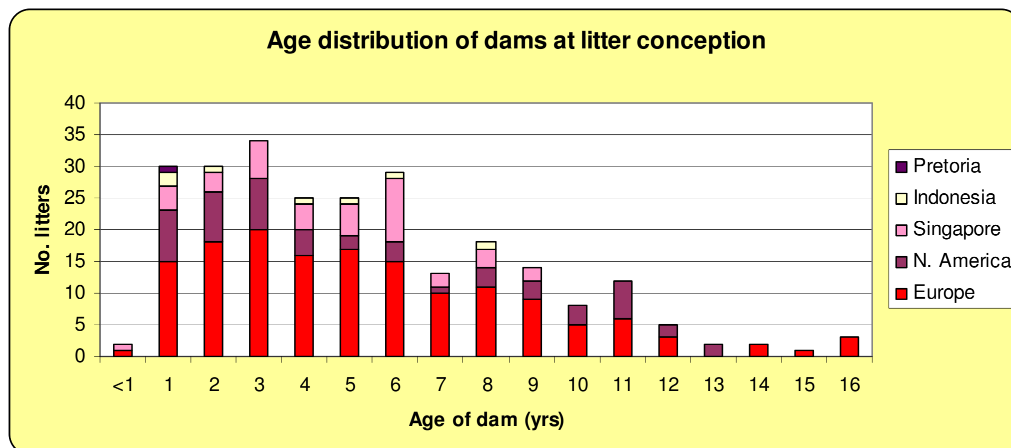


Figure 18: Graph to show the age distribution of dams at litter conception, displayed as stacked bars, divided between the geographical regions.

You can see the distribution of conception ages for the combined dams in each age category is clearly skewed towards the lower ages. The majority of litters are born to dams between 1yr and 6yrs, with a peak in births for dams aged three years and a steady decline thereafter. Note also that only dams in Europe and North America are seen to conceive aged 10yrs and above. Perhaps this is a reflection of having to remove so many Indonesian animals from the analysis – there were only eleven litters suitable to be included. Alternatively, it may reflect overall shorter lifespan for animals kept in the SE Asian region, or due to population management differences, and this is assessed later. Overall, numbers are too small to make any definitive claim for older dams being *unable* to conceive in the SE Asian region (see later analyses).

Youngest dams

The four youngest dams were analysed further to more accurately establish their age at delivery, and thus their age at conception. The results are shown in ***Table 4.***

Studbook #	Region	Dam's date of birth	Date of delivery	Interval (days)	Age at conception (months)
4	Europe	05-Apr-75	24-Jun-76	446	9.6
262	Singapore	17-May-95	15-Sep-96	487	11.0
160	Europe	17-Sep-91	25-Feb-93	527	12.3
T15	N. America	12-Sep-02	21-Feb-04	527	12.3

Table 4: Details of the four youngest dams at litter conception.

This data would suggest that puberty in babirusa females held in captivity can be as early as 9.6 months of age, and certainly by one year. However, it is interesting to note that the single piglet to dam 4 died after 34 days, one of the twins born to dam 160 died after 7 days (the other lived to 5 years), and the single piglet born to dam

T15 lived only one day. All of these dams had subsequent successful litters. The offspring to dam 262 survived and was still alive at the end of 2008, aged 12yrs.

Oldest dams

The six oldest conceptions were also analysed further to more accurately establish the dam's age at delivery, and thus their age at conception. The results are shown in ***Table 5***.

Studbook #	Region	Dam's date of birth	Date of delivery	Interval (days)	Age at conception (yrs)
8*	Europe	26-Mar-75	03-Mar-90	5456	14.5
34	Europe	30-Aug-81	08-Jan-97	5610	14.9
8*	Europe	26-Mar-75	06-Apr-91	5855	15.6
4*	Europe	05-Apr-75	11-Nov-91	6064	16.2
142	Europe	16-Jul-90	03-Jun-07	6166	16.5
8*	Europe	26-Mar-75	15-Aug-92	6352	17.0

** estimated birth dates*

Table 5: Details of the six oldest dams at litter conception

Three of the six oldest conceptions, including the overall oldest at 17yrs are attributed to female #8. In fact, these three litters were the last of eight from a female who produced six sets of twins and two single piglets in total, all of whom survived beyond 30 days. Female #8 was from the F1 generation of original founder animals in Surabaya, with a hypothetical inbreeding value of 0. She was held at the Stuttgart Zoo in Germany from her arrival in Europe until her death at an estimated 22yrs old.

Female #4 was one of the F2 generation ($f = 0.125$), held in Antwerp Zoo in Belgium. There she produced 4 litters in total, the last one shown here being conceived ten years since her previous one. Although these twins are recorded as 'births', they were actually discovered decomposed in the uterus of the female on autopsy after her death.

Female #34 was also held in Antwerp Zoo, another of the F2 generation ($f = 0.125$). She consistently conceived from the age of 4 years until this last one, producing 15 piglets in ten litters. All but two of these survived beyond 30 days.

Female #142 is one of the offspring from #34 (making her F3 generation), by a mating with an F1 male, #49 ($f = 0.1875$). She had a long reproductive lifetime on transfer from Antwerp to the Vienna Zoo in Austria, with first conception aged 1year 4months, producing six piglets in five litters. Three further single piglets in her last three pregnancies (aged 11, 12 and 16 years), conceived with a new sire at the Fuengirola Zoo in Spain, all died before 30days

These data would suggest there is a potentially long reproductive lifespan in the babirusa female. However, in this *ex-situ* setting, it would seem that this applies only to the least inbred females from the earliest days in the pedigree. Unfortunately, the lack of reliable data from the early females in Surabaya Zoo doesn't allow this hypothesis to be tested in that region.

Oldest dams at first conception

It is also interesting to look at the oldest dams at *first* conception. There are concerns that females who do not breed early in their lives are unlikely to do so later on (anecdotal). However, there is some evidence that older dams can breed for the first time. The oldest first conceiver from the studbook is #279. She was 8yrs and 5 months old at first conception, and produced a single piglet which is still living. The next two oldest first conceivers (#92 and #127) first conceived aged 8yrs and 6yrs 11 months respectively. The single piglet to #92 died immediately, but one of the twins

to #127 survived and is still living today. Unfortunately, none of these females produced further litters.

3) Age distribution for sires

As for the dam analysis, a number of litters were removed from the 311 available in order to look at sire ages. 33 litters were removed due to unknown sire and 36 where the sire date of birth is not known, leaving 242 available for analysis. Similarly, a number of sires (#s 1, 5, 7, 15, 47 & 49) have been retained despite having only estimated dates of birth. Combined, these males contributed 48 litters to the total of 242 (20%).

Age at conception (in days) is given as an output from SPARKS reporting, and is expressed here by dividing by 365 days to express sire age in years. The remaining data for the conception of 242 litters from 64 sires are shown in ***Figure 19***. Each age category includes all sires of the age shown up to one day less than the next category.

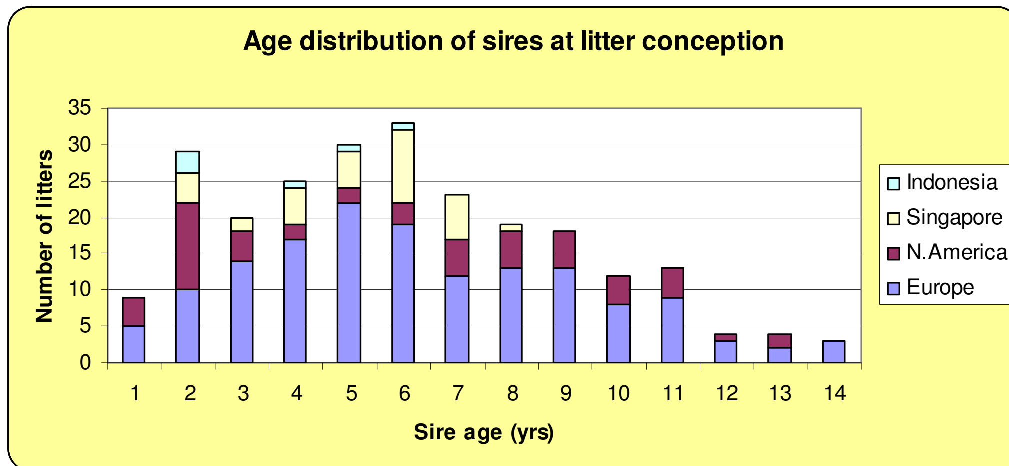


Figure 19: Graph to show the age distribution of sires at litter conception, displayed as stacked bars, divided between the geographical regions.

Here, there is a roughly normal distribution of conception ages, particularly when the largest data set for Europe is considered in isolation. There is a peak in litters born to sires at 2 years, primarily due to the large number of litters in North American zoos. There is an additional peak period for sires between the ages of four and six years of age. Note also that, similar to the dams, only sires in Europe and North America are seen to conceive aged 11yrs and above. Again this may be the result of having to remove so many Indonesian animals from the analysis (there were only six litters included), the impact of generally shorter lifespan in the SE Asian region or management decisions not reflecting true reproductive potential.

Youngest sires

The four youngest sires were analysed further to more accurately establish their age at conception. Based on the data shown in ***Table 6***, males would seem to reach sexual maturity later than the females in a similar *ex-situ* setting, almost 18 months at the earliest.

Studbook #	Region	Sire's date of birth	Date of delivery	Age at conception (months)
T88	N. America	19-Aug-04	06-Jul-06	17.6
134	Europe	29-May-90	25-Feb-93	18.1
165	N. America	24-Dec-91	21-Feb-04	20.4
146	Europe	22-Oct-90	15-Sep-96	21.8

Table 6: Details of the four youngest sires at conception.

The single piglets produced by the first three youngest sires all survived into adulthood, despite sire inbreeding coefficients of 0.3125 for #s T88 and 165. #134 was the most successful of these four, going on to produce a further four litters (six piglets) all of whom survived to adulthood. #146, with an inbreeding coefficient of

0.3906, was less successful. Despite siring seven piglets in five litters, only one survived to adulthood.

In comparison to these European and North American animals, it is interesting to note that the youngest male to sire a litter in Indonesia was almost two years of age, and the youngest in Singapore was 2yrs and 9months.

Oldest sires

The oldest sires known from the studbook are presented in ***Table 7***.

Studbook #	Region	Sire's date of birth	Date of delivery	Age at conception
195	N. America	23-Apr-93	16-Aug-07	14yrs 1month
23	Europe	08-Jun-79	28-Nov-93	14yrs 3months
47*	Europe	28-Apr-82	08-Jan-97	14yrs 6months
23	Europe	08-Jun-79	11-Jul-94	14yrs 10months

* estimated birth date

Table 7: Details of the four oldest sires at conception.

Despite sire #23 having a long reproductive life, from age 5yrs to the oldest siring at almost 15yrs, he was not particularly successful. From 10 matings with the same female (#35) over the ten year period, there were four single piglets, four sets of twins and two of triplets born. Only three of these 18 piglets survived into adulthood, and none of the nine sired after #23 was 10yrs old. It is interesting to look at the lineages of this sire and dam to discover possible reasons they were so successful in conceiving litters but not in their survival. #23 was born to female #16 and male #15, both original animals brought to Europe from Surabaya, so had a hypothetical generation level of 2 and inbreeding coefficient of 0.125. The dam #35, however, was born of a father-daughter mating, with a higher inbreeding coefficient

of 0.1325 despite a mean generation level of only 2.5. Could it be the female who was responsible for the poor survival rate of their offspring?

More successful was sire #47, one of the European founder animals brought from Jakarta to Antwerp. From the (estimated) ages of 1yr 10months and 1yr 11months (the 7th and 9th youngest sirings) to 14yrs 3months, he sired thirteen litters with four different dams. Of the resulting 19 piglets, all but four survived into adulthood. This is an indication of how intensively the babirusa was bred in Europe at the start of the breeding programme, and how much of a contribution single animals like #47 made to the population.

Equally successful in more recent times is sire #195. Despite only starting his breeding career aged 6yrs 8months in 2000, he has already sired 5 litters. Of the nine resulting piglets, all have survived except one of a set of twins.

Oldest sires at first conception

Unlike the females, three males from this data set have been able to sire their first litters beyond 9yrs of age, the oldest being 13yrs 6months. Having said that, this single piglet did not survive post-partum and there were no further litters (0.1875). The other two sires were 12yrs 6months and 10yrs 6months old at conception (#s 29 and 241 respectively). Neither of these litters survived either but both males went on to sire further piglets who survived to adulthood.

4) Birth intervals

There were 181 birth-birth intervals available for analysis, ranging in length from 169 to 3666 days. Considering a typical gestation length of 158 days, this makes the

earliest post-partum conception at around 11 days i.e. during the first post-partum estrus. The mean interval +/- SD was 622 +/- 527 days. Eight litters were conceived within 30 days of the previous parturition (4.4% of the 181 intervals examined), three of those to dam #440 who conceived easily but had a poor parenting record. In six of these cases, the previous litter typically died very soon after birth (between 0 and 5 days), allowing the dam to come into estrus again and be fertilised in this period.

However, there are a couple of exceptions to this. In one case, the dam is reported to have had twins – one died at 3 days post-partum but the other is recorded as living to adulthood. In another, the previous single piglet is reported to have lived for 34 days. Both are recorded as being ‘parent-reared’, suggesting that the dam should have been lactating and unable to become pregnant again so soon. Perhaps there is some inaccuracy in the records here, or that the piglets were only parent-reared for a short time then removed. Either that or perhaps prolactin levels were not sufficiently high to suppress fertility in these females.

5) Survival and lifespan

The previous four analyses were concerned with describing what has happened historically, and identifying typical conditions for pregnancy and birth of offspring. More importantly, how long do those offspring survive, and what are the optimal conditions for longevity? If they are to contribute to the overall success of the conservation breeding programme, they have to survive into adulthood and successfully reproduce themselves.

Considering the small founder number that established this population and the lack of exchange of animals between regions, there can be no doubt that the captive

babirusa population is inbred to some extent, and is likely to have been increasingly so from founder to current times. If inbreeding is considerable, there may be detectable signs of inbreeding depression. As described in the first chapter, inbreeding effects have typically been noted in relation to juvenile survival rates but are likely also to have implications at other life-stages (Koeninger-Ryan, Lacy et al. 2003). These authors suggest that inbreeding may also have effects on adult survival, mate acquisition, fecundity (through effects on gamete production, fertilisation, implantation and gestation/incubation), and parental care.

The analyses presented in this thesis assess the effect of various factors, including inbreeding, on juvenile survival rates and lifespan as these are the two outcomes most easily investigated from studbook data. Survival is measured as the percentage of individuals living beyond three critical time periods: 30 days; 90 days; and one year. All three measurements have been calculated to assess when deaths are most likely and so identify the critical times for survival.

There are two possible ways of assessing lifespan in this data set. To get the most accurate measure of lifespan, only those animals already dead should be included. However, this excludes a large number of animals who have passed critical survival stages and beyond, and who are still living. In fact, in still living, they may represent some of the oldest animals recorded in the studbook. A study of lifespan in captive elephants (Wiese and Willis 2004) was criticised by Hutchins (Hutchins 2006) for not including living animals in their calculations. A possible solution would be to include all animals for which data is available (living or dead) and consider their 'survival time' rather than age at death. However, this method would only be valid if

the two sets of data had similar distributions. To assess whether or not this is the case here, living animals have been assigned an age as of the end of February 2008 and dead animals their age at death. Animals surviving <365 days were removed then both data sets compared. The results are shown in ***Figure 20***.

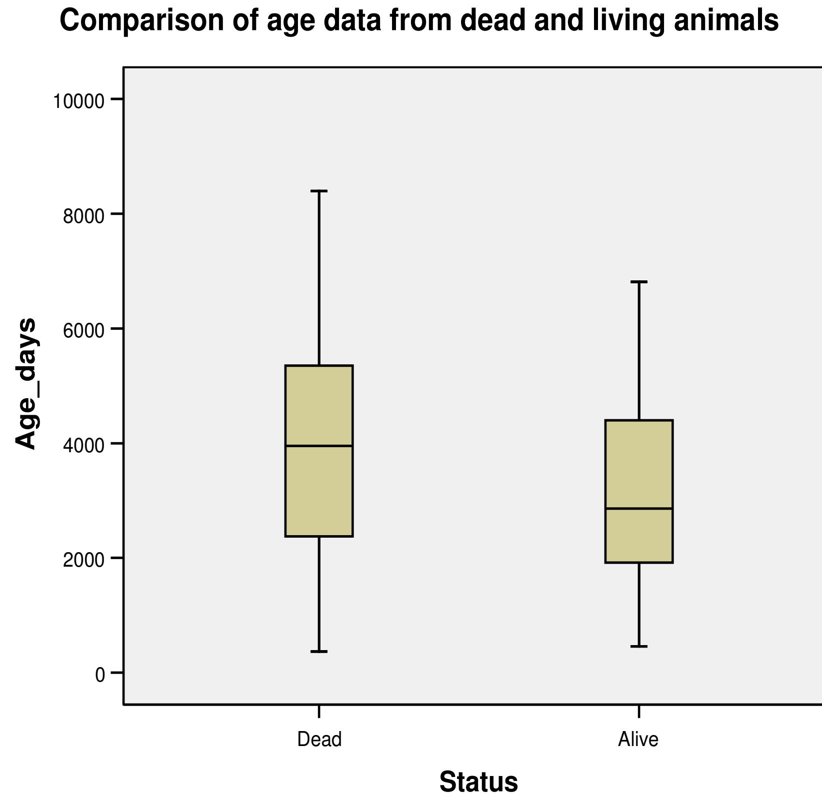


Figure 20: Boxplots comparing the age distribution of all 'dead' and 'alive' animals, with maximum and minimum, upper and lower quartiles and mean values for each group.

The boxplots illustrate that the age distribution of the living animals is less than those who are dead, as are their mean age, 25 and 75 percentile values. There is also skewing +/- SE of 0.377 +/- 0.208 of the 'alive' data set compared to -0.103 +/- 0.209 in the 'dead' set. To assess any significant different between mean values, an independent t-test was carried out. Equal variance was assumed from Levene's Test

(*p=0.098*), and the pooled t-test showed that the mean values for survival time (age in days) for 'dead' and 'living' animals are significantly different beyond the 1% level: t (269), p (two-tailed) = 0.009.

As a result of these analyses, it would not be considered appropriate to combine the two sets of data for measurement of potential lifespan. Accordingly, only the data from animals already dead is used in the following analyses of mean lifespan, calculated based on those living beyond a year. However, dead and living animals *have* been pooled in the calculation of *survival rates* as they only consider survival up to 1 year. In this situation, to exclude the living animals would be to ignore the fact of their survival, regardless of their final age at death. To summarise, survival rates are based on all suitable animals (living or dead) but mean lifespan measures are based only on animals that are already dead, and who lived beyond 1 year. After exclusions (those with unknown dates of birth or death), there were 420 individuals available for analysis. Of these, 277 were dead and 143 still living at the end of February 2008.

Overall survival and lifespan

The overall survival rate (*n=420*) beyond 30 days was 70.7% (*n=297*), beyond 90 days was 67.6% (*n=284*) and beyond 365 days was 64.5% (*n=271*). For those who lived beyond 365 days and since died (*n=135*), mean age at death +/- SD was 3868 +/- 2034 days i.e. 10.6 +/- 5.6 years.

Effect of generation level

There are anecdotal accounts from animal keepers and managers that through the course of the breeding programme there has been a general decline in breeding

success and survival rates. To investigate this, 69 animals were removed from the 420 available (38 due to hypothetical parents and 31 due to unknown parentage) leaving 351 available for analysis by generation level. 248 of these were already dead and 103 still living (including one <30days old and four between 90 and 365 days). Animals were grouped by generation level and % survival calculated for each of the three key life stages. Results are presented in ***Table 8***.

	% survival by generation level*				
	≤2.0 (*n=35*)	≤3.0 (*n=89*)	≤4.0 (*n=86*)	≤5.0 (*n=101*)	>5.0 (*n=36*)
>30days	84.6%	78.7%	54.7%	76.2%	55.6%
>90days	82.1%	67.4%	54.7%	75.2%	55.6%
>365days	79.5%	67.4%	54.7%	71.3%	47.2%

*there were no entries for generation level below 1.0

Table 8: Table showing % survival beyond 30, 90 and 365 days, presented by generation level.

Although the % survival levels over 1 year are greater in the earliest generation category, and lowest in the highest generation category, there is no clear trend from low to high generation levels. In addition, these categories have the lowest sample sizes so are also the least reliable.

After removal of individuals still living, and those with unknown or uncertain parentage, 224 individuals were suitable for lifespan analysis. Note that ALL animals from Indonesia and Singapore were excluded due to the knock-on effects of unknown parentage early in the pedigree, removing any animals with generation levels <2.0. The plot therefore represents the populations from Europe (*n=176*) and North America (*n=45*) and three animals from Pretoria. The relationship between age at death and generation level is shown in ***Figure 21***.

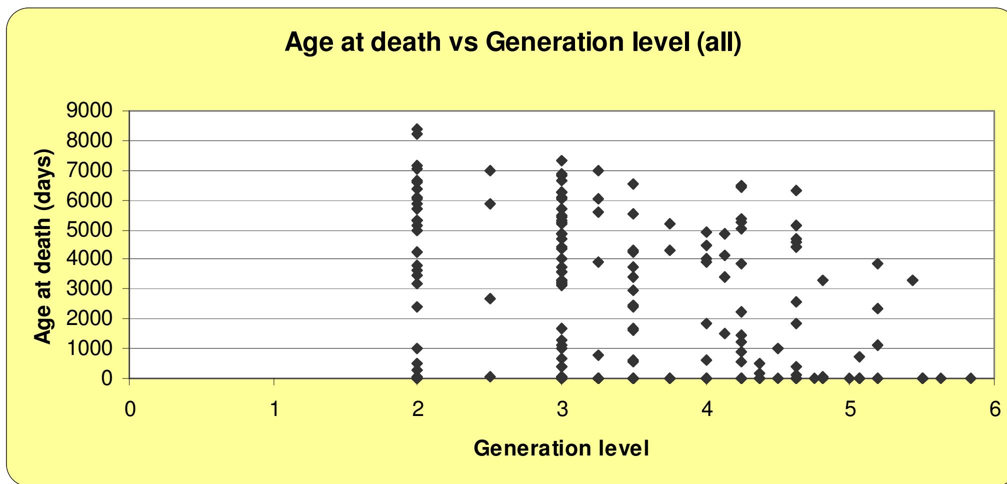


Figure 21: Scatter plot to show the relationship between age at death and generation level.

This scatter plot shows that beyond a generation level of around 3, there is a general trend towards declining age at death. When tested, these data had a Pearson Correlation value of -0.344, which is significant at the 0.01 level (two-tailed).

Effect of inbreeding

So it looks like there is indeed something affecting life expectancy as animals are born further and further down the generations. Could this be the result of inbreeding? To test for this, the same subset of 351 suitable animals used above was similarly analysed according to inbreeding coefficient and the results shown in ***Table 9*** and ***Figure 22***.

	% survival by inbreeding coefficient				
	≤0.1 (*n=9*)	≤0.2 (*n=150*)	≤0.3 (*n=83*)	≤0.4 (*n=84*)	>0.4 (*n=25*)
>30days	77.8%	70.0%	78.3%	65.5%	60.0%
>90days	77.8%	66.7%	75.9%	59.5%	60.0%
>365days	77.8%	65.3%	72.3%	56.0%	60.0%

Table 9: Table showing % survival beyond 30, 90 and 365 days, presented by inbreeding coefficient.

The two least inbred groupings show greater percentage survival than the two highest inbreeding groups but there is no overall trend from least to most inbred. It could be said therefore that inbreeding is likely to be having some impact on juvenile survival but is not the only factor.

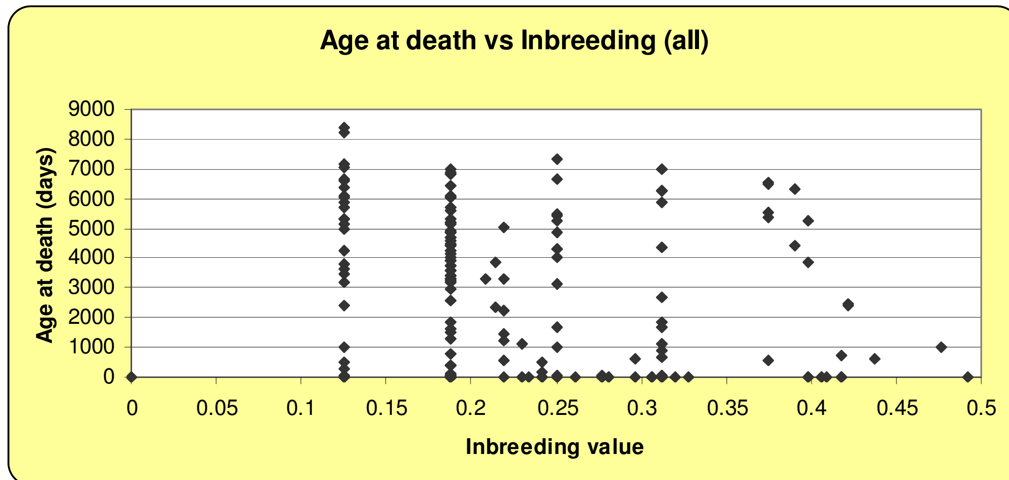


Figure 22: Scatter plot to show the relationship between age at death and inbreeding coefficient.

In addition to the effects on juvenile survival, the scatter plot shows that there is a decline in lifespan as inbreeding values increase, most notably beyond 0.35. The Pearson Correlation value was calculated to be -0.176, again significant at the 0.01 level (two-tailed).

Relationship between generation level and inbreeding

From the analysis above, inbreeding could be a factor contributing to reduced survival as generation levels increase, i.e. as the population grows through continued breeding of existing lineages. To test this relationship between generation level and inbreeding, the two factors were plotted against each other (*n=224*), as shown in ***Figure 23***.

Inbreeding vs Generation level

Inbreeding value

Generation level

Figure 23: Scatter plot to show the correlation between inbreeding coefficient and generation level in the 'dead' population.

You can see a general trend towards increasing inbreeding values as generation level increases. The Pearson Correlation value was calculated to be 0.599, significant at the 0.01 level (two-tailed), indicating a positive relationship between generation level and inbreeding.

In summary, the populations analysed here (a sample of those already dead in Europe, N. America and Pretoria) have been becoming more inbred through the generations, and this is having an effect on survival times as measured by age at death. However, not all of the effect can be attributed to inbreeding: the effect of size of generation level $(-0.344^2) = 0.118$, which is considered to be a 'large' effect (Kinnear and Gray 2004) is much greater than that of inbreeding $(-0.176^2) = 0.031$, a 'medium' effect. It would seem that other factors are also contributing to decreased survival times through the generations, and these are now further investigated.

Effect of gender

For the 420 animals represented above, gender is recorded as male, female or unknown. Survival rates for each group at key stages are shown in ***Table 10***.

	% survival		
	Female (*n=165*)	Male (*n=192*)	Unknown (*n=63*)
>30days	83.0%	82.3%	3.2%
>90days	79.4%	78.6%	3.2%
>365days	77.0%	75.0%	0.0%

Table 10: Table showing % survival beyond 30, 90 and 365 days, presented by gender.

Survival rates at key stages are very similar between males and females, with females scoring slightly higher at each. Those marked as unknown are unknown simply because they didn't survive long enough to be identified. It is therefore logical that survival rates are essentially zero for this group.

Despite similar survival rates, longevity was seen to be higher in females. For those who survived beyond 365 days then subsequently died, the mean age at death +/- SD was 4374 (12.0yrs) +/-2166 days (*n=51*) for females, 3561 (9.8yrs) +/-1898 days (*n=84*) for males and 3868 (10.6yrs) +/-2034 days (*n=135*) for both combined, as illustrated in ***Figure 24***. Females also show a larger range of ages at death compared to males, with a maximum age of 8399 days (23.0yrs) compared to 7312 days (20.0yrs) for males. There is also a skewness +/- SE of -0.367 +/- 0.333 in the female data compared to -0.036 +/- 0.263 for males. Levene's test showed no significant difference in variances (*p=0.200*) between the two data sets, and a pooled t-test showed that the mean age at death was higher for females than males, significant beyond the 0.05 level: t (133) = 2.287, p (two-tailed) = 0.024.

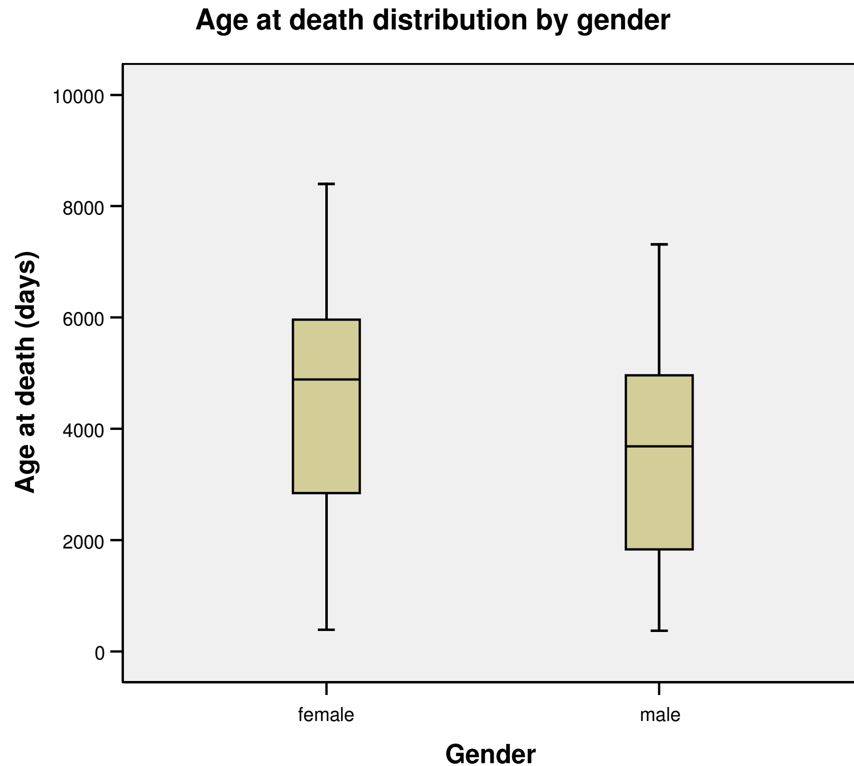


Figure 24: Boxplots comparing the distribution of age at death by gender, showing maximum and minimum, upper and lower quartiles and mean values for each group.

Effect of litter size

33 of the 453 individuals assigned to litters were removed due to unknown or hypothetical birth date. Survival rates for the remaining 420 individuals, assigned to either the singleton, twin or triplet group, are summarised in ***Table 11***. There was no recorded litter size greater than three.

	% survival		
	Singletons (*n=176*)	Twins (*n=208*)	Triplets (*n=36*)
>30days	68.2%	74.0%	63.9%
>90days	63.6%	72.6%	58.3%
>365days	61.9%	67.8%	58.3%

Table 11: Table showing % survival beyond 30, 90 and 365 days, presented by the litter size it was born into.

The data seems to suggest that twins are most likely to survive at each key stage. For those that survived beyond 365 days and subsequently died, the mean age at death +/- SD was 3692 (10.1yrs) +/- 2075 days (*n=54*) for singletons, 3908 (10.7yrs) +/- 1973 days (*n=66*) for one of twins, and 4323 (11.8yrs) +/- 2210 days (*n=15*) for one of triplets. The distribution of age at death for each group is shown in ***Figure 25***.

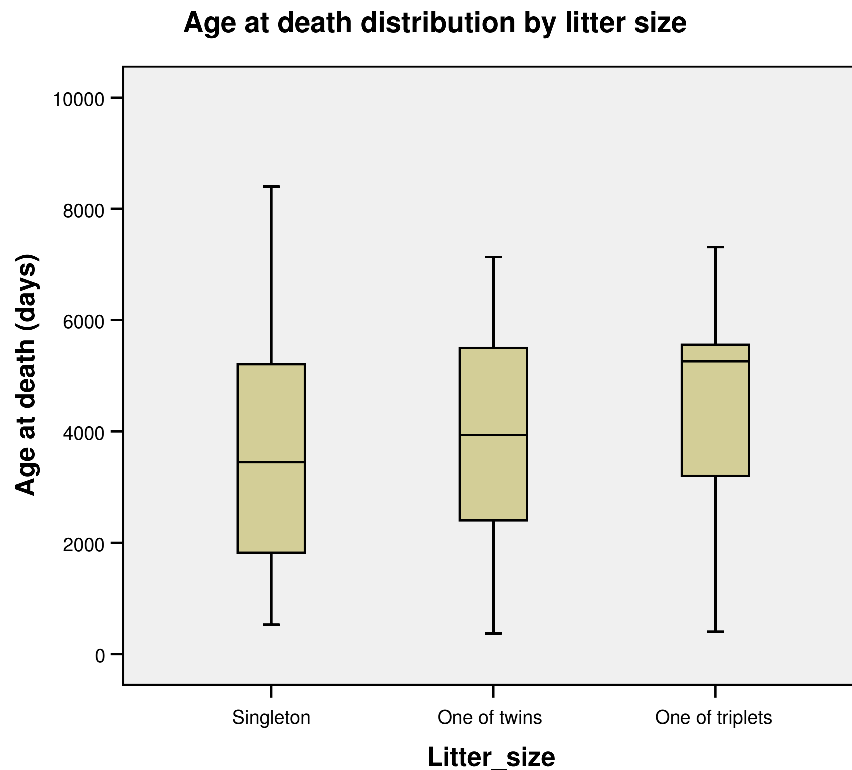


Figure 25: Boxplots comparing the distribution of age at death by litter size, showing maximum and minimum, upper and lower quartiles and mean values for each group.

Despite the apparent differences in distributions patterns for the three groups, an independent t-test showed that mean values were not significantly different between any two of them: between singletons and twins, t (118) = -0.584, p (two-tailed) = 0.560; between singletons and triplets, t (67) = -1.027, p (two-tailed) = 0.308; or between twins and triplets, t (79) = -0.718, (two-tailed) = 0.475. As in previous

analyses, Levene's Test had shown no significant difference in variance between any pair of groups ($p=0.535$, *0.836* and *0.548* respectively), so the pooled t-test was used.

Effect of geographical region

The survival rates in each region may be influenced by many factors, including latitude, photoperiod and climate that are fairly similar between zoos in Indonesia and Singapore but highly variable between institutions within Europe or North America. There are also potential effects of periods of overcrowding (seen in Surabaya Zoo at times), repeated matings of incompatible pairs (seen with at least one pairing in Europe), nutrition or even disease that was undiagnosed or untreated. This analysis cannot offer reasons for differences between regions, only if differences exist. Survival rates are summarised in ***Table 12***.

	% survival			
	Europe (*n=220*)	N. America (*n=89*)	Singapore (*n=51*)	Indonesia (*n=55*)
>30days	61.4%	88.8%	62.7%	83.6%
>90days	58.2%	87.6%	54.9%	81.8%
>365days	56.4%	84.3%	52.9%	72.7%

Table 12: Table showing % survival beyond 30, 90 and 365 days, presented by geographical region in which it was born.

In addition, five animals were born in Pretoria Zoo, and all survived past 1 year. It is interesting to note the overall lowest % survival for all the regions is in Singapore, closely followed by Europe. Also conspicuous is the larger drops in % survival between 30 days and one year in Singapore and Indonesia (by 9.8% and 10.9%, respectively) compared to Europe and North America (5.0% and 4.5%, respectively). Could nutrition, disease or some other aspect of husbandry be affecting the

likelihood of transition from early life to adulthood? The drop is most marked between 30-90 days in Singapore and between 90-365 days in Indonesia.

The mean age at death +/- SD for those surviving beyond a year was 4043 (11.1yrs) +/-2054 days (*n=85*) in Europe, 4099 (11.2yrs) +/-1999 days (*n=33*) in North America, and 2881 (7.9yrs) +/-1610 days (*n=11*) in Singapore. The distribution of age at death for these groups is shown in ***Figure 26***. Numbers were insufficient to include Indonesia (*n=2*) or Pretoria (*n=4*) in this analysis.

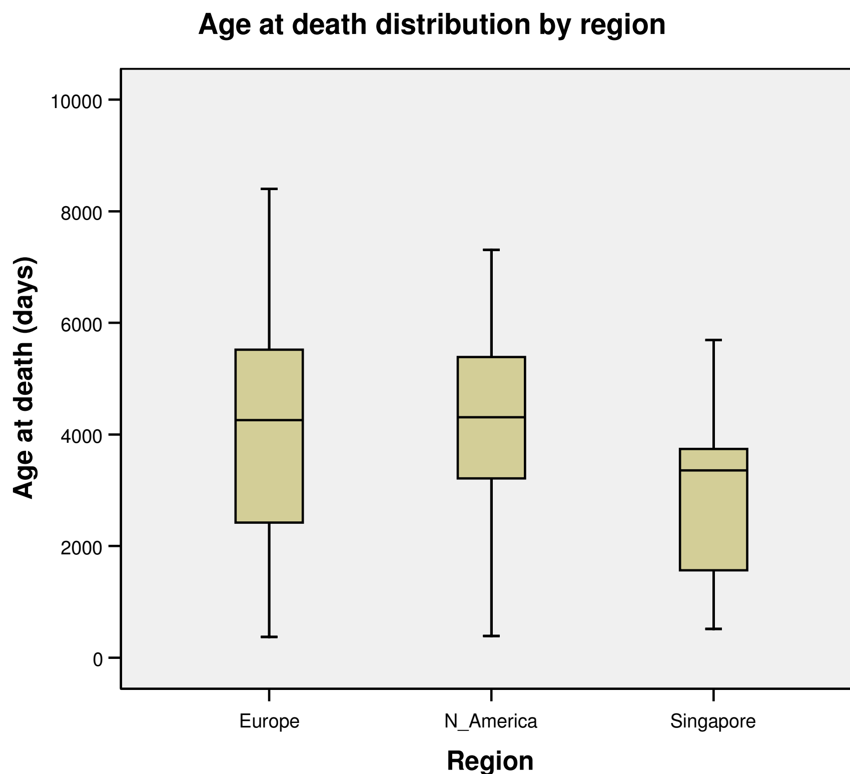


Figure 26: Boxplots comparing the distribution of age at death by litter size, showing maximum and minimum, upper and lower quartiles and mean values for each group.

No significant difference in variance was detected between pairs of group using Levene's Test (*p=0.672*, *0.240* and *0.406*) so the pooled independent t-test was again

employed to compare means between groups. Despite the obvious difference in distribution for the Singapore group, mean values were not quite significantly different at the 5% level, although only by a small margin: between Europe and N. America, t (116) = -0.135, p (two-tailed) = 0.893; between Europe and Singapore, t (94) = 1.911, p (two-tailed) = 0.059; between N. America and Singapore, t (42) = 1.934, p (two-tailed) = 0.06. It is likely that the culling of 16 animals in 1998 could account for this lack of older animals within the Singapore population. At the time of culling, the mean age of animal +/- SD was 1885 (5.2yrs) +/- 1327 days, ranging from 7 to 3768 days. If these animals had not been culled and all survived to the end Feb 2008, their mean age would have been 5290 (14.5yrs) +/- 1327 days, ranging from 9.3 to 19.7 years old. By culling, 16 of the total 51 animals who died (31.4%) were not able to reach their full lifespan potential, likely resulting in the lower mean age at death seen in the Singapore Zoo and Night Safari population.

Effect of age of dam

It is well-known in humans that embryos developed from ageing ova have a higher incidence of genetic defects and associated mortality than younger ones. Could this also be a factor for survival in the babirusa? Survival rates for the 364 individuals available for analysis (dead and living) are summarised by age of dam in ***Table 13***.

	% survival by dam age at conception				
	≤1000days (*n=52*)	≤2000days (*n=117*)	≤3000days (*n=89*)	≤4000days (*n=62*)	>4000days (*n=44*)
>30days	67.3%	74.4%	68.5%	64.5%	70.5%
>90days	65.4%	72.6%	60.7%	64.5%	65.9%
>365days	63.5%	70.9%	57.3%	62.9%	63.6%

Table 13: Table showing % survival beyond 30, 90 and 365 days, presented by age of dam at conception.

There is no obvious trend towards reduced survival rates with increasing age of dam. However, when survival times are considered separately for dead and living animals, the picture is slightly different. ***Figures 27*** and ***28*** show the relationship between the age at death/age at end Feb 08, respectively, and the age of their mother when she conceived.

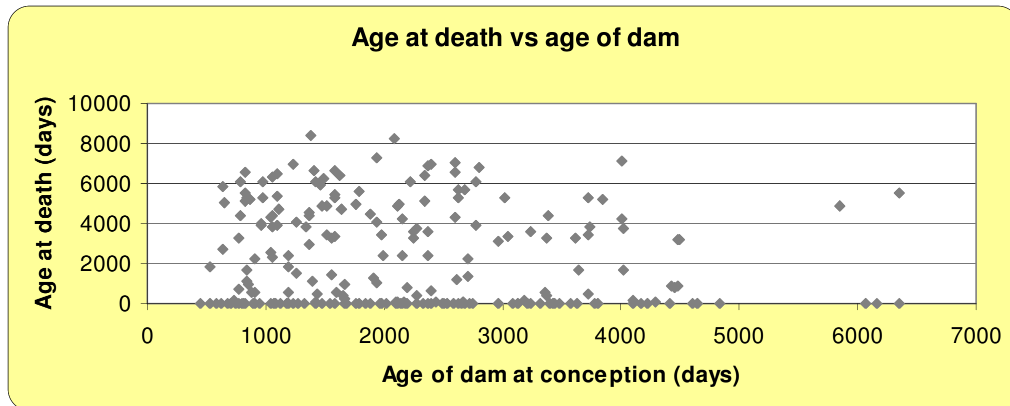


Figure 27: Scatter plot to show the relationship between the age at death for those no longer living at the end of February 2008 and the age of dam when she conceived that individual.

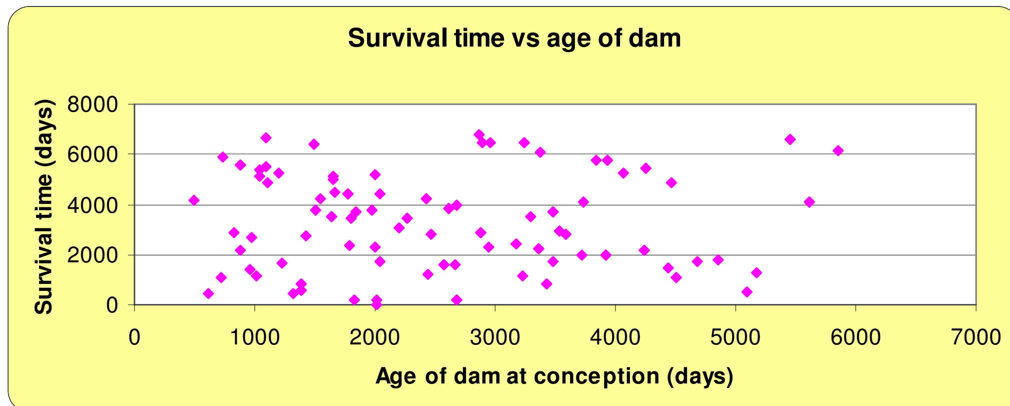


Figure 28: Scatter plot to show the relationship between the age of those still living at the end of February 2008 (survival time) and the age of dam when she conceived that individual.

Looking only at the data for animals now dead, there is a significant correlation between the age at death of the individual and the age of dam (*n=255*), with a noticeable drop beyond 3000 days. Distribution was assumed to be normal, giving a Pearson correlation value of -0.137, and two-tailed significance at the 5% level (*p=0.028*). It could also be significant to note that the % survival rates past 365 days for these 'dead' animals was lower than the overall rates for dams (as shown in ***Table 13***): 47% (compared to 57.3%) in those aged up to 3000 days (*n=65*); 37.8% (compared to 62.9%) in those aged up to 4000 days (*n=37*); and 40.7% (compared to 63.6%) in those over 4000 days (*n=27*). A significant correlation was not seen between the age of dam of those animals still living and their age at end Feb 08 (*n=109*, Pearson correlation=0.031, *p=0.747*), as shown in ***Figure 28***.

Effect of age of sire

Similar logic was applied to the possible effects of aging sires on the survival and lifespan of their offspring. Survival rates for the 351 individuals available for analysis (dead and living) are summarised by age of sire in ***Table 14***.

	% survival by sire age at conception				
	≤1000days (*n=32*)	≤2000days (*n=103*)	≤3000days (*n=103*)	≤4000days (*n=80*)	>4000days (*n=33*)
>30days	78.1%	80.6%	66.0%	63.8%	48.5%
>90days	78.1%	76.7%	60.2%	62.5%	45.5%
>365days	75.0%	73.8%	60.2%	61.3%	36.4%

Table 14: Table showing % survival beyond 30, 90 and 365 days, presented by age of sire at conception.

From this crude analysis, there seems to be reduced survival chances among those animals sired by older males, especially those over 4000 days old (~11yrs) at conception. As for the dam age data, this relationship is further analysed by looking

separately at the group now dead and those still living, and illustrated in ***Figures 29*** and ***30*** Especially for those animals already dead, you can see that the age at death appears to decrease as the age of sire increases above and beyond around 4000 days.

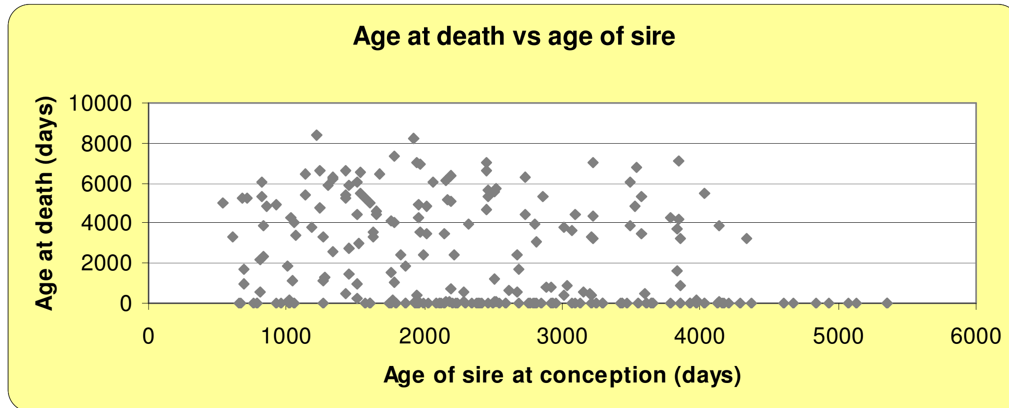


Figure 29: Scatter plot to show the relationship between the age at death for those no longer living at the end of February 2008 and the age of sire when that individual was conceived.

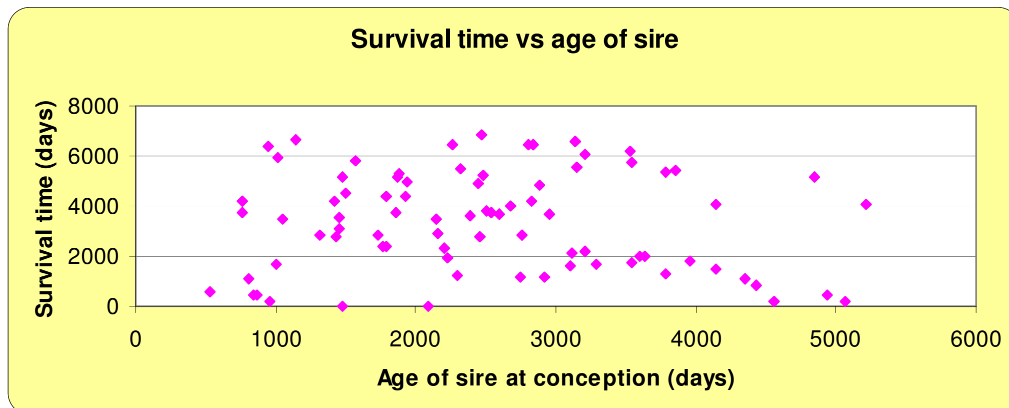


Figure 30: Scatter plot to show the relationship between the age of those still living at the end of February 2008 (survival time) and the age of sire when that individual was conceived.

Similar to the dam age analysis, the Pearson correlation value (-0.274) was significant at the 0.001 level (two-tailed) for the 'dead' population ($n=249$) but not for those still living ($n=102$, Pearson correlation=-0.112, $p=0.261$).

Multiple factor analysis

The previous analyses suggest that the factors of gender, age of dam, age of sire and inbreeding (*f*) can all contribute to survival and lifespan in a sample representing a large portion of the historical *ex-situ* babirusa population. However, each analysis was conducted on a slightly different subset of animals, depending on where data was available in relation to the factor of interest. In order to identify which of these factors are most important, a multiple factor analysis was performed on animals for which data was available in relation to all factors. The analysis was conducted with three data sets: with all animals for which data was available in relation to all factors (*n=246*); then excluding animals with hypothetical sire and dam ages i.e. sires 1, 5, 7, 15, 47 & 49 and dams 2, 6, 8, 16, 48 & 50 as mentioned in sections 2) and 3) above (*n=177*); then further excluding any animals from Singapore due to the possible knock-on effects of unknown parentage on generation levels and inbreeding values (*n=155*). Only lifespan was considered here as it didn't require the use of groupings and could retain the exact age at death. The results are presented in ***Tables 15***, ***16*** and ***17***.

	Factors affecting age at death (*n=246*)			
Factor	**Correlation**	**Sig. (one-tailed)**	**Effect size (r^2)**	**Effect size**
Gender	-.396	.000	0.157	large
Sire age	-.277	.000	0.077	medium
Dam age	-.157	.007	0.025	medium
Inbreeding	-.217	.000	0.047	medium

Table 15: Factor analysis to determine relative importance of gender, sire age, dam age and inbreeding coefficient on age at death, based on all animals with sufficient data available.

Of course, as from previous analyses, all the correlation values were negative, i.e. the factors all had a detrimental effect on age at death, or longevity. From this analysis,

all correlations were significant: gender, sire age and inbreeding to beyond the 0.001 level, and dam age beyond the 0.05 level. The relative order of importance by correlation value and therefore effect size is:

gender >> sire age > inbreeding > dam age

	Factors affecting age at death (*n=177*)			
Factor	**Correlation**	**Sig. (one-tailed)**	**Effect size (r^2)**	**Effect size**
Gender	-.370	.000	0.137	large
Sire age	-.293	.000	0.086	medium
Dam age	-.239	.001	0.057	medium
Inbreeding	-.152	.021	0.023	medium

Table 16: Factor analysis to determine relative importance of gender, sire age, dam age and inbreeding coefficient on age at death, based on all animals with sufficient data, excluding sires 1, 5, 7, 15, 47 & 49 and dams 2, 6, 8, 16, 48 & 50.

This time, the correlation values are significant to or beyond the 0.001 level for gender, sire age and dam age and at the 0.05 level for inbreeding. The order of importance by correlation value and effect size is:

gender >> sire age > dam age > inbreeding

	Factors affecting age at death (*n=155*)			
Factor	**Correlation**	**Sig. (one-tailed)**	**Effect size (r^2)**	**Effect size**
Gender	-.421	.000	0.178	large
Sire age	-.325	.000	0.106	large
Dam age	-.229	.002	0.052	medium
Inbreeding	-.119	.071	0.014	medium

Table 17: Factor analysis to determine relative importance of gender, sire age, dam age and inbreeding coefficient on age at death, based on all animals with sufficient data, excluding sires 1, 5, 7, 15, 47 & 49 and dams 2, 6, 8, 16, 48 & 50 and also animals from Singapore.

Now the correlation values are not significant for inbreeding and the order of importance by correlation value and effect size is:

gender >> sire age >> dam age > (inbreeding)

It is clear from all three data sets that gender will have the greatest effect on the longevity of a captive babirusa, followed by sire age (significant correlation to the 0.001 level in all three cases). Dam age and inbreeding also show a significant correlation with longevity but not to as great an extent as the other two factors.

Analysis of living population

Looking only at the 'current' population i.e. those still living at the end February 2008, can give an insight into the potential future for this babirusa *ex-situ* breeding programme. Without further addition of wild founders, what is present in zoos now would be the only genetic material available to further expand the population, and to act as the reservoir of animals for potential reintroduction at a later date. Effective management of this population is therefore paramount, and information gathered from the analysis of the now dead population can be used to inform the decision-making and action-planning process.

1) Population census (to end Feb 2008)

At the end of February 2008 there were 201 babirusas recorded as living in the worldwide *ex-situ* population: 85 males, 113 females and three of unknown gender (too young to be identified at that time). Of these, 98 (36.60.2) were held between five institutions in Indonesia, the largest groups in Ragunan Zoo in Jakarta (11.17) and Surabaya Zoo (12.31.2). 22.20 were held in European zoos, 20.23.1 in North American zoos, 7.9 in Singapore Zoo and 0.1 in Pretoria Zoo.

2) Origins of animals living in Europe, N. America and Singapore

These regions are of particular interest as they are committed to working together to maintain the *ex-situ* conservation of the babirusa outside of Indonesia. All animals

from these three regions were derived from a selection of the original founder population in Surabaya Zoo, but have received contributions from slightly different individuals, making each sub-population unique. As detailed earlier in _**Figure 16**_, the European population was founded from 6.6 animals brought in directly from Surabaya Zoo between 1974 and 1977 and through Ragunan Zoo in Jakarta in 1984. The North American population was founded over a period of 11 years (between 1984 and 1995) solely from animals bred within European zoos, and the Singapore population from a single transfer of 3.5 animals directly from Surabaya Zoo in 1992. The transition through the generations from these founding events through the maternal line to the population alive at the end of February 2008 is presented in _**Figures 31**_, _**32a**_ and _**32b**_

Note that animals designated '90—' are hypothetical and the relationships between them were hypothetical in producing F1 animals. They are all shown in dotted boxes/lines. Despite the artificial nature of these founder relationships, the information beyond them is known to be correct, and can be used to follow the divergence of maternal lines into the different regions. Animals now dead are shown only where they lead directly to those alive, they are otherwise omitted. Sires are shown on the lines connecting females, and in some cases two numbers are shown in one box: one is the International Studbook number, the other a regional one.

You can see from _**Figures 31**_ and _**32a**_ that there was early segregation of some lineages into the European and North American regions. The third figure is interesting in showing a more complex picture between the regions, including a small number of transfers between regions after the founding events.

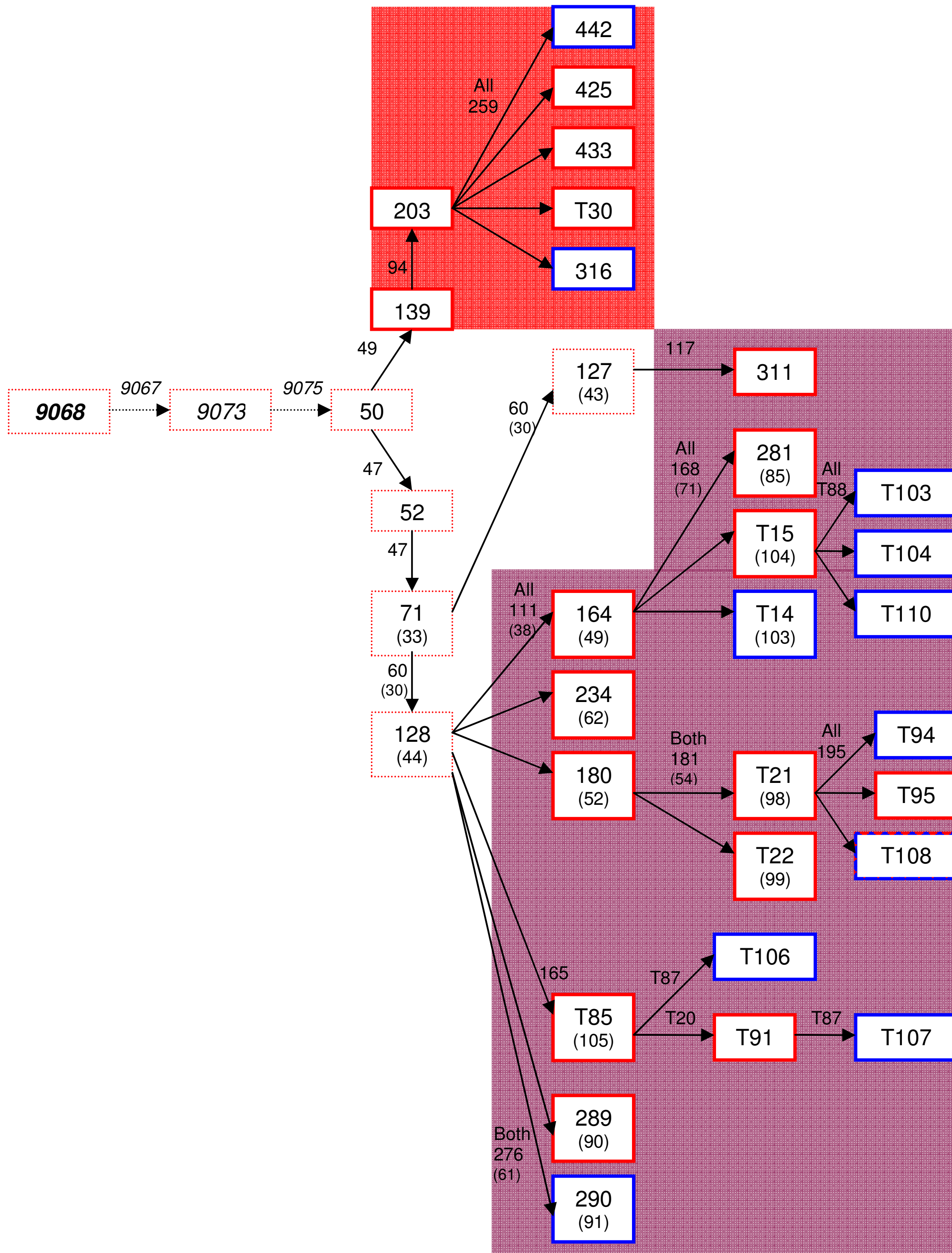


Figure 31: Diagram to show the female lineage proposed to derive from founder female 9068 to animals in the living population at the end of February 2008.

Notes: red box = female, blue box = male, thin box = dead, thick box = alive
red area = Europe, purple area = N. America

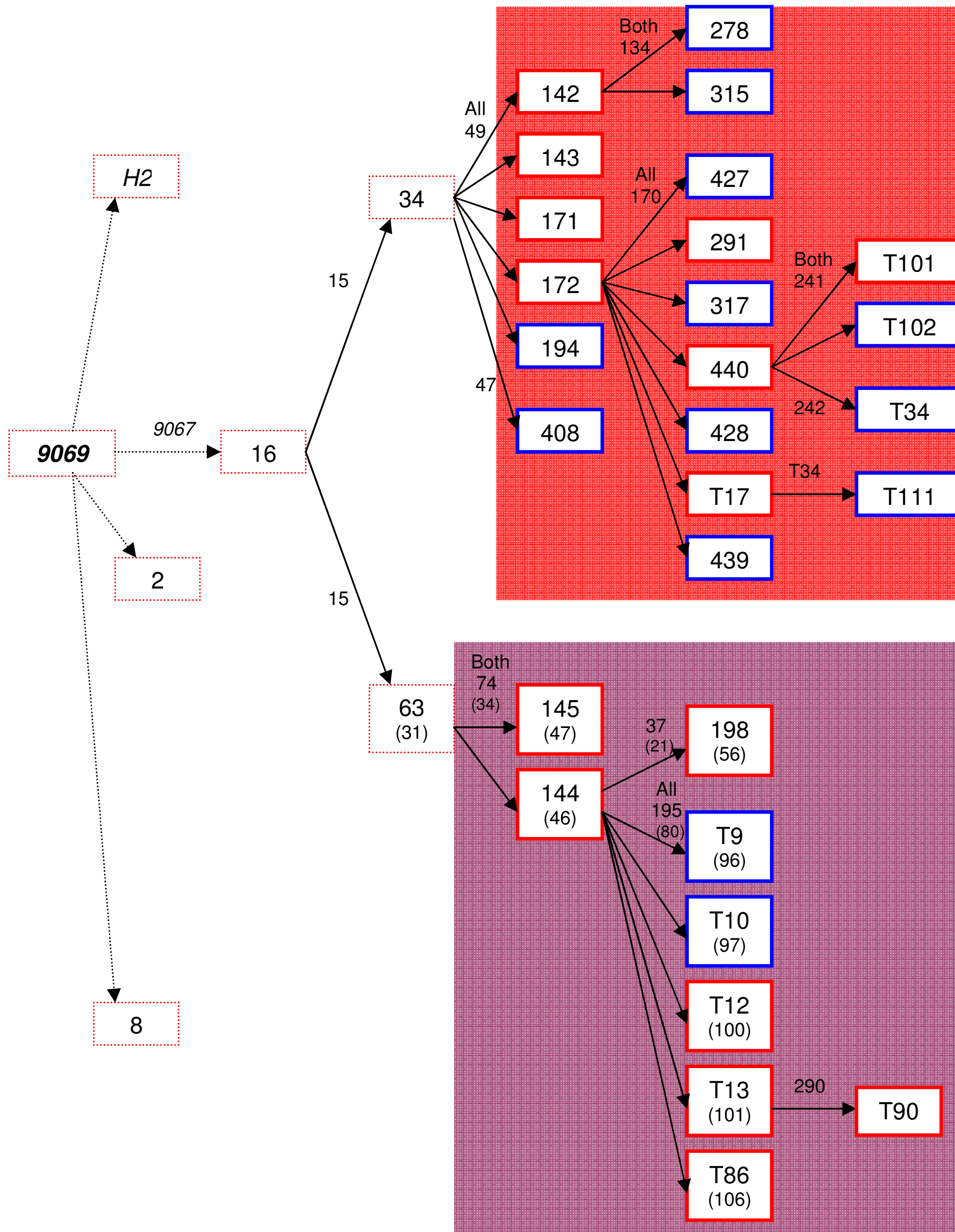
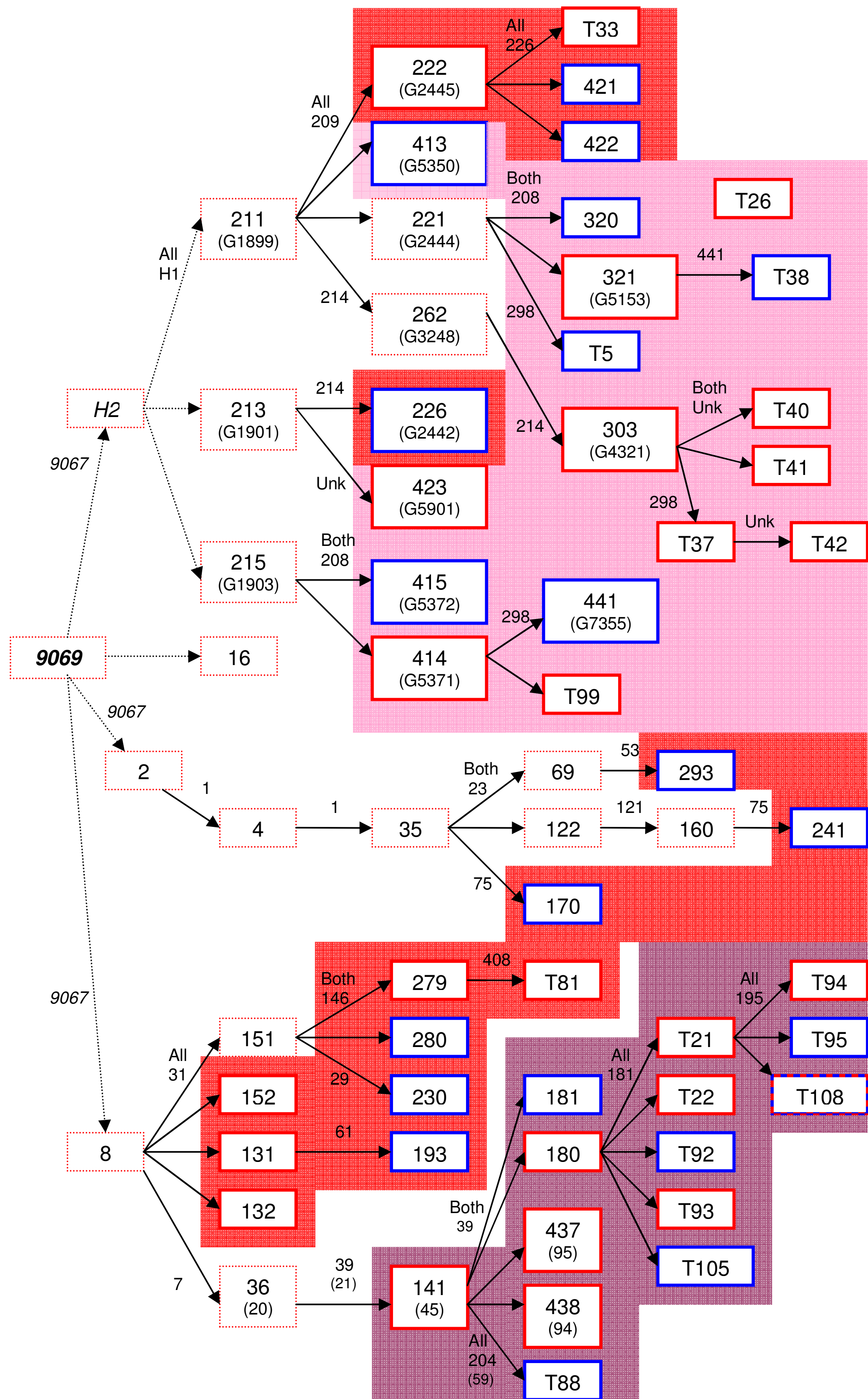


Figure 32a and 32b (next page): Diagram to show the female lineage proposed to derive from founder female 9069 to animals in the living population at the end of February 2008.

Notes: red box = female, blue box = male, thin box = dead, thick box = alive
red area = Europe, purple area = N. America, pink area = Singapore



All
226
T33
222
(G2445)
421
422
All
209
413
(G5350)
Both
208
T26
211
(G1899)
221
(G2444)
320
All
H1
321
(G5153)
441
T38
214
262
(G3248)
298
T5
214
Both
Unk
H2
213
(G1901)
226
(G2442)
214
303
(G4321)
T40
9067
T41
Unk
423
(G5901)
298
T37
Unk
T42
Both
208
215
(G1903)
415
(G5372)
441
(G7355)
298
9069
16
414
(G5371)
T99
9067
53
2
Both
23
69
293
1
1
121
75
4
35
122
160
241
75
170
408
Both
146
279
T81
9067
All
195
T94
151
All
181
T21
T95
All
31
280
29
152
230
181
T22
T108
61
8
131
193
180
T92
132
Both
39
T93
437
(95)
7
39
(21)
36
(20)
141
(45)
T105
438
(94)
All
204
(59)
T88

Combined, the figures give the impression that the European population represents a greater number of lineages that either of the other two. In fact, it a clear to see that the living Singapore population represents only three female founders (#s 211, 213 and 215) and two males (209 & 214).

3) Assessment of breeding-age animals

Considering the outcome of the dam and sire age analysis, it is important to establish the current age distribution of the animals still alive in the breeding programme. It is important not only to see how many are likely still to be of reproductive age, but also to assess the possible implications of ageing dams and sires on survival and longevity of their offspring. The following age pyramid (***Figure 33***) represents the worldwide population at the end of Feb 2008.

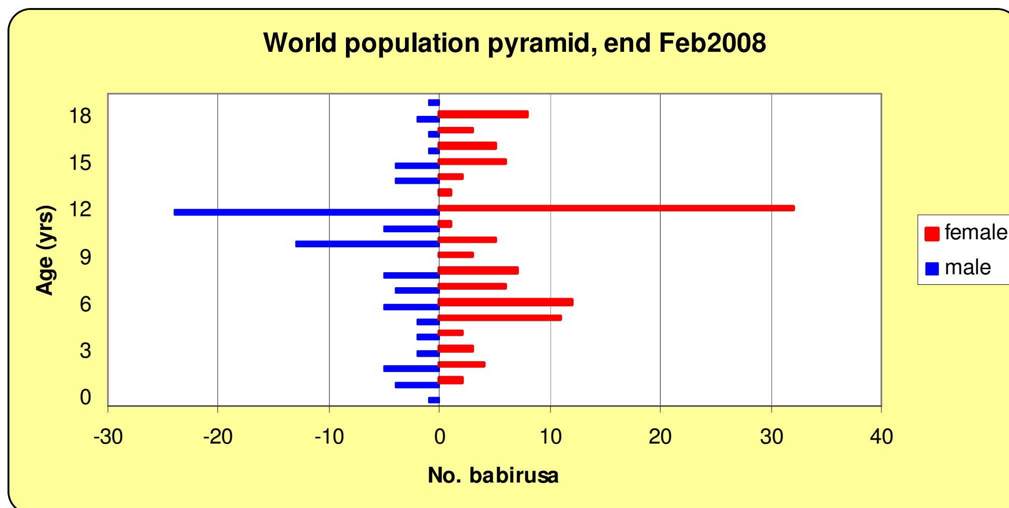


Figure 33: Age pyramid showing the age distribution of males and females living in the worldwide ex-situ population at the end of February 2008.

Note that the large bars at age 12 represent the large number of Indonesian animals with arbitrarily assigned birth dates, as previously described. Other than this artefact,

there is no particular age 'bulge' in the males, but two of them in the females, one between ages 5-8yrs and the other between ages 15-18yrs.

From previous analyses of the dead population, considering the number of births and subsequent effects of the longevity of their offspring, the optimal age range for breeding females is from puberty up to 6 years. Beyond this age, there is reduced likelihood of success i.e. a proportion of females in this 'bulge' is already beyond prime reproductive age. However, there is likely to be only a limited effect on offspring survival and longevity should a pregnancy occur. For males, it is a little different: breeding up to age 8, 9 or even 10 years is likely to be successful, but from around 11 years there is likely to be lower success and significant effects on offspring survival and longevity. In an ideal situation, therefore, the large number of animals in the 'artefact' group aged 12 years should probably not be used for breeding purposes.

When this data is presented by geographical region the story becomes even more interesting, and is shown in ***Figure 34***. Note the different x-axis scale for the Indonesian population compared to the three others. Looking at each region in turn, the potential for future population growth is also assessed, assuming each existed in isolation.

Indonesia: there is an excess of females over males, but with a good range of female ages, peaking at 6 years. Males are limited in number and the majority are past their prime age. Although males can be used to sire multiple females at any given time, it is likely to result in over-representation of a few males.

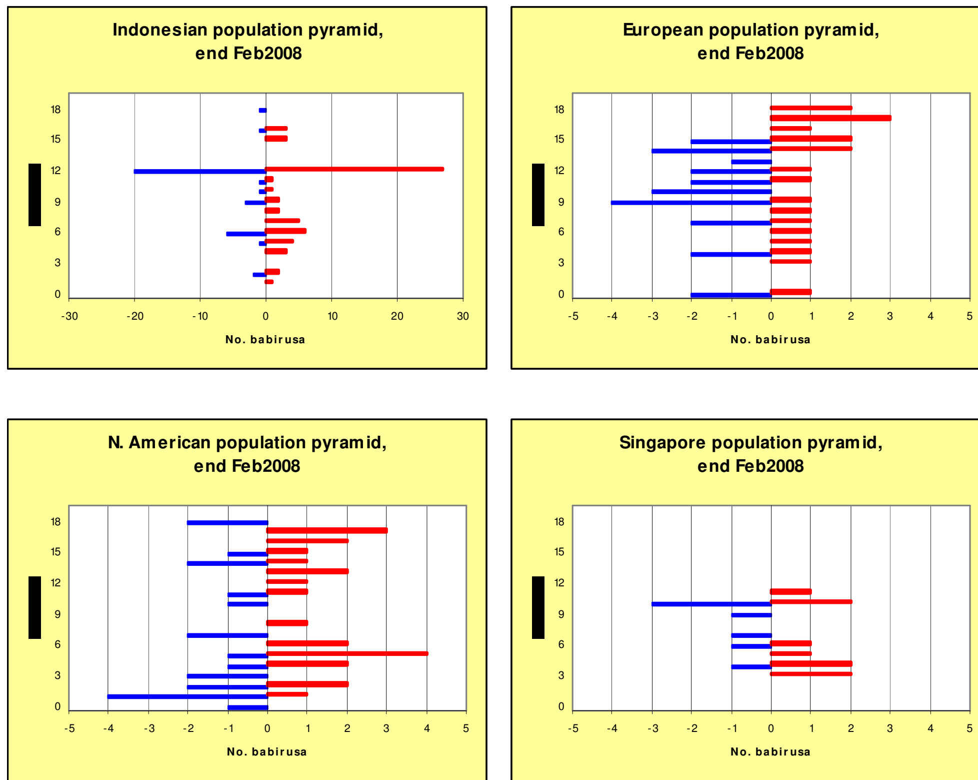


Figure 34: Age pyramids showing the age distribution of males and females living in the worldwide *ex-situ* population at the end of February 2008, shown separately by region.

Europe: age-distribution is top-heavy in both sexes, but especially in females, with only eight of them under 10 years. Many of the males are also past optimal breeding age, with three aged 10 years and only ten others below this age. Limited numbers of optimally-aged animals, particularly females means population growth will be limited. In addition, the use of older males may impact on offspring survival and longevity.

North America: around half of the population is below nine years, with almost equal numbers of males and females. Although these numbers are still small, they offer good potential for further population growth.

Singapore: although this population is not as old overall compared to the others, the small number of animals makes the risk of inbreeding particularly high. This may eventually have an impact on juvenile survival and longevity of any offspring.

Clearly each region has its own potential limits to population growth, the least problematic perhaps being in North America. In theory, of course, all animals in all regions are part of the International Breeding Programme and should be able to be considered as a single population, as shown in ***Figure 33***. In reality, however, there are many practical and political obstacles to being able to truly consider the population as a whole. Most obvious, perhaps, are the huge distances between regions, and even within them, with the resulting costs and risks of animal transportation. There is also the limitation on space that zoos can provide to a single species. In particular, there are 'fashions' with regard which species zoos keep and that doesn't always match to the conservation needs of any given species. There is also the limitation of mate choice for any given animal, due to keeping small numbers of animals dispersed between institutions, leading in some cases to incompatibility. This has been cited as a factor limiting population growth of cheetah (*Acynonyx jubatus jubatus*) at the San Diego Wild Animal Park (Augustus, Casavant et al. 2006) and in rhesus macaque (*Macaca mullata*) (Goy 1979).

Incorporation of additional stock from Surabaya

From the analysis of population pyramids above, it is clear that the largest single population of babirusa is still in Surabaya, Indonesia. A large part of this population is made up of 'new' founder stock brought into Surabaya in 1998 and their multiple offspring of F1 and F2 generations. As well as contributing considerably to the

lower age range of the pyramid, they represent new genetic lines not found anywhere else in the worldwide population. As such, they are vital to the successful continuation of the International Conservation Breeding Programme.

4) Assessment of European, N. American and Singapore populations

Efforts to release some of these 'new' animals to the population outside of Indonesia have so far been unsuccessful. It is uncertain when this situation might change and the invaluable injection of new genetic lines into existing populations can begin. In the meantime populations are ageing and becoming more inbred. It is therefore prudent to consider the options for the programme with the existing animals available only in Europe, North America and Singapore. The population pyramid representing these regions combined in shown in ***Figure 35***.

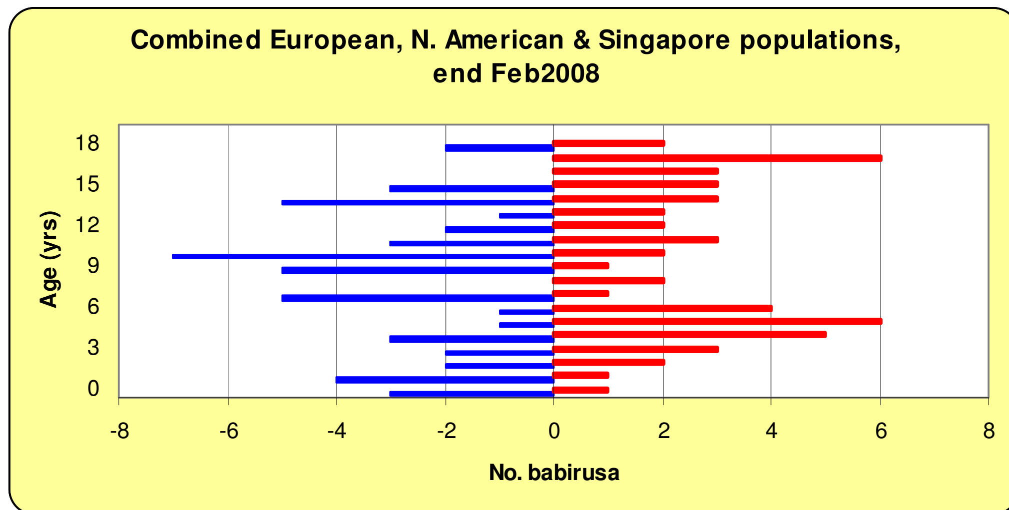


Figure 35: Age pyramid showing the combined age distribution of males and females living in the European, N. American and Singapore populations at the end of February 2008.

Already, the lower age range of the pyramid looks healthier, with increased numbers and roughly balanced male and female numbers. It also makes clear however, just

how much of the population is already beyond likely breeding age and thus cannot be considered as having an active role to play in the continued growth of the population. In other words, the $N_e < N$ based on the age pyramid alone.

In addition to the age pyramids for each region, other details are important to inform the management strategy for these populations. ***Table 18*** summarises the main factors of mean age, generation level and inbreeding value for each of the three regions, and also combined. This will help to identify the benefits to each regional population by being considered a part of the larger combined population, and so to assess the potential of each in turn.

Region	**Males Mean age (days) +/- SD**	**Females Mean age (days) +/- SD**	**Mean generation**	**Mean inbreeding**
Europe	3713+/-1532 (*n=22*)	4300+/-2058 (*n=20*)	4.12	0.24
N. America	2682+/-2249 (*n=20*)	3466+/-2070 (*n=23*)	4.52*	0.28*
Singapore	3089+/-791 (*n=7*)	2429+/-1138 (*n=9*)	3.76**	0.34**
Combined	3203+/-1828 (*n=49*)	3608+/-2018 (*n=52*)	4.26*	0.27*

* Note that there was 1 of unknown sex and this was included in these calculations
**Based on 11 animals: 5 were excluded with unknown sire (*n=4*) or dam (*n=1*)

Table 18: Summary of mean values for age of males and females, generation level and inbreeding coefficient, presented for the European N. American and Singapore populations, and for all three combined.

From ***Table 18*** you can see some of the benefits to each region of combining with the others to be considered as a whole. For example: the mean age of animals in Europe is reduced in the combined population (from 10.2 to 8.8yrs for males and from 11.8 to 9.9yrs for females); the high mean generation level of the North American population is reduced from 4.52 to 4.26 combined; and the high inbreeding

level of 0.34 in the Singapore population is reduced to 0.27 combined. These numbers are of course theoretical, and would depend on efficient exchange of animals into and out of each region. They do show, however, that even without 'new' stock from Indonesia, the three regions can improve their current situation by considering themselves part of a larger amalgamated population.

Inbreeding vs generation level in the living population

Analysis of the dead population highlighted the positive correlation between generation level and inbreeding, suggesting that the population was becoming more inbred over time. Does this correlation exist in the living population? If so, there is a serious possibility that inbreeding will become a greater risk to the health and success of this population. The correlation between these factors for 97 living animals for whom data is available is show in ***Figure 36***.

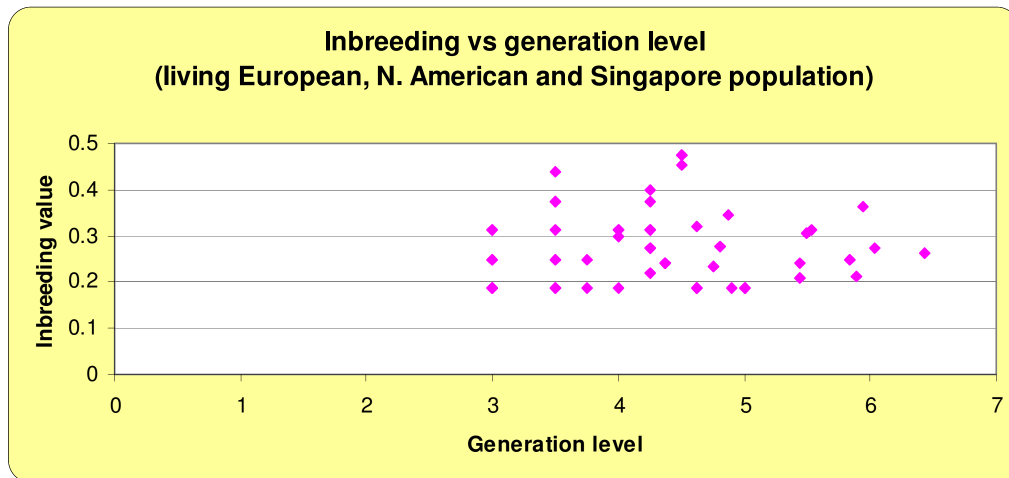


Figure 36: Scatter plot showing the relationship between inbreeding coefficient and generation level combined for the population living in Europe, N. America and Singapore at the end of February 2008.

You can see that all of the animals are from generation level 3 or above and that the lowest inbreeding values are just below 0.2. However, the Pearson correlation value

between these factors was 0.076, not significant with a 2-tailed p-value of 0.459. So, in the living population, generation levels are increasing, but without increasing inbreeding. Perhaps animal pairings likely to produce offspring of higher inbreeding levels were simply never successful. Whatever the reason, this is obviously good news, although there may still be an impact on lifespan of these animals through their relatively high generation levels and inbreeding values.

From the previous analyses based on the 'dead' population, there was a significant negative correlation between generation level and age at death. The scatter plot (***Figure 21***) showed a clear reduction in age at death between generation levels 3-6. Considering the distribution of generation levels in the living population (almost up to level 7), it is clear that individuals living in the population today may not enjoy the same longevity as those before them. In addition, the negative correlation between inbreeding and age at death (although not so strong) was most obvious beyond inbreeding values above 0.35 (***Figure 22***). Looking at the spread of inbreeding values for this living population there is also some cause for concern, with 12/102 individuals (11.8%) inbred beyond the 0.35 level: 2/42 (4.5%) in Europe, 2/16 (12.5%) in Singapore, and 10/44 (22.7%) in North America.

5) Assessment of 'new' Indonesian stock

In 1998, 5 male (#s 404, 405, 406, 410 and 419) and 2 female babirusa (#s 411 and 420) were introduced to the population held in the Surabaya zoo. Of these, all of the males and one female have successfully bred and produced numerous F1 and a few F2 animals, giving a founder base of this 'new' stock of 5.1. A total of 43 animals (6 wild founders, the non-breeding female and 36 offspring) can be considered part of

this ‘new’ stock. 33 of the offspring are F1 animals, 4 from the mating of two wild parents and the others from mating new males with original stock females (9 in total). Of the other 3 offspring, two were from the mating of a new F1 animal with another of the wild males, and the third from the mating of a new F1 male with an unknown female.

Living population from ‘new’ stock

Of the 43 ‘new’ stock animals, 34 were still living at the end of February 2008, including 2.1 of the founders (and the non-breeding female). All were still at the Surabaya Zoo except for one female who had been transferred to Jajasan Babirusa on the Indonesian island of Bali. A population pyramid for this ‘new’ population is shown in ***Figure 37***. More details of this population are summarised and compared with the combined European/North American/Singapore population in ***Table 19***.

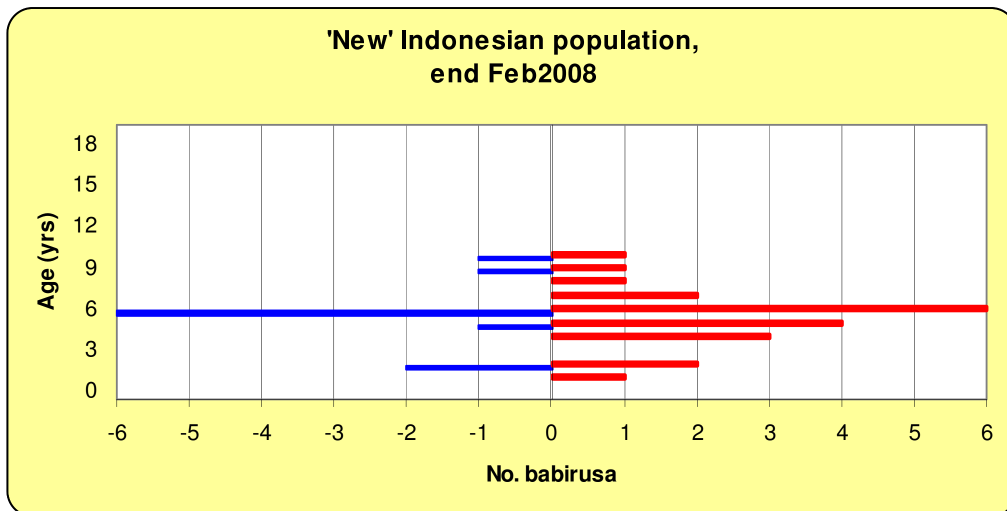


Figure 37: Age pyramid showing the age distribution of males and females at the end of February 2008, derived from the ‘new’ founder animals introduced to the Surabaya population in 1998.

From the pyramid of the 'new' population shown in isolation, you can see that it is still early in its development with all animals under 11years of age. You can also see the large difference between numbers of the two sexes, with females outnumbering males 21 to 11. There are two additional animals of unknown sex (aged 2 days and 92 days at the end Feb 2008) not included in the pyramid. If only this 'new' population is added to those from the other regions, it has a significant impact on the overall mean status of the *ex-situ* population, as shown in ***Table 19***.

Region	**Males Mean age (days) +/- SD**	**Females Mean age (days) +/- SD**	**Mean generation**	**Mean inbreeding**
'New' Indonesian	2267+/- 933 (*n=11*)	2142+/-825 (*n=21*)	1.36*	0*
Europe, N. Am & Sing	3203+/-1828 (*n=49*)	3608+/-2018 (*n=52*)	4.26	0.27
All Combined	3033+/-1731 (*n=60*)	3186+/-1876 (*n=73*)	3.51*	0.20*

* Note that there were 2 of unknown sex and they were included in these calculations

Table 19: Summary of mean values for age of males and females, generation level and inbreeding coefficient, presented for the 'New' Indonesian population, the European N. American and Singapore populations grouped together, and for all four populations combined.

So, with the inclusion of these 'new' animals, as well as the obvious increase in numbers (40.4% more males and 22.4% more females), there are improvements in mean ages, mean generation level (reduced by 17.6%) and mean inbreeding coefficient (reduced by 25.9%). An added benefit of considering adding only this 'new' population is that studbook data for these animals is much more accurate (see earlier assessment) than had been previously. Therefore, population management could be planned much more effectively than if all Indonesian animals were included. The final benefit to adding these populations together is the positive effect it has on the population pyramid, as shown in ***Figure 38***.

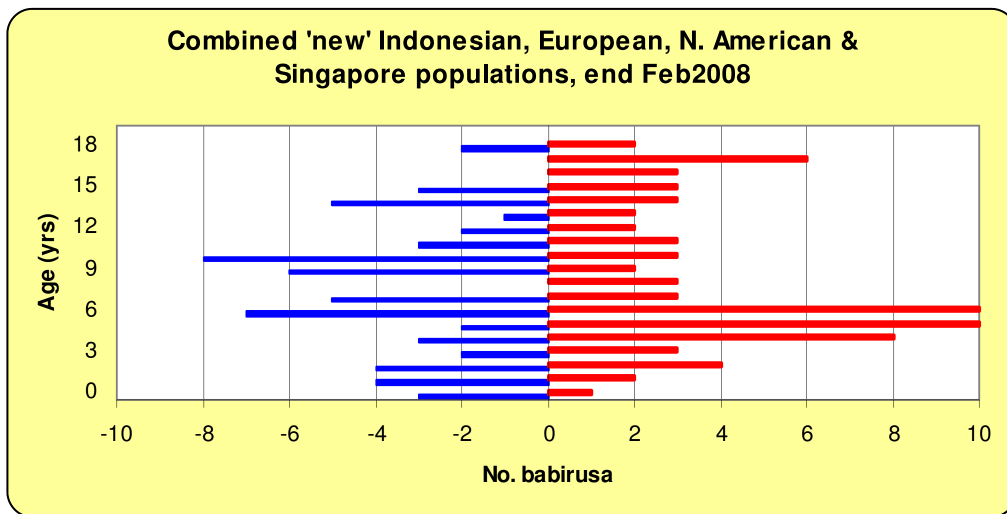


Figure 38: Age pyramid showing the age distribution of males and females at the end of February 2008, showing the 'new' Indonesian populations combined with those from Europe, N. America and Singapore.

Looking at the pyramid, it is clear that male and female numbers are more balanced, although still with an excess of females between four and six years. However, this is peak breeding age for females and would offer huge potential for rapidly growing the population. The male population up to age 10 is also looking good, representing 46 animals, 76.7% of the total male population.

DISCUSSION

The purpose of this chapter was to interrogate the International Studbook for the babirusa to assess in detail past population parameters and apply them to the population currently living. In this way, it is possible to predict the impact of factors such as age, generation level and inbreeding on the likely survival and reproductive capacity of the population. There are a number of discussion points with respect to the methodology of the analysis, the overview of the breeding programme, findings from the historical population, the application of historical findings to the current living population, and the recommendations that can be made for future management of this species in an *ex-situ* context.

Reproductive parameters

Litter sizes are recorded as the number of piglets witnessed by keeping or other zoo staff, usually on the morning after parturition. Mean litter size was calculated at 1.46, with single piglets being the most common, followed by twins then triplets. It is likely in a number of cases that there were in fact more piglets born than recorded, with one or more being stillborn or killed and eaten peri-partum. There are anecdotal reports of this happening with the babirusa and it is also reported in domestic sows (Chen, Gilbert et al. 2008). There is also evidence from post-mortem reports that females have many more corpora lutea on their ovaries than there are piglets born (Alastair Macdonald, personal communication), suggesting that more conceptions occur than piglets are born and survive.

There are obvious differences in the distribution of ages at conception for sires and dams. Females are capable of conceiving young much younger than their male

counterparts, with the youngest recorded conceptions aged 9.6 and 17.6 months respectively. The peak ages for conception are also different, with most litters recorded to females aged 3 years and to males at 6 years, with females less likely to produce young beyond 7 years although both clearly drop beyond 11 years. Although both sexes can breed for the first time later into life, there is a risk that they will only be successful once, or that their offspring may not survive.

There is also an interesting case of a female (#142) who was a very successful breeder with one sire (producing six piglets who all survived to adulthood) but whose three piglets born to another sire all died within 30 days of birth. How could this be explained? Perhaps it is just a result of her ageing beyond 11years with the second sire, or could there be an issue of individual incompatibility? An additional factor may have been the transfer from one institution to another with all the associated stresses due to changes in management, diet, climate, etc. Could this have affected her temperament so much that it affected her parenting skills? Even if this is possible, it is unlikely to be a long-term effect.

Factors affecting survival and lifespan

There are multiple factors affecting survival and lifespan in this population. Survival beyond the critical periods of 30 days, 90 days and one year is seen to be affected in this population by two factors: the region in which they were born, with survival rates from North America > Indonesia > Europe > Singapore; and by the age of sire, particularly when he is >11yrs old at the time of conception. Other factors affecting survival and lifespan were not investigated but have been shown to affect other species. For example, parity in female Indian rhinoceros has been shown to affect

infant mortality, with primiparous females less likely to successfully raise offspring than more experienced mothers (Pluháček, Sinha et al. 2007). This has been attributed to lack of mothering skills in primiparous females.

With increasing generations of this *ex-situ* breeding programme, there is evidence that individuals' lifespan is reducing overall. This is obviously a concern with respect to the ongoing success of the programme as fewer individuals may live long enough to contribute to it. Although they may live well into their prime breeding age, they have to be given the chance to breed early to ensure they are able to contribute. Multiple factors appear to be affecting typical lifespan, with gender having the greatest effect as females are living significantly longer than males. This of course cannot be modified but should be taken into account when considering the relative likelihood that an individual will survive beyond a certain period.

Other factors that are affecting lifespan have also been identified and could potentially be corrected for. The most important of these from the multiple factor analysis is that of sire age at conception. It has already been noted that the male babirusa is capable of siring offspring well into later life, even if only mating for the first time. However, the impact of this is not necessarily on breeding success or offspring survival but on their likely lifespan, particularly as the sire ages beyond 11 years or so.

Similarly, but to a lesser extent, female age at conception can have effects on the lifespan of her offspring, particularly as she ages beyond 8 years. In some respects this is less of a potential problem as female breeding events are uncommon beyond this age anyway, as previously noted. In both of these cases, there is not seen to be a

significant correlation between parent age and lifespan for those currently still living. Could this be a sign that this will be less of a problem in the future population, or just that the living population is not yet old enough to see any significant effects? Inbreeding has also been identified as a factor affecting lifespan, although less so than parental age at conception. At the population level, mean inbreeding coefficients beyond 0.25 start to increase the risk of extinction (Frankham 1995).

Population management effects

Although it is possible to describe trends and associations within the babirusa studbook, it is important to acknowledge that this population is not a naturally-breeding population. Any analyses undertaken here are done so on the basis of the population being actively managed in multiple locations, not as a free-living, freely-mating population. As a result, breeding between individuals is inevitably restricted by the availability and suitability of mates.

For example, measures of breeding ages and success are certainly affected by which animals are 'allowed' to mate and who is available to mate with (Lacy 1994). Some animals may be prioritised for breeding by age or prior success, or by which animals are easiest to transport between institutions. These decisions may limit the likelihood of any individual being allowed to mate and may result in inability to breed when they are then given the opportunity. Success may also be affected by the management strategy employed by a given institution. Some have traditionally held large groups together while others have only kept a single pair or small numbers (Kauffels 2008). This is likely to have affected pair interactions and dynamics with respect to access to each other and contact by sight and/or smell.

On occasion, for reasons of health and/or welfare grounds, animals have to be euthanased. This has implications on a number of levels. Firstly, deaths noted in the studbook and reported by SPARKS are not distinguished from 'natural' deaths. Because of this, it is not always certain under what circumstances a death has occurred. In the case of the babirusa studbook, it was known from ARKS records that a number of animals in Singapore were euthanased around the same time in 1998 after positive diagnoses with swine fever. This has been noted and taken account of in these analyses, but other similar situations may have existed that are not accounted for. Secondly, practices may vary between regions or over time in zoo management attitudes towards euthanasia, leading some to euthanase more readily than others. Finally, there are reports of increasing incidence of lameness and spinal problems within the *ex-situ* breeding population (personal communication Alastair Macdonald). If this is really increasing through the generations, perhaps this is contributing to increases in euthanasia (the only option once standard treatments are no longer effective). Could these health problems be related to inbreeding?

Record-keeping

As well as the previous example of records not distinguishing natural deaths from instances of euthanasia, or litter sizes only recording those 'left alive', there are likely to be other examples of information not being readily captured from the studbook by SPARKS reporting. Another encountered in this analysis was where twins were recorded as 'births', but on closer inspection of ARKS records were found to have been discovered decomposed in the uterus of the female on autopsy. Should these strictly-speaking have been recorded as births?

Exclusions

It has already been argued that in order to make best use of the studbook data available that some exclusions have been made. Firstly, animals with unknown parentage are automatically considered to have generation levels and inbreeding coefficients of 0. Not only might this be untrue but it also has knock-on effects on the whole lineage that follows. In some cases, the whole lineage has then been removed from the analysis (e.g. the whole Singapore population). In others however, where generation levels and inbreeding are not being investigated, these individuals have remained in the analysis. This results in each analysis being based on a slightly different subset of animals from the pedigree and although this makes direct comparison of analyses difficult, it does allow the use of as many data points as possible in each scenario. The possible problem with comparing different data sets has been addressed in the section dealing with factors affecting survival and lifespan by using a single data set to investigate the relative effects of the various factors. It is also important to note here that founder uncertainties have less of an effect as generation level increases through the pedigree (Rudnick and Lacy 2008).

Inbreeding effects

Increasing generation number need not result in increased inbreeding. Maintaining a large enough population and/or careful genetic management can limit its occurrence by allowing breeding between unrelated individuals or at least only between those distantly related. This is the Mean Kinship (MK) strategy which has been shown to be the most effective way to manage small populations for optimal retention of genetic variation (Montgomery, Ballou et al. 1997).

In the case of the *ex-situ* babirusa population, where the MK strategy has not always been applied, there is a correlation between the two measures, at least when considering those animals who have since died. This confirms that mean inbreeding levels have been increasing as the programme has progressed and are associated to some extent with negative effects on post-natal survival and lifespan. However, inbreeding was the least important of factors in multiple factor analysis and cannot fully explain the effects on decreasing survival and lifespan. There is more evidence pointing to an association with 'generation level', but this in itself is not a factor.

The good news is that there is no significant correlation between generation level and inbreeding coefficient in the 'living' population. This could perhaps be the result of management efforts aimed specifically at limiting inbreeding, or could there have been a degree of 'purging' of inbred animals from the population? This phenomenon has been noted in relation to both wild and captive mammal populations (Ballou 1997) and in simulation studies (Boakes and Wang 2005).

The need for new stock

There is no doubt that even if inbreeding is not having significant measurable effects on the current babirusa population, it still represents a threat to the long-term survival of the species *ex-situ*. Continued breeding of a relatively small population, with multiple barriers to free reproduction will inevitably lead to inbreeding depression (Frankham, Ballou et al. 2002). This will then further limit the ability of the population to maintain itself or grow (Koeninger-Ryan, Lacy et al. 2003). The ultimate goal of maintaining this species as a 'reservoir' for potential reintroduction

to the wild cannot be satisfied by a small, inbred population as it will have reduced fitness and be more prone to extinction (Dudash and Fenster 2000).

As has been previously stated, the vast majority of the captive babirusa population was derived from the hypothesised 1.2 founder animals brought to the Surabaya Zoo in 1972. This population has been bred into its seventh generation mainly within distinct geographical confines and is showing some signs of potential problems ahead. The problem of small founder number was acknowledged officially in 1996 when the Conservation Breeding Specialist Group (GBSG) suggested at a Population and Habitat Viability Assessment meeting (Manansang, Macdonald et al. 1996) that more founder be incorporated into the population. Of the recommended 20 animals, seven (5.2) were successfully brought to the Surabaya Zoo and 5.1 of them have bred well since that time. 2.1 of these founders and their 30 surviving offspring represent 'new' genetic lineages. The new founders are recorded as having been caught in 'North Sulawesi', compared to reported origin of 'North Central Sulawesi, near Poso' for the original ones. This may or may not represent a significant difference in location but it does represent a group of animals collected 26 years after those who founded the original *ex-situ* population. During those years, there has been a significant decline in babirusa numbers in the wild (Lee 2001; Milner-Gulland and Clayton 2002; Lee 2005).

Theoretical combined populations

Although the theoretical population composed of current living animals in Europe, North America, Singapore and the 'new' Surabaya stock has been shown to give a good basis for continued growth and recovery of genetic health, there is still a major

obstacle. Due to ongoing problems with exportation of animals out of Indonesia, the new animals have not yet been able to contribute to the worldwide *ex-situ* population. This stems from the intense difficulties in moving CITES-protected species, and also due to reluctance on the part of the Indonesian government to allow any transfers. Because of this, due consideration has been given to a theoretical population consisting of only European, North American and Singapore populations. In real terms, these are the global areas willing to cooperate and exchange animals in order to see improvement in the *ex-situ* conservation breeding population in the immediate future. Although they all originate from the initial founder stock from Surabaya Zoo, they may still offer some hope of continued breeding until a better solution can be found.

Long term, however, there MUST be injection of new stock to the population outside Indonesia. Even the introduction of a single genetically distinct individual per generation can limit the effects of inbreeding and inbreeding depression (Frankham, Ballou et al. 2002), although up to 10 has also been recommended . Otherwise the future of the *ex-situ* population will be threatened and all the efforts of zoos worldwide to support conservation of this species will have been in vain.

Summary

The recommendations resulting from this analysis are:

1) to breed animals as early in life if possible, taking into account the peak ages for reproduction and the differences in mean longevity between males and females. This will maximise successful breeding and reduce the risk of limiting the lifespan of any offspring. It may also reduce the risk of females becoming irreversibly non-reproductive or developing pathologies related to non-parity, a has been seen in captive white rhinos (Hermes, Hildebrandt et al. 2006).

2) to breed NOW in order to fill up the lower end of the population pyramid. Even if it means breeding closely related individuals, numbers are most important at this stage where population growth is the priority (Lacy 1994).

3) to continue managing the population by minimising mean kinship as the most effective way to avoid inbreeding depression with increasing mean generation level (Rudnick and Lacy 2008). This is more easily achievable now than ever before with the ‘cleaned’ International Studbook and improved record-keeping.

4) to integrate Indonesian stock with the other populations. This will serve to even out sex ratios and increase numbers in the Indonesian population as well as reduce mean age, generation level and inbreeding coefficient in the other regions.

Muhibah's place in the babirusa pedigree

Muhibah, the female babirusa that resided for many years at Edinburgh Zoo but never reproduced, had an interesting place in the babirusa pedigree. Her parents, sire #75 and dam #160 had a total of seven offspring, Muhibah being the only female and from the second of four litters. Three others died within days of birth, and a fourth aged five years. The two surviving males, twins #241 and #242, successfully produced their own litters with the same female at the Southlakes Wild Animal Park in the UK. #241 sired six, with two surviving and #242 sired three with one surviving. The story of the Southlakes animals is described later in **Chapter 4**.

Muhibah and her siblings were of mean generation level 4 and had inbreeding coefficents of 0.1875. Their sire was one of the first generation bred in Europe from stock sent from Jakarta in Indonesia (from sire #49 and dam #50, hypothetical half-sibs), with an inbreeding coefficient of 0.25. Their dam was the product of a full-sib mating between sire #121 and dam #122 (both from the early Surabaya Zoo lineage) and had an inbreeding coefficient of 0.3984 as a result. Muhibah's inbreeding coefficient was lower than those of her parents as their lineages were unrelated. Looking at dam #160, could the high inbreeding coefficient (through a full-sib mating) be a factor in her post-natal loss of three from seven offspring? What effect might this have had on Muhibah's fertility? It clearly didn't affect the ability of her (male) siblings to reproduce but could there have been more damaging effects on her female reproductive system? It is not possible to know for sure, but this analysis gives an indication of how inbreeding coefficient alone can't give all the information that might be relevant to understanding the background to any individual animal.

Chapter 3 Genetics

INTRODUCTION

As mentioned in the previous chapter, genetic analyses are becoming an increasingly utilised tool to assess studbook clarity and as an adjunct to pedigree analysis. There are many other possible applications of genetic techniques, and these will be outlined later in this chapter.

DNA, inheritance and evolution

Long before the DNA molecule was identified, the concept of inheritance had already been proposed. Charles Darwin (and others) had noted that characteristics were passed from one generation to the next, and that subtle changes occurred within populations over time. These observations culminated in the publication of Darwin's 'On the origin of species' on 24th November 1859 in which he described how every organism was engaged in a 'struggle for existence' and that only those most suited to their environment could survive. He further explained how this can lead to the process of 'natural selection' where changes in environments lead to changes in the character of the individuals who live there, driven by 'survival of the fittest'. He then went on, controversially at the time, to outline how these fundamental processes could explain the abundance and variety of life, and of how species were the product of 'divergence of character' of ancestral species, i.e. through evolution.

In April 1953, Crick & Watson published their identification of the deoxyribonucleic acid (DNA) molecule (Watson and Crick 1953). They described the molecular structure of the double-stranded helix, and also alluded to the coding possibilities

derived from the strict purine-pyrimidine base pairings i.e. adenine with thymine, and guanine with cytosine.

These two key events in biology paved the way for what we now think of as population genetics, Darwin providing the framework for the theory of speciation and Crick and Watson with the physical model of the molecule that facilitates it.

Genetic markers

Before the development of true DNA-based tools, the primary technique for assessing differences at the 'genetic' level was allozyme analysis, using differences in protein sizes (the allozymes) as a reflection of differences in DNA sequences. Although this technique gave insights into genetic diversity in over 1000 species (Ward, Skibinski et al. 1992), it has limited powers of resolution. The reasons for this are twofold: firstly, not all changes at the DNA level result in changes in amino acid sequence due to the multiplicity of DNA-amino acid translation; and secondly, not all amino acid changes result in measurable changes in allozymes as their size/charge may remain the same. In contrast, DNA-based markers allow the detection of single base changes, thus are more likely to accurately reflect 'true' differences between individuals. Conservation genetics "utilizes the tools and concepts of genetics and applies them to problems in conservation biology" (Hedrick and Miller 1992) and this is the context in which genetic analyses are discussed in this thesis.

DNA-based methods

Within almost all animal cells, there are two sources of DNA – the nucleus and the mitochondria. Over time, each of these sources has been exploited in different ways

to yield information about the fundamental genetic differences between individuals, populations and species.

Numerous methods for using assessing nuclear DNA have been used, but microsatellites have been the markers of choice as of the early 2000s (Selkoe and Toonen 2006). They are now the basis of huge numbers of genetic studies across diverse species and taxa. Equally, mitochondrial DNA analyses are increasingly popular, particularly for investigating divergence of populations of ecological significance and in assigning sub-species and species divisions. Entire journals, for example Conservation Genetics (ISSN: 1566062, published by Springer, accessible at www.springerlink.com) and Molecular Ecology (ISSN: 09621083 , published by Wiley-Blackwell, accessible at www.wiley.com) are now dedicated to the topic of genetic analyses and their role in conservation planning and management.

Examples of studies based on genetic techniques include those assessing wild populations in terms of population genetic structure and founder effects (Broders, Mahoney et al. 1999), changing spatial genetic structure in a wild population (Nussey, Coltman et al. 2005), population structure in marine mammals to inform culling strategies (Graves, Helyar et al. 2009), estimation of genetic bottleneck size in an endangered sub-species (Culver, Hedrick et al. 2008), development of domesticated breeds (Larson, Dobney et al. 2005) and the diversity both between and within them (Laval, Iannuccelli et al. 2000), and the genetic variation and relatedness between wild and domestic breeds (Silva, Guan et al. 2008).

Increasingly, DNA-based analyses are also being used to assess *ex-situ* populations, for example to add detail to studbook data (Jones, Glenn et al. 2002), overcome

problems with significant missing pedigree data (Russello and Amato 2004), assess genetic diversity within the population (Gautschi, Müller et al. 2003; Peng, Liu et al. 2008), estimate genetic variability present in the founder population (Toro, Barragán et al. 2003), determine where animals in the programme originated (Gautschi, Jacob et al. 2003; Tzika, Remy et al. 2009), identify hybridisation between sub-populations (Miller, Chapple et al. 2009), compare allele representation *ex-situ* with wild populations (Henry, Miquelle et al. 2009), inform strategy for species reintroduction (Olech and Perzanowski 2002), evaluate the success of genetic management programmes (Wisely, McDonald et al. 2003) and to investigate the possiblilty of recovering genetic diversity (Doyle, Perez-Enrique et al. 2001). Genetic analysis recently brought into question the proposed pedigree for the Przewalski's Horse, with the suggestion than *more than* one domestic mare has contributed, and some parentage is questionable (Frankham, Ballou et al. 2002).

This is a necessarily small selection of studies conducted over the last 10 years using molecular genetic techniques, but gives an idea of the vast range of questions that can be answered using microsatellite and/or mitochondrial markers.

Microsatellites (Msats)

Microsatellites are small segments of non-coding nuclear DNA found dispersed throughout the genome of a cell. They are composed of repeating di-, tri- or tetra-nucleotide motifs, with typically between 5 and 40 repeats (Selkoe and Toonen 2006). These multiple repeats are the result of 'slippage' of the DNA strand during replication leading to an increase or decrease in the number of repeats copied from the template strand (Levinson and Gutman 1987). The number of repeats varies

between individuals, giving them different lengths (alleles), and allele numbers at a microsatellite marker site can be highly variable (Ellegren 2000). As such, they can be very useful markers in the study of genetic diversity *within* individuals, relatedness *between* individuals, and the genetic diversity of populations and sub-populations within them (Selkoe and Toonen 2006).

Mapping of nuclear DNA has been undertaken in several studies for the domestic pig (*Sus scrofa*), e.g. (Rohrer, Alexander et al. 1994), culminating in an extensive multi-centre study conducted by the PigMAP Consortium who produced a full cytogenetic and linkage map for multiple known coding (Type 1) and non-coding (Type 2) markers, including microsatellites (Archibald, Haley et al. 1995; Yerle, Lahbib-Mansais et al. 1995).

Mitochondrial DNA (mtDNA)

Excepting the gametes, 100 – 10, 000 separate copies of circular mitochondrial DNA (mtDNA) are found in most mammalian cells, with two to ten copies per mitochondrion (White, Wolff et al. 2008). Each mtDNA molecule is made of 15,000-17,000 base pairs, and encodes for 37 genes: 13 proteins, 22 transferRNAs and the small and large rRNA subunits, including those involved in the oxidative phosphorylation pathway, as shown in ***Figure 39***, taken from www.wikipedia.org/wiki/Mitochondrial_DNA, accessed on 6th August 2008).

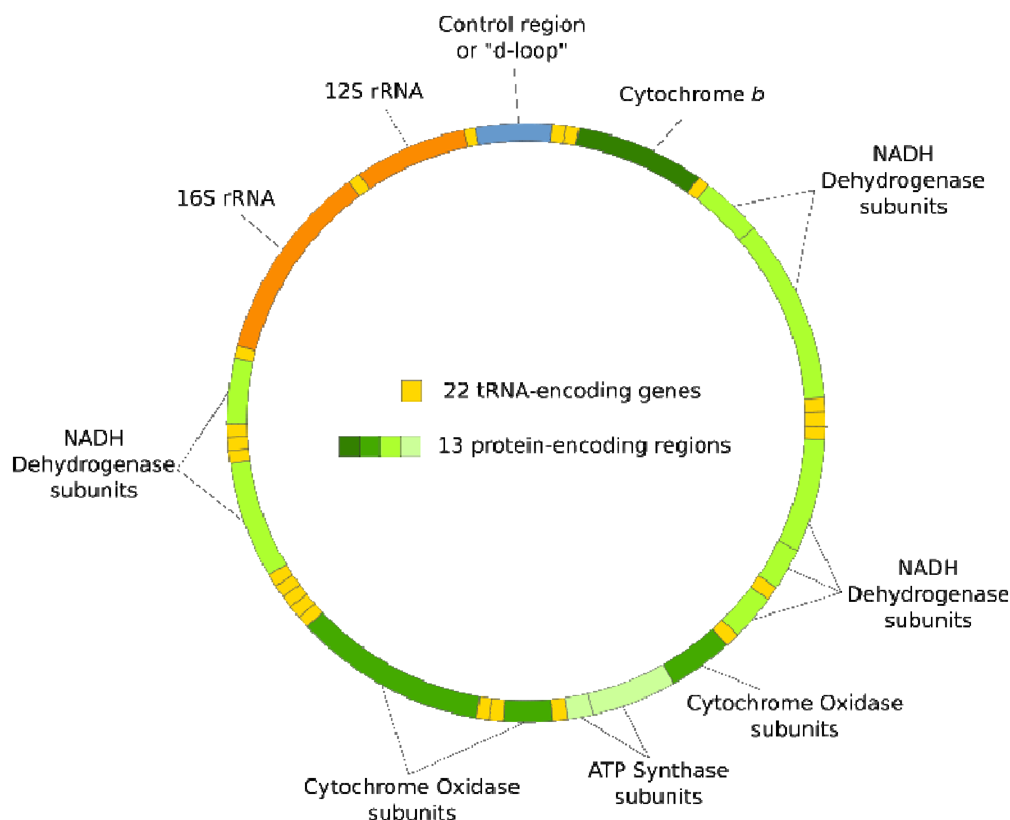


Figure 39: Illustration of the structure of the circular mitochondrial DNA molecule, which is well-conserved across eukaryotes (Saraste 1999).

mtDNA replication is semi-autonomous, and under the control of the triple-stranded area called the D-Loop. It has a relatively high mutation rate compared to nuclear DNA (Brown, George et al. 1979) and is thus a useful marker for use in population genetics (White, Wolff et al. 2008). Frequently used marker areas are the Cytb and D-Loop domains. These markers were both used, for example, in the investigation of taxonomic status of the Pygmy Hog in relation to other suidae, as mentioned in **Chapter 1** (Funk, Verma et al. 2007). Multiple mtDNA markers have also been used to identify multiple centres of pig domestication (Larson, Dobney et al. 2005), and to follow the dispersal of pigs through south-east Asia (Larson, Cucchi et al.

2007), and is currently being used to further understand the phylogeography of the babirusa in its natural range (personal communication Greger Larson). The full mtDNA sequence in the domestic pig (*Sus scrofa*) was published in 1998 and has been used to place the pigs within the Artiodactyla and to estimate their time of divergence (Ursing and Arnason 1998; Lin, Sun et al. 1999).

Mitochondrial DNA is almost exclusively passed through the generations in the maternal line, although there is some evidence of rare incidence of paternal leakage, heteroplasmy and recombination events (Ballard and Whitlock 2004; White, Wolff et al. 2008). There are two principle mechanisms that are responsible for this largely uni-parental transmission. Firstly, there are numerically much greater numbers of maternal mitochondria, owing to the much larger size and greater cytoplasmic content of an ovum compared to a single spermatozoan (White, Wolff et al. 2008). Secondly, mechanisms exist to prevent the survival of any mitochondria that may enter the oocyte with the sperm head, with chemical pathways leading to the breakdown of ubiquitin-tagged paternal mitochondria (Sutovsky, Moreno et al. 1999). It is thought that these mechanisms have developed to reduce the potential for competition between differing haplotypes within the conceptus (White, Wolff et al. 2008).

Over time, mutations occur in the mtDNA leading to multiple haplotypes. It is possible to follow these mutations back through generations of the maternal line, and can, for example, be used to infer the miniumum number of female founders to a population (Hill, Bradley et al. 2002).

DNA amplification and visualisation

For both marker types (Msats and mtDNA), using the Polymerase Chain Reaction (PCR) to ‘amplify’ the section of marker DNA allows the use of very little starting material collected through non-invasive sampling. It means that even very small samples e.g. from buccal swabs or highly degraded DNA extracted from museum or fossil specimens (Larson, Cucchi et al. 2007; Miller, Drautz et al. 2009) can be successfully incorporated into genetic studies. In theory, a single copy of the DNA strand should be sufficient for analysis if it is of high enough quality.

‘Markers’ of interest are amplified selectively using oligonucleotide primer pairs which flank either end of the sequence required to amplify. They are typically less than 20 base pairs long and usually one of the primers is ‘labelled’ e.g. with a fluorescent dye to allow the PCR products to be visualised. This is often achieved using standard gel electrophoresis, or through capillary electrophoresis using a DNA sequencing apparatus. Both techniques take advantage of the slower migration of larger DNA fragments through the gel, allowing separation of alleles of different size. For any marker, homozygotes i.e. individuals with two copies of the same allele will show one band, and heterozygotes with two different alleles will show two bands.

Once suitable genetic markers have been identified, samples have been analysed and genotypes assigned, genetic data is then suitable for analysis at the population level.

Population Genetics

Population genetics is the application of genotyping methods such as those using microsatellites or mitochondrial DNA applied to the study of whole populations.

Normally many individuals are sampled from a population and subjected to genetic analysis for multiple markers, giving a genotype profile for each. These profiles can either be used to compare individuals or, more commonly, combined to assess the genetic diversity of the population. There are various measures that are used to describe the genetic characteristics of populations and to allow comparison between them, and those employed in this study are described below. Many of the measures depend on the concept of heterozygosity and the Hardy-Weinberg equilibrium.

Heterozygosity & Hardy-Weinberg Equilibrium

Heterozygosity (H) is used as a measure of genetic diversity, and describes the situation where a marker site contains two different alleles, distinguished by size or sequence (as opposed to homozygosity where they are both the same). Overall heterozygosity levels for individuals or populations are calculated from multiple markers and give an indirect measure of inbreeding, as inbreeding is associated with reduced heterozygosity (Frankham, Ballou et al. 2002; Slate, David et al. 2004).

For any marker site, there is expected to be a specific proportion of heterozygotes within a population. The proportion of expected heterozygosity (H_{exp}) depends on the number of alleles present and their relative occurence and is calculated from the Hardy-Weinberg equation. ***Figure 40*** illustrates the simplest situation where there are two alleles present at a single locus with frequencies of p and q. The expected proportion of homozygotes is p^2 and p^2 and of heterozygotes is $2pq$.

$$p^2 + 2pq + q^2 = 1$$

Figure 40: The Hardy-Weinberg equation describes the relationship between the number of alleles present at a locus (p and q), and their frequency to the expected levels of heterozygosity (H_{exp}) at that locus within a population.

Differences between the expected heterozygosity (H_{exp}) and heterozygosity observed from genetic analysis of the population (H_{obs}) can indicate inbreeding (if $H_{obs} < H_{exp}$) or a recent bottleneck event (if $H_{obs} > H_{exp}$). There are multiple conditions required to be satisfied for the HW expectations to be valid: that the population is large and freely breeding with all members contributing equal offspring numbers; and there is no immigration or emigration, no mutation, selection or drift. Inevitable any real population cannot satisfy all of these conditions, and in particular, conservation breeding populations are often small and inbred, leading to likely $H_{obs}<H_{exp}$. However, it can still be useful to assess all markers for HW equilibrium to identify any that behave differently from the rest. For example, an unusually low H_{obs} could indicate that a marker locus has a null allele or large-allele drop-out leading to heterozygotes being wrongly scored as homozygotes (Wattier, Engel et al. 1998; Dewoody, Nason et al. 2006).

Population dynamics

Population dynamics describes various parameters associated with populations and how they change over generations. It is concerned particularly with the transmission of genetic information over time, and ultimately with its retention or loss. It is useful to assess changes over time as this can estimate the long-term potential of a population or species. Genetic changes in a population over time can be the result of various mechanisms:

1. Genetic drift

Genetic drift is the random loss of alleles from generation to generation. It is inevitable in all but the largest populations as each offspring only inherits ½ of its

genetic material from each parent. If each allele is present in a large number of individuals, it has a greater chance of persisting in the population. It follows then that small populations are at greater risk of genetic drift as there are fewer individuals available to ensure that *all* alleles are retained.

2. Mutation

Excepting migration of unrelated individuals, the only way new alleles can be introduced into a population is through mutation, typically through single base-pair changes caused by insertions, deletions, inversions or translocations. Depending on the type of mutation and the base change that occurs, there may or may not be a resulting change in any protein coded for by the mutated sequence.

3. Selection

Selection (natural or otherwise) increases the frequency of alleles that confer an advantage for the individual, resulting in survival of the fittest and transmission of the advantageous genotype.

Genetics and conservation breeding

As the medium of inheritance, genetic material has proved very useful in studying and understanding the structure and development of populations. In particular, it is becoming more widely used in the study of small populations such as those of conservation breeding programmes, often in conjunction with existing pedigree information from studbooks. In isolation, it can be a more reliable and useful technique than studbook and pedigree analyses, especially where there is uncertainty regarding relationships between individuals within the population.

It was stated in the Introduction chapter that it is possible to improve studbook integrity with genetic studies, for example by allowing you to "clarify suspect studbook data, indicate likely relatedness of founder animals, suggest minimum founder numbers where unknown/uncertain, assess true diversity within a population and assess the genetic 'value' of individuals". Examples of studies investigating these questions have been given earlier in this chapter. In more detail, genetic information was used to enhance studbook data for the Whooping Crane (*Grus americana*), where founder relationships were clarified (Jones, Glenn et al. 2002) and for the Jamaican yellow boa (*Epicrates subflavus*), where founders were matched to natural populations, parental allocations were clarified, and genetic diversity and inbreeding levels were calculated for the captive breeding population (Tzika, Remy et al. 2009). A similar study was conducted for the captive bearded vulture (*Gypaetus barbatus)* population (Gautschi, Jacob et al. 2003).

What can genetics do that pedigrees can't?

In addition to their usefulness as adjuncts to pedigrees, genetic analyses have further independent benefits. Although they are often used to measure the same *concept* of inbreeding as pedigrees, they do so using different *units*, i.e. heterozygosity (*H*). This is a measure of the genetic diversity within an individual or population which gives an insight into the genetic consequences of breeding schemes. Genetic diversity is the basis for natural selection i.e. the raw material of evolutionary potential, so this insight is important in the assessment of extinction risk for a population based on the level of inbreeding and thus population fitness.

Genetic analysis allows the estimation of inbreeding coefficient (*F*) using neutral markers to follow the dispersal of alleles through generations. Essentially, any DNA sequence could be used as a marker but in order to be informative each must show some degree of variability between individuals (i.e. be polymorphic) (Sefc and Koblmuller 2009). It is also important that the chosen sequences are neutral i.e. not under selection as this would bias their transmission from one generation to the next, and that they represent a wide range of chromosomes to ensure fair coverage of the genome (Selkoe and Toonen 2006). Increasing marker numbers leads to increased measurable variation, thus greater powers of resolution and so increasing ability to differentiate between species through sub-species to populations or individuals. However, due to constraints of time and resources, most genetic studies use only a few markers, often developed for related species or taxa. This was the case for microsatellite markers developed and assessed in *Sus scrofa* (Laval, Iannuccelli et al. 2000) then used in wild pigs (Lowden, Finlayson et al. 2002) and peccaries (Gongora, Chen et al. 2002).

Statistical methods for population genetics

Statistical methods are required to convert raw genetic data into useful descriptions of overall trends and associations, in a format that is suitable to inform management of the population under investigation. Commonly employed methods are F-statistics and Principle Component Analysis (PCA).

F-statistics

F-statistics in population genetics are used to look at population sub-division and how it impacts on genetic variation, and are based on levels of Heterozygosity (*H*),

as described previously. They are used to compare inbreeding coefficient (F) between individuals (F_{is}), between sub-populations (F_{st}) and for individuals in the context of the whole population (F_{it}). The relationship between these F-statistics is:

$$(1 - F_{it}) = (1 - F_{is})\ (1 - F_{st})$$

This illustrates the relationship between heterozygosity it the different elements of the fragemented population: the individuals, the sub-populations and the population as a whole i.e. total inbreeding (F_{it}) is the product of the inbreeding within individuals (F_{is}) and that between sub-populations (F_{st}). F_{st} is the statistic used in this study as a way of assessing population differentiation, comparing the *ex-situ* populations in Europe, North America and Surabaya, and is further described:

> F_{st} is sometimes referred to as the Fixation Index, and is used to compare the genetic diversity of a subpopulation compared to the total population. It is a measure of the mean reduction in heterozygosity of a sub-population due to genetic drift, with values from 0.0 (no differentiation) to 1.0 (complete differentiation).

Principle Component Analysis (PCA)

PCA is a statistical method used to identify the most influential factor(s) responsible for an observed effect. In this case, the ‘factors’ are each of the microsatellite markers, and the effect under investigation is the differentiation of populations. Once the factors are ‘condensed’, and the most influential markers duly weighted, they are then plotted graphically to visualise each sample in relation to the population it was taken.

Genetic studies in the babirusa

Genetic studies in the babirusa have previously been confined to simple karyotyping (Bosma and de Haan 1981; Bosma, de Haan et al. 1991) and chromosome painting (Bosma, de Haan et al. 1996) to establish the general phylogenetic position of the species in relation to the domestic and other wild pigs.

The use of 'new' genetic techniques to investigate the status of the babirusa provided the opportunity to work beyond uncertain studbook data and assess past and current genetic health of the *ex-situ* population. Multiple polymorphic marker sites had already been identified for domestic pig breeds (Laval, Iannuccelli et al. 2000) and assessed for their suitability in the babirusa and other wild suiformes (Lowden, Finlayson et al. 2002).

A preliminary analysis of a small sample of the *ex-situ* babirusa population was initiated by the Wildlife Conservation Center, the research arm of the Bronx Zoo in New York (Amato 2001). Four porcine genetic markers (one mitochondrial and three nuclear microsatellites) were used to assess twelve North American population founders, fifteen EEP animals, and four wild-caught animals in Surabaya Zoo. All the North American founders were shown to be monomorphic for all four markers, as were the sample of fifteen from Europe. The four wild-caught animals in Surabaya had three different mitochondrial haplotypes, but only showed one additional allele at one of the three nuclear loci. This suggested either that the markers used here (developed for domestic pig breeds, and highly variable between them) were tightly conserved in the babirusa, or that there was little genetic variability within the animals investigated.

The current study

Uncertainty over founder numbers and relationships, and of increasing inbreeding within the *ex-situ* babirusa population are issues in need of clarification, and the preliminary study outlined above confirmed there was probable cause for concern. Uncertain knowledge regarding founder numbers and identities makes estimates of inbreeding unreliable and makes genetic planning very difficult. At this stage in the conservation breeding of the babirusa, it is vital that accurate information is available on the genetic makeup of the various populations to allow sensible decisions to be made regarding their future breeding. The goal of retaining 90% genetic variation in the *ex-situ* babirusa population over 100yrs ((eds.) Manansang, Macdonald et al. 1996) is difficult to achieve when there is no baseline knowledge of what genetic variability you have to work with, and how that compares with wild populations.

The world zoo population is considered to be a single breeding population, but this is of course not practicable and the world's geographical regions manage their zoo populations with relative independence. This has already been highlighted in the Studbooks and Pedigrees chapter, as has the need for this to change to maximise the potential success of the breeding programme. However, the *status quo* of independent regional management has led to the genetic analyses presented here being considered both separately and combined. Note that there are no samples analysed from the North American population, primarily as their animals were all derived from the European population and were thought not to represent any 'new' genetic variation as identified in the WCS study (Amato 2001). However, as this study will show, it may in fact be prudent to assess these animals as they may retain alleles now lost from the rest of the *ex-situ* population.

SAMPLES

Eighteen whole blood samples were collected from all the babirusas living in Singapore Zoo and Night Safari at the end of 2007. These were added to twenty samples of tissue, blood and buccal swabs from a selection of babirusa currently or historically living within the European Zoo population (EEP), and twenty from the current/historical population living in the Surabaya Zoo. EEP and Surabaya Zoo samples were already extracted and mostly analysed as part of an ongoing project at the Centre for Research and Conservation (CRC) at the Antwerp Zoo. Singapore Zoo samples were new and were treated from extraction to analysis. See ***Table 20*** for the full sample list.

Note that all Singapore animals were still living at the end of 2007. By comparison, many of the sampled animals from the EEP and Surabaya populations were already dead by this time. This raises questions about how comparable the populations are, and results must be treated with caution. What this data set can tell us, however, is the minimum level of genetic diversity that was once present in the Surabaya and EEP populations, as compared with the Singapore population, and also compared with a small selection of samples from wild babirusas (note that these were brought into Antwerp as *Sus celebensis* but later found to be from babirusa). Also note that there has been the addition of 'new' wild-caught animals to the breeding population (as discussed in **Chapter 2**) but that none of these or their progeny are included in this study. This means that the Surabaya population at this moment in time will certainly be more genetically diverse than is presented here.

Singapore				EEP				Surabaya			
Stud #	**Sex**	**mt msat both**	**Status end '07**	**Stud #**	**Sex**	**mt msat both**	**Status end '07**	**Stud #**	**Sex**	**mt msat both**	**Status end '07**
301	M	both	alive	**4**	F	both	dead	**324**	F	both	alive
303	F	both	alive	**19**	F	both	dead	**325**	F	both	alive
319	M	both	alive	**47**	M	both	dead	**327**	F	both	alive
320	M	both	alive	**50**	F	both	dead	**329**	F	both	dead
321	F	both	alive	**61**	M	both	dead	**333**	M	both	alive
413	M	both	alive	**66**	F	msat	dead	**340**	F	both	dead
414	F	both	alive	**67**	F	mt	dead	**341**	F	both	alive
415	M	both	alive	**115**	M	mt	dead	**345**	M	both	alive
423	M	both	alive	**122**	F	both	dead	**347**	M	both	dead
441	M	both	alive	**131**	F	both	alive	**348**	M	both	dead
T5	M	both	alive	**139**	F	mt	alive	**350**	F	both	dead
T26	F	both	alive	**142**	F	mt	alive	**351**	F	both	alive
T37	F	both	alive	**146**	M	both	dead	**352**	F	both	dead
T38	M	both	alive	**147**	F	msat	dead	**353**	F	both	dead
T40	F	both	alive	**151**	F	msat	dead	**354**	M	both	dead
T41	F	both	alive	**160**	F	both	dead	**355**	F	both	dead
T42	F	both	alive	**170**	M	msat	alive	**356**	F	both	alive
T99	F	both	alive	**172**	M	msat	alive	**359**	M	both	alive
				183	M	both	dead	**361**	F	both	alive
				193	M	mt	alive	**365**	M	both	alive
				194	M	msat	alive				
				203	F	mt	alive				
				206	F	both	dead				
				230	M	both	alive				
				280	M	mt	alive				
				294	M	both	dead				
				313	M	mt	dead				
				315	M	mt	alive				
				408	M	mt	alive				
				422	M	mt	alive				
				430	F	both	dead				
				439	M	mt	alive				
				442	M	mt	alive				
				T34	M	mt	alive				
n=18	**9M 9F**	**18 mt 18 msat**	**All alive**	***n=34***	**19M 15F**	**25 mt 20 msat**	**16 alive 18 dead**	***n=20***	**7M 13F**	**20 mt 20 msat**	**11 alive 9 dead**

mt = mitochondrial DNA analysis was completed
msat = microsatellite analysis was completed
both = both mitochondrial and microsatellite analyses were completed

Table 20: Table showing details of all animals sampled, and the analyses completed for the genetic study from the Singapore, EEP and Surabaya populations.

METHODS

DNA Extraction

From whole blood (Singapore samples)

All Singapore samples were of whole blood, collected in blood tubes with either heparin or EDTA, and transported on ice from Singapore to Antwerp in January 2008. Samples were thawed by quickly raising the temperature to 37°C in an oven then storing on ice until use. DNA extraction of 300µL thawed whole blood was carried out using the PureGene® DNA Purification Kit (Gentra Systems) following the instructions of the manufacturer. The procedure consisted of cell lysis, with one solution to lyse erythrocytes and destroy non-DNA containing cells and another to lyse the DNA-containing leucocytes, an RNAase treatment to destroy any RNA present, followed by protein precipitation and removal. The remaining DNA was precipitated into a pellet which was rehydrated in 100µL of Hydration Solution and stored at-20°C until use in the Polymerase Chain Reaction (PCR).

From tissue samples and buccal swabs

Most of the European (EEP) and Indonesian (Surabaya and wild) samples were of different tissue types. In these cases, genomic DNA had been isolated from 5-10mg solid tissue or Puregene® buccal swabs using the PureGene® DNA Purification kit (Gentra Systems) following the instructions of the manufacturer. The procedure was broadly similar to that for whole blood, with cell lysis, RNAase treatment, protein precipitation, DNA recovery and DNA hydration stages.

Microsatellite amplification and allele identification

Thirteen microsatellite markers (twelve dinucleotide loci and one tetranucleotide) isolated from *Sus scrofa* were originally selected for their degree of polymorphism in the babirusa (Lowden, Finlayson et al. 2002).

Microsatellite analysis was conducted using Polymerase Chain Reaction (PCR) to amplify ten marker loci ranging from 82-349 base pairs in length, representing nine of the 19 babirusa chromosomes (see ***Table 21***). Amplified bands were visualised using gel electrophoresis and alleles were sized using an ABI sequencing apparatus.

Locus	Genbank Accession #	Chromosome arm	Annealing temp (°C)	No. cycles	Size range (base pairs)
S0386	U78012	11q	50°C	32	158-170
S0215	L31358	13	55°C	30	147-163
S0214	-	4	55°C	30	125-131
S0026	L30152	16	55°C	35	90-108
S0149	-	15	55°C	30	315-349
S0228	L29195	6q	55°C	30	264-324
SW72	AF235346	3p	58°C	30	120-134
SW632	AF225099	7q	58°C	30	182-196
SW951	AF235412	10q	58°C	30	134-154
SW936	AF225107	15	58°C	30	82-90

Table 21: Details of the ten genomic microsatellite markers successfully amplified in all three populations. Details of chromosome positions and Genbank Accession numbers were obtained from Peter Galbusera of the CRC, Antwerp.

Note that three additional loci were tried (SO155, SW911 and SW857) but Singapore samples were not successfully amplified. The reasons for this were unresolved during the study and these loci are not included in the analyses presented here.

Msat amplification

PCRs were performed in an Eppendorf Mastercycler® gradient apparatus. In general, the 10µL PCR mixture consisted of 1µl DNA (about 50-100ng), 1 x Eppendorf Taq

buffer containing 1.5mM $Mg(Oac)_2$, 200µM dNTP's (Eppendorf), 1µM forward primer (0.5µM for S0214 and S0149), 10µM reverse primer (5µM for SO214 and SO149), 10µM FAM/HEX-labelled M13 probe (Boutin-Ganache, Raposo et al. 2001; Tomasulo-Seccomandi, Schable et al. 2003) and 0.25U Taq DNA polymerase (0.5U for S0214 and S0149). Thermal cycling was preceded by 5 min at 94°C and consisted of 30/32/35 cycles (see ***Table 21***) of 94°C for 1 min, optimal annealing temperature for 1 min (see ***Table 21***), and 72°C for 1 min then was followed by 5 min at 72°C. For each locus, 2-4 positive samples were run with each PCR, representing known alleles with which new samples could be compared. Negative samples (no DNA) were also included with each PCR to check for possible contamination.

Band visualisation using gel electrophoresis

PCR products were separated on a 1.5% agarose (Acros Organics) gel, prepared in 75mL 30mM (stock) TAE buffer. 40x stock TAE buffer consisted of 145.37g Tris (Acros Organics), 11.16g DiSodium EDTA (Acros Organics) and 34.4ml Acetic Acid (glacial, VWR) prepared in 1L distilled water. Sample wells contained 4µL PCR product with 2µL loading buffer and products were run for 40-45mins at 100V, 20°C. 100-355bp size standard ladders were run with each gel (see preparation method below). Bands were stained using 5% ethidium bromide (Merck) prepared in 10mM TAE buffer and photographed using the Dolphin-DOC system (Wealtec). An example of a gel visualised using Dolphin-DOC is shown in ***Figure 41***.

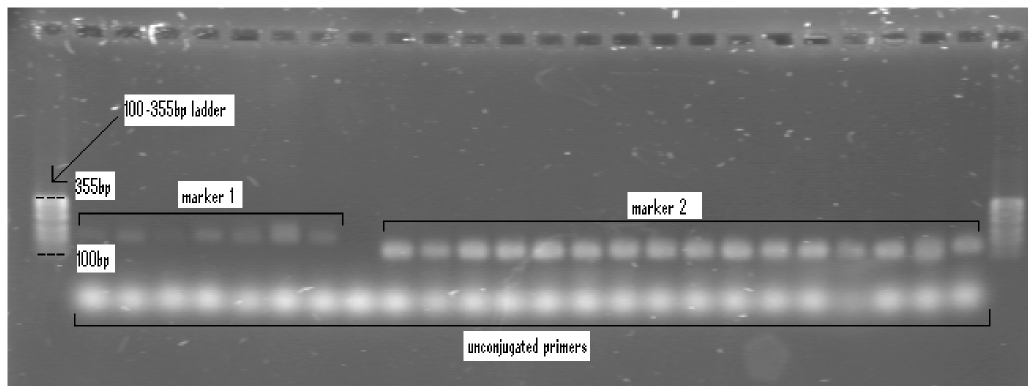


Figure 41: Gel image showing bands for two different microsatellite markers and the bands of unconjugated primers that have run ahead of the marker bands. The 100-355bp ladder is shown at both ends of the gel.

Preparation of base pair ladder (for gel electrophoresis)

In order to size the DNA fragments amplified during PCR, fragments of known length are required. These internal markers constitute a 'base-pair ladder' that is run with every gel electrophoresis to compare with the bands produced for each sample. In this case, known lengths of DNA were made using a vector (M13mp18) which produces fragments of 100, 154, 201, 260, 313 and 355 base pairs. These fragments constitute the 'rungs' of the base-pair ladder. The fragments are produced using a PCR reaction with the M13 Universal Primer for forward extension and a reverse primers specific for each fragment (van Oppen, Rico et al. 1997).

100µL PCR reactions were prepared for each fragment. 100ng vector in 1µL was added to 99µL mix containing 1x Eppendorf Taq buffer with 1.5mM $Mg(Oac)_2$, 200µmol of each dNTP (Eppendorf), 0.5µmol forward and reverse primers and 2Units Taq DNA polymerase. PCR reactions were carried out using an Eppendorf Mastercycler® gradient apparatus with the following conditions: 3mins denaturation at 94°C; 29 cycles of 1min at 94°C, 1min at annealing temperature (AT), 1min at

72°C; then 5mins final extension at 72°C. The ATs for M13 reverse primers were: 50°C (100bp); 55°C (154bp); 53°C (201bp); and 60°C (260, 313 and 355bp fragments). All PCR reactions were run simultaneously using a temperature gradient to provide suitable ATs to each reverse primer.

Primer sequences:

M13 Universal Primer	3'-TTGTCGATACTGGTACT-5'
100bp Reverse Primer	5'-CTATGACCATGATTACGAATTC-3'
154bp Reverse Primer	5'-GGCTCGTATGTTGTGTGGAATTGT-3'
200bp Reverse Primer	5'-GTTAGCTCACTCATTAGGCACCC-3'
259bp Reverse Primer	5'-CTGGCACGACAGGTTTCCCCACTG-3'
312bp Reverse Primer	5'-CGCCCAATACGCAAACCGCCTCTC-3'
353bp Reverse Primer	5'-GCTGTTGCCCGTCTCGCTGGTGAA-3'

Fragment size identification

Using the method of Tomasulo-Seccomandi (Tomasulo-Seccomandi, Schable et al. 2003), an M13Forward tag (5'-CACGACGTTGTAAAACGAC-3') was added to the 5' end of the forward primer for each marker locus. PCR products with this M13 signature were then labelled using an M13-HEX or M13-FAM fluorescent dye which can be detected using an ABI Prism® 310 Sequencing apparatus. Briefly, PCR products were denatured at 95°C for 3 minutes then 0.5-2.0µL (depending on the DNA content from agarose gel electrophoresis) was loaded onto the sequencer with 9µL Hi-Di Formamide (Applied Biosystems) and 0.2µL 500 LIZ® Size Standard (GeneScan™) per reaction tube. Laser-induced fluorescence capillary electrophoresis was conducted using POP-4 Acrylamide (Applied Biosystems) and cycles consisted of 15.0kV for 40 mins at 60°C.

This method allowed automatic identification of allele size using GeneMapper v4.0 software based on comparison with previous known samples. The use of two different flourescent dyes allowed the identification of multiple fragment sizes in the same sequencing run.

Genotype analyses

Using a combination of genetics software packages (Genetix, Genepop, and Fstat), a number of analyses were performed: genetic variability by allelic number (Genetix) and richness (Fstat); testing for Hardy-Weinberg equilibrium (H_{exp} and H_{obs}) in each population at each marker site (Genetix); and assessing population differentiation using F_{st} (Genetix and Genepop) and Principle Component Analysis, PCA (Genetix).

Mitochondrial DNA amplification and sequencing

Mitochondrial DNA analysis was conducted using PCR to amplify two overlapping short fragments on the D-loop of the control region (Larson, Dobney et al. 2005). Each fragment was then sequenced, combined using the common 125bp overlap and single base polymorphisms identified in the typical combined fragment of ~450bp (with losses at fragment ends due to inefficient PCR amplification).

mtDNA amplification

Two primer sets (BabyF-RuminR and L15387-H648n) were used to amplify the two overlapping fragments (442bp and 253bp respectively). PCRs were performed in an Eppendorf Mastercycler® gradient apparatus. The 25 µl reaction mixture consisted of 1µl DNA (about 50-100ng), 1 x Eppendorf Taq buffer containing 1.5mM $Mg(Oac)_2$, 1µM of each primer, 200µM dNTP's (Eppendorf) and 1.25U Taq DNA

polymerase. Thermal cycling was preceded by 1.5 min at 94°C and consisted of 35 cycles of 94°C for 45 sec, 53°C for 45 sec, and 72°C for 1.5 min then was followed by 10 min at 72°C. A positive and negative control was included with each reaction.

Band visualisation using gel electrophoresis

PCR products were visualised using the same method as for the Msats, except that a 100-1000bp SMART ladder (Eurogentec) size standard was used. Problems with non-specific band amplification were resolved by making slight changes to the PCR reaction conditions

Fragment sequencing

The two fragments were amplified and sequenced separately by the "Vlaams Instituut voor Biotechnologie" (VIB) in Antwerp, Belgium. Sequences were manually checked to ensure correct residue assignment by the sequencing software and then combined using BioEdit (Tom Hall, Ibis Biosciences) to produce a single fragment per sample for analysis.

Sequence and haplotype analyses

Full fragments were aligned using BioEdit and haplotypes were identified and assigned based on single or multiple residue substitutions or rearrangements. Female founder numbers were identified from the number of haplotypes present.

RESULTS

The work of this thesis primarily concerns the identification of Msat and mtDNA profiles for the eighteen babirusa held at the Singapore Zoo and Night Safari. Genotypes from the ten successful microsatellite markers are shown in ***Table 22***.

Singapore Stud#	Int SB#	Marker									
		S0215		SW72		SW632		SW951		SW936	
G5126	**319**	163	163	120	120	196	196	134	148	88	90
G9926	**T42**	147	163	124	130	182	196	148	148	88	90
G10150	**T99**	147	163	120	130	182	196	148	148	88	90
G4319	**301**	153	163	?	?	196	196	148	148	88	90
G5152	**320**	147	163	134	134	196	196	148	148	88	90
G4321	**303**	153	163	124	130	182	196	134	148	88	88
G9720	**T40**	147	163	?	?	182	196	148	148	88	88
G8455	**T5**	153	163	120	120	196	196	148	148	88	90
G8737	**T37**	147	163	124	134	196	196	148	148	88	88
G5153	**321**	147	147	120	120	182	196	148	148	88	90
G7355	**441**	147	163	120	130	182	196	148	148	88	90
G5901	**423**	163	163	120	130	182	196	134	134	90	90
G9234	**T26**	147	163	124	134	196	196	134	148	88	90
G9721	**T41**	147	163	124	130	196	196	134	134	88	90
G5371	**414**	147	163	120	130	182	196	144	148	88	88
G5372	**415**	147	147	130	134	196	196	144	148	88	88
G9594	**T38**	147	163	120	120	196	196	148	148	88	90
G5350	**413**	147	163	130	130	196	196	134	144	90	90

Singapore Stud#	Int SB#	Marker									
		S0214		S0386		S0026		S0149		S0228	
G5126	**319**	129	129	164	164	106	106	315	321	312	316
G9926	**T42**	125	129	164	164	106	108	343	343	?	?
G10150	**T99**	125	129	158	164	106	106	331	343	312	316
G4319	**301**	125	129	158	164	106	106	321	321	?	?
G5152	**320**	125	125	164	164	106	106	331	343	298	316
G4321	**303**	129	129	158	164	106	106	321	343	?	?
G9720	**T40**	125	129	158	158	106	108	315	321	312	316
G8455	**T5**	125	129	158	158	106	108	315	331	298	316
G8737	**T37**	125	129	164	164	106	108	321	343	264	298
G5153	**321**	125	129	158	164	106	106	331	343	298	312
G7355	**441**	125	129	158	164	106	108	343	343	264	312
G5901	**423**	129	129	158	158	106	106	315	343	264	298
G9234	**T26**	129	129	158	158	106	108	321	343	264	312
G9721	**T41**	129	129	158	170	106	106	343	343	298	312
G5371	**414**	129	129	158	158	92	106	343	343	312	316
G5372	**415**	129	129	158	158	106	108	315	343	312	316
G9594	**T38**	125	129	164	164	106	108	343	343	312	312
G5350	**413**	125	129	158	170	106	106	315	343	298	312

? indicates the genotype is unknown due to unsuccessful amplification or fragment analysis

Table 22: Genotype results at ten Msat markers for all Singapore samples.

The overall genotyping success with Singapore Zoo samples was 97.2%. These results have been analysed in the context of two populations already genotyped (Msats) and haplotyped (mtDNA) at the CRC in Antwerp (EEP and Surabaya, See **Appendix 2** and **Appendix 3** for raw data) and a few wild animal samples. This necessitates the inclusion of data not generated by me for this thesis but required to put this new data into context. All analyses presented here are new.

Microsatellite genotype analyses

Genetic variability: allelic number and richness

Table 23 shows the number of alleles present at each locus in each of the *ex-situ* populations. To account for variable sample size, and for missing values from the data set, allelic richness was calculated based on a sample size of twelve diploid individuals. The limiting factor was locus SW936 in the Surabaya population.

	Singapore		**EEP**		**Surabaya**		**Combined**	
Locus	No.	Richness	No.	Richness	No.	Richness	No.	Richness
SO215	3	3.000	3	2.980	3	2.969	3	2.990
SW72	4	3.844	4	3.914	4	3.999	6	5.342
SW632	2	2.000	3	3.000	2	2.000	3	2.853
SW951	3	3.870	5	4.562	4	2.969	5	4.683
SW936	2	3.000	3	2.989	3	2.000	3	2.709
SO214	2	2.000	4	3.833	2	2.000	4	3.386
SO386	3	4.967	5	4.705	5	2.895	5	4.707
SO026	3	2.000	3	3.000	2	2.667	4	3.166
SO149	4	5.870	7	6.755	6	3.991	8	6.382
SO228	4	5.714	5	4.626	6	3.999	7	5.472
Mean	**3**	**3.627**	**4.2**	**4.036**	**3.7**	**2.949**	**4.8**	**4.169**

Table 23: Allele number and allelic richness at ten microsatellite loci in the three populations, presented individually and combined.

By looking at allele numbers, all populations are polymorphic (there are at least two alleles, neither of which is represented in ≥ 95% of cases) for all markers, although

few loci contain five or more alleles. The EEP population appears to be the most diverse followed by Surabaya then Singapore. When allelic richness is considered (taking account of different sample sizes at some loci in one or other population), the EEP remains the most diverse but followed by Singapore then Surabaya.

However, because the EEP population was founded from Surabaya stock, it should not have higher genetic diversity than the Surabaya population derived from the same original founder stock (unless new mutations have occurred). This apparent contradiction almost certainly reflects a sampling artefact rather than true greater diversity in the EEP population: the EEP animals sampled here were alive at different times so do not represent a snapshot of diversity at any given moment in time, rather a glimpse of how much genetic diversity *has* existed within the EEP at some time. In contrast, the Surabaya and Singapore populations were sampled as a whole population around a short time-period, although not at the same time as each other. In addition, both the EEP and Surabaya populations analysed here are only a subset of the total population either at a given point in time, or over the duration of the breeding programme.

Table 24 gives further details of the alleles present at each marker site in each population, and provides further evidence of this sampling effect. There are 11 alleles (highlighted in yellow) represented in either the Singapore population ($n=2$), the EEP population ($n=8$) or both Singapore and EEP populations ($n=1$), but NOT in the sampled Surabaya population. This confirms that, in the absence of recent mutation, there must be progeny of Surabaya founder animals sampled in the Singapore and EEP populations but not in the Surabaya sample population itself.

Locus	Singapore	EEP	Surabaya
SO215	*n=18*	*n=20*	*n=20*
147	0.4167	0.3750	0.3000
153	0.0833	0.1000	0.2750
163	0.5000	0.5250	0.4250
SW72	*n=16*	*n=19*	*n=20*
120	0.3750	0.5263	0.3000
122	-	-	0.1500
124	0.1563	-	-
126	-	0.3158	0.0500
130	0.3125	0.0789	0.5000
134	0.1563	0.0789	-
SW632	*n=18*	*n=20*	*n=20*
182	0.2222	0.3500	0.3000
186	-	0.2000	-
196	0.7778	0.4500	0.7000
SW951	*n=18*	*n=18*	*n=19*
134	0.2222	0.1944	0.2895
140	-	0.0278	0.1842
144	0.0833	0.0556	0.0526
148	0.6944	0.3056	0.4737
154	-	0.4167	-
SW936	*n=18*	*n=16*	*n=12*
82	-	0.0938	0.0417
88	0.5833	0.5938	0.5833
90	0.4167	0.3125	0.3750
SO214	*n=18*	*n=20*	*n=20*
125	0.3333	0.0500	0.3000
127	-	0.1250	-
129	0.6667	0.7000	0.7000
131	-	0.1250	-

Locus	Singapore	EEP	Surabaya
SO386	*n=18*	*n=17*	*n=18*
158	0.5278	0.3824	0.2500
162	-	0.2353	0.1667
164	0.4167	0.2059	0.3611
168	-	0.0294	0.1389
170	0.0556	0.1471	0.0833
SO026	*n=18*	*n=20*	*n=20*
90	-	0.3250	-
92	0.0278	-	-
106	0.7500	0.3000	0.7250
108	0.2222	0.3750	0.2750
SO149	*n=18*	*n=15*	*n=19*
315	0.1667	0.3000	0.2105
321	0.1944	-	0.1842
331	0.1111	0.1000	0.0789
333	-	0.0333	0.0789
339	-	0.0667	-
341	-	0.1333	0.0789
343	0.5278	0.2667	0.2684
349	-	0.1000	-
SO228	*n=15*	*n=19*	*n=19*
264	0.1333	0.1579	0.0526
298	0.2333	0.1842	0.0526
312	0.4000	0.5000	0.3684
316	0.2333	0.1316	0.3158
318	-	-	0.1053
320	-	-	0.1053
324	-	0.0263	-

Yellow highlight = allele not found in the sampled Surabaya population
Red = allele unique to only one of the sampled populations

Table 24: Details of the alleles present at each of the ten microsatellite loci in all three populations.

Accepting the sampling effect, ***Table 24*** provides further evidence of higher genetic diversity in the EEP population (as sampled), and the presence of a greater number of

genetic lineages not found in the others: of the 13 unique alleles (in red), eight belong to the EEP, three to the Surabaya and two to the Singapore population.

Minimum founder numbers

From maximum allele numbers, the sampled Surabaya population must have had at least three founders (six alleles at loci SO149 and SO228), the sampled EEP at least four (seven alleles at locus SO149) and the current Singapore population at least two (four alleles at loci SW72, SO149 and SO228). The interesting point here is again with respect to the EEP population, with its minimum four founder animals. Considering the EEP populations was founded from the Surabaya population, it must also be the case (unless mutation has occurred), that four founders contributed to the Surabaya population. From anecdotal accounts, one male and two females founded the population in 1972, and the studbook for the species is rooted as such using 1.2 hypothetical founder animals. However, this assumption is now in doubt as a result of these genetic data, and considering they represent only a sample of the historical and extant population, there may even be additional founders not yet identified.

With respect to the Singapore population founding, maximum allele numbers suggest that there were at least two founders, supporting the decision to assign hypothetical animals H1 and H2 during the studbook clean-up in 2004 (Leus 2004).

From ***Table 24***, you can see that the Singapore population has two unique alleles, allele 124 at the SW72 locus, and allele 92 at the SO026 locus. The significance of this is that these alleles have not been detected anywhere in the sampled EEP or Surabaya population. It may be that this allele is just no longer represented in these sampled populations, as discussed above regarding unique alleles in the EEP

population, or they may represent alleles only present in the Singapore founding animals.

Allele 124 at locus SW72 represents 15.6% of all alleles detected at this locus in the Singapore population, found in five of the nine females, always as heterozygotes. Due to its uniqueness and distribution, this allele was traced back from the five females (SB#s 303, T26, T37, T41 and T42) to the founding of the Singapore population, as illustrated in ***Figure 42***.

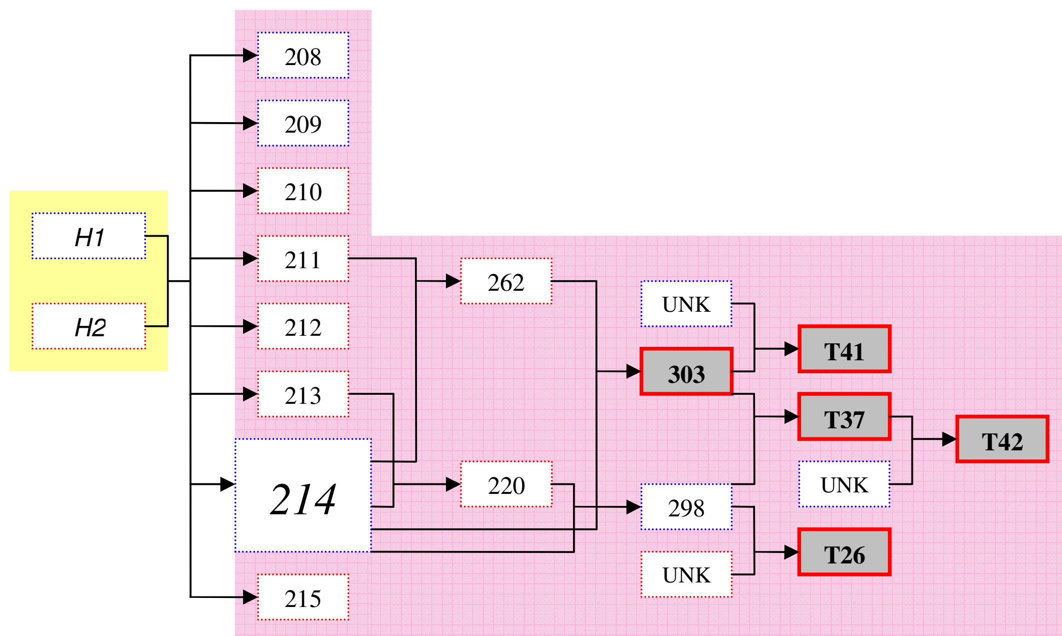


Figure 42: Diagram tracing the origin of the 124 allele at locus SW72, found only in the Singapore population (animals in grey), traced back to male #214.

Allele 124 can be traced back to male #214 in the Singapore lineage, so could have been contributed by either H1 or H2. Its absence in the other sampled populations could be due either to its true uniqueness in this population i.e. from a unique

founder that didn't contribute to the other populations, or simply due to it being missed through inadequate sampling.

Only one sample, female 414, was shown to carry allele 92 at locus SO026. Neither of her parents (#s 208 and 215) are included in this study so it is not possible to further trace its origin. Male 415, the full-sib brother of this female, and her only living relative does not carry allele 92.

Hardy-Weinberg equilibrium

Table 25 shows expected and observed heterozygosities by locus and population.

	Singapore		EEP		Surabaya		Combined	
Locus	H_{exp}	H_{obs}	H_{exp}	H_{obs}	H_{exp}	H_{obs}	H_{exp}	H_{obs}
SO215	0.57	0.78	0.57	0.5	0.65	0.75	0.61	0.67
SW72	0.71	0.63	0.61	0.32	0.63	0.45	0.72	0.45
SW632	0.35	0.44	0.64	0.70	0.42	0.50	0.50	0.55
SW951	0.46	0.33	0.69	0.67	0.66	0.89	0.68	0.64
SW936	0.49	0.61	0.54	0.63	0.52	0.58	0.52	0.61
SO214	0.44	0.56	0.48	0.35	0.42	0.50	0.47	0.47
SO386	0.54	0.39	0.73	0.65	0.75	0.78	0.71	0.60
SO026	0.39	0.50	0.66	0.65	0.40	0.45	0.56	0.53
SO149	0.64	0.67	0.80	0.60	0.77	0.89	0.76	0.73
SO228	0.71	0.93	0.67	0.74	0.74	0.89	0.73	0.85
Mean (*excSW72*)	**0.53** ***(0.51)***	**0.58** ***(0.58)***	**0.64** ***(0.64)***	**0.58** ***(0.61)***	**0.60** ***(0.59)***	**0.67** ***(0.69)***	**0.63** ***(0.62)***	**0.61** ***(0.63)***

Table 25: Expected (H_{exp}) and observed (H_{obs}) heterozygosity at each locus and at all loci in the Singapore, EEP and Surabaya populations, and in all three combined.

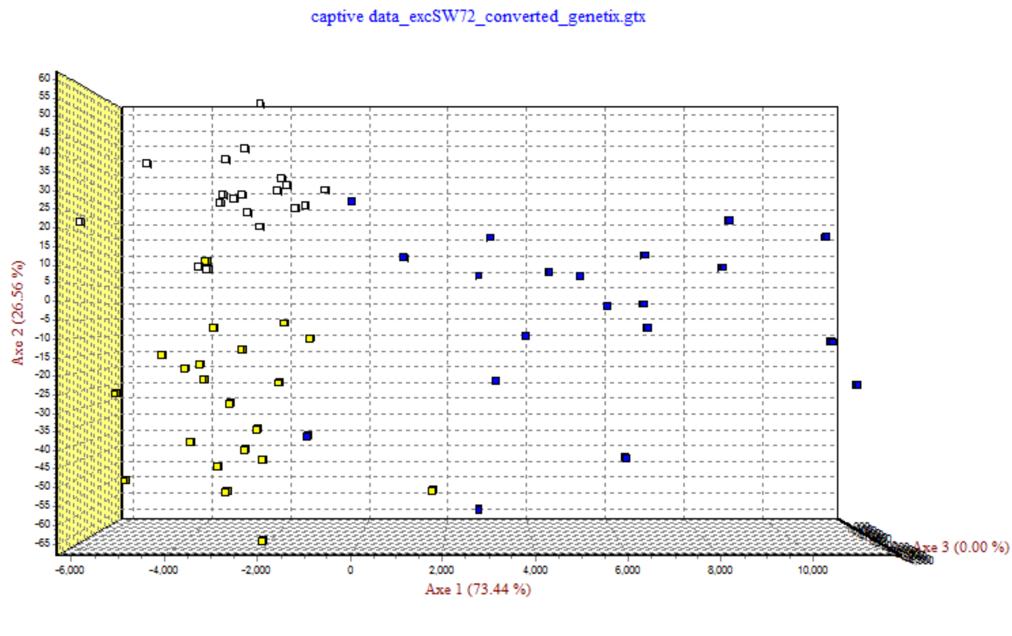
In general terms, there is reasonable correlation between expected and observed heterozygosities. The main exception to this is with locus SW72 where in all three populations H_{obs} is lower than H_{exp} (by 11.3%, 47.5% and 28.6% respectively) and overall by 37.5%. In some respects this is not unexpected due to these populations being small, thus prone to inbreeding and excessive homozygosity. However, the

other nine loci sampled are in reasonable agreement between expected and observed heterozygosities, with no consistent positive or negative difference for all three populations. For this reason, the SW72 locus has not been included in the following genotype analyses as it is likely there is something unusual about it. It may be that there is a null allele (Dewoody, Nason et al. 2006), i.e. an allele that does not amplify at the SW72 locus. This can be caused by a mutation in the primer sequence flanking the marker region, meaning the primer will not be able to anneal with the sequence and amplification by PCR will be unsuccessful. If this is the case, heterozygotes with a null allele will be counted as a homozygote in error (only one band will be seen instead of two), lowering the observed heterozygosity. Another possibility is large-allele drop-out (Dewoody, Nason et al. 2006), where large alleles at a locus are less effectively amplified than smaller ones, again resulting in scoring a single allele (homozygote) where there are actually two (heterozygote) (Wattier, Engel et al. 1998). This can be a problem with differences in allele sizes above 150 base pairs (Wattier, Engel et al. 1998). However, this is unlikely at this locus as the allele sizes range from only 120 to 134 base pairs.

Population differentiation: PCA

The results of the PCA analysis (*excluding locus SW72*) are shown below (***Figure 43***) and in greater detail in **Appendix 4** The PCA gives a visual representation of genetic diversity represented in each *ex-situ* population, and illustrates differentiation between the three based on the samples available from each. The plot is based around two axes: axis 1 (the *x*-axis) represents 73.44% of the variation present; and axis 2 (the *y*-axis) represents the other 26.56%. Each box represents a single sample, with Singapore samples in white, Surabaya samples in yellow and EEP samples in

blue. It is clear from this that there is a degree of differentiation between the three populations, with greater sample spread and distance from the other populations for the EEP samples. The Singapore and Surabaya populations cluster together more tightly, and share similar values on the *x*-axis.



white points = Singapore samples
blue points = EEP samples
yellow points = Surabaya samples

Figure 43: Visual representation of population differentiation between the sampled Singapore, EEP and Surabaya populations, as determined by Principle Component Analysis (PCA).

Population differentiation: F_{st} values

Genepop was used to estimate the significance of this apparent population differentiation using an exact G test for individual loci (*excluding SW72*), and the results are summarised in ***Table 26***. From this, you can see that there is significant differentiation between the EEP and Surabaya populations at 6 loci, between the Singapore and Surabaya populations at 3 loci and between the Singapore and EEP populations at 6 loci.

	P-value (±SE) for population differentiation		
Locus	**EEP & Surabaya**	**Singapore & Surabaya**	**Singapore & EEP**
SO215	0.15307±0.00166	0.08195±0.00131	0.94343±0.00050
SW632	**0.00230**±0.00017*	0.60214±0.00143	**0.00113**±0.00012*
SW951	**0.00001**±0.00001*	**0.01786**±0.00060*	**0.00000**±0.00000*
SW936	0.69730±0.00152	0.65768±0.00158	0.12755±0.00130
SO214	**0.00011**±0.00005*	0.80857±0.00082	**0.00010**±0.00004*
SO386	0.21399±0.00238	**0.00104**±0.00013*	**0.00143**±0.00015*
SO026	**0.00001**±0.00001*	0.59844±0.00174	**0.00001**±0.00001*
SO149	**0.01926**±0.00075*	0.15582±0.00189	**0.00022**±0.00007*
SO228	**0.00298**±0.00035*	**0.01801**±0.00067*	0.71020±0.00223

*significant below the 5% level

Table 26: p-values (±SE) for population differentiation at all loci tested.

Genetix was used to confirm the significance of population differentiation based on all loci combined by calculating pairwise comparisons of mean heterozygosity between sub-population. For the analysis, calculations were made both with and without locus SW386 as it was found to contribute significantly to differentiation between the Singapore and Surabaya populations. F_{st} values were calculated using the method of Weir and Cockerham (Weir and Cockerham 1984) and significant differences in pairwise comparisons were estimated by comparing observed values with 1000 permutated values using the following Markov-Chain parameters: 10,000 Dememorisations; 100 Batches; 5000 Iterations per batch. The results are presented in ***Table 27***.

		F_{st} value	**% permuted values < actual**
EEP & Surabaya	Inc SW386	0.05897	100.00
	Exc SW386	0.06614	100.00
Singapore & Surabaya	Inc SW386	0.01501	94.70
	Exc SW386	0.00775	78.90
Singapore & EEP	Inc SW386	0.08305	100.00
	Exc SW386	0.08621	100.00

Table 27: F_{st} values comparing the three *ex-situ* populations for differentiation, with significance determined by comparing actual with 1000 permuted values.

It is clear that the F_{st} values are much lower for the Singapore-Surabaya comparison than either of the other two, with or without locus SW386. In addition, less than 95% of the permuted values were less than actual values i.e. differentiation between these populations is not significant at the 5% level.

As increasing F_{st} values indicate increasing levels of differentiation, there is greater differentiation between the EEP and the Singapore population (F_{st} = 0.08305 or 0.08621) than the EEP and Surabaya population (F_{st} = 0.05897 or 0.06614). In addition, 100% permuted values were less than actual values for the comparison between the EEP population and the other two i.e. the actual value in each case falls outside the entire set of permuted values, and is therefore significantly higher than would occur by chance alone.

This would fit with the pedigree information in that there are few links between the EEP and Singapore populations (as illustrated previously in ***Figures 31***, ***32a*** and ***32b***). The Singapore population was established from only two Surabaya animals (H1/H2), neither of which is known to have directly contributed to the EEP population. There was a later movement of Singapore animals to Berlin in 1995 (SB#s 217, 222 and 226) but none of these animals are represented in the EEP sample, or had any offspring, and only one of the Singapore animals (SB#413, a full-sibling of SB#222) is included in this study. This is in comparison with the multiple movements of larger numbers of animals from Surabaya to the EEP, therefore more Surabaya lineages are likely to be represented in the EEP sample.

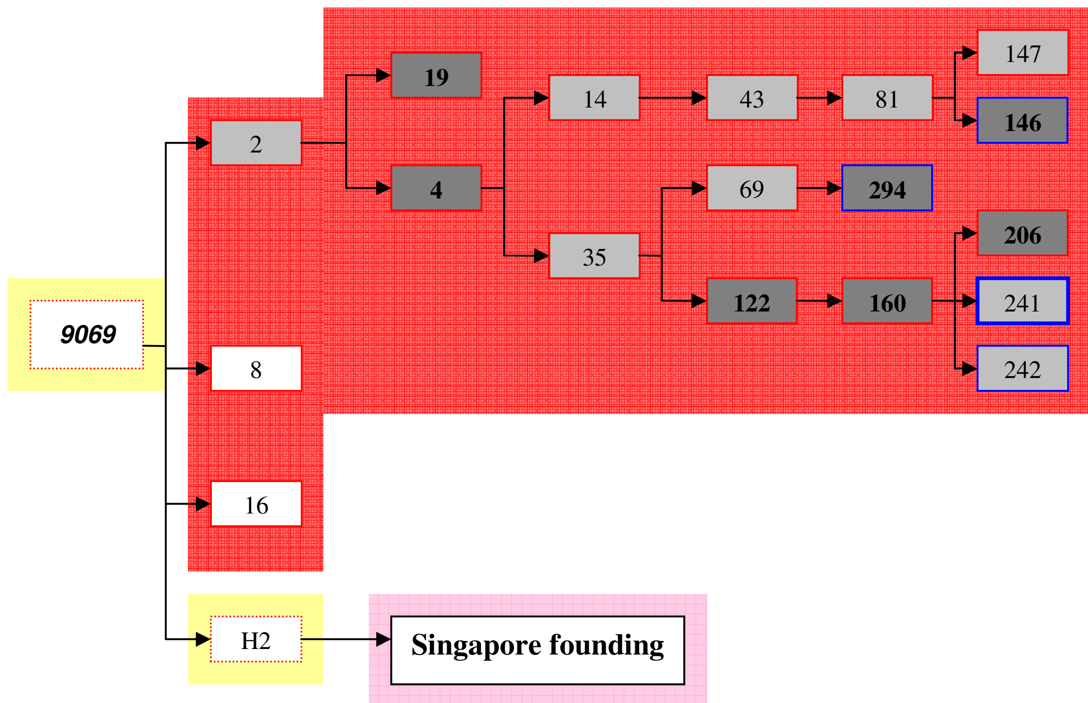
Mitochondrial haplotype analysis

All Singapore samples were monotypic for the mtDNA marker. This haplotype is referred to as Haplotype A (hA) and its 454bp sequence is shown in ***Figure 44***. This haplotype was also found in the Surabaya population (all samples) and the majority of EEP samples.

AAACCCCCACATTTCATGGGCCCGGAGCGAGAAGAGGGATCCCTGCCAAGCG
GGTTGCTGGTTTCACGCGGCATGGTAATTAAGCTCGTGATCTAGTGGTGGTGA
TACGCATGTTGACTTGAACTTATTTAATATAAATGTGCTATGTACGATCAATAATT
ATATGTACTATGTAATATTAATAATATTATGTACATGCTTATATGCATGGGGACTA
GCGATTAATGCACGACGTACATAGGGTTATAATTATACTATATTGTGGTTTTTTA
ATGGTTAGGTTTTTAGAGTTGTATATTTCTATGTTTGTGCGAGATTTTTTTGTAAA
ATTTTGCTTTAAGGATAGTAATAATGTACTTGATGTTTTGGCGTACATACGAATG
TGATCGTTGGAATTTTGATTGTCAGGGAATAGTTTAGTTAGAATTTCAGCTTTGG
GTGCTGATGGCGGAGA

***Figure 44*:** Nucleotide sequence for 454bps of Haplotype A

A further haplotype (hB) was identified within the EEP, representing a single lineage of animals, as illustrated in ***Figure 45***. This lineage was thought to have been part of the female founding of the EEP population (through female 9069), but this cannot be true based on these mtDNA results. Therefore, it is possible that there was an additional female founder that established a separate lineage through female #2, raising the female founder number to at least three, not two as has been suggested. Alternatively, the hypothetical founding should assign the #2 lineage to one of the two females (9068 or 9069), and all other lineages to the other, retaining a female founder number of two. However, referring back to ***Figure 16***, this would result in one female founding a single lineage (through F1 female #2) and the other founding the remaining fourteen F1 lineages! This would seem unlikely and gives weight to the likelihood of at least three female founders.



dark grey = hB animal
light grey = not tested but must be hB
white = not tested but no evidence on hB in this lineage

Figure 45: Diagram tracing the female lineage of the rare Haplotype B (animals in grey) back to female #2, one of the original founder animals for the European population. There are no hB animals known in any other lineage, suggesting that female #2 had a different mother to females 8, 16 and H2, i.e. NOT hypothetical female 9069 as has been suggested.

Haplotype variable sites

There are ten variable sites between the sequences of haplotypes A and B, as illustrated in ***Figure 46***.

	Nucleotide site number									
	15	***96***	***101***	***124***	***136***	***220***	***262***	***265***	***312***	***392***
hA	C	G	G	C	A	G	G	G	C	G
hB	T	A	A	T	G	A	A	A	T	A

Figure 46: Positions of nucleotide differences between hA and hB

From this, the order of mutation could be A → B
or B → A

From only two haplotypes it is not possible to speculate on the direction of mutation, or which base pair changes occurred before others. What is clear, however, is that considering the number of base-pair differences between hA and hB, it is highly unlikely that this is a mutation event from female 2 to female 4. This must represent two well-established separate lineages, indicating at least two female founders each contributing a different haplotype. This does not contradict the anecdotal account of two female founders, but neither does it confirm that there were *only* two. If there were additional female founders (which is possible from the Msat results), they would not be detectable in this analysis if they were also haplotype A or B.

DISCUSSION

It was stated in the introduction that there a numerous benefits of using genetic techniques as an adjunct to pedigree analysis from studbooks. In the case of the babirusa, this genetic study has been able to challenge suspected founder numbers, to compare the genetic diversity of the populations in each of three geographical regions, and to identify some degree of differentiation between them. The main practical outcome of these analyses has been the recent resumption of breeding from the Singapore population, previously thought to be genetically indistinct from the other populations (based on pedigree analysis, see **Chapter 2**). With concerns about limited genetic variability in the *ex-situ* population as a whole, it is important that any possible breeding occurs to preserve what little is there, and breeding the Singapore animals is a positive step towards this.

Genetic diversity

Heterozygosity

There is a large body of evidence that threatened species have consistently lower mean heterozygosity than their non-threatened counterparts, as reviewed in (Frankham, Ballou et al. 2002) and typical heterozygosities at polymorphic loci are between 0.6 and 0.8 (Frankham, Ballou et al. 2002). Mean observed heterozygosity in the three populations sampled here were at the lower end of this range, with a combined value of 0.63.

From Hardy-Weinberg calculations relating to the combined population, observed heterozygosity was very similar to that which was expected. This doesn't tell us anything about the genetic health of the population, but simply that heterozygosity is

similar to that expected based on the number of alleles present (discussed below). In two of the regions, $H_{obs} > H_{exp}$: in Singapore by 13.2% and in Surabaya by 16.7%. This could be the signature of a recent bottleneck effect (Luikart and Cornuet 1998) where rare alleles are lost faster than heterozygosity. As H_{exp} is derived from allele number, this underestimates the true expected value leading to a relatively high observed value.

Allele number/richness

The comparison of allelele number and richness gave slightly different results from each other, although both identified the EEP sample population to be most diverse of the three. However, there are a couple of considerations here. First, only nine loci were available for the analysis. Considering the vastness of the nuclear genome, this is a tiny fraction of the possible variation contained within it, although it is not unusually small compared to similar studies that used between five and fourteen loci (Broders, Mahoney et al. 1999; Jones, Glenn et al. 2002; Gautschi, Müller et al. 2003; Wisely, McDonald et al. 2003; Miller, Chapple et al. 2009; Tzika, Remy et al. 2009). Equally, the level of power inherent in the analysis depends on the natural levels of polymorphism at each locus within the species of interest (Sefc and Koblmuller 2009). Although the polymorphic nature of these loci was confirmed in the studies of Lowden (Lowden, Finlayson et al. 2002) and early in this study (by the CRC, Antwerp), there could also be other unknown msat loci that are more or less so, potentially offering a different insight.

So, although allele number and richness are useful measures for comparing populations, they are rather arbitrary in themselves. In addition, they don't reflect

any element of fitness as they are non-coding neutral sequences that cannot offer any selective advantage. However, what they have confirmed in this study is that there are unique alleles present in all three sampled regions, furthering the argument that they *must* be managed as a single population in order to preserve the maximum genetic diversity possible.

Mitochondrial haplotypes

It is important to note that there are additional living descendents of the hB lineage who have not been sampled for this genetic study and therefore are not shown in ***Figure 45***. Referring back to the living population presented in ***Figure 32b***, you can see that the female lineage 9069 → 2 → 4 → 35 → → leads to three living males, SB#s 293, 241 and 170. In the same Figure, you can see twins 279(f) and 280(m) from a mating between female 151 and male 146 (known to be hB, from ***Figure 45***). There is also living female SB# T81 from female 279, who cannot be hB but is still related to the hB lineage through male #146. Similar examples of living individuals *indirectly* related to the hB lineage can be seen in ***Figure 32a***. For example, there are living offspring of males, SB#s 241 (female T101 and male T102) and 242 (male T34), both through matings with female SB# T17. Male T34 has then gone on to sire male SB# T111 with female T17.

Although the three direct descendents still living in the population cannot pass on the B haplotype itself, other unique alleles related to this lineage may continue through them and their offspring. Unfortunately there is insufficient genetic data at present to investigate the uniqeness or otherwise of these animals and their offspring.

Comparison with samples from wild babirusa

There were eight samples extracted from meat samples and partially analysed from wild animals found at markets in northern Sulawesi. These were used as a comparison with the captive population. Even with *n=8*, there is a higher number of alleles present at four of ten marker sites, and three with the same number (although not the same alleles). In a large, outbreeding population, you would expect to see between five and ten alleles per microsatellite locus (Frankham, Ballou et al. 2002) and ***Table 28*** summarises the number of shared and unique alleles within the 58 animals from the *ex-situ* population compared with the eight wild samples.

Locus	*Ex-situ* (*n=58*)	Wild (*n=8*)	Shared alleles	# Unique *Ex-situ* alleles	# Unique Wild alleles
SO215	3	4	2	2	1
SW72	6	5	3	2	3
SW632	3	7	3	4	0
SW951	5	5	4	1	1
SW936	3	5	3	2	0
SO214	4	6	4	2	0
SO386	5	5	4	1	1
SO026	4	5	4	1	0
SO149	8	6	4	2	4
SO228	7	7	3	4	4
Mean	**4.8**	**5.5**	**3.4**	**2.1**	**1.4**

Table 28: Comparison of allele numbers and uniqueness between a number of *ex-situ* and wild babirusa samples.

As well as the relative abundance of alleles in the small sample of wild animals, there were also 3 mitochondrial haplotypes present in addition to hA and hB from the *ex-situ* animals sampled. This illustrates the relative lack of variation present in the *ex-situ* population, and this is compared with only a small number of wild animals from the north of Sulawesi. This is likely to be a result of the founder effect at the start of the programme as well as subsequent inbreeding through the generations.

The founder effect

The founder effect, as described earlier, occurs when the gene pool of a species/population is not fully represented in a sample population, resulting in a population bottleneck (Frankham, Ballou et al. 2002). In this population, the reasons for the founder effect are two-fold. In the first instance, all founders were said to have been sourced from the same geographical area (Northern Sulawesi), a small part of the suspected range of *Babyrousa celebensis* (refer to ***Figure 9***). Even the second group of founder animals were sourced from a similar area (although these animals are not part of this genetic analysis). In addition, very few founders contributed to the original *ex-situ* population. Anecdotal accounts suggested that only 1.2 founders established the original population, although msat data suggested that there were at least four, and mtDNA data suggests that three of these may have been female. Obviously more is better, but the *real* impact of having four rather than three founders should not be overestimated, for the reasons outlined below:

Firstly, the founder assumption has been made i.e. it is assumed that these founders were unrelated. However, the only evidence for this is the existence of the two mtDNA haplotypes confirming two unrelated female lineages. Beyond this, there is no concrete evidence that all four were unrelated.

Secondly, the unique mitochondrial haplotype (hB) is only present in three living babirusa, all male as highlighted above. This means that this hB will no longer be represented in the known *ex-situ* population.

Thirdly, and perhaps most importantly, the recommended founder number for a conservation breeding population is 20-30 (Frankham, Ballou et al. 2002) making

three or four founders seem unimportant in the bigger picture. Even if we can add the 5.1 additional founders from 1998, this still gives a likely founder base of ten animals. Limited founder number is common in conservation breeding programmes (Frankham, Ballou et al. 2002) and results in limited genetic diversity due to serious bottleneck effects. In this case, the brief comparison between the 58 animals sampled in this study and the eight wild samples (***Table 28***) confirms that the *ex-situ* population is seriously limited in this respect. The knock-on effects of this are inbreeding and inbreeding depression, both of which were suggested from the pedigree analysis of **Chapter 2**.

Historical vs current range

Despite the limited genetic diversity of the *ex-situ* population, it is possible that it actually represents genetic lineages no longer found in the wild population and is therefore still vitally important. Since the capture of the founder animals in 1972, there has been extensive habitat destruction and hunting in Northern Sulwesi (Macdonald, Burton et al. 2008), driven by palm oil plantations and demand for meat in the Christian North (Milner-Gulland and Clayton 2002; Fitzherbert, Struebig et al. 2008; Macdonald, Burton et al. 2008) and it may be that the source population for these founders has been reduced in size or no longer exists. A study of the Amur tiger found that there were unique alleles present in the *ex-situ* conservation breeding population that were not identified in the wild (Henry, Miquelle et al. 2009). The authors concluded that "the captive population may be a reservoir of genetic variants lost *in situ*": could this also be the case for the babirusa?

Population differentiation

As well as each containing unique alleles, the three sampled populations show evidence of differentiation, significantly between the EEP and the two others. This will have driven each sub-population further from the wild gene pool as well as each other, and has occurred in the space of only 36 years over less than seven generations. This divergence will have been driven by random genetic drift and inbreeding within each small population. The concern with this is that if it continues, not only will inbreeding become more severe in each sub-population, but it may eventually lead to genetic incompatibility between them and possible 'outbreeding depression'. This results in reduced fitness of individuals and is just as detrimental as that caused by inbreeding. The best way to mitigate this risk is to allow 'migration' i.e. transfer of animals between the regions, as would be recommended for small fragmented populations in a wild situation (Spieth 1974; Lacy 1994).

Retaining genetic variation

Regardless of how small the population is, or how genetically bereft it is, it is vital that the remaining *ex-situ* population is retained and improved to be of any conservation value. As already mentioned, the MK strategy for minimising mean kinship is considered the best for conserving genetic variation (Montgomery, Ballou et al. 1997) and relies on good studbook management. An additional tool for population management is the PM2000 software package (Pollack, Lacy et al. 2005). This allows predictions to be made for future growth and genetic health of a population based on hypothetical mating situations and variations in population parameters.

Limitations in this study

Despite the undoubted usefulness of genetic markers, there are also precautions that must be taken to ensure the appropriate identification of genotypes and haplotypes, and the subsequent interpretation of these data.

Technical problems with genetic analyses

From human error such as mis-labelling samples or mis-reading genotypes/sequences (van Oosterhout, Hutchinson et al. 2004; Bakker, Sinke et al. 2005) to methodological problems such as large allele drop-out and null alleles (Dewoody, Nason et al. 2006), there are many points during sample collection, preparation and analysis where errors can occur. None of these are thought to have been a significant problem in this study, except for the possible null allele present at locus SW72 and this was accounted for by removing data from the analysis.

There may also be errors associated with missing data (Nakagawa and Freckleton 2008), but in this study it was only ~3% and is unlikely to have significantly affected the resulting analyses and conclusions drawn from them.

Conceptual problems with genetic analyses

When an individual has two copies of the same allele at any given locus, it is identified as homozygous. However, this assignment is based on the assumption that alleles are 'identical by descent' i.e. that they have come from the same common ancestor, but this it not necessarily the case. In reality, DNA sequences can mutate to create 'new' alleles or can revert back to alleles already present in the gene pool. If this occurs, the allele is identical to the existing one but is *not* identical by descent, a situation known as homoplasy (Estoup, Jarne et al. 2002). Although the origin of

an allele will not affect the resulting phenotype, homoplasy is an important mechanism to consider in term of genetic analysis: as inbreeding coefficients are calculated based on levels of homozygosity, homoplasy will lead to assignment of a higher inbreeding coefficient than is appropriate by incorrectly assuming alleles are identical by descent.

Another assumption with respect to the mutation process leading to ‘new’ alleles is that related to the mutation model. Many genetic statistic rely on either the SSM (Simple Stepwise Model), which assumes that mutations can only occur one base pair or tandem repeat at a time, never returning to a previous variant, or on the Infinite Allele Model (IAM), which assumes that any mutation is possible in any direction (Estoup, Jarne et al. 2002). In reality, mutation mechanisms probably lie somewhere between these two models, and this can be taken into account when applying statistical methods to data sets.

A final theoretical consideration is that of potential complexities of mtDNA inheritance. Although strict maternal inheritance is considered to be very much the norm, there are examples where paternal contribution or recombination events have occurred (White, Wolff et al. 2008). However, documented incidences of non-maternal inheritance are still very rare.

Sampling issues

By far, the biggest limitation in this study is the differing sampling considerations of each population. In an ideal scenario, the living population would have been fully sampled at a given point in time making comparisons between populations more valid, and also more applicable to the current situation and how to manage it.

Although this kind of sampling was possible from the Singapore population, it was not so for the other two regions. If only the living population had been used, there would have been only five Msat and 13 mtDNA samples for the EEP population, and 11 each for the Surabaya population.

In terms of the Surabaya population, exporting new DNA samples from Indonesia required Governmental permission which is not routinely granted. The only feasible option for the current study was to use samples already in Europe, collected for other purposes. This has meant that none of the 'x.y new' lineages from wild founders added in 1998, representing fresh blood lines vital to the continuation of the breeding programme, could be included.

For the EEP population, there are continued efforts by the researchers in Antwerp to obtain samples from all living babirusa, but before this is achieved, a mixture of living and historical samples is the only way of increasing sample numbers for comparative purposes.

Future genetic studies

Considering especially the differentiation seen with the Singapore population, it would be worth adding North American animals to the study to look for a similar effect. From the pedigree analysis (see ***Figures 31***, ***32a*** and ***32b***), there are lineages represented in North American not widely found for example in Europe, so breeding in this population is another key to continued success. There may be additional hB lineages present in this population, or even others derived from so-far unidentified founders.

From populations to individuals

Of the 201 animals living in the worldwide babirusa conservation breeding programme at the end of February 2008, many are not in a position to breed. Either they are too old, especially those within the European population (refer to the population pyramids in ***Figures 43*** and ***44***), or they are not in a breeding situation, or they may even have been castrated. For those animals currently held in Indonesia, representing almost 50% of the total, there may eventually be problems of overcrowding (a problem in the past, personal communication, Alastair Macdonald) or poor health (personal observation, 2008) and transfer out to other populations is not possible. On top of this, some animals who perhaps should be breeding are not due to other factors such as having been prevented from breeding they have not resumed, they are being kept in small groups, or have reproductive pathologies. The combined result of these problems is a limited effective population size (N_e).

Summary

The recommendations resulting from this analysis are:

1) to resume and continue breeding within the Singapore population as it represents some genetic diversity not present in the sampled EEP and Surabaya populations.

2) to ensure the continuation of the remaining hB lineages within the EEP. Even though the haplotype itself is lost, there are at least ten living individuals who are derived either directly or indirectly from hB females.

3) to assess the complete living population for genetic diversity, including all those in Surabaya and Ragunan Zoos, and within the North American population (who

may carry unique alleles no longer present elsewhere in the population, and may provide evidence of additional female founder lines). The ‘new’ stock in Surabaya currently represent the best chance of introducing completely new genetic lineages into the worldwide population.

4) as before, to truly consider the various regional sub-populations as a single entity. This will give the conservation breeding programme the best genetic diversity and so the best chance of long-term survival.

Muhibah’s genetic profile

Muhibah was part of the study conducted at the CRC in Antwerp, as sampled from the EEP population. In fact, she was one of the animals identified with the rare mitochondrial haplotypeB, studbook #206 as seen in ***Figure 45***. It might be tempting to assign Muhibah’s reproductive failure in some way to her unusual haplotype but there is no evidence that others from this lineage have been unable to breed. In fact, her siblings, males #241 and #242 were the two held at Southlakes Wild Animal Park, UK, both of whom sired litters with the female SB #T17, Kota, who is featured in the next chapter.

Chapter 4: Faecal steroid analysis

INTRODUCTION

Detailed knowledge of reproductive physiology in threatened species, particularly those supported in conservation breeding programmes, is becoming increasingly important. With limited time and resources, it is vital to maximise breeding efficiency to rapidly increase numbers, whilst maintaining genetic diversity to safeguard a species with evolutionary potential. Founder lineages may be lost if valuable individuals are unable to breed within their ideal breeding parameters, or their success rates are low. It is important to identify reproductive insufficiency of individuals as early as possible, and to assess the reasons for it, to avoid investing resources in animals that are simply not able to breed or investigate the potential for using assisted reproductive techniques.

Assisted reproductive techniques (ARTs)

As increasing numbers of species become threatened in their wild habitats, many more are likely to require the support of conservation breeding. Some scientists are looking to new ways of improving breeding success, including the use of assisted reproductive techniques (ARTs) such as sperm-sorting, artificial insemination (AI), in-vitro fertilisation (IVF) using either intra-cytoplasmic or sub-zonal injection of individual spermatozoa (ICSI or SUZI), embryo transfer (ET), and cloning (Wildt and Wemmer 1999; Andrabi and Maxwell 2007; Durrant 2009; O'Brien, Steinman et al. 2009). As well as a good understanding of reproductive anatomy, the success of ARTs will depend on sufficient knowledge of the physiological peculiarities of each species in its own right. It is therefore important that there is an understanding of the hormonal control of fertility, the effect of external factors on fertility, and a method

of monitoring the effects of various interventions. Undoubtedly, one of the most useful techniques, developed over the last 20 years, is the monitoring of reproductive steroids in faeces (Schwarzenberger 2007).

Control of reproduction in the female

The control of reproductive status in the female ultimately depends on changes along the hypothalamo-pituitary axis (HPA), resulting in the production and secretion of steroid hormones from the ovary. The principal releasing hormone in relation to reproduction is Gonadotrophin Releasing Hormone (GnRH), synthesised in the parvocellular neurones of the hypothalamus and secreted into the primary portal plexus of the anterior pituitary. GnRH is released in a pulsatile manner from the onset of puberty until death. On arrival at the anterior pituitary, GnRH effects the release of Follicle Stimulating Hormone (FSH) and Luteinising Hormone (LH) in an accordingly pulsatile manner. These endocrine hormones travel systemically, and exert control over the cyclical functioning of the ovary by stimulating complex biochemical pathways that lead to the production and secretion of steroid hormones. Multiple feedback systems operate between the reproductive organs, pituitary and hypothalamus, 'fine-tuning' the overall functioning of the female reproductive system.

Hormone production

The reproductive hormones are produced primarily in the ovary, with the placenta playing an additional role in progestagen production during pregnancy in many species, including the domestic pig during late pregnancy (Johnson and Everitt

2000). The precise hormones produced by the ovary depend on species, but fall into the categories of estrogens and progestagens.

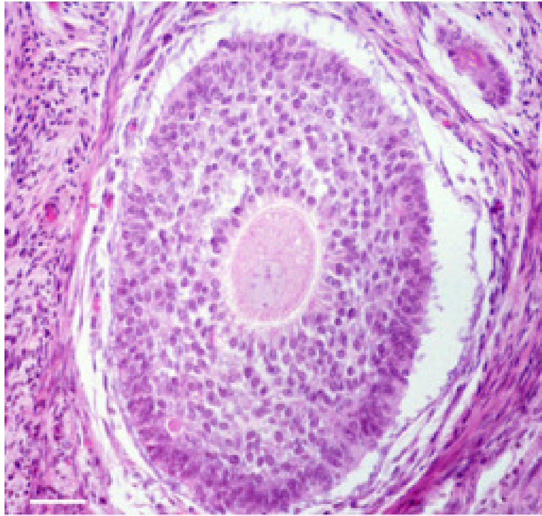
Estrous cycle

The estrous cycle describes the alternating structure and function of the mature non-pregnant ovary and reproductive tract, driven by the HPA and the hormones FSH and LH. The cycle reflects the dual purpose of the female reproductive tract: to create a suitable environment for oocyte transportation and fertilisation (the estrogen-mediated follicular phase): and to support the development of a conceptus should fertilisation occur (the progestagen-mediated luteal phase).

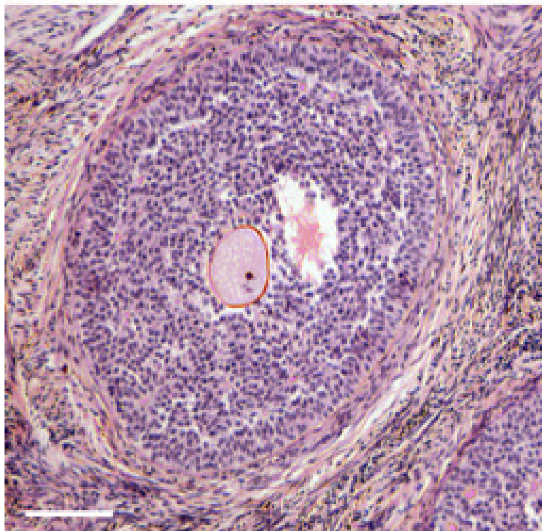
The 'follicular' phase

The follicle is the basic unit of reproductive function and consists of a central oocyte surrounded by supporting cells. The mitotic expansion of oocytes has already occurred by the time of birth, and the resulting primordial follicles remain in an inactive state until the onset of puberty. The active post-pubertal mammalian ovary contains vast numbers of follicles at differing levels of development.

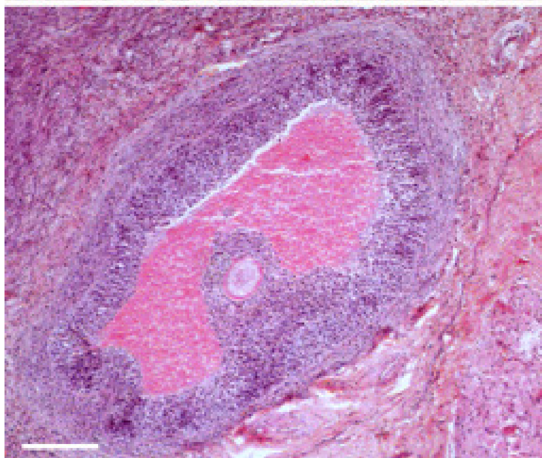
The follicular phase of the estrous cycle involves the development and maturation of one or more follicles to the point of ovulation. During this process, the granulosa cells surrounding the oocyte produce increasing quantities of estrogens. During maturation, the oocyte is surrounded by increasing numbers of granulosa cells, and eventually develops a central fluid-filled antrum. Early follicular development is gonadotrophin-independent until the stage of antral formation, when FSH is required to prevent follicular atresia. The key stages of follicular development in the babirusa are shown in ***Figure 47***, images from (Ziehmer, Ogle et al. 2009).



a) Secondary follicle showing the central oocyte surrounded by multiple layers of estrogen-producing granulosa cells.



b) Early antral follicle with fluid-filled antrum shown to the right of the oocyte. There is clear distinction between the outer granulosa and thecal cell layers.



c) Late antral follicle with a large fluid-filled antrum and the oocyte surrounded by a layer of cumulus cells.

Figure 47: A series of histological images of the Babirusa ovary showing key stages of follicular development, from (Ziehmer, Ogle et al. 2009).

Ovulation and the 'luteal' phase

Once the dominant follicles have been selected and matured into tertiary pre-ovulatory follicles, their substantial estrogen output has two main effects. Firstly, it mediates the emergence of physical signs of estrus and related behaviours i.e. "heat". Secondly, it induces a 'surge' of LH from the pituitary which induces ovulation and development of post-ovulatory follicles into corpora lutea (CLs). This involves significant changes in their structural and biochemical character with granulosa cells increasing in size, becoming 'luteinised' and producing large quantities of progestagens. If conception occurs, progestagen levels remain high and the estrous cycle is halted. If there is no conception, the corpus luteum regresses under the influence of $PGF_{2\alpha}$ and the follicular phase resumes.

Follicular and luteal phases in the domestic pig

In the domestic pig, follicles spend an unknown time at the pre-antral stage, ~10 days in the antral phase, 41 hours at the pre-ovulatory stage and 15-17 days at the luteal phase (Johnson and Everitt 2000). From this, the lifetime of a follicle from pre-antral growth through ovulation to luteal regression is ~27-29 days. However, the cycle length, from ovulation to ovulation is only 18-21 days (Corner 1920; Bazer, Geisert et al. 1982; Macdonald 2000). These numbers appear not to add up, but can be explained by the 'overlap' of cycles in the pig, where follicular development occurs during the previous luteal phase, due to the maintenance of sufficient levels of circulating FSH. The result is a follicular phase length of only 5-6 days, the early stages 'hidden' in the previous luteal phase. This is typical of domestic animal species, in contrast to humans and other primates where there is strict separation between the follicular and luteal phases, each of similar length (Baird et al.,1975;

Baerwald 2009). In contrast with other domestic species, follicular development in the domestic pig (*Sus scrofa domestica*) is not wave-like, and it produces a relatively large number of follicles with over a hundred of small/medium size typically present during the luteal phase, reduced to around 20 ovulatory follicles during the follicular phase (Evans 2003).

Ovarian steroid production

The production of steroid hormones by the ovary depends heavily on co-operation between the granulosa and thecal cells of the follicle. The steroids they produce are described below.

The steroid hormones

From the description above, the steroid hormones important in female reproduction clearly fall into two classes: the estrogens and progestagens. Both are cholesterol-derived based on a four-ring carbon structure, with variations in saturation levels and side-groups. The general structure of the steroid hormones is shown in ***Figure 48***, with the rings labelled A-D. All chemical structures in this section are taken from 'Steroid Analysis' by Makin, Gower and Kirk (Makin, Gower et al. 1995).

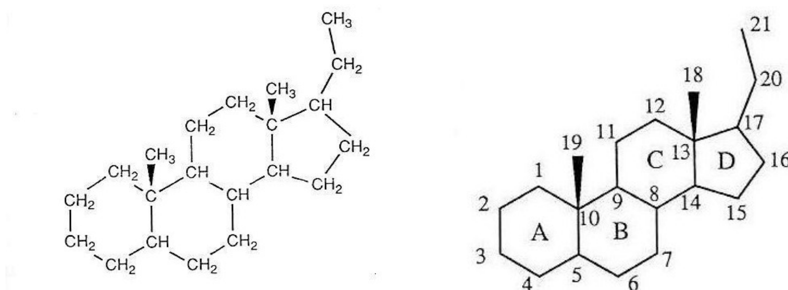


Figure 48: The general structure common to steroid hormones. The diagram on the right shows the carbon numbering system, used to identify the many possible steroid structures.

The estrogens are based on the structure of estrane, and the progestagens on pregnane, as illustrated in ***Figure 49***.

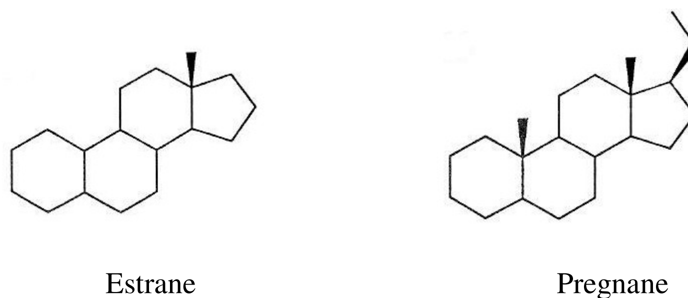


Figure 49: Parent structure of the two reproductive steroid classes, the estrogens and progestagens, based on estrane and pregnane respectively.

The common origin of the two steroid classes is obvious here, the primary difference being the additional carbons at the C19, 20 and 21 positions in the pregnanes. In addition, the A-ring is fully aromatised in the estrogens. These hormones are described in the plural (estrogens and progestagens) due to the multiple possible forms of each, with differences conferred by variations in side-groups resulting in molecules with variable potency at the respective hormone receptor.

The estrogens

There are three estrogens with significant potency at the estrogen receptor, as shown in ***Figure 50***.

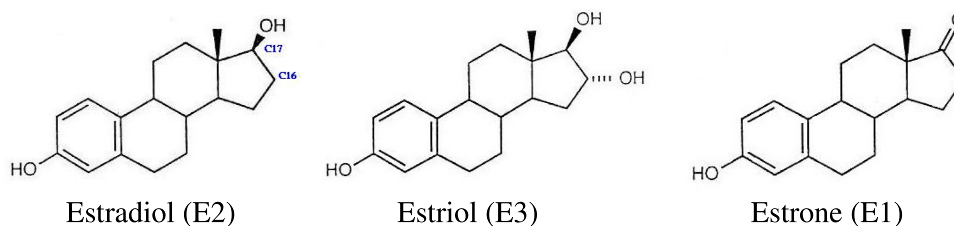


Figure 50: Structures of the active estrogens.

Estradiol 17β (also known as estradiol or E_2), with a fully aromatised A ring and hydroxyl group at C17 is normally considered at 100% potency. Estriol (E_3) and estrone (E_1) both have a fully aromatised A ring but differ in their side groups at C16 and C17. They have relative potencies compared to estradiol of 10% and 1%, respectively (Johnson and Everitt 2000).

The progestagens

The progestagens are 21-carbon structures, partially saturated in the A ring (1 double bond), with an oxo- (=O) group at C3. As with the estrogens, there are three principle progestagens with potency at the progesterone receptor, as illustrated in ***Figure 51***.

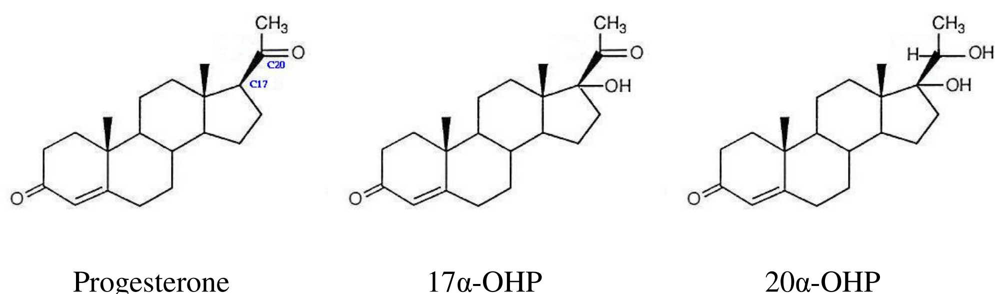


Figure 51: Structures of the active progestagens

Setting the natural progestagen progesterone at a potency of 100%, 17α-hydroxyprogesterone (17α-OHP) has an additional hydroxyl group at C17, conferring 40-70% relative potency. 20α-dihydroprogesterone (20α-OHP), also with a hydroxyl group at C17 *plus* an HCOH configuration at C20 has a relative potency of only 5% (Johnson and Everitt 2000).

Hormone conversions and metabolism

After production, steroid hormones are subject to numerous interconversions, resulting in a complex network of biologically active and inactive intermediaries, as summarised in ***Figure 52***, adapted from (Johnson and Everitt 2000).

The specific types of estrogens and progestagens and their relative proportions in a given species depends in part on the most active metabolic pathways responsible for producing and degrading each (Taylor 1971; Schwarzenberger, Mostl et al. 1996) and can vary considerably, even between related species (Brown, Wasser et al. 1994; Berger, Leus et al. 2006).

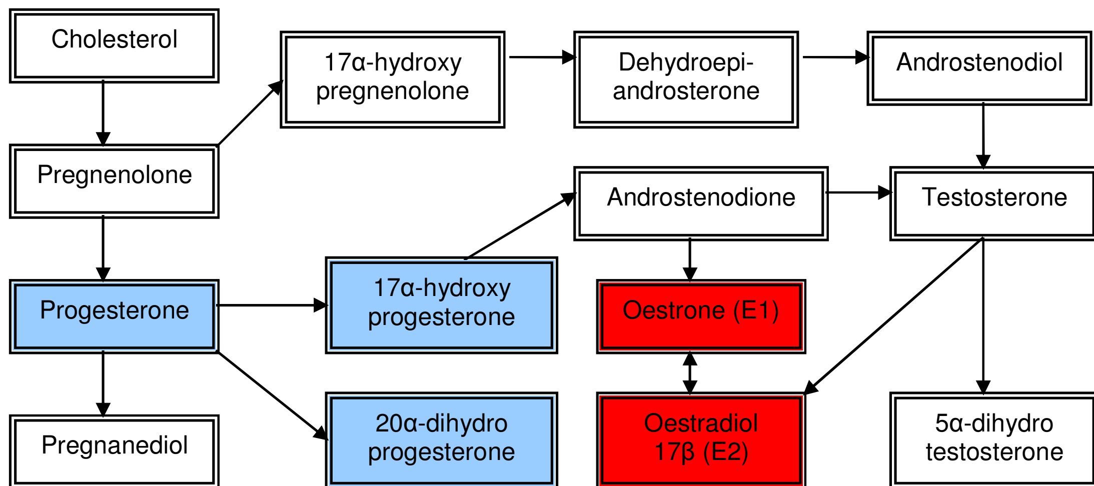


Figure 52 : The major steroid interconversion pathways, with progestagens shown in blue, and estrogens in red. Diagram adapted from (Johnson and Everitt 2000).

There are numerous possible sites of steroid production, metabolism and excretion, and these are summarised in ***Figure 53***, from (Schwarzenberger, Palme et al. 1997).

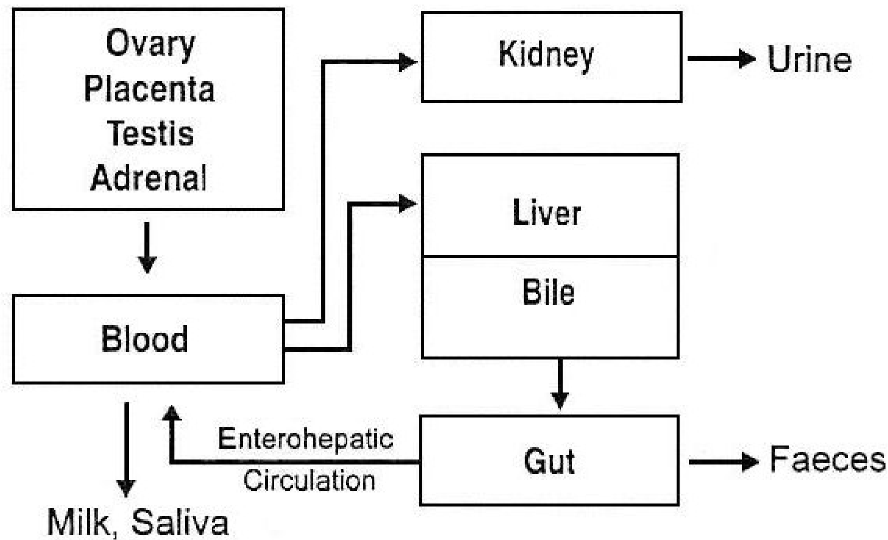


Figure 53: Overview of the sites of production, metabolism and excretion of steroid hormones, and the pathways between them. Taken from (Schwarzenberger, Palme et al. 1997).

Once produced, the steroids are transported in the blood and eventually end up in the liver where they are metabolised and/or conjugated and released in bile into the gut. Here, deconjugation, transconjugation and/or further metabolism can occur, aided by gut bacteria and mucosal enzymes (Taylor 1971; Schwarzenberger, Palme et al. 1997). Enterohepatic circulation is the re-uptake of metabolites from the gut after initial processing by the liver. It can result in de- or re- conjugation on second passage through the liver and was previously thought not to occur in the pig. A later study of progesterone clearance rates suggested that this enterohepatic circulation does in fact occur in domestic swine (Symonds, Prime et al. 1994).

The major excretion pathways in the typical mammal are into urine and faeces (Williams 1965; Schwarzenberger, Palme et al. 1997), although small quantities can be secreted into saliva (Gómez, Jewell et al. 2004) and milk (Williams 1965).

The balance of excretion by urinary and faecal routes is steroid-dependent and again species-dependent (Taylor 1971). In a study of excretion of ^{14}C-steroid hormones in domestic livestock (Palme, Fischer et al. 1996), the ratio of faecal : urinary excretion of progesterone was 34 : 66% in the pig, 77 : 23% in sheep and 75 : 25% in ponies. For estrone, the ratios were 4 : 96% in pigs, 89 : 11% in sheep and 2 : 98% in ponies, demonstrating the wide variation even between this small number of species for only two of the steroid hormones. However, the parent hormones progesterone and estrogen are generally excreted in very small quantities, if at all, into the faeces (Schwarzenberger 2007) and their detection relies on picking up their associated metabolites. A study of the domestic sow suggested that progesterone itself was found in high quantities in faeces (Hultén, Forsberg et al. 1999) but the authors conceded that this was likely due to the antibody also detecting multiple metabolites.

Reproductive events under hormonal control

1. The estrous cycle

The estrous cycle is a continuous alternation between the two related but differing functions of the female reproductive tract. The first is to produce and release mature gametes into the tract, the second to aid fertilisation, implantation and development of the conceptus. These two different tasks require different physical and hormonal environments to succeed, and are governed by the action of estrogens and progestagens, respectively. Their fluctuations during the estrous cycle in the domestic pig are illustrated in ***Figure 54***, from (Johnson and Everitt 2000). From this, you can see that when estrogen levels are high, progesterone levels are low, and vice versa. This reflects the regular changes in ovarian structure and function related

to the development of follicle (high estrogens) to ovulation (subsequent to the LH surge) and development of the corpus luteum (high progestagens).

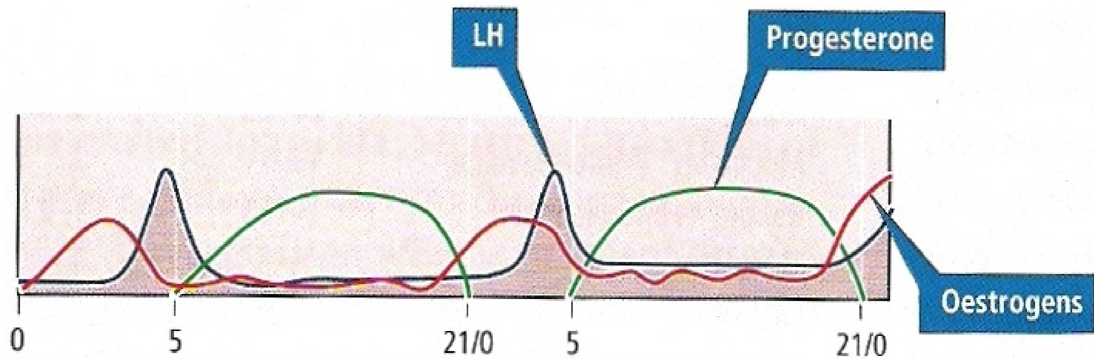


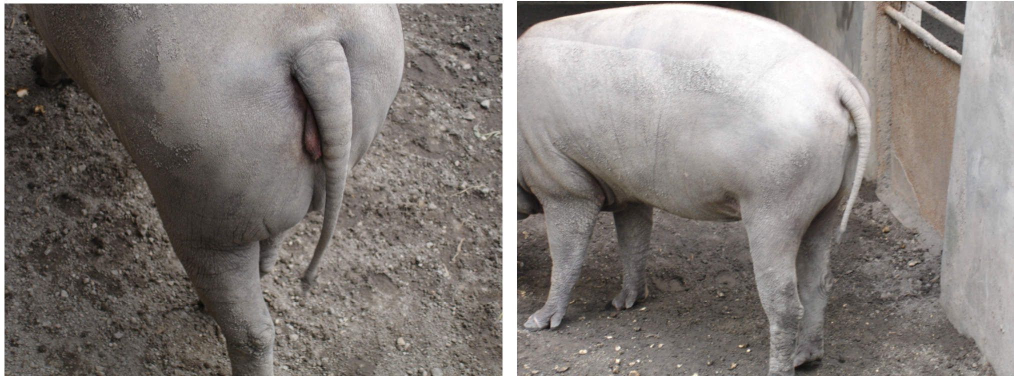
Figure 54: Illustration showing the typical changes in LH, progesterone and estrogen levels during the 21-day estrous cycle of the domestic pig. There is clear cycling between estrogen and progesterone dominance, with the LH surge occurring between the two (day 5), triggering ovulation and effecting the change from follicular to luteal phase. Taken from (Johnson and Everitt 2000).

Estrus signs

The reproductive status of a female babirusa, or indeed any female mammal is not always obvious. In species with intermittent periods of fertility, such as the pig and other domestic species, females express a period of 'estrus', driven by high levels of estrogens produced from developing ovarian follicles. During this receptive period, there may be physical signs such as vulval swelling, reddening and/or eversion (Soede and Kemp 1997), or behavioural signs such as increased proceptive and receptive behaviours (Soede and Kemp 1997). The classic sign that the female is near to ovulation is when she exhibits 'lordosis', or the 'standing reflex' where the female will stand still in response to pressure applied to the rump (Signoret 1970).

Typically in the babirusa, estrus signs include vulval changes (see ***Figure 55***) and soliciting behaviours towards the male (Leus, Bowles et al. 1992). In full oestrus,

the babirusa female has been seen to approaches the male, rub against him, back off and stand still, allowing him to mount her (Bowles 1986).



Pink, swollen vulva   Grey, flattened vulva

Figure 55: Differences in the vulva of the female Babirusa when she in is estrus (left) and non-estrus (right). Images from Surabaya Zoo, Indonesia.

In some individuals there are few obvious signs of estrus, resulting in so-called 'silent heat' (Leus, Bowles et al. 1992), likely due to insufficient estrogen production during the follicular phase or resistance to its effects. Slow follicular growth has been proposed as a reason for silent estrus in the water buffalo (*Bubalus bubalis*). In cases like this and where possible, hormonal monitoring is useful to assess whether or not the female is actually cycling.

2. Pregnancy

If implantation of a fertile conceptus occurs, hormonal support is required from the the ovaries and placenta to proceed with the pregnancy. The normal luteolytic effect of $PGF_{2\alpha}$ is prevented during pregnancy, allowing continued functioning of the luteinised follicle(s), the CL(s). Progestagens are maintained around or above luteal-phase levels until 1-2 weeks before parturition. At this time, rapid luteolysis is

induced by $PGF_{2\alpha}$ and there is a precipitous fall to baseline levels, as illustrated for the domestic pig in ***Figure 56***, from (Baldwin and Stabenfeldt 1975).

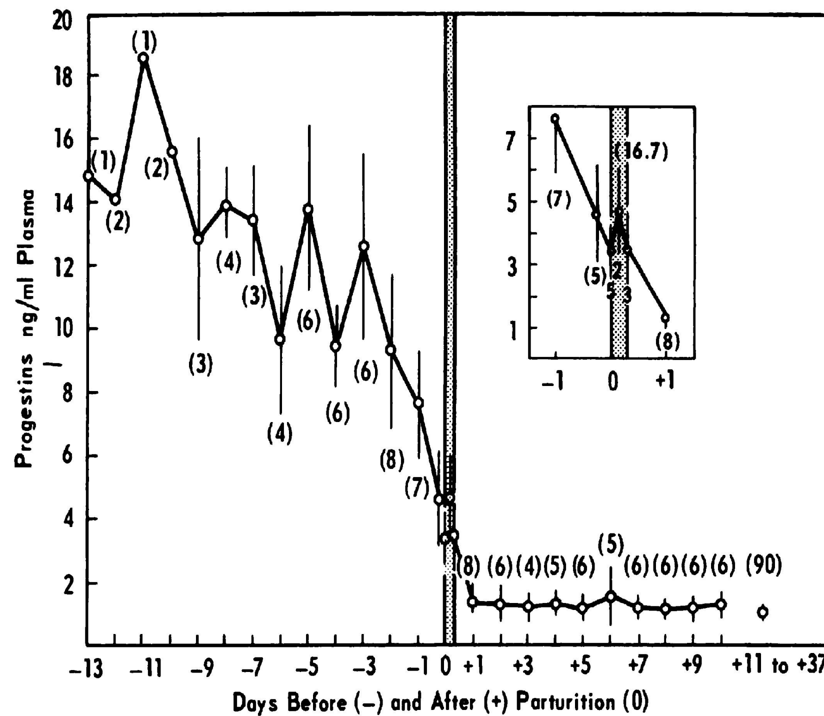


Figure 56: Diagram to illustrate the pre-parturient fall in progestins related to involution of the CL(s), reaching baseline levels almost immediately post-partum. Taken from (Baldwin and Stabenfeldt 1975).

During this same period, a rapid rise in estrogens is seen, also falling to baseline levels peri-partum (Baldwin and Stabenfeldt 1975). A post-partum estrus within a few days of birth is often seen, but is generally not considered to be fertile (Anderson 2000).

What is known of reproduction in the babirusa?

Information on the anatomy of the non-pregnant reproductive tract has been summarised for the Common warthog, the Forest hog (*Hylochoerus meinertzhageni*), the Babirusa, the Sulawesi warty pig, the Pigmy hog, the Eurasian wild pig and the

domestic pig by (Macdonald, Kneepens et al. 1984) and (Macdonald 2000). A summary of known reproductive parameters for the Suidae is given in ***Appendix 5***.

The majority of published information regarding reproduction in the Babirusa is either from records related to breeding parameters and behaviours or is derived from anatomical and post-mortem specimens. There are records of investigations into the functional anatomy of the babirusa (Macdonald and Leus 1995), placental structure (Macdonald 1994) and accessory organs such as the eye glands (Macdonald 1991). Much of the recent published data has relied heavily on observations of and data collected from the conservation breeding population.

A recent study was completed on the anatomical and histological characteristics of the male and female Babirusa reproductive tracts (Ziehmer 2008; Ziehmer, Ogle et al. 2009). The female study compared nine adult Babirusa tracts (of variable age and condition) with ten tracts from adult European Wild boar, and looked at ovarian features in some detail for the first time.

The relative lack of information of reproduction in the Babirusa beyond anatomical and behavioural observations may be attributed to a number of reasons: a lack of animals to work with; limitations of working with a CITES-listed threatened species; ethical issues on working with zoo animals; and research competition with other more 'charismatic' species such as rhino and elephants.

Like other pigs…

In common with other Suidae (Macdonald, Kneepens et al. 1984), the female Babirusa has paired ovaries, roughly ovoid in shape with multiple surface

irregularities resulting from underlying follicles, corpora lutea and corpora albicantes. They are partially enclosed by a wide conical bursa. The narrow convoluted uterine tubes and bicornate uterus are suspended within the abdomen by the extensive broad ligaments. The relatively long cervix has a spiral lumen to engage with the coiled penis of the male and there is a relatively short vagina (Macdonald 1991; Ziehmer 2008).

During pregnancy, and similar to other suidae, the Babirusa develops a diffuse, non-invasive chorioepithelial placenta (Macdonald and Bosma 1985; Macdonald 1994; Macdonald and Fowden 1997). From the information available, the histological features of the female reproductive tract are also similar between pig species (Macdonald, Kneepens et al. 1984; Macdonald 1991).

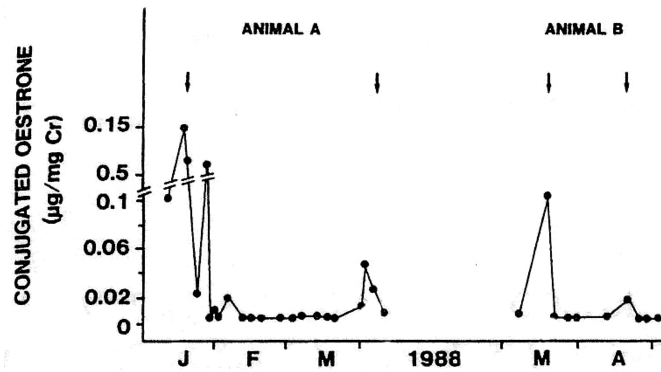
Unlike other pigs…

The gross anatomy of the female Babirusa is similar to that of other suidae, except for their relatively short uterine horns (Macdonald and Fradrich 1991). This is likely to be a limiting factor for litter size in the babirusa, and is also true of the Common Warthog (Macdonald, Kneepens et al. 1984; Macdonald 2000). The size of the Pigmy hog uterus, although small in absolute terms (Macdonald 2000), is still sufficiently long to accommodate up to seven fetuses (Macdonald 2000).

The estrous cycle

Estrous cycle length in the babirusa has been reported from 28 to 42 days (Macdonald 1993; Macdonald 2000). Other than that, very little more is known. A small study of two animals (Chaudhuri, Carrasco et al. 1990) related conjugated estrone levels in urine with estrus signs, as shown in ***Figure 57***. This study

determined that both females had a regular estrus cycle and their estrus periods are indicated in the figure. However, no more accurate values were given for cycle length in either case.



Urinary concentrations of conjugated oestrone in two Babirusas *Babyrousa babyrussa* during two oestrus periods. The arrows indicate the days that oestrous behaviour was observed.

Figure 57: Results of a small study investigating the urinary excretion of conjugated oestrone in two Babirusa females, showing variable cycle length. Taken from (Chaudhuri, Carrasco et al. 1990).

Nothing more was uncovered regarding estrus cycling in the Babirusa until the study of Berger *et al.* (Berger, Leus et al. 2006). Of the 155 studies involving 100 species listed in the most recent review of faecal steroid analysis by Schwarzenberger (Schwarzenberger 2007), this is the only one to have involved the babirusa.

The Berger study included five female Babirusa living in European zoos (International Studbook numbers 206, 152, 8, 142 and 132), that were investigated for faecal steroid excretion patterns during estrous cycling. They were investigated for excretion of two progestagens (20α-OH-pregnanes and 20-oxo-pregnanes), total estrogens and 17-oxo-androstanes and a typical trace is shown in ***Figure 58***.

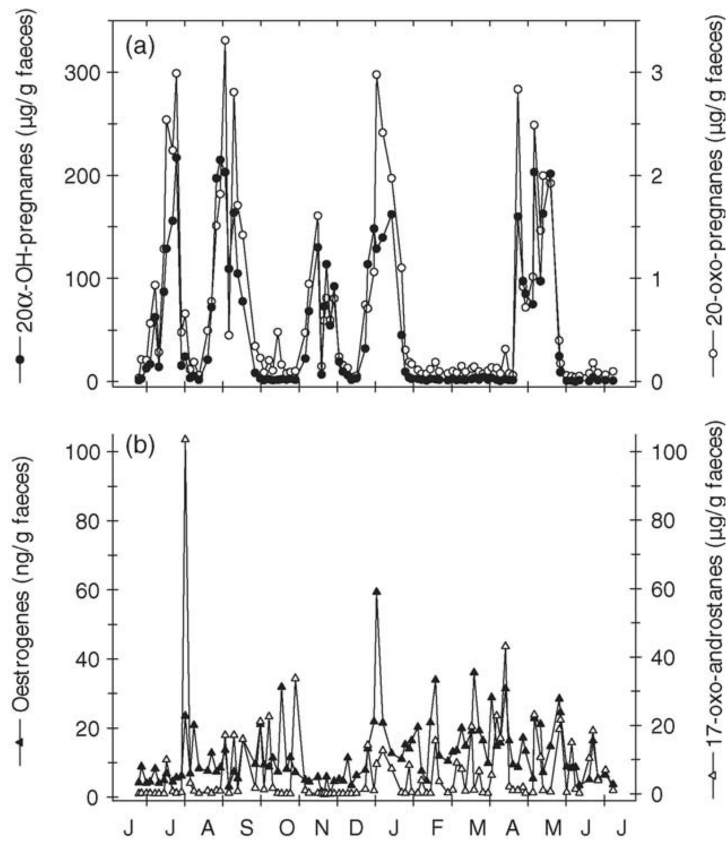


Figure 58: An example of the typical changes in faecal levels of four different steroids as seen in a babirusa during multiple estrus cycles. Taken from (Berger, Leus et al. 2006).

In summary, the Berger study found the progestagen assays to be the most useful in determining oestrus cycles and defined levels found in the luteal phase as shown in ***Table 29***.

	Typical luteal phase progestagen levels (µg/g faeces)	
Species	**20α-OH-pregnanes**	**20-oxo-pregnanes**
Warthog/Red River Hog	3-10	0.5-3
Babirusa	30-200	0.5-3

Table 29: Threshold levels for ‘luteal phase’ progestagens using two different assays for three different pig species as determined by (Berger, Leus et al. 2006).

It is interesting to note that the 20α-OH-pregnane assay detected higher progestagen levels in all three species, and that the Babirusa showed 10-20-fold higher luteal-phase 20α-OH-pregnane levels than the other two species, but similar levels of 20-oxo-pregnanes.

Pregnancy

The duration of pregnancy varies considerably between the different suidae, and ranges from ~ 114 days in a number of the *Sus* species (including the domestic pig and wild boar), to ~ 170-175 days in the warthogs (Macdonald, Kneepens et al. 1984; Macdonald 1993; Macdonald 2000).

There is very little known about pregnancy in the babirusa, except that gestation is considerably longer than in other pigs (Macdonald 2000), litter sizes are relatively small, (Macdonald, Kneepens et al. 1984; Macdonald 2000), the young seem to develop relatively slowly (Houston, Hagberg et al. 2001) and are relatively under-developed at birth (Macdonald 2000). As a direct comparison, the domestic pig can produce up to 20 piglets in a single litter after a 115 day gestation, whereas the Babirusa typically produces one or two piglets after a 158 day gestation. The study of Chaudhuri (Chaudhuri, Carrasco et al. 1990) also followed two pregnancies and and these are illustrated in ***Figure 59***. Note that oestrone levels only start to rise around 90 days before parturition, i.e. around 68 days into the pregnancy. The further increase in the period preceding parturition, and the rapid drop peri-partum, is similar to that shown for the domestic sow in ***Figure 56***.

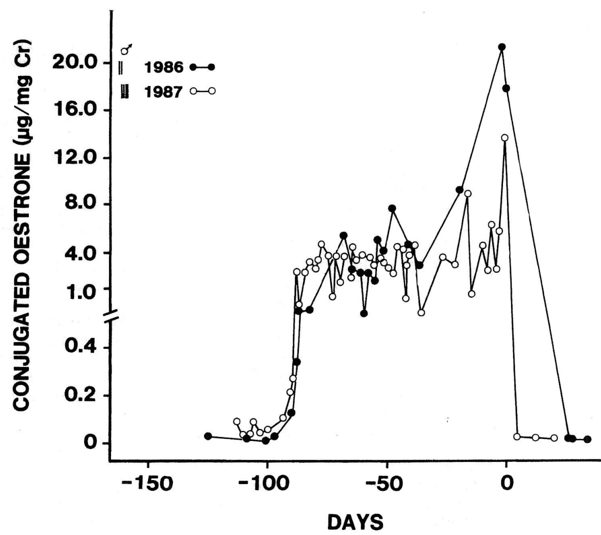


Figure 59: Changes in urinary oestrone levels during two pregnancies in the Babirusa. Taken from (Chaudhuri, Carrasco et al. 1990).

The female Babirusa signals imminent parturition in a number of ways: teat swelling may be seen from 2 months (Kalk and Holland 2002) to 3 weeks pre-partum (Bowles 1986), as shown in ***Figure 60***.

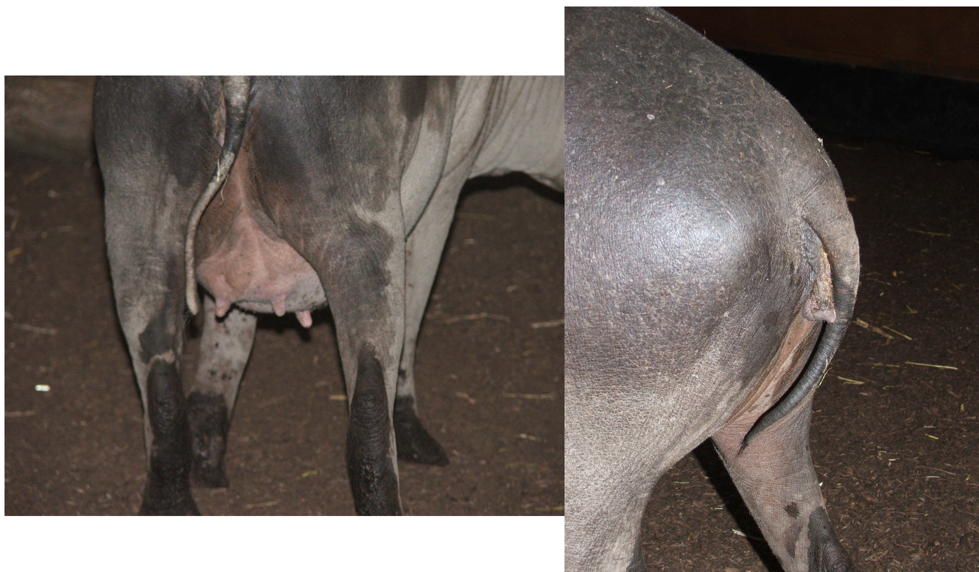


Figure 60: Obvious teat swelling (left) and vulval eversion (right) in female Babirusa Majene, two days before giving birth. (Pictures from Tim Rowland, Chester Zoo).

Vulval swelling is obvious 10 - 14 days pre-partum; and vaginal opening and discharge can be seen 1 week before parturition (Kalk and Holland 2002) with vulval swelling and eversion as the mucus membranes are exposed 2-4 days before birth (Leus, Bowles et al. 1992). These physical changes are accompanied by typical pre-parturient behaviours: nest-building may be seen a few days in advance of parturition (Macdonald 2000), mediated by $PGF_{2\alpha}$ and critically dependent on a supply of straw (Burne, Murfitt et al. 2000; Burne, Murfitt et al. 2001); and agitation may increase, with the female pacing or rolling over, sometimes associated with antagonistic behaviours (Leus, Bowles et al. 1992; Kalk and Holland 2002) .

After parturition, the female has been noted scent-marking her den area for several days, a behaviour rarely seen in the female under normal circumstances (Bowles 1986). It has also been observed that new mothers may cannibalise the piglet(s), as is known to occur in domestic pigs (Chen, Gilbert et al. 2008) and wild boar (Harris, Bergeron et al. 2001). It may be related to elevated stress levels, for example during longer birth periods (Harris, Bergeron et al. 2001), or as a symptom of a more general increased excitability and not directly piglet-related (Chen, Gilbert et al. 2008). Other adult male and female Babirusa have also been known to cannibalise the piglets from sows soon after parturition (Bowles 1986; Leus, Bowles et al. 1992). It is likely, therefore, that in the wild the sow separates from the other pigs during the farrowing period and this would be appropriate husbandry *ex-situ*.

Unknown…

What is *not* known about the Babirusa is any significant detail of normal ovarian function or typical follicular dynamics. For example, nothing is known of follicular

recruitment patterns, rates of follicular development and atresia, or rates of ovulation. Saying that, Zeihmer recently reported that up to four corpora lutea have been found on the ovaries of Babirusa females in preserved specimens (Ziehmer 2008). With a maximum litter size of three, and more typically one or two, this would suggest that more CLs may be present during pregnancy than the number of fetuses. Oliver (1980) reported this phenomenon for the Pygmy Hog, where five corpora lutea were found on the ovaries of a sow which had only two fetuses. This could be the result of failed fertilisation of released ova, or failed development at some point after fertilisation, resulting in fetal resorption, as is known to occur in other mammal species particularly in response to 'stress' (Westlin, Soley et al. 1995). An example of this in the Babirusa was the discovery of two placentae during a caesarean section for a single piglet, one of which was poorly developed (Macdonald, 1994). Resorption was also suspected during each of three babirusa pregnancies monitored by ultrasound at the St Louis Zoo (Houston, Hagberg et al. 2001).

Overall, prenatal losses are common in pigs and have been reported to range from 11-15% in the European Wild Boar (Boye 1956; Stubbe and Stubbe 1977; Mauget 1982), from 9-18% in the warthog (Child, Roth et al. 1968; Clough 1969; Mason 1982) to 28.5% in the Bushpig (Seydack and Bigalke 1992).

Why is steroid analysis important for the babirusa?

In order to maximise the reproductive potential of *any* vulnerable species held in conservation breeding programmes, their underlying anatomy and physiology has to be well understood. Simply diagnosing reproductive insufficiency or anomaly is dependent on knowing the 'norms' for a species. This is even more important when

considering the use of artificial reproductive techniques such as AI or IVF. For example, the Black, White *and* Indian rhino species (*Diceros bicornis*, *Ceratotherium simum* and *Rhinoceros unicornis*) have been subject to various reproductive interventions in a bid to maximise successful breeding, supported by extensive fundamental research into their normal reproductive function (Schwarzenberger, Francke et al. 1993; Schwarzenberger 1996; Schwarzenberger, Rietschel et al. 2000; Graham, Schwarzenberger et al. 2001; Gómez, Jewell et al. 2004; Hermes, Hildebrandt et al. 2006; Swaisgood, Dickman et al. 2006; Pluháček, Sinha et al. 2007; Hermes, Goritz et al. 2008; Behr, Rath et al. 2009; Hermes, Goritz et al. 2009).

The Babirusa may seem well suited for the use of ARTs as a member of the pig genus: the pig industry is huge and much effort has been made to understand the finer points of reproductive output and the factors that influence it. However, as was illustrated in the opening chapter, the Babirusa is by no means a close relative of the domestic *Sus scrofa* so the applicability of details regarding reproduction in *Sus scrofa* or other Suinae is not certain and cannot be assumed without further investigation.

What to measure?

The study of Berger *et al* (Berger, Leus et al. 2006) demonstrated the usefulness of faecal progestagens in following estrous cycles in three exotic pig species, including the babirusa. Estrogens and androgens were not closely correlated with the cycle so are not of use in these species. As progestagens are the steroid of choice to follow cycling and pregnancy in the babirusa, their metabolism is more fully described.

Figure 61 shows the principal metabolites produced by the breakdown of progesterone in the liver, adapted from (Schwarzenberger, Palme et al. 1997).

In all cases, progesterone metabolism leads to a reduction at the C5 position, resulting in complete saturation of the A-ring and formation of the pregnanediones. Additional hydroxylation at the C3 and/or C20 positions leads to the production of various isotypes of the pregnanolones (hydroxylation at either C3 or C20) and the pregnanediols (hydroxylation at both C3 *and* C20).

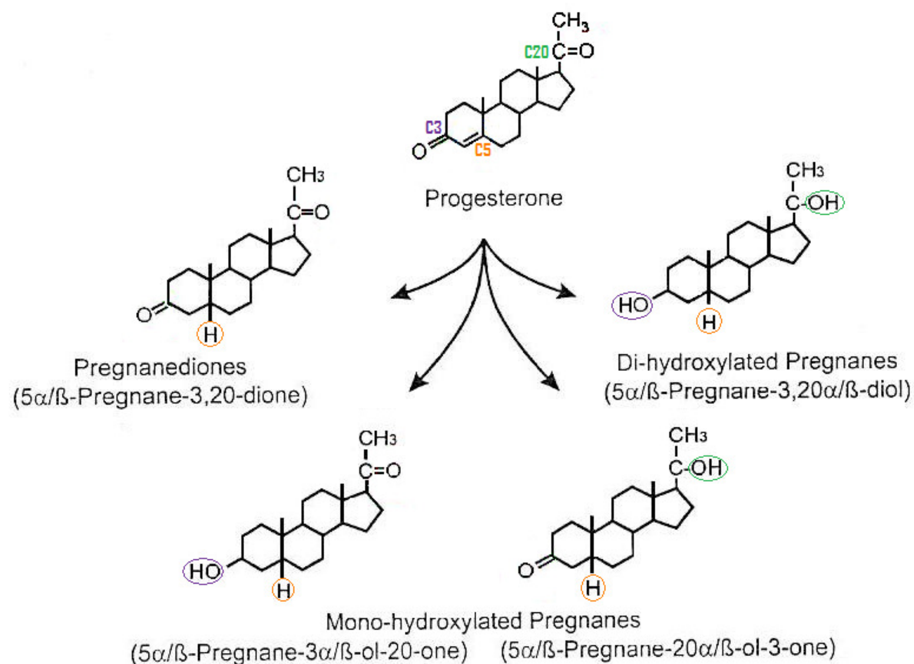


Figure 61: Major metabolites from the breakdown of progesterone, adapted from (Schwarzenberger, Palme et al. 1997).

FAECAL STEROID ANALYSIS

Faecal steroid analysis has primarily been employed to investigate female reproductive physiology, especially during onset of puberty, estrus cycling and pregnancy in a vast range of species, but also in relation to male reproductive

physiology and dominance behaviour, and to monitor ‘stress’ through faecal cortisol measurement. The large range of studies conducted using faecal steroid techniques have been reviewed by Dr Franz Schwarzenberger of the University of Vienna (Schwarzenberger, Möstl et al. 1996; Schwarzenberger, Palme et al. 1997; Schwarzenberger 2007). The most recent review refers to 155 studies of 100 species, and this is not a complete list of all published works available, highlighting the huge scope of this technique. The underlying principles of the technique are in the production, metabolism and excretion of steroid hormones in relation to reproductive status, and these are described below.

Considerations for faecal steroid analysis

Faecal steroid analysis is an increasingly common tool used to monitor reproductive cycling in wild and zoo animals (Schwarzenberger 2007). It usually depends on measuring estrogens and/or progestagens to follow the cycle of changes within the functional ovary, or to identify abnormalities. The non-invasive nature of faecal sampling has seen it replace blood/serum analysis as the method of choice for wild and zoo animals as it avoids the need for ‘stressful’ invasive procedures.

Despite its increasing usefulness, however, faecal steroid analysis should be undertaken with due consideration to the numerous variations in techniques and their relative merits and pitfalls, as discussed below.

1. What do you want to know?

What you want to know will determine which hormone class will be most useful to monitor. As estrogens are related to follicular growth and progestagens to luteal activity, either could be useful to investigate estrous cycling, with the most useful

dependent on the species under investigation. For example, progestagens have proved useful in the maned wolf (*Chrysocyon brachyurus*) (Velloso, Wasser et al. 1998) and various pig species (Berger, Leus et al. 2006) and estrogens in yellow baboons (*Papio cynocephalus*) (Wasser 1996).

Both steroid classes may also be employed to investigate pregnancy, and have been used to do so in a number of species, including the feral horse (*Equus caballus*) (Kirkpatrick, Shldeler et al. 1991), the macaque (*Macaca fascicularis*) (Shideler, Ortuño et al. 1993), the maned wolf (*Chrysocyon brachyurus*) (Velloso, Wasser et al. 1998), the right whale (*Eubalaena glacialis*) (Rolland, Hunt et al. 2005), the sea otter (*Enhydra lutris*) (Larson, Casson et al. 2003), the sun bear (*Helarctus malayanus*) (Schwarzenberger, Fredriksson et al. 2004), the leopard cat (*Felis bengalensis*), cheetah (*Acinonyx jubatus*), clouded leopard (*Neofelis nebulosa*) and snow leopard (*Pantheris uncia*) (Brown, Wasser et al. 1994), the Asian and African elephant (*Elaphus maximus* and *Loxodonta Africana*) (Hodges 1998; Feiß, Heistermann et al. 1999), the black rhino (*Diceros bicornis michaeli*) (Schwarzenberger, Francke et al. 1993), the okapi (*Okapia johnstoni*) (Schwarzenberger, Patzl et al. 1993), and the brown brocket deer (*Mazama gouazoubira*) (Pereira, Polegato et al. 2006). It has also been used in the Babirusa (*Babirusa celebensis*) (Chaudhuri, Carrasco et al. 1990) and the domestic sow (Sus *scrofa*) (Choi, Kiesenhofer et al. 1987; van de Wiel, Vos et al. 1992; Vos, van Oord et al. 1998; Isobe and Nakao 2004).

In addition to steroid levels, it is useful to have records of behaviours related to estrous and pregnancy as they can be used to help validate the steroid profiles and also link back to husbandry practices.

2. What species are you investigating?

As a consequence of differing steroid production, metabolism and excretion pathways, different species may well have unique faecal steroid profiles in relation to female reproduction. Although even closely-related species may show significant differences, they may be a good starting point in assessing the relative merits of metabolites to assay. It can also be useful to submit faecal extracts for mass spectrometry (MS) analysis to identify the range and relative abundance of steroid metabolites present. Reproductive steroids and their metabolites have been successfully separated for MS analysis using Liquid Chromatography (LC)/High Performance LC (HPLC) (Schwarzenberger, Speckbacher et al. 1994; Schwarzenberger, Son et al. 1996; Hauser, Deschner et al. 2008) and Gas Chromatography (GC) (Santen, Demers et al. 2007; Tripp, Dubois et al. 2009).

Species (or individuals) may also vary in terms of their digestive transit time, from 12-48 hours (Schwarzenberger, Mostl et al. 1996; Wasser, Papageorge et al. 1996). This is important in analysing faecal profiles as they impose a delay between steroid production and action, to metabolite processing in the liver, to excretion from the gall bladder into the gut, and then to final defecation. This passage through the gut also means that faecal steroid levels represent pooled endocrine activity over the period of several hours (Schwarzenberger 2007). Despite the delay, strong correlation between serum and faecal steroid level has been shown in multiple studies (Schwarzenberger, Möstl et al. 1992; Larter, Rajamahendran et al. 1994; Schwarzenberger, Speckbacher et al. 1994; Morrow and Monfort 1998), including for the domestic sow (Hultén, Forsberg et al. 1999) where the delay between serum and faecal progestagens is reported at ~2 days. Much of this can be accounted for by

the digestive transit time of 25-39 hours in domestic pigs from the proximal duodenum to excretion, depending on levels of dietary fibre (Wilfart, Montagne et al. 2007).

3. How will samples be collected, identified and stored?

Where faecal collections are required from a number of animals in the same collection, some care must be taken to ensure that each sample can be definitively attributed to a specific animal. Obviously, defecation can occur at any time of the day and keepers are unlikely to witness each event. The easiest way to assign faeces to specific animals is to have them kept separately, even just overnight to make collections in the morning. However, for some species this is not appropriate or there may not be the space to do so. In these cases, methods can be employed to match faeces to animals, for example by feeding harmless dyes or glitter to individuals to 'mark' their faeces (personal communication Amy Plowman), or by using coloured water marked with dyes during hand-feeding (Cerda-Molina, Hernández-López et al. 2006). Where animals are free-roaming, DNA markers can be used instead, as recently employed in a study of wild otters (*Lutra lutra*) (Kalz, Jewgenow et al. 2006).

The storage method of choice for faecal samples is immediate freezing until analysis (Khan, Altmann et al. 2002; Schwarzenberger 2007). In this state, degradation by faecal bacteria will be minimal and samples can be stored indefinitely (Khan, Altmann et al. 2002). Where freezing is not possible, Schwarzenberger recommends storage in ethanol until analysis (Schwarzenberger 2007), and has shown to be sound in some studies (Terio, Brown et al. 2002). However, this method has been related to

degradation of steroid hormones in others (Pettitt, Wheaton et al. 2007). Drying was suggested as a best alternative to freezing in this case and in other studies (Lynch, Khan et al. 2003; Galama, Graham et al. 2004), although this can also be associated with some steroid loss (Ziegler and Wittwer 2005). Alternatives such as storage in 80% methanol (Galama, Graham et al. 2004) and solid-phase extraction (SPE) (Ziegler and Wittwer 2005) have also been proposed for use in the field. It seems clear from these somewhat conflicting reports that if freezing is not possible the alternative method of choice should be validated for each study.

4. How will steroids be extracted?

Being sterol-based, the reproductive steroids can easily be extracted from faeces using simple alcohol-based procedures. Extraction protocols usually suggest the use of a solvent with a high percentage of alcohol (Schwarzenberger, Palme et al. 1997), typically using at least 30% ethanol or methanol to ensure extraction of both conjugated and unconjugated metabolites (Ziegler and Wittwer 2005). In some cases, an additional ether extraction is carried out in order to further purify the sample extract (Schwarzenberger, Möstl et al. 1991; Shideler, Ortuño et al. 1993; Larter, Rajamahendran et al. 1994; Velloso, Wasser et al. 1998; Schwarzenberger, Fredriksson et al. 2004; Busso, Ponzio et al. 2007). This is especially important if an HPLC/GC procedure is being used with mass spectroscopy to specifically identify the metabolites present.

There is some evidence that steroids are not evenly distributed within the faecal ball in some species, e.g. the pony (Palme, Fischer et al. 1996). There are two ways to deal with this: either homogenise each sample, as some protocols recommend e.g in

(Cerda-Molina, Hernández-López et al. 2006); or take samples routinely from the same part of the faecal ball, assuming that the differences exist between the inside and outside of the sample.

It is not recommended that alcohol-extracted steroids are stored for any length of time in plastic. In some cases, alcohols will degrade plastics, and a recent study suggested that the use of plastic can have a negative effect on subsequent RIA quantification (Ocvirk, Bisson et al. 2009).

5. What method will be used for quantification?

Immunoassay is the method of choice for faecal steroid quantification. Increasingly, assays based on radioactively-labelled steroids (radio-immunoassay, RIA) are being replaced with enzyme-linked assays (enzyme-immunoassay, EIA), for example see (Graham, Schwarzenberger et al. 2001). The major benefit of EIA is that it removes the risks related to working with radioactive substances, and does not require a specialist laboratory set-up. Whichever type of assay is used, however, the method depends on competitive inhibition between labelled and intrinsic steroids for access to antibodies against the substance of interest. Sample values are compared against a standard curve of known concentrations, allowing quantification of each.

Assay validation is vital to faecal steroid analysis (Schwarzenberger 2007) and should be conducted to ensure that there are dose-dependent changes in quantification in keeping with dilution factors of the stock sample. Typically a parallelism is conducted, comparing curves produced using the same dilution factors of stock standard of the steroid of interest and a pooled faecal sample extract.

It is the antibody and its particular cross-reactivity with multiple metabolites within the sample that determine the specificity of the assay, regardless of the use of RIA or EIA. Antibodies can be very specific to only one or a few metabolites, or can be designed to pick up related groups of metabolites, similar in certain side-group structures or configurations e.g. the 'group-specific' antibodies used in the Vienna laboratory (Schwarzenberger 1996; Schwarzenberger, Son et al. 1996; Schwarzenberger, Palme et al. 1997). The sensitivity of the assay will depend on the antibody as well as the conditions under which the assay is conducted. Intra- and inter-assay variation will also depend on the conditions of the assay, as well as a degree of experimenter error. Typically, intra-assay variation is assessed by comparing duplicate/triplicate values for each sample and inter-assay variation by running a number of samples of known high and low value with each assay and comparing between them.

SAMPLES

Faecal samples were collected every two or three days from eleven female Babirusa held in zoological collections in Indonesia and the United Kingdom (see ***Table 30***). In addition, keepers were asked to observe and record any signs of estrus, either physical (swelling and/or reddening of the vulva) or behavioural (soliciting a male, standing reflex, mating). In some cases, details of changes to the enclosure or daily routine, or of any veterinary/keeper procedures were also recorded.

Local ID	Global SB#	Location	No. samples	No. Days	No. cycles	No. pregnancies
Sur 9 *'Kunti'*	330	Surabaya	45	60	1	0
Sur 53 *'Dewi'*	411	Surabaya	34	57	1	0
Sur 59 *'Pahing'*	T47	Surabaya	48	61	0	0
Sur 66 *'Sepon'*	T54	Surabaya	50	58	1	0
Sur 86 *'Jane'*	T74	Surabaya	53	60	1	0
Sur 1 *'Suci'*	322	Surabaya	54	60	0	0
Sur 20 *'Lala'*	341	Surabaya	49	61	1	0
Sur 3 *'Kiki'*	324	Surabaya	59	66	2	0
Sur 81 *'Sari'*	T69	Surabaya	55	61	2	0
440 *'Majene'*	440	Southlakes, UK	153	347	1	2
T17 *'Kota'*	T17	Chester, UK	355	579	1	2
104* *'Beret'*	none	Los Angeles	140	687	2	2
90* *'Greta'*	289	Los Angeles	265	687&430	19	0

*N. Am SB#s. Steroid assays were carried out at the St Louis Zoo, Illinois, USA

Table 30: Samples for whom faecal samples were collected (Surabaya/UK animals) and for whom steroid data were already available (Los Angeles animals).

Further raw data was kindly made available by Dr Joan Bauman at the St Louis Zoo on two Babirusa females who had been monitored for faecal progestagens over a number of years. These samples had also been extracted using a methanol-based procedure and analysed using a commercial RIA for progesterone but the results had not been analysed in any detail or correlated with physical/behavioural signs of estrus. Additional husbandry and behavioural information was provided for the two female babirusas held in the Los Angeles Zoo by Jeff Holland, Curator of Mammals.

Animal histories

Surabaya females

These females in Surabaya Zoo represent animals held in conditions broadly comparable with those in the wild, i.e. within the same climate and photoperiod. They are held closely together in a breeding compound with separate pens for breeding pairs, mothers and piglets or individual animals (see ***Figures 62*** and ***63***). There is sight, sound and smell communication between individuals of both sexes. Breeding is not considered to be difficult in this setting, with many successful pregnancies and births since it was established as the official Babirusa breeding institution in Indonesia. As such, the female population here could be considered optimally suited to breed. However, due to the large number of animals being sampled and limited freezer storage here, faecal collections were continued for a maximum of 70 days. This was considered enough time to look at one or two estrous cycles per female and to establish 'baseline' measurements of steroid levels. Collections were made between 14th January and 20th March 2005. A description of each animal involved in faecal steroid analysis is given below.

Figure 62: Example of typical pens in Surabaya Zoo with single animals, breeding pairs or mothers and piglets held in each.

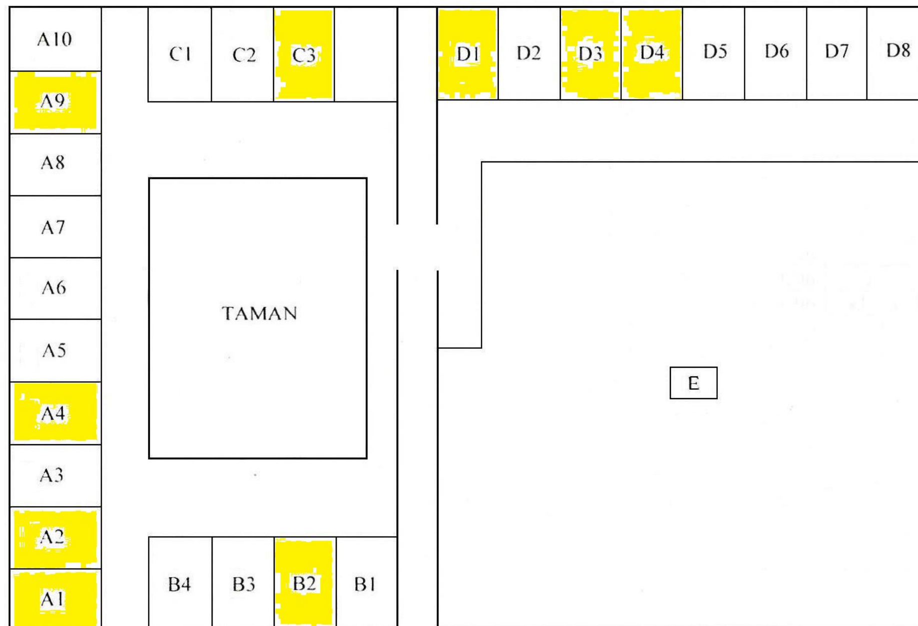


Figure 63: Layout of pens in Surabaya Zoo – those in yellow housed the females sampled for this study.

Sur 9 (Pen B2) – 'Kunti'

Kunti was born in Surabaya to unknown parents on an unknown date, estimated around 1st Jan 1993. This made her ~12yrs old at the start of the study. Kunti had four previous pregnancies between 1996 and 2003, two of which produced twins.

Sur 53 (Pen A1) – 'Dewi'

Born wild in Sulawesi (estimated at 1st Jan 1997), Dewi arrived in Surabaya in November 1998 and was penned with a wild-caught male. She had four successful single-piglet births between 1999 and 2002, all sired by another of the wild-caught male babirusa. She was ~8yrs old at the start of the study.

Sur 59 (Pen A2) – 'Pahing'

Pahing was born on 19th August 2000 to Sur 53 'Dewi'. She was 4yrs 5months old at the start of the study and had not produced any piglets up to that time.

Sur 66 (Pen A4) – 'Sepon'

Sepon is another of Sur 53's offspring, born on 1st June 2001. That made her 3yrs 7months old at the start of the study, and she had not been pregnant up to that time.

Sur 86 (Pen A9) – 'Jane'

Born to Sur 9 and a wild-caught sire on 5th January 2003, Jane was 2yrs old at the start of the study.

Sur 1 (Pen C3) – 'Suci'

Suci was born in Surabaya to unknown parents on an unknown date, estimated around 1st Jan 1996. This makes her ~9yrs old at the start of the study. She finally gave birth to a single piglet after the study, on 15th December 2005.

Sur 20 (Pen D1) – 'Lala'

Lala was born in Surabaya on 15th June 1996, making her 8yrs 7months at the start of the study. She had two previous single births in 2001 and 2003, and gave birth to another after the study, on 27th October 2005.

Sur 3 (Pen D3) – 'Kiki'

Kiki was born in Surabaya to unknown parents on an unknown date, estimated around 1st Jan 1996. This makes her ~9yrs old at the start of the study. She already had two single piglets, in 2001 and 2003, and produced another after the study on 31st January 2006.

Sur 81 (Pen D4) – 'Sari'

Sari was born in Surabaya on 2nd December 2001, making her 3yrs 1month old at the start of the study. At the time of writing she had not produced any piglets.

UK females

The two females in the UK (Southlakes Wild Animal Park and Chester Zoo) were much easier to sample for a longer period as batches of faecal samples were collected from the institutions at regular intervals. There was significantly more additional information regarding the Chester female as keepers kept very good records of both reproductive and non-reproductive events.

T17 (Chester Zoo) – 'Majene'

Majene was born in Twycross Zoo, UK on 23rd August 2002 making her 3yrs 7 months at the start of faecal collection. On her move to Chester Zoo on 2nd March 2005, she was introduced to a male for the first time with the hope that she would

breed with him. This happened within a year but the piglet was stillborn on 15th April 2006. Unfortunately, she didn't become pregnant again with this male before he died (in August 2006), or with another that only survived 6 months between January and June 2007 before being euthanased due to ill-health. These events left Majene without a male for five months then a further three months until, in September 2007, a young male was introduced from the Southlakes Wild Animal Park. He was quickly accepted, and she became pregnant during her first cycle after he was introduced, resulting in a single piglet on 26th February 2008.

440 (Southlakes Wild Animal Park) – 'Kota'

Kota was born in Twycross Zoo on 1st May 2000, making her 6yrs 7months at the start of this study. Between 2002 and mid-2006 she had a total of eight pregnancies, two with twins and six with single piglets. All of these pregnancies went to term and were delivered successfully but only a single male survived and was reared normally (he then went on to sire piglets to Majene at Chester Zoo). This is due to Kota's highly stressed and aggressive post-partum behaviour resulting in her killing and/or eating the piglets within a few hours of birth. After a series of incidents, the decision was taken to remove and hand-rear any further piglets for the best chance of survival. This has resulted in healthy twins born in August 2007 and a further female mid-2008.

North American females

Information was made available regarding two females held in the Los Angeles Zoo. These animals had been monitored over a period of years for husbandry purposes but

the full data set had never been analysed. There was also extensive reproductive and non-reproductive information available to allow a comprehensive analysis.

104 (Los Angeles Zoo) – 'Beret'

Beret was born on 12th September 2002 in Oklahoma Zoo, and was transferred to the Los Angeles Zoo in December 2005. Soon after, faecal collections began, making her 4yrs 7months at the start of the study. She had not previously been pregnant.

90 (Los Angeles Zoo) – 'Greta'

Greta was born on 13th August 1996 and was transferred through a series of zoos to arrive in Los Angeles in May 2002. She was monitored for faecal steroid levels from October that year, at 6yrs 2months old and throughout her stay until her final movement to the Bronx Zoo in March 2006. She had not previously been pregnant.

Faecal sample collection, transportation and storage

Whole faecal balls were collected from animal enclosures, usually in the morning, every two/three days where possible. All females in this study were either held in separate pens or at least separated overnight so there could be no confusion over whose faeces were present (except in one case where there was also a youngster present, but the faecal ball was noticeably smaller compared to the mother). Samples were collected into individual plastic bags or specimen bottles and stored frozen until transportation to the relevant university for analysis. In Indonesia, this was from Surabaya Zoo to the Institute Pertanian Bogor, and in the UK from Dalton-in-Furness (Southlakes Wild Animal Park) and Chester to Edinburgh. A degree of thawing may have occurred during this time but this was minimised using polystyrene cool boxes for transportation. Samples were re-frozen and stored at -20°C until extraction.

Steroid extraction and analysis

Steroids were extracted from the Indonesian faecal samples in the Department of Reproductive Physiology at the Institut Pertanian Bogor (IPB), Indonesia, and from the UK samples at the Centre for Reproductive Biology, the Queens Medical Research Institute (QMRI) in Edinburgh, UK. All extracted samples were analysed for progestagens at the QMRI, using an RIA procedure modified for use with faecal samples.

METHODS

The faecal steroid analysis method consisted of a methanol extraction of steroids followed by quantification of progesterone (P4) and progestagen metabolites using a radioimmunoassay. Extraction efficiency was estimated using recovery of radio-labelled progesterone, the anti-P4 antibody was tested for cross-reactivity with a number of progestagens and assay validity was tested using a parallelism method. Inter-assay QCs and intra-assay duplicates were used as control measures.

Steroid Extraction

In all cases, 0.5g of defrosted faeces was taken from the outer layer of the faecal ball and mixed with 0.5ml double distilled water (DDW) and 4ml 100% methanol in Pyrex glass test tubes (Fisher). They were shaken for 45 minutes at 1500rpm in an IKA Vibrax® XVR orbital shaker, then centrifuged for 15 minutes at 4000rpm. Extracts from the Indonesian samples were then shipped to the UK and treated as for the UK extracts, as follows: 0.5ml supernatant containing the steroid metabolites was decanted, dried (under mild heat and nitrogen flow) and reconstituted in 1.0ml PGBS assay buffer. This was used as the basis for analysis in the immunoassay, with further dilutions made as appropriate.

Note: PGBS assay buffer contains 7.75g citric acid (Sigma, C7129), 17.85g Na_2HPO_4 (Sigma, S9763) and 1.00g gelatin (Sigma, G9382) in 1L de-ionised water, with 0.10g thiomersol (Sigma T5125), adjusted to pH 6.0.

Extraction efficiency

The extraction procedure was carried out as previously described with the addition of 100µL radio-labelled progesterone (I^{125}-Progesterone-11α-glucoronide, Amersham

Pharmacia) to the faeces at the same time as the methanol and distilled water. The radio-labelled progesterone tracer was prepared by diluting stock in PGBS assay buffer to give a starting value of 5772 counts/20s i.e. 17316cpm. This diluted tracer solution was used for all extractions.

Three 0.5g samples (a, b & c) were taken from each of two faecal balls belonging to a single animal, one with a high intrinsic P4 level (pre-parturient) and another with low intrinsic P4 (post-parturient). Gamma counts were recorded using an LKB 1261 Multigamma apparatus before addition to the sample and after spinning (A), drying (B) and reconstitution in buffer (C) to calculate the % radioactive progesterone retained after each stage of the extraction procedure. There was a dilution factor of 9 as only 0.5mL extract was dried down from the total volume of 4.5mL. For each sample extract, two or three 0.5mL aliquots were dried, reconstituted and measured. Glass tubes were used throughout, as γ-counts will differ depending on the substrate it is being measured through, and glass was required for the drying stage.

Progesterone radio-immunoassay (RIA)

Stock solution of progesterone (Sigma P9776) was prepared in ethanol to 1µg/ml from solid progesterone. This was then diluted in PGBS assay buffer to prepare a top standard concentration of 1000 pg/50µl and double diluted out to give 9 standards with concentrations ranging from 1000pg to 3.9pg/50µl. These were stored at 4°C.

For each RIA, 10x75mm polystyrene tubes (Sarstedt) were prepared for samples, QCs and progesterone standards in duplicate as follows: 50µL sample, QC or standard; 150µL PGBS assay buffer; 100µL P4 Tracer; and 100µL rabbit anti-P4

antibody (SAPU) added in that order. Samples were diluted 1:25 at this stage, giving a total dilution factor of 1:50 (including the initial x2 dilution of 0.5mL dried sample reconstituted in 1.0mL buffer). Subsequent dilutions were made where necessary.

Total counts (TC) tubes were prepared with 100µL tracer to measure maximum radioactivity values at the end of the assay. To test the maximum binding of P4 tracer, duplicate tubes were prepared with sample substituted by buffer (Bo tubes). To test the non-specific binding of P4 tracer to the plastic tubes without a specific binding antibody, duplicate tubes were prepared with both sample and antibody substituted by buffer (NSB tubes). Total assay volume was 400µL in all cases (except for TCs). Once all tubes were prepared, they were shaken to mix and incubated either overnight at 4^oC or for 3 hours at room temperature.

After this first incubation, 100µL each of goat anti-rabbit serum (GARS, ICN, 1:100 dilution) and normal rabbit serum (NRS, SAPU, 1:60 dilution) was added to each tube, shaken then incubated either overnight at 4^oC or for 3 hours at room temperature. After the second incubation, 1mL wash buffer was added to each tube then centrifuged for 45mins at 3000rpm. Wash buffer contains 4% w/v PEG, 0.2% v/v Triton-X (Sigma X-100) and 0.9% w/v Saline (sodium chloride, Sigma S7753). The supernatant was decanted and pellets dried then γ-counted, and data analysed using AssayZap software (Universal Assay Calculator for Windows, Biosoft 1996).

Assay validation

1) Parallelism

Parallelism is used to check that there is a concentration-dependent change in output with a similar profile to the standard curve. 100µL of four sample extracts (2 high

and 2 low concentrations) were combined to give a starting stock assumed to be similar to the top standard 1000pg/50µL. This was serially diluted as for the standards and assayed using the standard RIA protocol. Resulting concentrations were plotted alongside the standard curve to look for parallelism.

2) Intra-assay control

All samples were run in duplicate. Where values showed >10% variance, they were usually repeated. The exception to this was when the values were both considered 'low', usually less than 50pg/50µL. Accuracy of measurement was not considered vital for these samples as small variations in concentration could not be attributed any physiological significance. Similarly, higher values were not always repeated for accuracy to 10% as it was sufficient only to know that they can be considered 'high' with the actual values being less important.

3) Inter-assay control

Four samples (two high and two low) were selected from the first RIA assay to be used as internal Quality Controls (QCs). The output from AssayZap also gave information on % binding and 20, 50 and 80% binding points and added each standard curve to a 'graveyard' of pooled curves to check for correlation between them.

4) Reproducibility between samples from the same faecal ball

Two samples (one high and one low) were selected to assess any impact of uneven metabolite dispersal within the faecal ball. All 0.5g test samples were taken from the outside of the faecal ball, as was the standard procedure during sample preparation. It was also standard to allow test samples to weigh +/- 0.02g (i.e. +/- 4%) and this

was again observed here. Samples were extracted at the QMRI in Edinburgh then assayed for pregnenolone by the laboratory of Dr Franz Schwarzenberger at the University of Vienna.

Cross-reactivity of SAPU anti-P4 antibody

The anti-progesterone antibody used in this RIA was obtained from Dr Ian Swanston of the MRC Centre for Reproductive Biology, QMRI, Edinburgh. The cross-reactivity of this antibody was unknown and was tested using the standard RIA protocol with progesterone and a series of progesterone metabolites. Pregnanediones, pregnanediols and pregnenolone were chosen to reflect the likely metabolites present in faeces (Schwarzenberger, Palme et al. 1997). Each progestagen was assayed at a concentration of 100pg/50µL and the relative outcome values compared. 100pg/µL was chosen as the stock concentration as it falls around the middle of the standard curve and is thus the most accurate measure.

Assay comparison with Vienna protocol

As an additional assay validation, a series of extracted samples were lyophylised and sent to the laboratory of Prof Dr Franz Schwarzenberger at the Department of Biochemistry, the University of Vienna. As the original laboratory to analyse faecal progestagens in the Babirusa it seemed appropriate to investigate how the RIA used in this study compared with the ELISA used in the original study. The samples sent to Vienna included the full sample series for Surabaya female Sur3 (SB#324) and for Chester female Majene (SB#T17) and these are used to compare the performance of each assay, and also to investigate the difference in metabolite levels found in Surabaya females and those from the UK.

RESULTS

Assessment of assay methodology

Extraction efficiency

Based on a starting value of 17316cpm in 100µL and a dilution factor of nine, 100% extraction efficiency would yield 1924cpm in 100µL of extract. Raw data of actual values are show in ***Appendix 6*** and summarised in ***Table 31***

	Extraction efficiency (mean%±SD)			
Sample	**Shaken & spun**	**Dried**	**Reconstituted**	**Overall**
High P4	80.65±5.40	78.21±5.40	76.78±3.78	78.55±4.47
Low P4	87.07±5.94	83.96±5.50	85.16±4.23	85.39±5.21
All	83.86±6.41	81.08±6.05	80.97±5.81	81.97±6.11
Coefficient of Variation (%)	6.23	5.37	6.83	6.14

Table 31: Summary of extraction efficiency for high and low intrinsic progestagen samples, assessed at each stage of the extraction process.

Overall, mean extraction efficiency was 82% with 6.14% replicate variation. On further analysis, the Friedman test showed there was a significant difference between extraction efficiency values during the extraction procedure (p=0.0289). Dunn's Multiple Comparison Test confirmed there was a significant difference between stages A (shaken and spun) and C (reconstituted) but not between the stages A-B or B-C i.e. there were minor losses during the drying and reconstitution stages. Secondly, there is some evidence that there is reduced extraction efficiency in the samples containing higher intrinsic P4, shown at each stage of the extraction process.

Radio-immunoassay validation

1) Parallelism

Mean values for duplicate results in the parallelism test comparing progesterone standards and a pooled faecal extract are shown in ***Figure 64***.

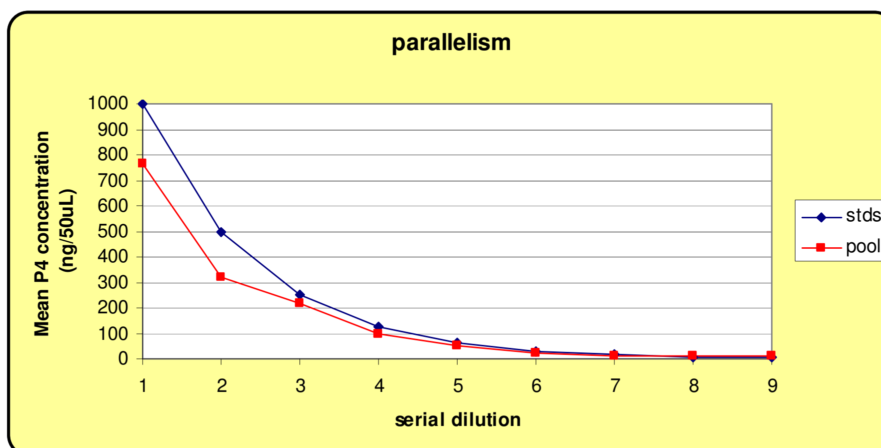


Figure 64: Results of parallelism between a progesterone standard curve and a serially-diluted pooled faecal sample.

The curve produced from dilution of pool samples shows a dose-dependent effect similar to the standard P4 solutions. However, there are large standard deviations for dilutions 1 and 2 in the pool samples (due to poor duplication) and it is clear that these points follow the standard curve least closely. ***Figure 65*** confirms that although the pool samples follow a roughly linear distribution, they curve of the graph is not identical to the standards, diverging with increasing concentration.

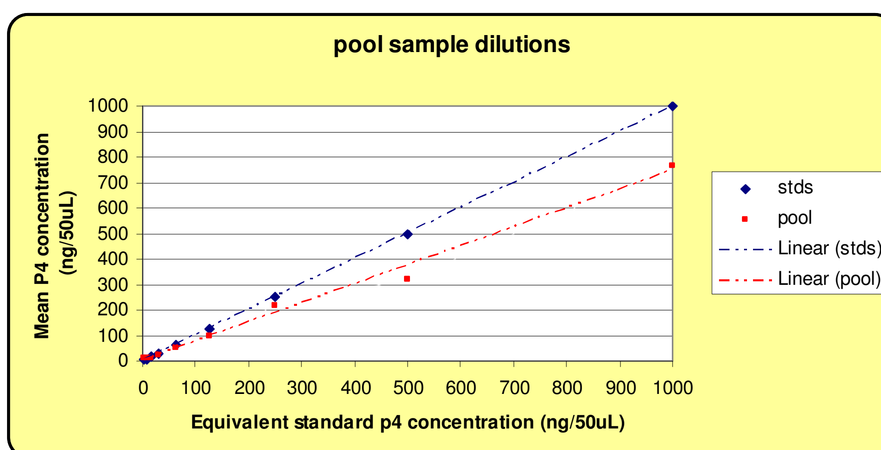


Figure 65: Comparison of *log*-transformed results of parallelism between standard progesterone and pooled faecal samples

2) Intra-assay control

Assay duplicate variation for each female ranged from 8.34% (Sur 53, *n=32*) to 19.84% (Muhibah, *n=18*) with a mean value 14.12% (all samples, *n=905*). A full list of values can be found in ***Appendix 7.***

3) Inter-assay control

In total, eleven RIA assays were run to obtain a full data set. In two cases, the standard curve did not sufficiently resemble those of previous runs so were adjusted manually to fit. In the final RIA, the standard curve was not ideal and was not easily adjusted. However, the curve had to be used as the radiotracer used in this assay had been discontinued and there were no further opportunities to repeat the samples. Overall, mean total binding value ± SD for Bo's (*n=11*) was 49.2% ±7.4, typical for this assay used in other contexts in this laboratory. Mean ± SD P4 concentrations at 20%, 50% and 80% binding were 300.3 ±30.4, 88.0 ±13.7 and 24.5 ±8.3 ng/50µL, within 10.1, 15.6 and 33.9% of each other. Lack of consistency was greatest for lower concentrations. QC values were within 18.5, 27.4, 25.6 and 19.1% of each other between assays. Full assessment of inter-assay control parameters are shown in ***Appendix 8***

4) Reproducibility between samples from the same faecal ball

The pregnenolone content of multiple test samples from the 'high' and 'low' faecal balls are shown in ***Table 32***. The data presented here was generated in the laboratory of Dr Franz Schwarzenberger at the University of Vienna. The table illustrates two points: firstly, that there is some degree of variance between samples taken from the same faecal ball, even when corrected for slight differences in mass of sample; and

secondly, that the level of variance is greater between samples where values are low (although the absolute mean difference is small). However, the 13-14% variance in samples with 'high' progestagen content is similar to the 14.12% intra-assay variation calculated above and is unlikely to be due to true differences between repeat samples. In the 'low' progestagen samples, although the variance between repeats is high, the absolute difference in content is very low.

Sample ref	Mass (g)	Pregnenolone content (ng/g faeces)	content/mass x 2
'High' sample			
1317	0.51	19318	18939
1318	0.5	14844	14844
1319	0.52	20212	19435
1320	0.5	20207	20207
Mean (+/- SD)		**18645 +/- 2569**	**18356 +/- 2399**
variance		***13.8%***	***13.1%***
'Low' sample			
1321	0.51	2229	2185
1322	0.5	1507	1507
1323	0.51	1566	1535
1324	0.49	762	778
Mean (+/- SD)		**1516 +/- 600**	**1501 +/- 575**
variance		***39.6%***	***38.3%***

Table 32: Raw data and summary of prenenolone content of multiple samples taken from two faecal balls, one each of 'high' and 'low' intrinsic P4 content.

It can be concluded, therefore, that the standard procedures of taking all test samples from the outside of the faecal ball, and allowing the small margins of error in weighing samples should not unduly influence the overall measured pattern of faecal excretion of progestagens in this study.

Cross-reactivity of SAPU anti-P4 antibody

Table 33 lists the metabolites tested against the SAPU anti-P4 antibody used in the assay, based on duplicate means.

Progestagen	**Mean reactivity of 100pg steroid solution**	**% cross-reactivity ±SD* (variance), *n=3***
Progesterone (P4)	113.34	100 ± 6.23 (6.2%)
5α-pregnan-3,20-dione	34.36	30.3 ± 3.36 (11.1%)
5β-pregnan-3,20-dione	42.26	37.3 ± 7.73 (20.7%)
5α-pregnan-3β-ol-20-one	7.19	6.3 ± 2.73 (43.3%)
5β-pregnan-3α-ol-20-one	15.45	13.6 ± 1.15 (8.5%)
5β-pregnan-3β-ol-20-one	12.89	11.4 ± 1.00 (8.8%)
5α-pregnan-3β,20α-diol	9.21	8.1 ± 0.20 (2.5%)
5β-pregnan-3α,20β-diol	0	0.00
5α-pregnan-3β,20β-diol	0	0.00
Pregnenolone	8.08	7.1 ± 2.20 (31.0%)

*normalised to progesterone (P4)

Table 33: Relative cross-reactivity of the SAPU antibody against various progestagens, normalised to P4.

The reactivity of the SAPU antibody against each progestagen was normalised to progesterone as it is the primary target for the antibody, having been raised in rabbits against 11α- progesterone-glucoronide-BSA. The value for progesterone itself should be 100 as only 100pg was available for the antibody to react with. This inconsistency is an indication of the inherent variation in this assay, and indicates that there is some degree of error in the % cross-reactivity values obtained here. However, each progestagen should be similarly affected by any error, making the normalised values likely to reflect the true cross-reactivity of this antibody. Clearly, there is greatest variance in the values for 5β-pregnan-3,20-dione (20.7% variance), 5α-pregnan-3β-ol-20-one (43.3% variance) and pregnenolone (31.0% variance).

The results indicate that, in addition to progesterone itself, the antibody is detecting the pregnanediones in significant quantities, and to a lesser extent the pregnanolones. It is also picking up 20α-pregnandiol but not the 20β isotypes, and also pregnenolone to a similarly small extent. This cross-reactivity profile can be explained in terms of

the molecular structure of the major metabolite groups, shown in ***Figure 66***, taken and modified from (Schwarzenberger, Palme et al. 1997).

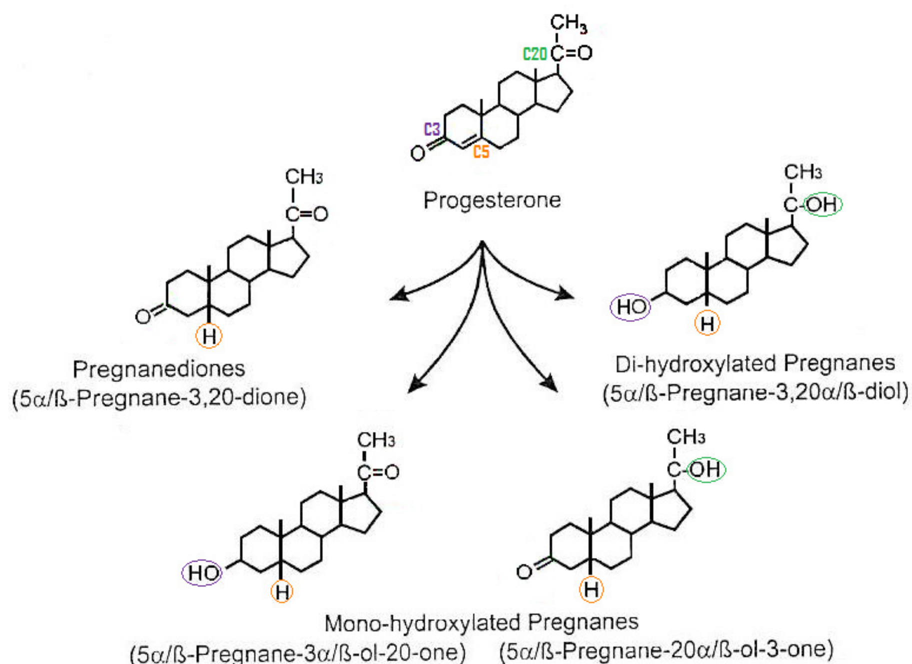


Figure 66: Major metabolites from the breakdown of progesterone, adapted from (Schwarzenberger, Palme et al. 1997)

From details of the chemical structure of the typical progestagen metabolites, it is clear that the antibody used in this assay is more likely to recognise a =O group rather than an OH at C3/C20. For example, the pregnandiones only differ from progesterone by reduction at C5, retaining the =O side-groups at C3 and C20. This would account for the high cross-reactivity of the antibody with the two pregnanediones tested. The low or absent cross-reactivity of the pregnanediols could be explained by the hydroxylation of both the C3 and C20 side-groups. The other metabolites tested have a cross-reactivity intermediate to the pregnanediones and pregnanediols, likely due to the hydroxylation of EITHER C3 or C20, but retention

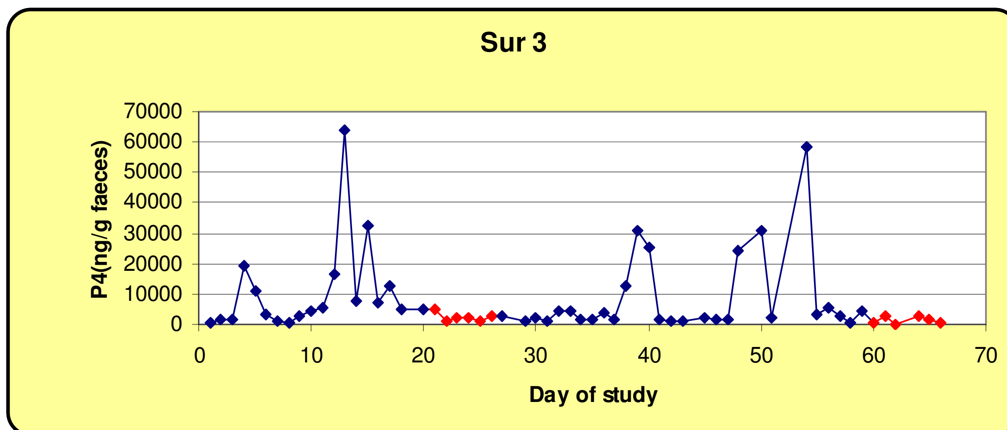
of the oxo group in the other position. Testing of the pregnane-20-ol-3-ones may have elucidated the relative importance of the C3 or C20 group structure in which is most likely to be recognised by the antibody used in this assay.

Assay comparison with Vienna protocol

As part of the assay validation, ***Figure 67*** shows the correlation between faecal progestagen content of samples from animal Sur3 and Majene as measured in Vienna by the ELISA method of Schwarzenberger (Berger, Leus et al. 2006) and the RIA used in Edinburgh. Progestagen levels are presented in ng/g faeces rather than pg/50μL extract to allow direct comparison with the Vienna assay. This required the application of a x9 factor to account for the combined effects of extracting 0.5g faeces into 4.5mL methanol, x50 dilution of raw extract during the assay and conversion of units from pg to ng. Note that the Vienna assay was also used to measure progestagens in a pre-pubertal male babirusa, giving a concentration of 771 ng/g faeces.

Comparison for female Sur3

All 59 samples from female Sur3 were analysed using both the progesterone RIA method in Edinburgh and the pregnanedione ELISA assay in Vienna, as illustrated in ***Figure 67***. The upper graph shows the Edinburgh results with estrus signs indicated in red. The lower graph shows the direct comparison of progestagen concentrations measured using each assay. Note that the Edinburgh assay values are x10 for the comparison to allow the correlation of data points to be more clearly seen.



Note: red = estrus signs

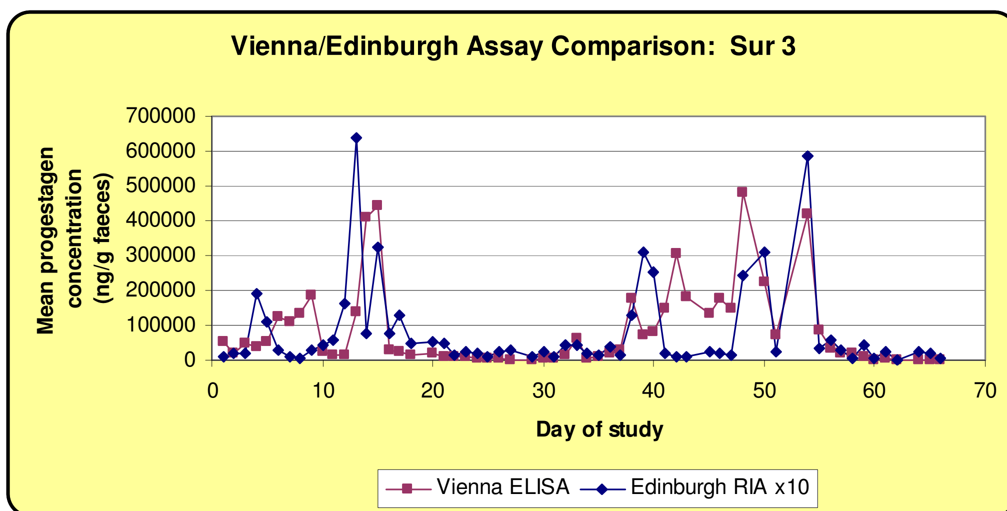


Figure 67: The upper graph shows the combined progestagen profile and timing of estrus signs in female Sur3. The lower graph compares the profiles obtained using the RIA in Edinburgh with the EIA (ELISA) in Vienna.

The correlation between samples appears reasonably good, with obvious luteal and non-luteal phases in both cases at the same time. The exception to this would be around days 40-47 where the Vienna assay is picking up metabolites not identified using the Edinburgh method. In addition, the Vienna assay is measuring ~x10 the quantity of metabolites making it a much more sensitive assay. Despite these anomalies, analysis of paired data points (*n=59*) confirmed the correlation was statistically significant with a Pearson correlation value of 0.506 and 2-tailed

significance beyond the 0.001 level, although this may be due to the larger number of lower values compared to higher ones.

Comparison for female Majene

Similarly, 310 samples collected for female Majene (T17, Southlakes Wild Animal Park) were analysed with both assays and the results are presented in ***Figure 68***.

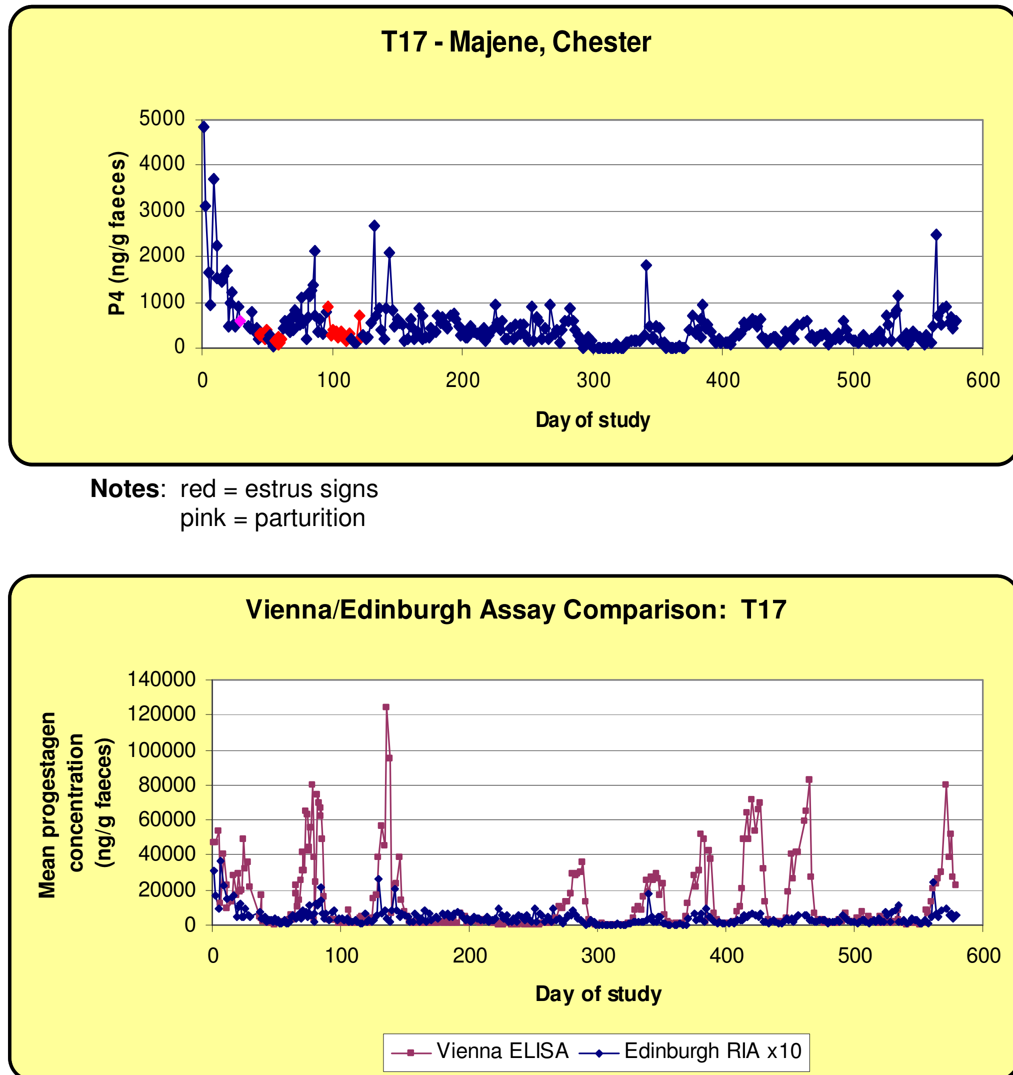


Figure 68: The upper graph shows the combined progestagen profile and timing of parturition and estrus signs in female Majene. The lower graph compares the profiles obtained using the RIA in Edinburgh with the EIA (ELISA) in Vienna.

With this data series, it is difficult to directly see the correlation between the two sets of data points in the lower chart due to low relative concentrations obtained in the Edinburgh assay, despite the x10 factor having been applied. However, analysis of data pairs revealed a significant correlation, with a Pearson correlation value of 0.340, and 2-tailed significance beyond the 0.001 level.

Comparison of females Sur3 and Majene

Overall, there are obvious differences in the level of progestagens measured between the two assays, and also between the two females. A summary of the values obtained for both females in both assays is presented in ***Figure 69***.

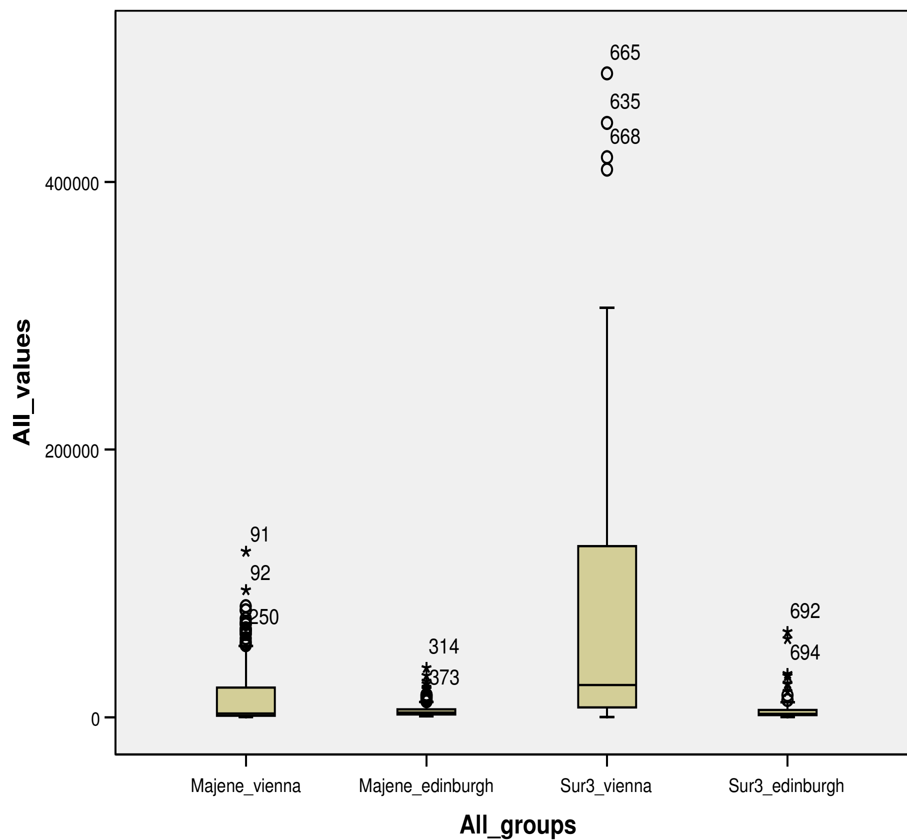


Figure 69: Comparison of progestagen values obtained for two different females (Majene and Sur3) using two different assays (Edinburgh RIA and Vienna EIA).

The comparison confirms that the higher levels of progestagens detected in the Edinburgh assay are also seen using the Vienna assay. This is suggestive the differences between females are 'real' but cannot be confirmatory that these differences are physiological rather than an artefact of the extraction or storage process. Even if they are physiological, they may reflect differential metabolite production of progesterone breakdown, perhaps the result of differing gut flora in these animals living on different continents.

Comparison of assay sensitivity

From the two data sets investigated above, it is clear that the Vienna assay is much more sensitive than the RIA conducted in Edinburgh. Minimum values were comparable with both assays but there were some samples with undetectable levels using the Edinburgh assay (*n=18*) where the Vienna assay was able to quantify them.

From a simple look at the maximum values measured by both assays for both data sets (in ng/g faeces), the Edinburgh assay detected a maximum value 29.8% that of the Vienna assay (36835 compared to 123755 ng/g) in Majene, and only 13.3% in Sur3 (63750 compared to 481125 ng/g). Although the maximum values in each case are not from the same sample, this crude analysis indicates that the Vienna assay has a greater ability to detect progesterone metabolites than has the Edinburgh assay, particularly at the higher end of the scale. It was already noted from the parallelism that the upper end of the detection scale was not as effective as it might be but this is not sufficient to explain the huge difference in values between the assays. It is more likely explained by the relative cross-reactivity of each antibody, as described in ***Table 33*** combined with the proportions of each metabolite excreted in the babirusa.

Comparison of assay specificity

As previously described, the antibody used in the Edinburgh assay is preferentially picking up progesterone and metabolites retaining the oxo-group at the C3 and/or C20 positions. This is in comparison with the antibody used in the Vienna assay (raised against 20α-hydroxypregnane) which it more likely to pick up metabolites with a hydroxylated C20 group (further analysed below). The different sensitivity of the two antibodies could be the result of higher levels of metabolites bearing the hydroxylated C20 group compared to the original oxo-group of progesterone. This is supported also by the findings of Berger where detection of progestagens was 100x higher using the antibody against the 20α-hydroxypregnanes than the 20-oxo-pregnanes (Berger, Leus et al. 2006). This is further evidence that hydroxylation of the C20 group is a major point of progesterone degradation in the liver and/or gut in this species.

Progesterone-specific vs Group-specific antibodies

Antibodies developed for diagnostic purposes tend to be very specific to the target molecule of interest, minimising cross-reactivity with related molecules. This is likely to be the case for off-the-shelf assays such as that used by the St Louis Zoo. By contrast, the two antibodies used in the Vienna laboratory are described as 'group-specific', designed to discriminate only on the basis of the C20 side-group (see ***Figure 70,*** adapted from (Schwarzenberger, Son et al. 1996), so increasing their potential for cross-reactivity with related molecules.

The two antibodies are specific to the C20 group configuration, directed against the 20-oxo-pregnanes (raised in rabbits against 5α-pregnane-3β-ol-20-one 3HS:BSA) or

20α-hydroxypregnanes (raised in rabbits against 5β-pregnane-3α,20α-diol 3HS:BSA). Because the antigenic group (the HS:BSA) is attached to the target molecule at the C3 end, antibodies will not 'see' the group structures found here, resulting in antibodies only specific to the C20 position. By comparison, progesterone-specific antibodies are produced by attaching the antigenic group around the C11 position, exposing all three groups specific to the progesterone molecule to antibody formation (C3, C4 and C20).

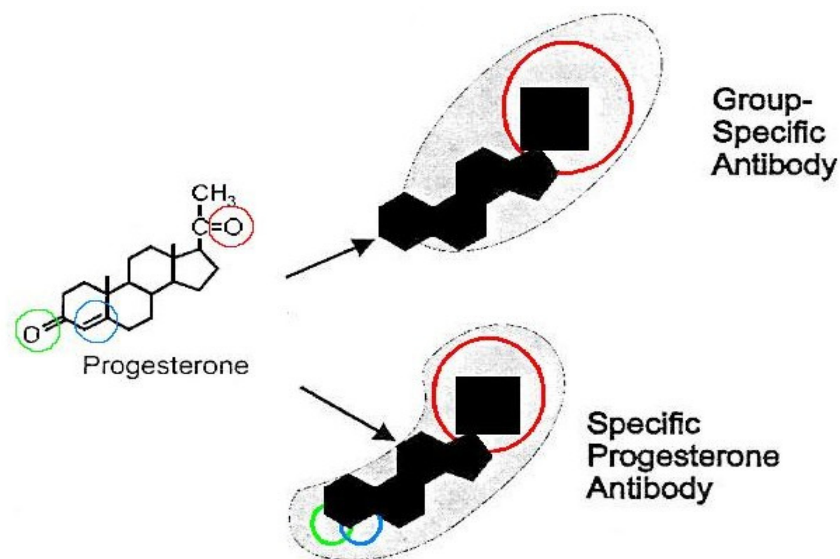


Figure 70: The arrows indicate the position of attachment of the antigenic group (the HS:BSA) and shows which group structures are then available for the antibodies to 'see'. Taken from (Schwarzenberger, Son et al. 1996).

The 20-oxo-pregnane antibody is reported to cross-react with 5α-pregnane-3, 20-dione (168%), Progesterone (100%), 5α-pregane-3α-ol-20-one (89%), 5β-pregane-3α-ol-20-one (88%) and 5α-pregnane-3β-ol-20-one (56%) (Schwarzenberger, Palme et al. 1997). The profile of the other is not reported but would be predicted to preferentially detect the pregnane-20-ol-3-ones and of course the pregnanediols, less

so progesterone itself, the pregnanediones or the pregnane-3-ol-20-ones i.e. those with the =O configuration at C20.

Using the same nomenclature, the Edinburgh antibody could be considered more of a progesterone-specific antibody as progesterone itself is the primary target of its reactivity. However, the significant cross-reactivity with progestagens retaining the 20-oxo group makes it more similar to the 20-oxo-pregnane AB of Vienna than the 20α-OH-pregnane one.

Which assay is best?

Despite the lack of sensitivity of the Edinburgh assay compared to the one used in Vienna, and its relative inability to detect the pregnanediols, it is still sufficiently good to detect temporal changes in faecal progestagens such that reproductive patterns can be discerned with some certainty.

Excretion of faecal progestagens in the female Babirusa

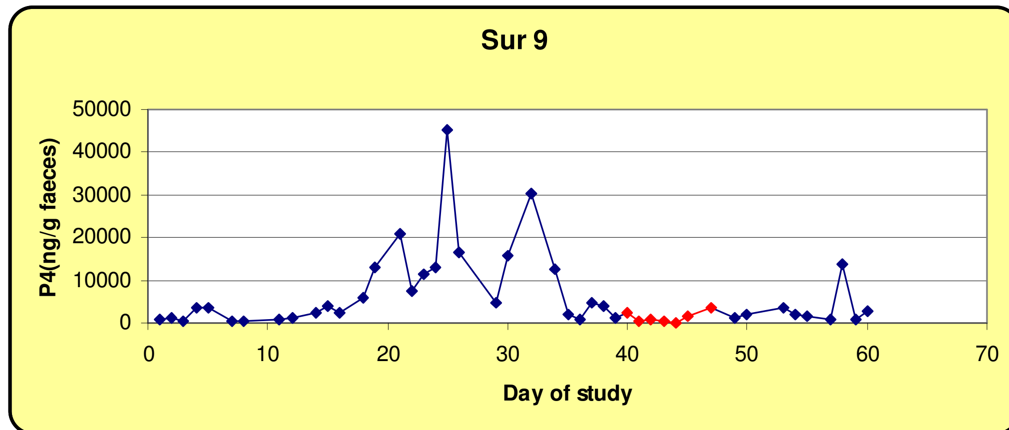
The results presented in this section are derived primarily from the RIA assay performed at the Centre for Reproductive Biology and the Queens Medical Institute in Edinburgh i.e. Indonesian ('Sur') and UK ('Kota' and 'Majene') samples. Those presented from North American zoos i.e. 'Beret' and 'Greta' are derived from steroid data kindly provided by Dr Joan Bauman of the St Louis Zoo and behavioural/husbandry details from Jeff Holland of the Los Angeles Zoo.

In all cases, data points have been plotted to correspond with the date of sample collection and no attempt has been made to account for the lag time associated with digestive transit. Points in red are associated with the recorded incidence of

behavioural or physical signs of estrus, points in pink with parturition, and in green with pre-parturient behaviours. These markers are intended to give an indication of when significant reproductive events occurred but they are not definitive due to the digestive time-lag and some difficulties in matching sample collection dates with specific events.

Progesterone levels vs estrus signs

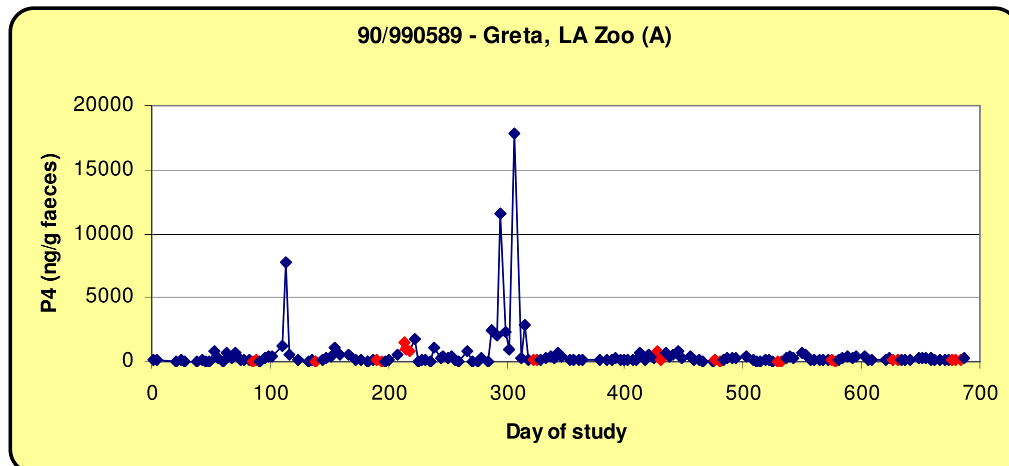
There are a number of examples of the relationship between progesterone levels and the onset of estrus (Sur 9, Sur 53, Sur 66, Sur 3, Sur 81, Kota, Greta), and full traces for each of these females is shown in ***Appendix 9***. In all cases, estrus signs begin soon after (within a week of) the drop in progesterone associated with the end of the previous luteal phase. In other words, estrus occurs during the follicular phase of the next cycle, when follicles are developing and preparing for ovulation. This allows insemination to coincide with ovulation. The progestagen trace from Sur 9 is shown in ***Figure 71*** as an example of a typical cycle.



Note: red = estrus signs

Figure 71: An example of a typical trace showing the relationship between progesterone peaks and estrus signs.

There are no good examples of long-term estrus cycling for either the Surabaya animals (too short a study time) or the two UK animals (both had pregnancies) using the Edinburgh assay. However, there is a good example from the North American female Greta (N.Am SB# 90), shown in ***Figure 72***.



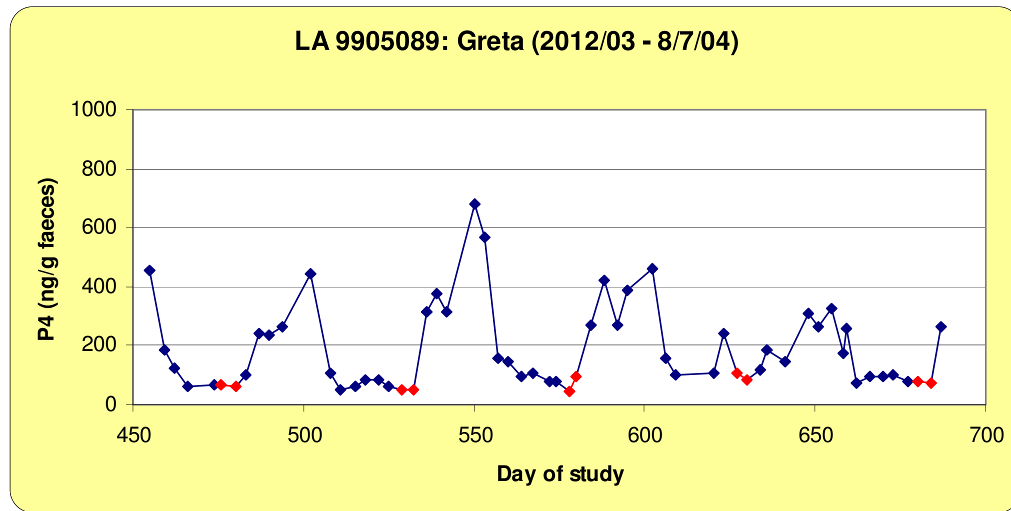
Note: red = estrus signs

Figure 72: An example of long-term monitoring of faecal progestagens in a female showing multiple, regular estrus signs.

This trace shows that Greta had eleven periods of estrus signs during the 687 day study, as recorded by her keepers. However, expected luteal increases in progesterone following estrus are not convincing. However, by simple manipulation, looking only at the second half of the data set (excluding the large peak around day 300) cycling becomes much clearer as shown in ***Figure 73***.

Not only is the cycle clearly regular but there are consistent increases in progesterone levels detected almost immediately after estrus signs, increasing baseline P4 levels ~100x. The mean estrus-estrus interval during this period is ~50 days (four cycles

over ~200 days which is somewhat longer that the expected maximum of 42 days (Macdonald 1993; Macdonald 2000).



Note: red = estrus signs

Figure 73: A more detailed examination of the relationship between P4 levels and estrus signs over multiple estrous cycles. The estrus signs are seem immediately before the increase in P4 levels, clearly illustrating the transition from late follicular to luteal phase of the cycle.

Without this kind of manipulation, the data presented in ***Figure 72*** may have been dismissed as poor quality and not very informative. Without information about estrus signs, it may not have been obvious to do the manipulation at all and the data could have been unduly discarded.

Estrous cycle length

The reported estrus cycle length in the domestic pig (*Sus scrofa*) and a number of wild Suidae is given in ***Table 34***, derived from the full table in ***Appendix 5***. From the data available, it is clear that the Babirusa has the longest cycle length, from 28-42 days, almost double of any *Sus scrofa* or the Pygmy Hog, and overlapping slightly with the suspected cycle length of the Red River Hog.

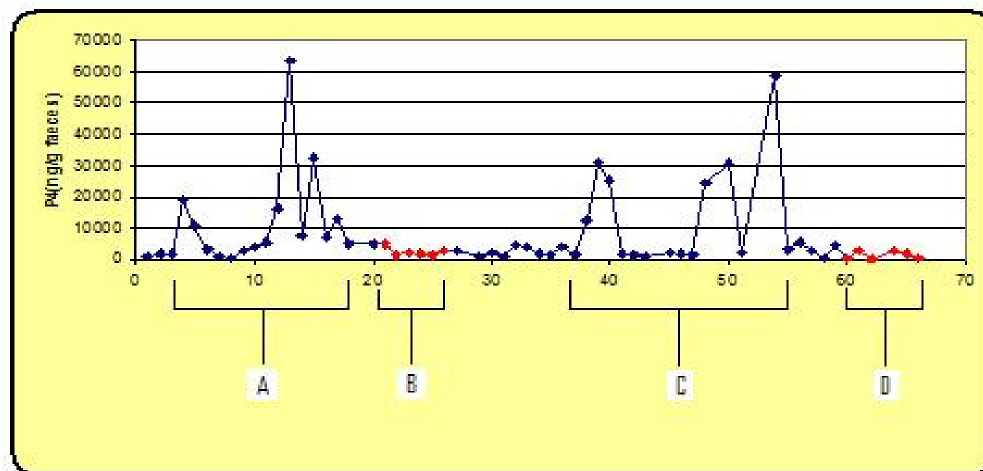
Species	Common name	Length of cycle (days)
Sus scrofa	**European Wild Pig**	21-23
	Asian Wild Pig	21-23
	Domestic Pig	19-21
Sus salvanius	**Pygmy Hog**	21
Potamochoerus porcus	**Red River Hog**	~30
Babyrousa celebensis	**Babirusa**	28-42

Table 34: Typical estrous cycle length in a selection of pig species

It is also interesting to note that in the domestic pig there is a relatively short follicular phase in relation to the whole cycle length (5 days out of total cycle length of 21 days) (Johnson and Everitt 2000). This is due to the active recruitment of follicles that still occurs during the luteal phase of the cycle, supported by sufficient baseline levels of FSH, as previously discussed.

Calculating cycle length

Estrous cycle length (from the onset of estrus signs/progesterone peaks) can be calculated from a number of females in this study. The tracing for Sur3 is shown in ***Figure 74*** and the calculations of phase and cycle lengths follow.



Note: red = estrus signs

Figure 74: Example trace (from Sur3) showing how various estrus cycle paramaters can be measured.

Luteal phase length calculated using increased P4 levels:
First luteal phase (A) = day 3 → day 18 = 15 days
Second luteal phase (C) = day 37 → day 55 = 18 days

Estrus length calculated using estrus signs:
First estrus (B) = day 21 → day 26 = 5 days
Second estrus (D) = day 60 → day 66 = 6 days

Estrous cycle length calculated using increases in P4 levels:
Start of A → start of C = day 3 → day 37 = 34 days
End of A → end of C = day 18 → day 55 = 37 days

Estrous cycle length calculated using estrus signs:
Start of B → start of D = day 21 → day 60 = 39 days
End of B → end of D = day 26 → day 66 = 40 days

Follicular phase length calculated using subsequent increases in P4 levels:
End of A → start of C = day 18 → day 37 = 19 days

In this case, it is relatively easy to see distinctive luteal and non-luteal phases and to estimate the cut-offs for each. However, in the other cases this is not so easy to do as phases may not be so easily distinguished or the sampling time is not sufficiently long to catch successive cycles. ***Table 35*** gives a summary of values obtained in this study for estrous cycle parameters, derived from full traces shown in ***Appendix 9***.

Note that samples were not always collected on consecutive days so these calculations can only be estimates. However, they suggest that cycle length in the females studied here is at the higher end of the range published for this species (Macdonald 2000).

Animal	Luteal phase length* (days)	Estrus length† (days)	Full cycle length‡ (days)
Sur 9	18 (days 18-35)	8 (days 40-47)	39 (days 18 & 57)[b]
Sur 53	19 (days 25-43)	5 (days 46-50)	-
Sur 59	-	-	-
Sur 66	19 (days 11-29)	5 (days 34-38)	42 (days 4 & 46)[b]
Sur 86	-	7 (days 48-54)	-
Sur 1	-	-	-
Sur 20	15 (days 25-39)	5 (days 4-8)	-
Sur 3	18 (days 3-20) 18 (days 38-55)	6 (days 21-26) 7 (days 60-66)	39 (days 21 & 60)[a] 35 (days 3 & 38)[b]
Sur 81	17 (days 38-54)	5 (days 16-20) 6 (days 56-61)	40 (days 16 & 56)[a]
Mean	**17.7 days**	**6.0 days**	**39.0 days**

*Measured from first to last day of P4 peaks, inclusive
†Measured from first to last day of estrus signs, inclusive
‡Measured from start of one estrus period to the next[a] or start of P_4 peaks to start of next peaks[b]

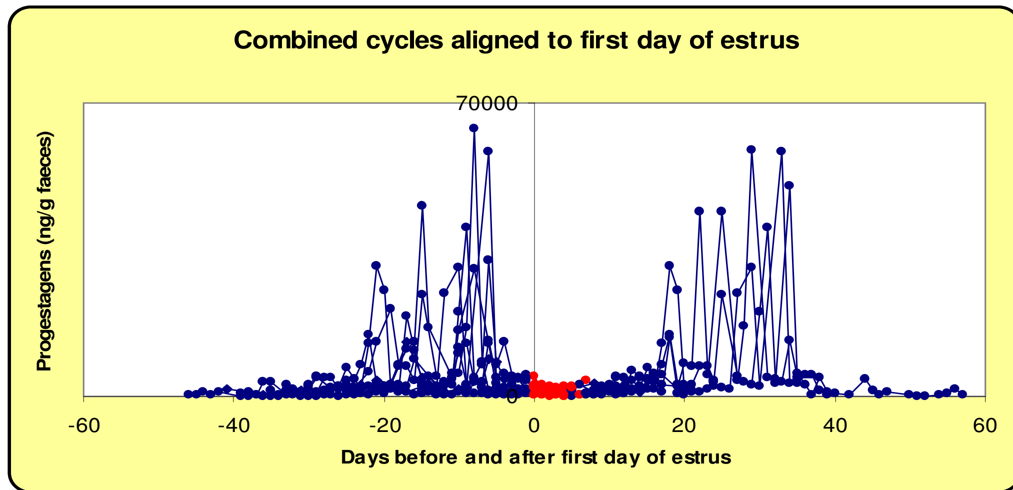
Table 35: Summary of estrus cycle parameters measured from Surabaya females.

What is interesting is the seemingly long follicular phase compared to the domestic pig. This analysis suggests that the follicular phase (non-luteal) accounts for 21.3 days of the 39.0 day cycle (55% of the cycle) compared to 5 days of the 21 days cycle (24%) in the domestic pig (Johnson and Everitt 2000). Does this mean that follicular development takes longer in the babirusa, or could it indicate that the follicular and luteal phases do not 'overlap' as is seen in *Sus scrofa* and other domestic species? Separation between follicular and luteal phases is seen in humans and other primates where CL-derived estradiol suppresses the gonadotrophins required for follicular development during the luteal phase (Baird et al., 1975).

Summary of estrous cycle

To get an overview of the faecal progestagen changes associated with the estrous cycle in the babirusa, multiple cycles were aligned by first day of estrous signs and plotted together. All nine estrus cycles were from Surabaya females, five individuals

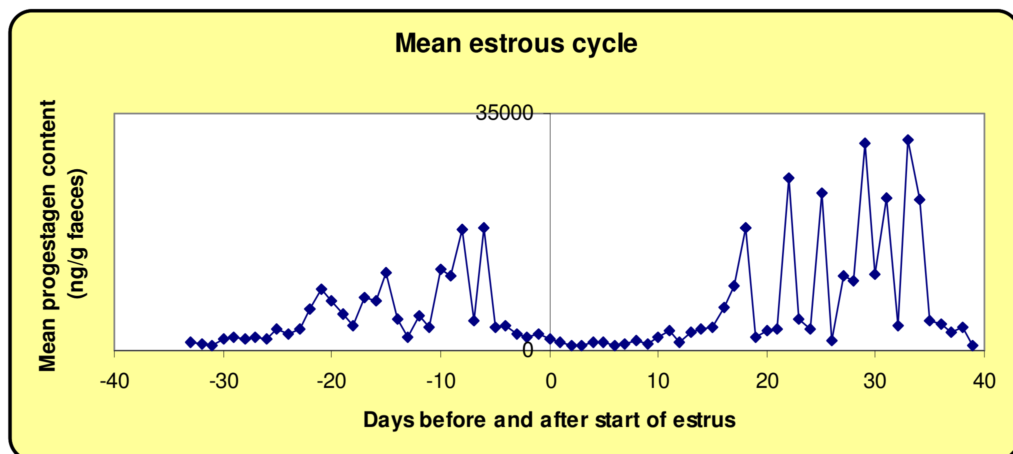
with a single cycle (Sur 53, 66, 9, 20 and 86) and two contributing two cycles each (Sur 3 and 81). The results are shown in ***Figure 75***.



Note: red = estrus signs

Figure 75: Chart showing nine estrus cycles combined from seven Surabaya females and aligned around the first day of estrus signs

When the aligned traces are combined for the nine cycles shown above, the overall trace gives mean values over roughly two cycles, as illustrated in ***Figure 76***.



Note: the values before day -33 and after day 40 have been removed as they have only one or two data points available.

Figure 76**:** Chart showing mean progestagen levels for nine combined estrus cycles from the Surabaya babirusa females.

The mean trace pre-estrus shows raised progestagen levels between days -23 and -5, giving a luteal phase length of ~18 days. There is a noticeable drop in progestagens between day -5 and the first day of estrus, indicating the end of the previous luteal phase and the development of new pre-ovulatory follicles. The mean trace post-estrus shows a typical interval of ~15 days until an increase in progestagens is seen. Combining this with the pre-estrus 5 days of low progestagens, this suggests a total follicular phase length of ~20 days. The second luteal phase lasts for ~30 days until day 35 when values drop again to near baseline. Overall, this suggests a mean estrous cycle period of ~40 days in the seven females sampled from the Surabaya population when calculated from the end of the first luteal phase (day -5) to the end of the second (day 35).

Typical progestagen values: luteal vs non-luteal

From the original study of Berger *et al.* (Berger, Leus et al. 2006) the Babirusa was characterised during luteal and non-luteal phases of the estrous cycle using the two different anti-progestagen antibodies. The cut-off values for luteal phase were 5000ng/g faeces for the 20α-OH-pregnanes and 270ng/g faeces for the 20-oxo-pregnanes.

In this study, it is difficult to assign luteal and non-luteal phases due to multiple peaks appearing in many cases. As an estimator of luteal progestagen levels for eight suitable animals in this study, the individual values for each day sampled during the ten cycles listed in the 'Luteal phase length' column of ***Table 35*** have been combined and compared with all other (non-luteal) values obtained for the same animals. The results are summarised in ***Table 36***.

			Progestagen concentration (ng/g faeces)		
Animal id	**Phase**	**N**	**Min value**	**Max value**	**Mean ± SD**
Sur9	Luteal	13	1801	45457	15283 ± 11730
	Non-luteal	32	162	13948	2165 ± 2531
Sur53	Luteal	13	1166	12864	4791 ± 3320
	Non-luteal	21	331	3720	1616 ± 976
Sur59	Luteal	17	1159	47101	9346 ± 11546
	Non-luteal	31	426	15217	2992 ± 3502
Sur66	Luteal	16	471	12561	4583 ± 3492
	Non-luteal	34	196	4524	1355 ± 1055
Sur1	Luteal	9	1117	40169	15774 ± 14264
	Non-luteal	45	178	22548	2241 ± 3710
Sur20	Luteal	11	2917	58678	21851 ± 22398
	Non-luteal	38	82	7827	1509 ± 1857
Sur3	Luteal	31	579	63750	12724 ± 16327
	Non-luteal	28	212	5557	2349 ± 1559
Sur81	Luteal	19	2361	40411	9119 ± 9832
	Non-luteal	36	101	11544	2399 ± 2388
Combined	Luteal	129	471	63750	11188 ± 13573
	Non-luteal	265	82	22548	2084 ± 2529

Note: these were all animals from Surabaya and their metabolite levels are consistently much higher than any samples taken from the two UK animals

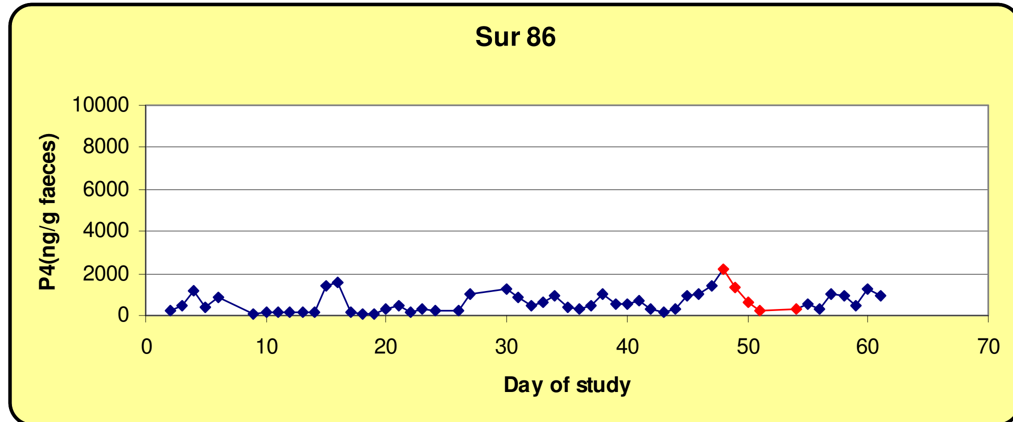
Table 36: Summary of luteal and non-luteal phase faecal progestagen content

As the data sets were not normally distributed, a non-parametric Mann-Whitney test was used to confirm significantly higher mean values during luteal (*n=129*) over non-luteal (*n=265*) phase samples, with 2-tailed significance beyond the 0.001 level. The difference in mean values for all animals combined suggests a 5.4x increase in faecal progestagens detected with this assay during the luteal phase.

Age at sexual maturity

From studbook records, the youngest age at conception for the Babirusa is recorded at 9.6 months, although this is highly atypical. Previous studies have suggested an age at puberty of 5-10 months for the female babirusa (Macdonald 1993), although this lower value seems now to be unlikely. This is compared with 10 months in the Bearded Pig, 18 months in the Forest Hog (Macdonald 1993) and 3-6 months in the

domestic pig, depending on breed (Fuller, Ford et al. 2001). It is known that is the domestic pig, attainment of puberty depends crucially on body weight (Hughes 1982) and can be induced earlier with boar exposure (Bartlett, Pain et al. 2009). The youngest female included in this study was Jane (Sur 86) and her faecal steroid profile is shown in ***Figure 77***.



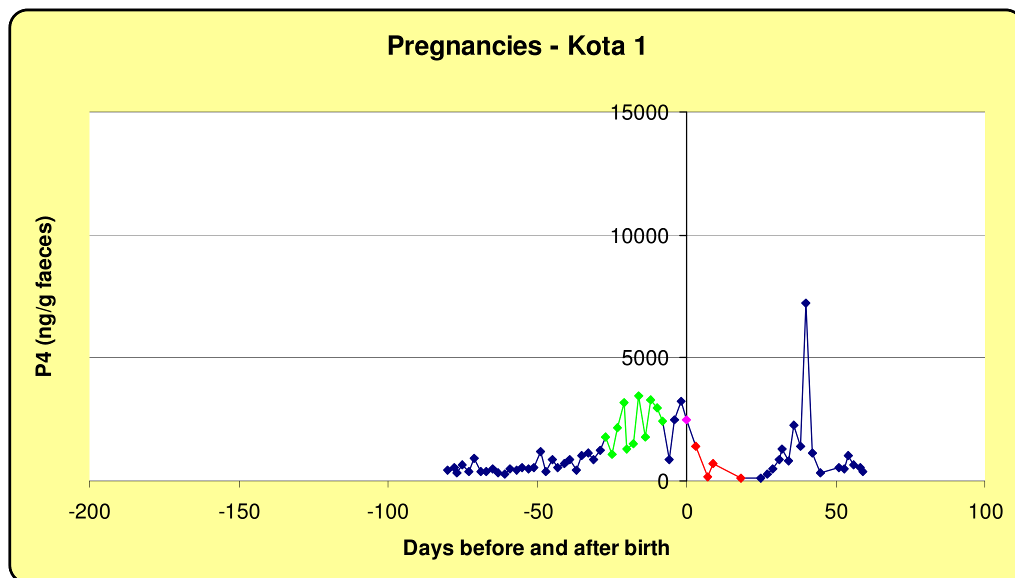
Note: red = estrus signs

Figure 77: Progestagen profile for a 2 year old female showing estrus signs but no indication of ovulation during the study period. Could this be typical around puberty?

At the age of 2 years at the start of this study, 'Jane' is showing signs of estrus (swelling and reddening of the vulva). However, her steroid profile indicates that she is not actually ovulating. Could this be normal around puberty, where estrus signs are driven by increasing estrogen produced by developing follicles, but no subsequent ovulation/luteinisation then occurs to produce progestagens. It is known that there is a high incidence of conception in females between the ages of 1-3 years, indicating that Jane should probably be ovulating. Could she have an underlying problem, or could poor nutrition be responsible for late-onset puberty? Alternatively, could this be a more typical age for puberty in this species in its home range, with animals held in other regions reaching puberty earlier due to better nutrition?

Pregnancy and post-partum estrus

There were five pregnancies followed in part or in full during this study. They show broadly similar patterns of progesterone excretion, including a sharp drop immediately after birth, but differences in actual P4 levels. Note that different raw values would be expected with the N. American animals as their steroids were quantified using a different method than those from the UK. Kota (SB# T17) was pregnant twice during this study and her progesterone profile for the first one is shown in ***Figure 78***.

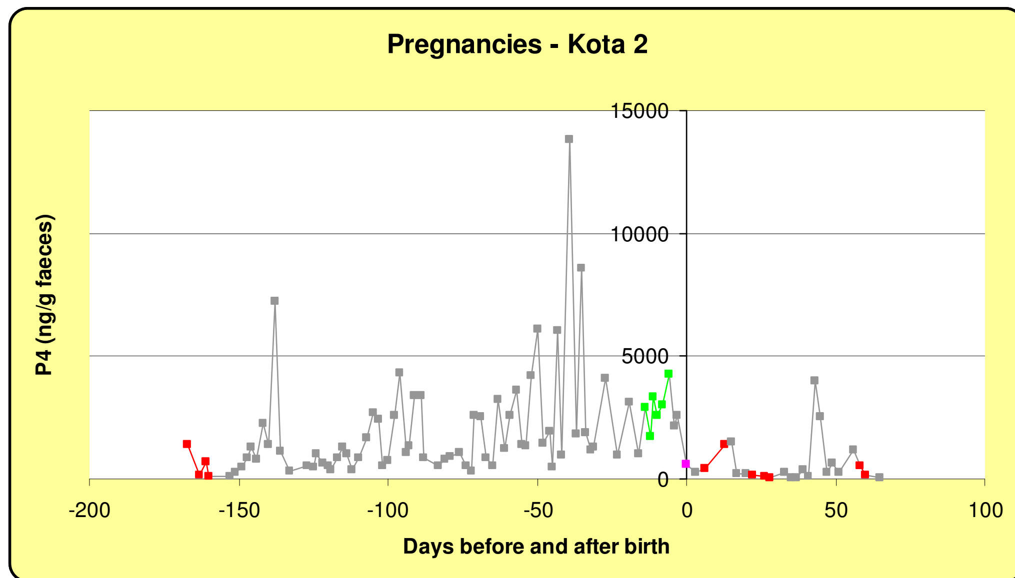


Notes: green = pre-parturient signs
pink = parturition
red = estrus signs

Figure 78: Progestagen profile for Kota (SB# T17) throughout her first pregnancy captured during this study

Increasing steroid levels are only really noticeable from ~50 days pre-partum. Green indicates pre-parturient behaviours (from day -27 to -8) such as increased aggression and nest-building. The piglet died within 48hrs (dob 3/3/07) and estrus signs are seen almost immediately. This post-partum estrus is typical in domestic pigs but is

not normally associated with ovulation and is thus not usually considered to be a fertile cycle (Anderson 2000). However, the progesterone peak around days 30-45, suggests that this was indeed a fertile cycle and that ovulation occurred. Kota's subsequent pregnancy showed a more sustained increase in P4 levels compared to the first one, to higher levels and from earlier in the pregnancy. The full progesterone profile is shown in ***Figure 79***.



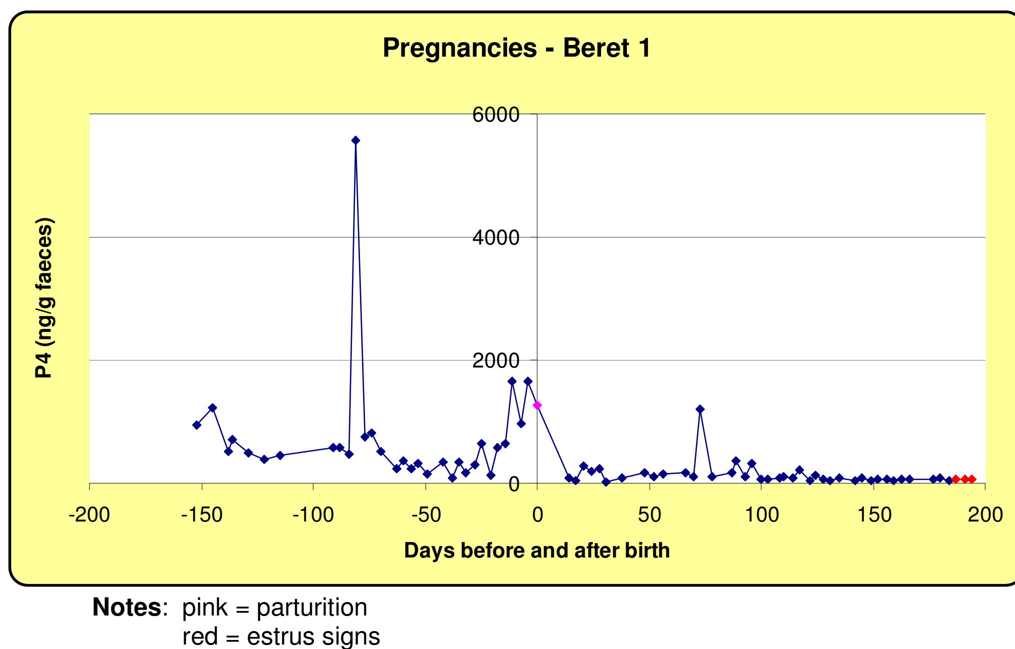
Notes: green = pre-parturient signs
pink = parturition
red = estrus signs

Figure 79: Progestagen profile for Kota (SB# T17) throughout her second pregnancy captured during this study

You can see the full pregnancy, where day -160 was the last day of estrus and fertilisation must have occurred during this cycle. Note that this was the first estrus cycle after giving birth to the previous piglet which died. Considering the short delay between estrus and fertilisation, this pregnancy was very close to the typical 158 days noted in the literature (Vercammen 1988; Macdonald 1993). Again, Kota showed clear pre-parturient signs (from days -14 to -6) which were similar but less

prolonged than the previous pregnancy. Kota gave birth to twins that were removed for hand-rearing and again she quickly showed signs of estrus after this second birth.

Because both of Kota's pregnancies resulted in death or removal of the piglet(s), it is not possible to assess the effect of lactation and weaning on fertility. However, data from one of the North American females, Beret (N. Am SB#104), allows this period to be assessed. ***Figure 80*** illustrates a full data series from Beret who successfully reared a single piglet to weaning.

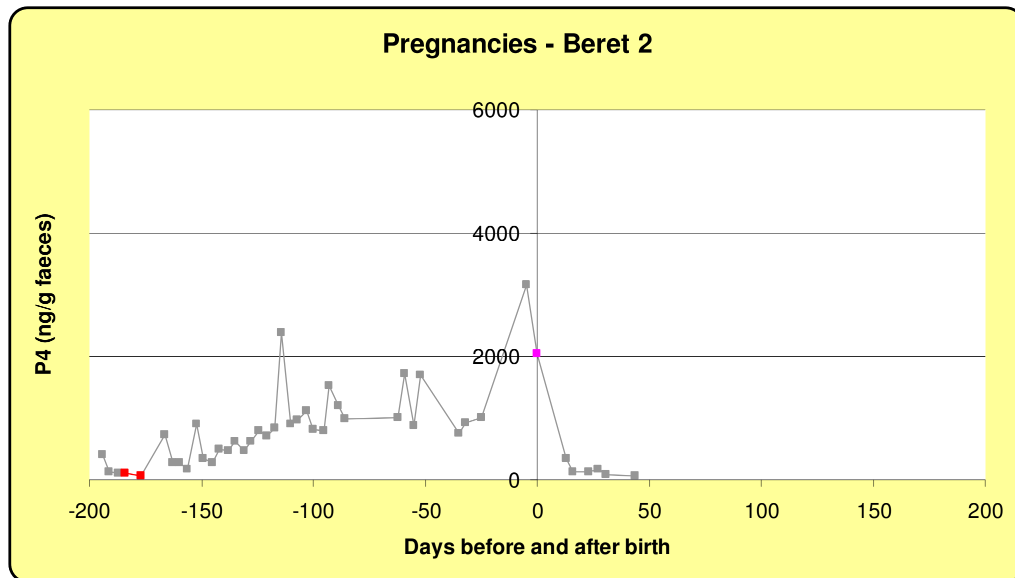


Notes: pink = parturition
red = estrus signs

Figure 80: Progestagen profile for Beret (N.Am SB# 104) during a pregnancy resulting in a single piglet that was reared to weaning around 6 months. Note that the raw P4 data for this analysis was supplied by Joan Bauman at the St Louis Zoo

There is a small sustained increase in P4 levels seen throughout the pregnancy, although it is only obviously above baseline when compared to the post-partum trace. There is a more sustained increase seen during the final month of pregnancy to parturition with the sharp drop immediately post-partum. The piglet was reared by

Beret, with consistently low progestagen levels during this time as suckling-induced prolactin levels will have suppressed milk production. No estrus signs were seen until 187 days (over 6 months) post-partum, during the period of weaning the piglet, and the second post-weaning estrus resulted in another pregnancy. This was also followed with faecal monitoring and the progesterone profile is shown in ***Figure 81***.



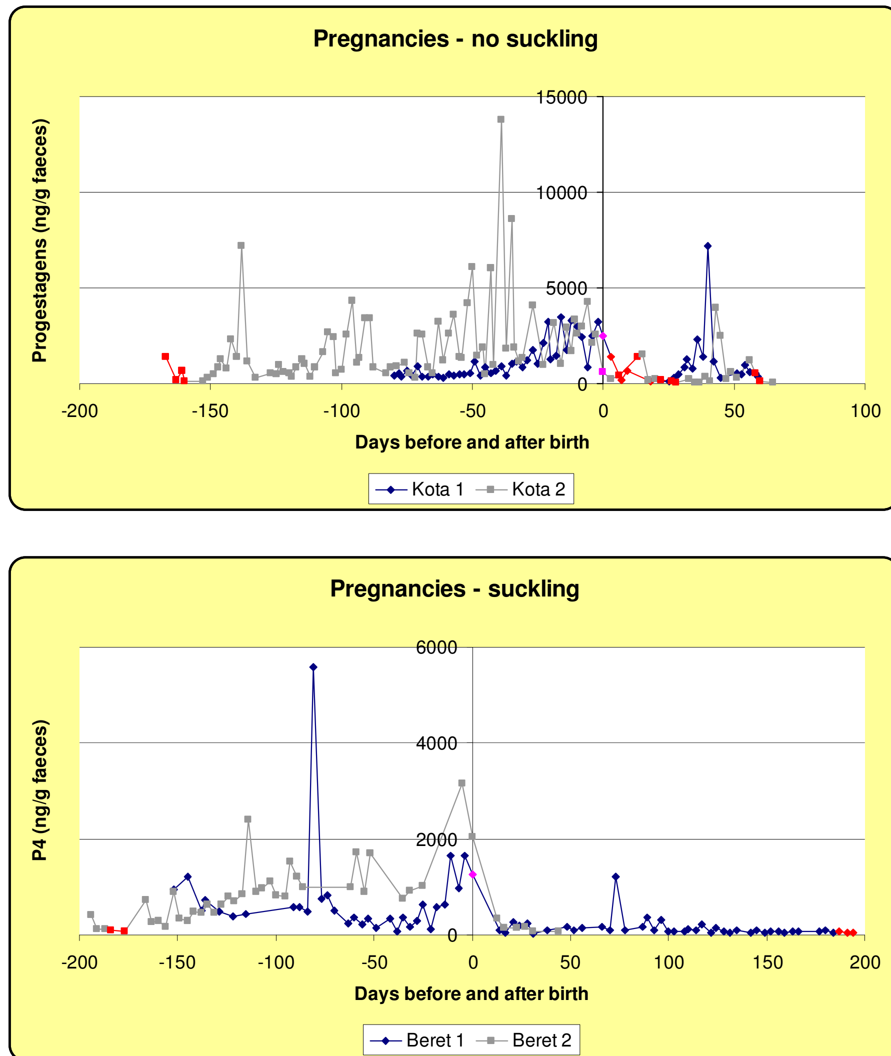
Notes: pink = parturition
red = estrus signs

Figure 81: Progestagen profile for Beret (N.Am SB# 104) during a second pregnancy resulting in a single piglet. Note that the raw P4 data for this analysis was supplied by Joan Bauman at the St Louis Zoo

In her second pregnancy, Beret shows more obvious sustained increase in P4 levels from early in the pregnancy. The estrus signs shown from day –184 to day –177 represent the cycle during which she must have become pregnant, making the full pregnancy slightly longer than 158 days. Is it possible that fertilisation actually took place during a subsequent cycle, around day -160 (where this is a clear dip in P_4 levels indicating possible estrus) but estrus signs were missed? If so, this would be a particularly short estrus-estrus interval at ~20 days.

Influence of suckling on return to estrus time

When the pregnancy traces are combined for Kota and Beret, it is easier to see the effect of removal of piglets post-partum compared to normal suckling and rearing of the piglet by the mother (see ***Figure 82***).

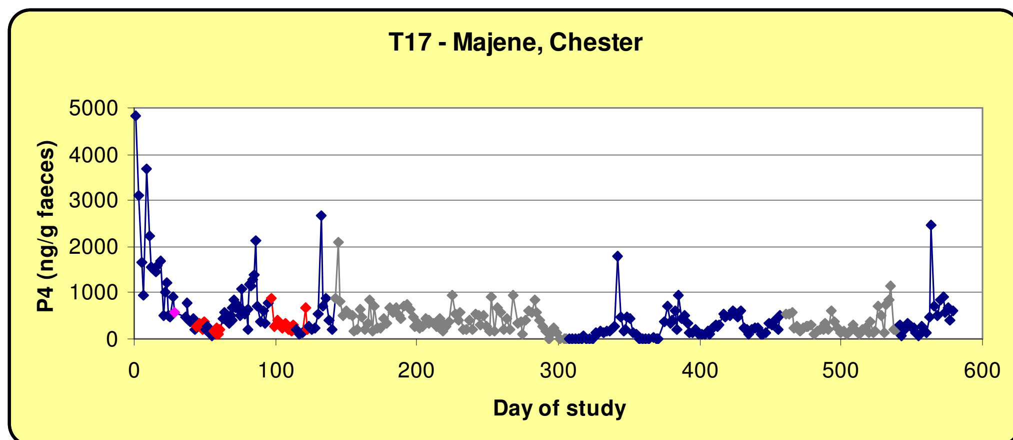


Notes: pink = parturition
red = estrus signs

Figure 82: Comparison of the effect of piglet loss/removal with natural rearing and weaning on progesterone profiles. The upper trace shows two examples where the piglets were killed or removed, and post-partum estrus occurs very quickly. The lower trace shows that when the piglet is parent-reared estrus is postponed until weaning

The importance of having a male around

In general, the relative breeding success of females in Surabaya could in some part be attributed to the close proximity of males and females. The importance of having stimulation from a male was seen clearly in the tracing for Majene when her long-term mate died and was not replaced for ~ 5 months, then a second male survived only 5 months and was not replaced for a further ~ 3 months. The impact this had on estrous cycling is shown in ***Figure 83***.



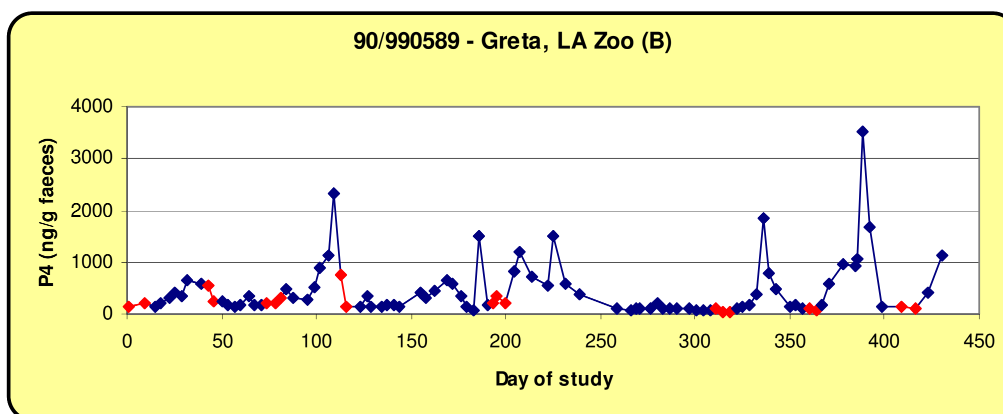
Notes: pink = parturition
red = estrus signs
grey = no male present

Figure 83: Progesterone profile for Majene (SB# T17) comparing estrus cycling when a male is present (grey) and when he is absent (blue)

During the times when a male is not present (grey), there were no estrus signs recorded by keepers, there is a distinct 'fuzziness' about the progestagen profile and a regular cycle is not obvious. It is interesting that between these two periods, when the second male was present, the profile seems to become more obviously cyclical although there are still no estrus signs or progesterone peaks suggesting ovulation has occurred. The lack of luteal activity in these periods is even more obvious from the results of the Vienna assay, previously presented in ***Figure 68***.

Effects of environmental factors

Female Greta from the Los Angeles Zoo showed regular signs of estrus cycling over two study periods (~700 days and ~430 days). In each of these periods, however, there was a notable instance of long estrus-estrus interval based on behavioural observations, confirmed by progesterone assay traces showing that progestagen levels were also reduced during these periods. An example of this is illustrated in ***Figure 84*** where there are two clearly extended inter-estrus intervals as measured from the start of one cycle to start of the next: one between days 113 and 193 (80 day interval, with a seemingly long delay before a rise in progestagens); and a second between days 193 and 311 (118 day interval, with an immediate post-estrus rise in progestagens followed by a long follicular period).



Note: red = estrus signs

Figure 84: Progesterone profile for Greta (N.Am SB#90) showing the effects of environmental disturbance (days 113 to 308) on estrus-estrus interval

During the first anoestrus period a new animal was introduced next door to the babirusa enclosure, a dwarf buffalo also native to Sulawesi. In addition, Greta had her feet trimmed and there was work going on in the exhibit and on the nearby golf course. During the second anoestrus period, Greta was being crate-trained in

preparation for moving to another collection. In conjunction with absent estrous cycling, she was also showing atypical behaviours at this time, suggesting that environmental factors were having an effect on both internal and external reproductive function. These examples provide evidence for the link between 'stress' associated with husbandry practices and/or environmental factors and reduced cyclicity in this species, although no stress-related markers were actually measured. A previous study also identified 'stress' as a possible cause of reduced fertility in a female Babirusa at the Antwerp Zoo (Chaudhuri, Carrasco et al. 1990). At least in Greta's case, estrus cycling and fertility resumed as normal after stressors were modified or removed.

Is there a Babirusa menopause?

Births have been known in the zoo environment to Babirusa females up to the age of 17 years, as highlighted in **Chapter 2**, yet some young animals currently in the EEP have been failing to breed despite being considerably younger than this. The oldest animal included in this study was Sur 9, 'Kunti' who was ~ 12yrs at the start of the study. As shown previously in ***Figure 71***, she was still having seemingly 'normal', regular cycles with estrus signs and a clear sustained period of elevated P4 during the luteal phase. There were no signs that she was in any way becoming reproductively sessile, although no further pregnancies are noted in the studbook for her after the study period. In addition, one of the original EEP females and a highly prolific one (from **Chapter 2**), SB#8 was found to have active corpora lutea on her ovaries at post mortem, despite being ~22yrs of age! (Ziehmer, Ogle et al. 2009). However, she is almost certainly an exception and two other females aged ~11 and 16 yrs (SB#s 48 and 14) had no CLs present.

Anomalies related to reproductive performance

There were a number of instances where progestagen traces do not follow the typical pattern as described previously, and these are described below.

P4 peaks but no estrus signs

Despite showing some elevated P4 peaks, Pahing (Sur 59) shows no clear distinction between luteal and non-luteal phases, as illustrated in ***Figure 85***.

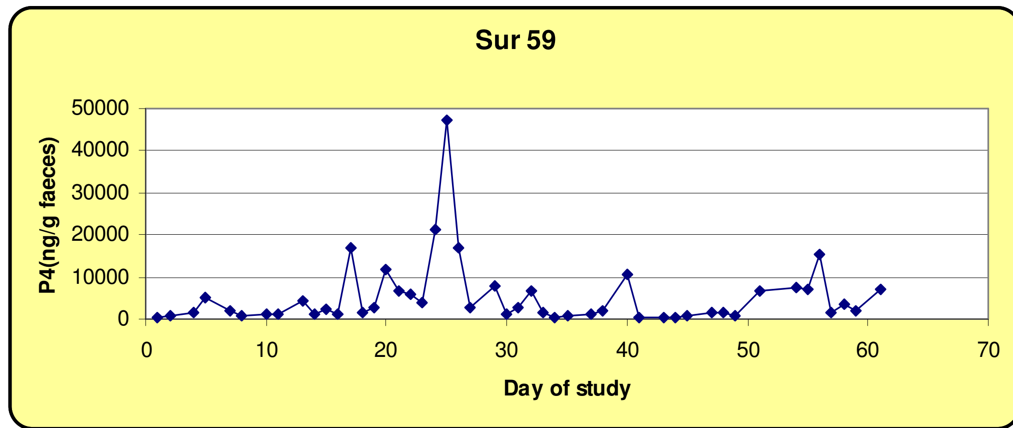


Figure 85: Progestagen profile of a female with no clear evidence of luteal activity and no estrus signs

Could this lack of sustained P4 be the reason she has not produced any piglets? She was 4yrs 5months old at the start of the study, prime reproductive age for the species. Equally, a lack of estrus signs suggests an estrogen insufficiency – is there something fundamentally wrong with Pahing's ability to produce the necessary steroids?

Similar to Pahing, Suci (Sur1, ***Figure 86***) has shown no estrus signs during the study. She has, however, shown the ability to produce sustained peaks of P4 in similar concentrations to the other Surabaya animals. It might have been suggested that at

~9 yrs old at the start of the study, Suci was past her prime breeding age but she finally gave birth to a single piglet not long after this study, on 15th December 2005.

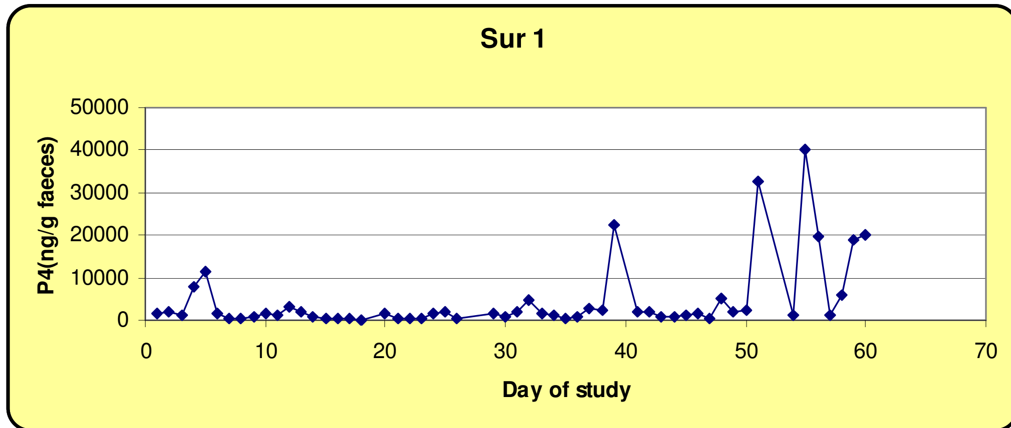
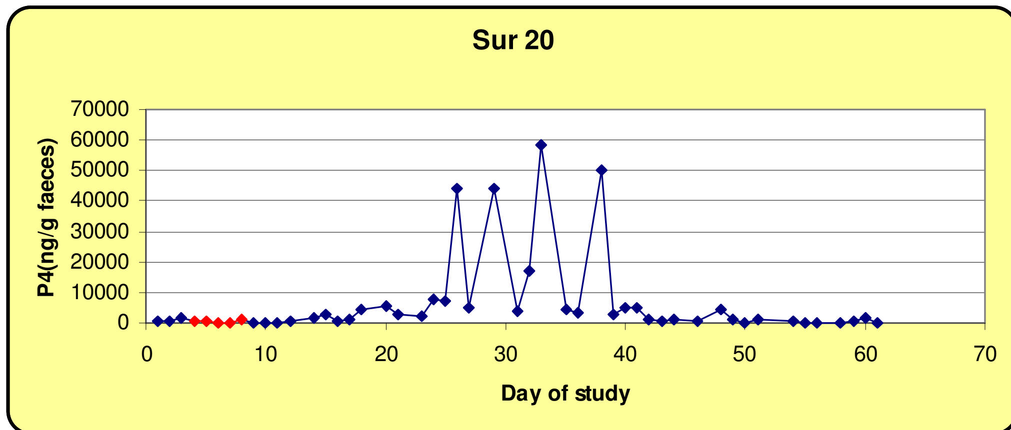


Figure 86: Progestagen profile of an older female showing no estrus signs

Unlike Pahing, Lala (Sur 20) shows very clear distinction between luteal and non-luteal phases, with sustained P4 elevation over ~ 16 days, as shown in ***Figure 87***.



Note: red = estrus signs

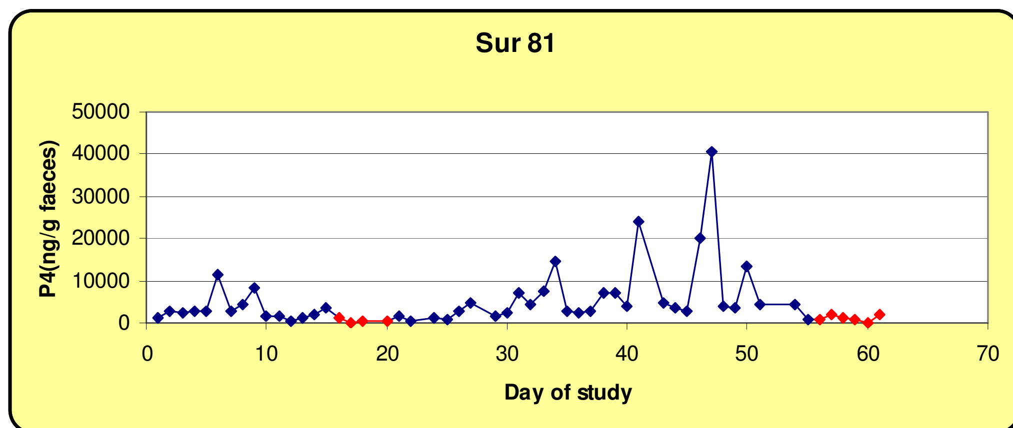
Figure 87: Progestagen profile for a female showing estrus signs before but not after a clear luteal period

It is surprising then that she showed no signs of estrus as expected from around day 40, especially considering her 'normal' signs in the previous cycle. Could the signs

have been missed or not recorded for some reason? Or could this be an example of a 'silent heat' where follicular estrogens are sufficient to induce the LH surge and ovulation but insufficient to stimulate estrus behaviours? Lala is certainly a successful breeder with two single births before and one after this study.

Small or no progestagen peaks followed by 'normal' estrus

Sari (Sur 81) was 3yrs 1month old at the start of the study and has not produced piglets before or since. It is not clear from her steroid profile (***Figure 88***) why she should not be successful.



Note: red = estrus signs

Figure 88: Progestagen profile for a non-breeding female in Surabaya Zoo

Sari is showing clear estrus signs for 5-6 days and shows a period of elevated P4 from days 30-50. Perhaps she is not consistently capable of maintaining the luteal phase, suggested by the lower P4 levels shown before the 1st estrus period. Alternatively, it may be that this female has simple not been mated as part of the management strategy for this population. Unfortunately, it is not possible to confirm this from the data available.

Estrus cycle synchronisation

Synchronisation of estrous can occur in species living in close proximity, including those in zoological collections. For example the four female elephants at the Vienna Zoo were synchronised to the matriarch (Weissenbock, Schwammer et al. 2009). Considering the close living quarters at the Surabaya Zoo (***Figures 62*** and ***63***), and the collection of faecal samples for many females over the same period of time, there was an opportunity to investigate whether estrus synchronisation occurred in the babirusa. ***Table 37*** compares the nine Surabaya females in terms of when progestagen peaks were evident and when external signs of estrus were recorded. The entries have been placed in the table columns to roughly represent their position in the 66-day study period to aid visual identification of synchrony.

Pen	Animal	Days of P4 peaks, inclusive	Days of estrus signs, inclusive
B2	Sur 9	18-35 57-	40-47
A1	Sur 53	25-43	46-50
A2	Sur 59	-	-
A4	Sur 66	11-29	34-38
A9	Sur 86	-	48-54
C3	Sur 1	50-60	-
D1	Sur 20	25-39	4-8 exp 40-
D3	Sur 3	3-20 38-55	21-26 60-66
D4	Sur 81	38-54	16-20 56-61

Note: Pens are arranged clockwise from B1→ B4→ A1→ A10→C1→C3→ D1→ D8

Table 37: Comparison of peak P4 levels and days of estrus for Surabaya females to look for evidence of synchronisation

There is some suggestion that there may be synchrony between females Sur 3 (aged ~9 years) and Sur 81 (aged ~3 years), housed in neighbouring pens at the time of the study, but there is no clear evidence otherwise. Interestingly, it was noted above that despite showing clear estrus signs, Sur 81 had not produced any piglets – could this be an indication of reproductive suppression by the older female next door?

DISCUSSION

Faecal steroid analysis for monitoring progestagens in the female Babirusa has proven useful in elucidating details of estrous cycling and pregnancy, and in relating physiological changes to external signs and behaviours. This study has gone beyond the small number of previous studies in this species by recruiting larger numbers of animals (representing three of the zoo regions), and by using data from three different assay methods. The incorporation of behavioural and environmental information, mainly through keeper records and ARKS, has allowed a greater degree of interpretation than would otherwise be possible.

What can faecal progestagen analysis tell you about individuals?

Faecal samples from the nine animals from Surabaya Zoo and the two UK animals were analysed using the Edinburgh radioimmunoassay for progestagens. From the results obtained using this method, it was possible to better characterise the estrous cycle, to link estrus signs to progestagen levels, and to follow typical changes during and after pregnancy. An alternative RIA was used to measure 'progesterone' in two North American females and these results added valuable insights into the return to estrus interval with normal piglet rearing and weaning, and also the profound effects that 'stress' can have on fertility.

Estrous cycle

Data on the estrous cycle were derived mainly from the Surabaya females. This data set is particularly useful for a number of reasons. First, nine females were available for study, more than has previously been reported. Secondly, they are held in their natural region of Indonesia with appropriate climatic conditions and photoperiod.

Thirdly, animals were held in close quarters to each other, with sight, smell and sound contact with multiple males, and this is thought to be key to their relatively high breeding success (Vercammen 1988) . From these nine females, seven were showing regular estrus signs and nine cycles in total were captured during the 2-month collection period. From these cycles, mean behavioural estrus length was calculated at 6.0 days (*n=9*) and mean cycle length was 39.0 days (*n=5*), with a mean luteal phase of 17.7 days (*n=7*).

Estrus length based on external signs and behaviours has previously been reported at 1-3 days (Bowles 1986) but this is clearly shorter than 5-8 days shown by the Surabaya females in this study. The 1-3 day estimate was based on females held in the UK, so may reflect a shortened period outside of Indonesia. However, additional data from the two UK females in this study suggested that they also express signs of estrus for greater than three days (see ***Figures 78***, ***79*** and ***83***), although the story is admittedly more complex with the multiple births in these cases.

Cycle length is reported in the literature at 28 to 42 days (Macdonald 1993; Macdonald 2000). Although the upper limit is in agreement with the data presented here, and there is some variation between females, there is no evidence of animals (either Surabaya or UK) having cycles shorter than this (see ***Table 35***). In fact, one of the North American females had a mean cycle length of 50 days spaced evenly over a five month period (see ***Figure 73***).

An interesting feature of the Babirusa cycle is the balance of time given to the luteal and follicular phases. With a mean cycle length of 39.0 days and mean luteal phase length of 17.7 days (45.4% of the cycle), the inter-luteal phase (follicular phase)

takes up the remaining 21.3 days (55% of the cycle). This contrasts significantly with the balance of phases seen in the domestic pig where 5 of 21 days (24%) are counted as the follicular phase. It is not the case that the follicular phase is actually this short, rather that follicular development can occur during the previous luteal phase due to the maintenance of sufficiently high levels of circulating FSH. This is typical of domestic species, but seems not to occur in the Babirusa from the data available here. Could this be the result of low FSH levels during luteal phase, meaning follicular growth is restricted, or does follicular development take significantly longer than in the domestic pig, requiring time during the luteal phase AND the dedicated follicular phase? It would be interesting to compare with the other non-domestic pigs: could this telescoped estrus cycle be an artefact of intensive selective breeding of *Sus scrofa domestica* and other domestic species over many generations?

From the North American data, there is seen to be a clear link between cycle length and 'stressful' events in the surroundings (see ***Figure 84***), where cycling became erratic during invasive events. The importance of having male stimulation was also illustrated for female Majene whose cycling ceased during periods of being alone (see ***Figure 83***). The close proximity of many babirusa of both sexes (as seen in ***Figures 62*** and ***63***) could explain why the females in Surabaya are considered to be efficient breeders (personal communication Alastair Macdonald), with stimulation from multiple males by sight, sound and smell. On the other hand, there may also be suppression of younger females by older ones, as proposed between Sur 3 and Sur 81 (see Table 37). In general, fertility of female Babirusa would appear to be very sensitive to environmental influences.

Pregnancy

The length of gestation inferred from this study is in keeping with the 158 days cited in the literature (Macdonald 2000). Despite the different assays used to measure progestagens, the four pregnancies presented here showed changes in keeping with the domestic sow, albeit over a significantly longer gestation time. Progestagens are clearly elevated above baseline levels until the pre-partum period where they are seen to drop rapidly. The clearest changes, especially pre-partum are seen during Kota's second pregnancy (***Figure 79***) as compared with the domestic sow (***Figure 56***). It is also possible to compare the profile of a female who suckled her piglets to one who didn't, illustrating the expected return to estrus either on removal of the piglets (***Figures 78*** and ***79***) or on weaning (***Figure 80***). In both cases, the first or second estrus post-partum proved to be fertile.

Influence of which assay is used

A number of assays have been shown to be suitable to monitor reproductive cycles in the babirusa. The study of Berger used four different antibodies to assess their relative usefulness and found that two different progestagen assays were most suitable, those against the 20-oxo-pregnanes and the 20α-OH-pregnanes. The study indicated that luteal-phase 20-oxo-pregnanes were detected at 0.5-3µg/g faeces in all three species studied, the 20α-OH-pregnanes at almost 10x that value in the Warthog and Red River Hogs (at 3-10µg/g faeces), and almost 100x that in the Babirusa (at 30-200µg/g faeces). It is not known why the Babirusa should show such significantly higher levels of luteal 20α-OH-pregnanes than the other two species.

In this study, results from three different assays have been used to generate data, two progesterone-specific (the Edinburgh and North American RIAs) and one group-specific, targeting the 20α-OH-pregnanes (Vienna ELISA) as in the Berger study. The higher faecal progestagens consistently obtained in the Vienna assay confirm that the 20α-OH-pregnanes are metabolites of progesterone found in particularly high levels in the faeces of Babirusa. Whatever the differences, however, the two other assays were still sensitive enough to follow changes in faecal progestagens during estrous cycles and pregnancy.

Edinburgh RIA vs Vienna ELISA

To further investigate the differences between the Edinburgh RIA and the Vienna 20α-OH-pregnane ELISA, a direct comparison was made using two complete series' of faecal samples, one from a Surabaya female (Sur3, *n=59*), and one from a UK female (Majene, *n=310*). From this comparison, it was clear that the Vienna assay is picking up ~10 times more progestagens than the Edinburgh one (see ***Figure 67***). So although the antibody used in the Edinburgh assay can cross-react with some of the progesterone metabolites (***Table 33***) it is not as efficient in this respect as the group-specific 20α-OH-pregnane antibody use in the Vienna assay. Despite this, and the potential sources of error identified in the assay validation, the RIA used in Edinburgh was good enough to clearly follow both estrus cycling and pregnancy in females from Surabaya and the UK.

The Surabaya conundrum

The progestagen levels found in the faeces of Babirusa females from the three geographical locations were considerably different. Mean luteal levels for Surabaya

females was ~ 5-60μg/g faeces compared to ~ 0.5-3μg/g in UK females and ~ 0.2-2μg/g in the two from North America. It is perhaps not surprising that the North American results are so different as they were assayed using a different method from the others. However, the difference between Surabaya and UK females is not so easy to explain, with Surabaya levels up to ten-fold higher than those from the UK as measured by two different assays.

It was hoped that the assay comparison (between the Edinburgh RIA and Vienna assay) may shed light on the reasons for such high progestagens measured in the Surabaya females compared to those from the UK. However, the comparison simply confirmed the x10 higher metabolite levels in Surabaya females. Could this mean that the differences are truly physiological? This would seem unlikely as animals in both locations are known to be successful breeders. It would seem unlikely that the Surabaya females are producing 10x the quantity of progesterone required for otherwise normal physiological functioning. Could the gut flora in these animals be so different from those in the UK that it leads to differential breakdown of progesterone into metabolites likely to be detected in the assays used? This would result in *apparently* higher progestagen levels, but would actually be an artefact of gut metabolism creating a different metabolite profile.

Alternative explanations relate to some stage of the extraction, storage and/or transportation process that has resulted in greater removal of progesterone and/or its metabolites from the Surabaya faecal samples or has changed the balance of metabolites present, making more available to both the Edinburgh and Vienna antibodies. These all rest on the fact that the samples were collected in Surabaya and

remained in Indonesia for a considerable time until extraction in Bogor and analysis in Edinburgh. This contrasts the UK samples that were collected at regular intervals from the relevant institutions and are known to have remained frozen until extraction.

There are a number of possibilities: perhaps samples were not collected or frozen promptly on defecation, exposing samples to very high ambient temperatures and humidity which may have resulted in rapid progesterone degradation by faecal bacteria; perhaps there were multiple freeze-thaw events during storage of samples or on their transportation from one end of Java to the other (Surabaya to Bogor, near Jakarta); perhaps high ambient laboratory temperatures or differences in methanol/water quality during the extraction process in Bogor resulted in greater extraction efficiency of the protocol. Alternatively, perhaps the long delays between collection (Jan-March 2005) and extraction (April 2006) then between extraction and analysis in May 2008, a further 2 years later, resulted in an artefactual change in the balance of metabolites present in the samples.

It is difficult to come up with a definitive answer, but mass spectroscopy analysis is currently underway (personal communication, Krissy Nicols) to at least identify which metabolites are most abundant in samples collected from Surabaya and UK animals. This will then answer the question of whether the metabolite balance is different between animals from the two regions, or if there is yet another explanation.

Added value of behavioural data

Behavioural data has been crucial in fully understanding and interpreting the results of faecal steroid analysis obtained during this study. It has allowed measurement of cycle lengths using both estrus behaviours and progestagen changes, confirming the

clear link between the two. It has also been useful to pick out cycles where they may have been missed from steroid data alone. In the case of Greta, looking only at the steroid data in isolation, you would likely conclude that there is little going on, with only a couple of seemingly random peaks. However, when behavioural data was included, and the scale of the graph reduced, there was clear indication of cycling. In addition, detailed information about the husbandry and happenings in the exhibit allowed factors to be identified that had seemingly resulted in long inter-estrous periods (see previous). This kind of information is useful in assessing the impact of environmental changes on fertility, and so to inform management practices.

Added value of North American data

The data collected on the North American females illustrates a common occurrence in zoos – huge data sets collected for husbandry purposes that have never been published. It was coincidental that this data set was found to exist, and it may never have been tied to other data sets so its contribution to a better understanding would have been lost. Instead, its inclusion in this analysis has provided another dimension to the study, especially with the long-term nature of the collections, and the inclusion of multiple pregnancies. Specifically, it gave an insight into progestagen levels post-partum when the female (Greta) suckled her piglet(s) and also into the time to weaning. This compared with the Southlakes female, Kota, who had her piglets removed and she quickly returned to estrus.

Is faecal steroid analysis useful in the babirusa?

Faecal steroid analysis has proved useful in the Babirusa in this study and has clearly linked external reproductive signs with internal physiological changes. The key

contribution that this technique can make to the conservation breeding of the babirusa is in its supportive role in ensuring that genetically valuable individuals are assisted to breed, and their alleles are not irreversibly lost from the gene-pool of the *ex-situ* population. This may be through individual interventions, or through better understanding the husbandry needs of the species.

Summary

The key contributions that faecal steroid monitoring can make to management of individuals within the conservation breeding programme are:

1) to assess if a female is cycling, or perhaps exhibiting 'silent estrus', which is important with respect to whether she can be considered a potential breeder. If she is considered sub-fertile but is genetically valuable, diagnosing the reasons for this and assisting her to conceive may be possible. However, techniques such as AI or IVF require a good knowledge of the estrous cycle and ovulation in order to effectively time any interventions (Andrabi and Maxwell 2007; Durrant 2009) and faecal steroid analysis would be useful in this case.

2) to more accurately predict date of conception/birth. Sometimes the date of conception is not exactly known, making it difficult to tell when a birth is due. This can be problematic as the pre-parturient sow requires a quiet area separate from the male with enough suitable nesting materials (Burne, Murfitt et al. 2000; Burne, Murfitt et al. 2001). Without these, sows under stress may give birth and savage the piglets (Curtis, Edwards et al. 2001; Harris, Bergeron et al. 2001). Long-term monitoring of females should increase the chances of identifying the date of conception so allow ample time to prepare for the birth.

3) to better understand the effects of environmental factors such as 'stress' or the grouping of animals on female fertility. This will aid the creation of ideal housing and husbandry practices to maximise the reproductive potential of all Babirusa females of breeding age.

Muhibah's progestagen profile

Muhibah was included in the faecal steroid study conducted by Berger *et al* in Vienna (Berger, Leus et al. 2006) as previously discussed. The results showed that Muhiba was producing only very low levels of progestagens, with indistinguishable cycling pattern as in the other animals sampled (see trace (b) in ***Figure 89***). As a result, she was 'diagnosed' with luteal insufficiency.

Ultrasound scanning of Muhibah's ovaries suggested that there may be a number of corpora lutea present, although the imaging was hampered by excess body fat. In response to this finding, and to her showing behavioural signs of estrus (personal communication Darren McGarry) Muhibah was stimulated with PG600 (Intervet) to try and induce ovulation. PG600 is a hormonal treatment widely used in the domestic pig industry to induce or synchronise ovulation or reduce the return to estrus time (Breen, Rodriguez-Zas et al. 2006; Vargas, Bernardi et al. 2006), or as an adjunct to boar exposure to induce early puberty (Bartlett, Pain et al. 2009). The PG600 preparation consists of 400IU eCG (equine chorionic gonadotrophin) and 200IU hCG (human chorionic gonadotrophin) which act in a similar manner to FSH and LH, respectively. Faecal samples were collected before and after this procedure and assayed for progestagen using the Edinburgh RIA method. In short, Muhibah

showed no response, behaviourally or hormonally and she was not further investigated.

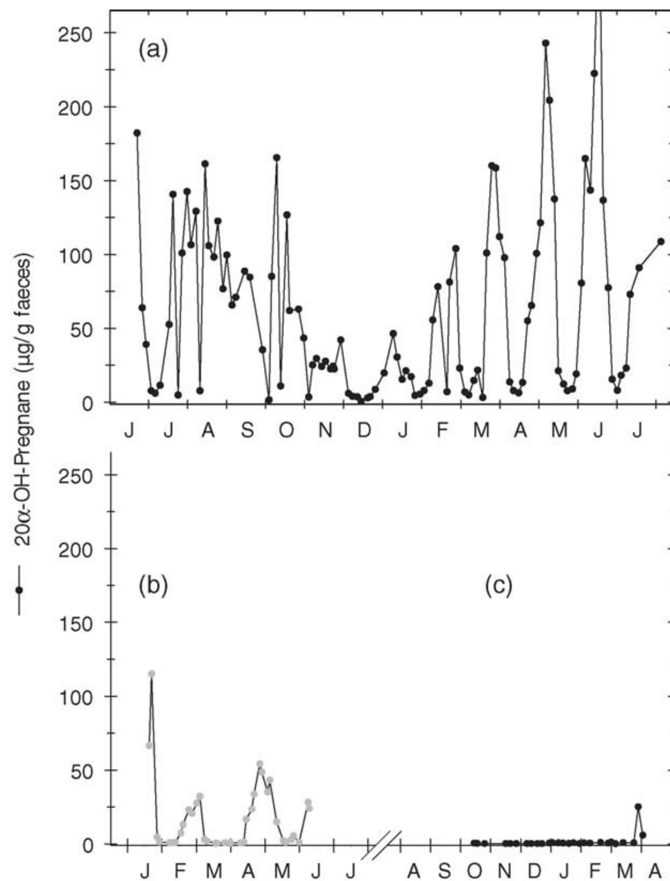


Figure 89: Trace (b) is that of Muhibah, showing regular estrous cycles but low luteal-phase progestagen levels. The other two traces show (a) Beroga (SB#132), a 14yr old female with persistent luteal activity followed by regular cycles and (c) Rifka (SB#142), a 3½ yr old female with no luteal activity at all. Taken from (Berger, Leus et al. 2006)

Interestingly, on post mortem at the age of 14years, Muhibah was found to have multiple corpora lutea present (personal communication Alastair Macdonald). However, she also had a large uterine leiomyoma with evidence of necrosis and mineralisation, a sign that this benign tumor had been present for some considerable time.

Chapter 5: Final Discussion

The aim of this thesis was to investigate the use of various techniques to assist the conservation breeding of the Babirusa (*Babyrousa celebensis*), a wild Indonesian suid. Three techniques were used to investigate different aspects of the programme: extensive studbook and pedigree analysis to assess the performance of the programme in the past, and to make suggestions for the future; genetic analysis using ten microsatellite markers and one mitochondrial region to assess genetic diversity and population differentiation within and between geographical regions; and faecal steroid analysis to investigate the 'normal' changes in progestagens during estrus cycling and pregnancy. All of these techniques are widely used in assessing populations and individuals, but here they have been used *collectively* to assess multiple aspects of the conservation breeding programme and individuals within it. There are many examples of analyses involving data derived from more than one analysis, and the case study of female Muhibah has followed a thread through all three techniques as well as using additional information to complete her story.

Detailed discussion has been presented in the chapters relating to each of the three techniques so this Final Discussion will serve as an overview of the major findings, recommendations for management and research, and suggestions for other techniques that may prove useful in future management of the babirusa conservation breeding programme.

CONSERVATION BREEDING OF THE BABIRUSA: Past & Present …

The conservation breeding programme for *Babyrousa celebensis* has been running for almost 40 years. As the programme continues, questions are being asked

regarding how well it is performing, and how it might be better managed. The use of extensive studbook analysis of historical and contemporary animals, of genetic analysis using microsatellite and mitochondrial markers, and of faecal steroid analysis has been combined to provide an insight into the various concerns regarding this population. These insights can inform management strategies and forward planning to ensure the continued existence of a suitable population for release back into the forests of Sulawesi should it become possible some time in the future.

Small founder numbers & studbook uncertainties

One of the concerns about the conservation breeding programme for the babirusa is the small founder number on which it was thought to be based. Anecdotal accounts suggested that 1.2 animals from Sulawesi contributed to the breeding programme on its establishment at the Surabaya Zoo in 1972 (as proposed in ***Figure 16***). However, poor record keeping at that time made this difficult to confirm, as well as how these founders were proposed to have bred to produce the first generation. The overview set out in ***Figure 16*** is based in part on hypothetical founders and relationships as proposed by Kristin Leus (Leus 2004).

In this study, a combination of genetics (using microsatellite and mitochondrial markers) and pedigree analysis has led to the conclusion that there were at least four founder animals (from ***Table 24***), three of which were likely to be female (***Figure 45*** and related discussion). This still, of course, falls well short of the suggested minimum founder number of 20-30 individuals (Frankham, Ballou et al. 2002)., meaning that concerns related to the founder effect, limited genetic variability and risk of inbreeding are still valid.

These combined methods of pedigree and genetic analysis have also been used to assess the past and present existence of a single female lineage containing a unique mitochondrial haplotype, hB (see ***Figure 45***), and to identify individuals still living from this lineage (see discussion on **Mitochondrial haplotypes**, pg175). They will be particularly important to maintain within the population as they likely contain unique genetic diversity not seen in any hA individuals.

The main contribution of this thesis to the genetic work of the CRC in Antwerp was the analysis of samples obtained from the babirusa population living in the Singapore Zoo and Night Safari. Genetic information from this sub-population was incorporated into the existing data set for animals sampled from the European and Surabaya populations. Having all been founded from the same Surabaya stock (see ***Figures 15*** and ***16***), it was thought that the Singapore population would be genetically indistinct from the others. However, genetic drift and inbreeding has led to some differentiation between all three populations tested, and significantly of the European population from the two others (see ***Figure 43***, ***Table 26*** and ***Table 27***). In particular, each population contained unique alleles (see ***Table 24***) making them equally important. As a result of these findings, animals in Singapore have resumed breeding to ensure the propagation of their unique gene pool.

Concerns over inbreeding

Multiple founder events and lack of contact between small sub-populations of the global conservation breeding programme has almost certainly led to some degree of inbreeding. This was confirmed using studbook analysis (see ***Figure 23***) and inferred also by limited allele numbers found in the genetic study (***Table 23***).

When the International Studbook for the babirusa was analysed based only on the historical population, there was a significant correlation between generation level and inbreeding coefficient (see ***Figure 23***). This indicated that the *ex-situ* population was becoming more inbred as the programme progressed. However, the *effects* of inbreeding i.e. inbreeding depression, were not so easy to ascertain. Although decreased longevity was seen for those of inbreeding coefficient 0.35 or above (***Figure 22***), the principal factor contributing to survival beyond key stages (***Table 8***), and to longevity (***Figure 21***) was generation level itself, with a particularly strong effect beyond the 5th and 3rd generation, respectively. However, it is still not entirely clear what element of increasing generation level is responsible for the impact on longevity. Additional factors identified as contributing to longevity were gender (females live longer than males, see ***Figure 24***), dam age and sire age (both associated with decreased longevity, see ***Figures 27*** and ***29***). Increasing sire age was also associated with reduced rates of survival at key stages (as shown in ***Table 14***). The relative importance of these factors on longevity was indentified as **gender >> sire age > dam age > inbreeding** (from ***Table 16***).

From this analysis, it is clear that inbreeding coefficients >0.35 can have effects of longevity whereas inbreeding coefficients of only 0.25 are though to increase the risk of population extinction (Frankham 1995). Encouragingly, however, was that analysis of only living animals suggested that inbreeding is *not* significantly correlated with generation level (the key factor in survival and longevity) in the 'current' population (***Figure 36***), although some individual coefficients are still worryingly high. From the other factors assessed here, it is not only inbreeding that is of concern with respect to long-term health of the population, but also the

inherently shorter lifespan of males, and the higher risks to survival and longevity of any offspring bred from older males and females.

Breeding success

So, what are the key parameters for *ex-situ* breeding success in this species? Although it must be remembered that this can be influenced in captive populations by management practices (Pelletier, Reale et al. 2009), some insight has been provided through the results of the studbook analysis, with further input from the faecal steroid analysis, and the findings are summarised below.

Although both males and female babirusa have been recorded as successfully breeding through most of their lifespan, peak litter production has been between the ages of one to six in females and two to six in males, as illustrated in ***Figures 18*** and ***19***, respectively. Mean litter size for this species in the captive environment is 1.46 (*n=311* litters), with the majority recorded as single piglets (58.2%), followed by twins (37.9%) and rarely by triplets (3.9%). The incidence of triplets is interesting as five of only twelve litters recorded in the International Studbook were born to female SB #50, and one to SB #71, #50's grand-daughter. This seems to indicate a genetic propensity towards triplet births.

From the pedigree analysis, individuals from the early generations of the *ex-situ* population (shown in ***Figure 16***) seem to appear in many of the analyses as examples of those at the extremes of breeding parameters measured. As well as the example of female SB #50 above, another of the rare triplet litters was born to SB #35, an early pedigree female, female SB #8 was responsible for three of the six oldest conceptions, and was one of the oldest animals ever in the programme (she died aged

~22yrs) and SB #4 was youngest *and* oldest female to conceive. One of the early generation males also appear in these extreme examples, with male SB #47 responsible for one of the three oldest conceptions and also two of the youngest.

All of the individuals listed above are from the early EEP breeding programme, three of them in the Antwerp Zoo, and all lived well beyond mean age at death. Why should they have been so successful compared to those later in the programme, or to the populations in other regions? It is very tempting to attribute this to their lack of inbreeding but there is insufficient evidence for this being the sole reason, and these are only a small representation of the whole population. The three animals included in the genetic analysis (SB #s 4, 47 and 50) did not stand out as unusual in the PCA analysis (see ***Appendix 4*** for details) and the later multi-factor analysis showed inbreeding to be only one of several factors related to survival and lifespan (shown in ***Tables 15***, ***16*** and ***17***). Unfortunately, there is insufficient reliable data on the original Surabaya animals (as identified in ***Tables 2*** and ***3***) to investigate the early generations of the original *ex-situ* population further.

Alternatively, rather than resulting from the biological factors investigated in the studbook analysis, the greater reproductive output and longevity seen in the early generations may be a result of changing management practices from the early days of the breeding programme to the present. When the first 4.4 animals were imported to Europe in the 1970s, the focus was very much on producing offspring to supply other zoos interested in keeping this unusual species (personal communication, Alastair Macdonald). This will likely have encouraged intensive management, with all available animals in breeding situations as early as possible, and for as long as

possible in order to rapidly increase population size. Having larger numbers of individuals in fewer institutions likely also enhanced breeding success, as alluded to previously.

In addition to maximising reproductive capacity, at the time of these early generations in Europe there was also more of a focus on keeping animals alive as long as possible (Kitchener and Macdonald 2004), with less emphasis on welfare concerns. The physical effects of this forced longevity have been documented in a number of large mammals, including the babirusa, and primarily manifest as skeletal and dental pathologies (Kitchener 2004; Kitchener and Macdonald 2004). Animals that were kept alive during these early years may otherwise have been euthanased at later stages of the programme, which would explain in part the apparently extended longevity seen in the earlier generations.

The faecal steroid method has also proved useful in relation to breeding success. It suggests that a female can be showing estrus signs without ovulating, like Sur 86, 'Jane', a two-year old from Surabaya Zoo (***Figure 77***) who is likely to be going through puberty. On the other hand, there can also be no external estrus signs but some evidence of ovulation, such as in the cases of Sur 1 'Pahing' and Sur 20 'Lala' (***Figures 86*** and ***87***), although these examples are not so definitive. In addition, there are clear indications that seemingly small perturbations in environmental conditions can be detrimental to estrus cycling in the female babirusa (see Greta example in ***Figure 84***) and that cycling can cease soon after removal of a male (as for Majene, ***Figure 83***). It has also been useful that three different progestagen assays have been used to contribute data for analysis. The extra sensitivity of the group-specific

antibody use in the Vienna assay has been shown to allow better resolution of progestagen traces (see ***Figures 67*** and ***68***), but all three assays have allowed identification of estrous cycles and pregnancy.

Demographics

A significant risk to the current population outside of Indonesia is likely to be the demographic problems of limited animals around suitable breeding age (see ***Figure 35***). Without the input of the 'new' Surabaya lineages or additional young animals, there are serious problems looming ahead with a lack of young animals of optimal breeding age, particularly for the European population (as shown in ***Figure 34***). Collectively managing the North American, European and Singapore populations will ease the problems in the short term, but unless there is a significant increase in birth rates in these regions combined, it will not be a long-term demographic solution

CONSERVATION BREEDING OF THE BABIRUSA: … the Future?

So, how should the conservation breeding programme continue into the future to ensure its success? The analyses presented in this thesis have led to various recommendations being made at the end of each chapter, and the key points are summarised below with justification of their importance.

In general, the recommendations fall into two categories: those related to ongoing management of the *ex-situ* population; and those related to further research required to better inform the breeding programme and maximise its long-term potential.

Recommendations from this study

Management recommendations

1) to increase founder numbers

There is no doubt that the *ex-situ* population is based on a small founder number, regardless of the actual value, and is likely to be the reason for relatively high inbreeding and low genetic diversity of the population, as illustrated in **Chapters 2** and **3**. The positive impact of incorporating the 'new' founder stock in Surabaya to the global population was illustrated in ***Table 19*** and ***Figure 38***, and similar effects on mean ages, generation levels and inbreeding coefficients would equally be expected with additional of further wild stock.

2) to manage a truly global *ex-situ* population

With or without additional founders, population size must be maximised to give the best chance of long-term survival by improving the demographic and genetic profile of the *ex-situ* population. This would be easier to achieve if the programme was truly managed as a single population, with regular movements of animals between regions to reduce any effects of inbreeding and hopefully improve reproductive fitness. There is clear evidence of the potential benefits of this from the studbook (as outlined above) and genetic analyses undertaken here (see ***Table 23***).

3) to increase the breeding population

In effect, the recommendation is to maximise N_e from the available N, to increase the population growth rate and boost the number of young animals by placing as many individuals in breeding situations as possible. This is especially important for the remaining animals related to the hB lineage within the EEP (as discussed in

Mitochondrial haplotypes, page 175) and any other unique lineages identified in the Surabaya or North American populations.

4) to allow individuals to breed early in life

Although there is evidence that both male and female babirusa can breed through much of their adult life, the pedigree analysis presented in this thesis has identified some risks associated with older parents, particularly sires, and that those breeding for the first time when older may only achieve a single litter (refer to ***Oldest dams at first conception*** on page 81). There is also the worrying incidence of reproductive pathologies in females who have never bred (Ziehmer 2008), as has also been seen in white rhino (Hermes, Hildebrandt et al. 2006).

5) to provide reproductive support for individual breeders

This is especially important for those of genetic importance to ensure their continued contribution to the breeding programme. It was a case of too little too late for Muhibah, the hB female whose genetic importance was not known and whose infertility was not fully investigated until she was already 12 yrs old.

Research recommendations

1. to complete genetic profiles for living population *ex-situ*

In order to best plan future management of the conservation breeding population, a comprehensive genetic analysis of all living individuals would be an invaluable additional resource. There are at least three microsatellite loci available but not yet analysed for the Singapore population (SO155, SW911 and SW857) and potentially others suitable from studies of *Sus scrofa* (Laval, Iannuccelli et al. 2000). Additional loci will increase the descriptive potential of the analysis and enable a more accurate

picture to be developed of the population and the individuals within it. In particular, it may allow further clarification of founder numbers and representation and so inform best management practice to equalise founder representation in the living population.

2. to further investigate female anatomy and physiology

Anatomical knowledge of the female babirusa will be especially important should ARTs become a real option. It is already well established that this pig species has a particularly small reproductive tract, with a long cervix and short uterine horns (Macdonald 1991; Ziehmer 2008), but much more detail would be required for instance to deliver sperm during artificial insemination. More detail of ovarian dynamics would also be useful, as would a direct comparison of follicular and luteal phase lengths with *Sus scrofa* and other pig species to establish just how unique the babirusa estrus cycle is. Ultrasound and faecal steroid analysis would be useful in these applications.

3. to further investigate male anatomy and physiology

Except for recent unpublished work of Ziehmer (Ziehmer 2008), there is a lack of information available regarding the reproductive function of the male babirusa. In the case of Muhibah, for example, there was no investigation made of the two males with whom she was housed. Until recently, babirusa sperm had never been described and even now has only been identified from fresh PM sections of testis (Ziehmer 2008), which showed that the babirusa sperm has a distinctive head, quite flattened unlike other mammals (Ziehmer 2008). To my knowledge, fresh ejaculates have

never been collected. Considering the findings of this thesis that there is reduced longevity in animals born to older sires, perhaps it is time to investigate further?

Artificial reproductive techniques (ARTs) for the babirusa?

As mentioned in the previous chapter, there is a general lack of knowledge of basic reproductive physiology in exotic species. This is a major obstacle to the use of ARTs (Andrabi and Maxwell 2007), and is no different in the case of the babirusa. Compare this with the domestic pig (*Sus scrofa*) for whom manipulation of fertility and ARTs have been a part of pork production for many years. In particular, early estrus induction and estrus synchronisation are induced using hormonal preparations such as PG600 (Paterson 1982), and artificial insemination is used to service large numbers of gilts with semen from a few valuable boars (Reed 1982). Perhaps a little more comparative detail of reproductive anatomy in the babirusa, together with honing the species-specific conditions required for semen preservation, embryo maturation or even surrogacy is all that is needed to convert technology for food production into technology for conservation?

The primary benefit of using ARTs are that it could increase breeding opportunities without moving animals around, so avoiding the perils of live animal transport and the costs and losses associated with it. It could also overcome any instances of mate incompatibility (Goy 1979) or concerns over aggressive males (Wildt and Wemmer 1999), as well as reducing the risk of disease transmission (Hermes, Goritz et al. 2008).

There are of course difficulties and limitations to the use of ARTs in non-domestic species. It can be an expensive, labour-intensive process, with no guarantee of

success, which relies on expertise not readily available in the zoo world. The added risk of putting these animals under anaesthesia, a procedure that is often more difficult and unpredictable than would be in domestic species (personal communication, Lesa Longley), may be considered too great for a rare species for too little chance of success.

However, the primary difficulty in applying ARTs to threatened species is the lack of species-specific knowledge required for success of any ART. As already stated, there is limited knowledge of reproductive anatomy and physiology in threatened species, as well as highly variable conditions required for electroejaculation, semen storage and delivery.

Some will argue that with all of these difficulties, pursuing ARTs as a method for conserving threatened species is a waste of resources that could be better spent preserving natural wild habitat. In reality, however, the funding streams for these two very different activities are unlikely to be available to both so they are not in direct competition with each other. It is also increasingly accepted that long-term species conservation will depend on efforts made to preserve both species and habitat (Conway 1995), as both are required for ecosystems to fully function. Reproductive technologies will not overcome these more fundamental problems (Wildt and Wemmer 1999).

Another argument against the use of ARTs is that it denies the individuals the opportunity for a natural mating experience, which is a typical concern for those in the zoo world. In welfare terms, animals should be given the opportunity to express as many of their natural behaviours as possible (Hosey, Melfi et al. 2009), and this is

a key behaviour in any animal's life experience. And here is the difficulty when it comes to balancing the needs of individuals with the needs of a species, and not an easy one to reconcile with even the best of intentions. Say, for example AI, or some other ART, was the only hope for maintaining an *ex-situ* population outside of Indonesia, would it be justified? What if it was the only hope for the species in any setting? Using ARTs in not just about technology, it's also about ethics and need, and ultimately about the willingness of the people who look after these animals to decide what they deem to be acceptable and what they don't.

ARTs in rhino conservation

As for it being a simple matter of a little more knowledge of anatomy and physiology, a bit of practice and then you have a fully-functioning ART, it is really not so simple. An example of where there has been long-term investment in ART development for an exotic species and its conservation is in the case of the rhinoceros, as outlined previously in **Chapter 4**.

All three rhino species have been the subject of investigation and experimentation in developing ARTs, particularly in Artificial Insemination. This has been in response to their threatened status in the wild, and their relatively poor breeding success in the captive environment (Swaisgood, Dickman et al. 2006). The combined efforts of zoo professionals and researchers culminated recently in the reported live birth and survival of a white rhino (*Ceratotherium simum simum)* after AI with freeze-thawed semen (Hermes, Goritz et al. 2008), a follow-up success to the first AI attempts with fresh semen where one of two calves survived (Hildebrandt, Hermes et al. 2007). This is of course a milestone achievement but has taken many years of basic

reproductive research, since the first steroid profiles produced for the black rhino (Schwarzenberger, Francke et al. 1993), and numerous failed attempts to get there.

How feasible is this type of reproductive assistance as an option for other threatened species? Perhaps some will be more easily manipulated, and the success will come relatively easily? Even then, the development of ARTs for wildlife is a long and expensive journey with no guarantee of success.

Gene banking

The potential of gene banking is to store the DNA of many more individuals that can be sustained in life, so can retain a greater breadth of variety and maintain it in a secure form, independent of living organisms and the requirements they have. In theory, it is a more efficient way of preserving genetic diversity than in living animals (in terms of space, costs, security, etc), but may be limited by the technology required to convert it back into living individuals when the time is right.

Gene banking is already established for plant species with over 1000 seed banks held worldwide, and a similar network is now being established for animal specimens. For example, the Frozen Ark project (www.frozenark.org) aims to "collect, preserve and store tissue, gametes, viable cells and DNA from animals in danger of extinction". It is 'banking' a variety of tissue types in a number of institutions, including the Zoological Society of London (ZSL), the North of England Zoological Society (NEZS at Chester Zoo) in the UK and other zoos and scientific institutions worldwide. There is also the Amphibian Ark (www.amphibianark.org), set up in response to the recent worldwide decline in amphibian species and numbers, as set

out in the IUCN Global Amphibian Assessment

(www.iucnredlist.org/initiatives/amphibians, visited 10/2/2010).

Unfortunately, there are concerns regarding a lack of funding for these initiatives (personal communication, Alastair Macdonald), and of long-term commitment to what may well become an extended period of time until the technology is available and reliable enough to reconstitute DNA back into animal bodies, and to have suitable habitat available for those animals to return to (Conway 1995). Is there also a danger in seeing this type of technology as a failsafe solution to preserving biodiversity, with the danger that it will reduce the sense of urgency in halting habitat decline and the other threats facing species today?

Genetic rescue?

The concept of 'genetic rescue' was introduced in **Chapter 1** as a way of reversing inbreeding and inbreeding depression by the addition of a few unrelated individuals to a population. Some successes were also highlighted, including that of an isolated wolf population (*Canis lupus*) in Scandinavia where the immigration of a single male led to "increased heterozygosity, significant outbreeding (inbreeding avoidance), a rapid spread of new alleles and exponential population growth (Vila, Sundqvist et al. 2003). With respect to the babirusa, 'genetic rescue' is a concept that could apply equally to the *ex-situ* and *in-situ* populations.

For the *ex-situ* population?

The level of inbreeding within the *ex-situ* population has been illustrated in the work of this thesis, although its relation to inbreeding depression has been more difficult to establish. There is clear evidence, however, that survival and longevity are reduced

in animals from later generations in the pedigree. It has already been suggested that additional wild founders are required to increase founder number to come closer to the 20-30 recommended individuals, and to improve genetic diversity. An added benefit to the addition of new founder lines may also be an increase in fitness associated with some degree of 'outbreeding' between individuals of different genetic lineages, such as that seen in the wolf population described above, or in the bighorn sheep population founded from only twelve individuals where there were "marked improvements in reproduction, survival and five fitness-related traits among descendents of 15 recent immigrants" (Hogg, Forbes et al. 2006). Although it probably wouldn't be strictly considered as 'genetic rescue' being applied to an *ex-situ* population, it follows the same principles and may bring the same kind of benefits.

The *in-situ* context

Only now are we beginning to understand some of the wider context to this species in its natural range, based on a number of studies currently underway. Hundreds of skull specimens, collected from all over the Indonesian archipaelago, are being investigated for morphometrics (personal communication Alastair Macdonald) and for mtDNA (personal communication Greger Larson) in order to clarify the true extent of differentiation between individuals from different geographical regions. The ultimate goal of this is to clarify the existence of multiple species and/or Evolutionary Significant Units for the babirusa. As mentioned in **Chapter 1**, the IUCN Red List report 2008 states that the threatened status of any *Babyrousa celebensis* population will depend of clarification of these boundaries (Macdonald, Burton et al. 2008), and so too will their potential level of protection.

Considering the ongoing threats to the wild population, could the individuals within the conservation breeding programme represent the remains of a now extinct wild population? Could it represent the last living vestiges of a gene pool no longer present in the wild? If this was the case, the *ex-situ* population may be vital not only to supplement a wild population, but also to reinstate genetic lineages no longer represented. However, the distinction of evolutionary significant units and sub-populations presents another dilemma: is it more important to save the *genetic integrity* of a species population, or to save it from extinction? This was integral to the decision to 'save' the Florida panther. A population of highly inbred individuals showing multiple signs of inbreeding depression including undescended testes and poor sperm counts had endured a very small bottleneck (Culver, Hedrick et al. 2008), and was 'rescued' by the introduction of individual Texas panthers. It meant the 'dilution' of the Florida panther lineage, but also likely saved the population from extinction.

It is a dilemma that is likely to become more and more pertinent as wild populations of many species become smaller, more isolated and ultimately more inbred. This will threaten their survival and decisions will have to be made as to whether populations considered distinct from one another e.g. as sub-species or ESUs, should be allowed to interbreed. This would seem to contravene a basic tenet of species conservation, and carries the risk of 'oubreeding depression', but it may be the only possibility for long-term survival of some species, where a degree of outbreeding may actually be what the species needs.

Outbreeding depression

There is thought to be an optimal ‘distance’ between parents that will result in maximum fitness of their offspring (Price and Waser 1979; Waser and Price 1989), where ‘distance’ refers to genetic distance, i.e. how closely related the parents are. This is sometimes referred to as ‘hybrid vigour’ or ‘heterosis’ where the fitness of the offspring is greater than that of the parents. At either extreme of this optimal distance there can be evidence of reduced fitness due to inbreeding or outbreeding.

The occurrence and effects of inbreeding are well documented and have been addressed earlier in this thesis. However, the phenomenon of outbreeding depression, where there is a ‘large’ distance between parents is much less well studied but evidence of reduced fitness has been reported in various plant species (for example, Montalvo and Elstrand 2001), salmonids (Emlen 1991), the snail *Physa acuta* (Escobar, Nicot et al. 2008), the song sparrow M*elospeza melodia* (Marr, Keller et al. 2002) and the common frog *Rana temporaria* (Sagvik, Uller et al. 2005).

The proposed mechanisms by which this reduced fitness occurs are: 1. by ‘dilution’ of local environmental adaptations and 2. by disruption of beneficial gene complexes (Price and Waser 1979). There is concern with respect to conservation efforts such as translocations and restoration of plant (or animal) populations where individuals may be from widely separated geographical or ecological locations and their hybridisation may have deleterious effects through outbreeding depression (for example see Sagvik, Uller et al. 2005). It is also possible, however that early depression effects may be outweighed in the long term by increased genetic diversity and heterozygosity although this may take many generations (Emlen 1991).

What more can Muhibah tell us?

The work of this thesis was initiated in part by the story of Muhibah and she has acted as a case study throughout. Late in her non-reproductive life, she was investigated using a number of methods, from partially successful ultrasound examination through baseline faecal steroid analysis and stimulation with PG600, to anatomical description at post-mortem. Her inclusion in the genetic studies of the CRC in Antwerp identified her as the last living female of the hB mtDNA lineage, and her death therefore sealed the fate of that haplotype lineage.

Despite these investigations, the reasons for her infertilitly are still not absolutely clear. She had shown signs of estrus and been mated on several occassions (personal communication Darren McGarry), yet had never conceived. She was shown to have luteal insufficiency (Berger, Leus et al. 2006) yet had corpora lutea on her ovaries at PM. She also had uterine tumors, however, and these may have affected her ability to the maintain a pregnancy. It is tempting to suggest that had she been in a suitable breeding situation at a younger age, she may have conceived and this may have protected her from the multiple reproductive problems she had.

THE NEED FOR CONSERVATION

The root cause

We are currently in the early stages of a 'global extinction crisis'. Estimates from the United Nations Environment Programme suggest that up to 50% of species may be lost in the next 100 years (www.unep.org/compendium2009/, accessed September 2009), driven by multiple anthropogenic factors including climate change (Zhang, li et al. 2009; Wright, Muller-Landau et al. 2009), and the synergistic interactions

between them (Laurance and Useche 2009). Can the species (*Homo sapien*) that is driving this extinction event by its increasing demands on the natural world employ its superior intellect to finding solutions (Mawdsley, O'Malley et al. 2009; Pettorelli, Katzner et al. 2009)?

Ultimately, any significant protection of species diversity can only be achieved by targeting the fundamental problems caused by human activity: by reducing logging and habitat destruction/conversion and creating more truly protected areas; by reducing the release of pollutants into the environment; by eliminating species exploitation; by addressing and reversing the increasingly harmful effects of climate change; and so on. However, these types of solutions are not easy ones, and ultimately conflict with the perceived needs of local human populations. In addition they will take considerable time to enact, and if we wait until they are in place much will have already have been lost.

The role of zoos and animal parks

It is clear that a multi-faceted approach is required at this vital stage in species conservation (Crandall 2009). One of the increasingly important tools is that of conservation breeding of threatened species in *ex-situ* populations such as those of zoos and animal parks (Mallinson 1995). Their impact on conservation will be maximised when species are selected an the basis of 'clear and rational criteria' (Balmford, Mace et al. 1996) and are managed in a sustainable way (Lees and Wilcken 2009) in co-operation with conservation efforts in the wild (Ebenhard 1995).

In addition to their activities in *ex-situ* breeding, zoos and animal parks can provide additional benefits to conservation in their role as centres for research and education and in their unique access to a wide demographic of visitors.

Conservation breeding

Conservation breeding is one of the tools being employed to establish temporary populations in a safe *ex-situ* setting, and has successfully resulted in the release of a number of species back into their natural habitat, either to re-establish populations previously extinct in the wild such as the Przwalski's horse (*Equus caballus*) and the black-footed ferret (*Mustela nigripes*).

Of course, conservation breeding programmes by necessity are established on relatively small numbers of founders, and can only remain as relatively small populations for practical reasons. As such, they are prone to the many problems seen in small wild populations, particularly with respect to genetic diversity. If genetic diversity is not sampled in the founder animals, or founders fail to breed, or subsequent generations lose diversity through inbreeding and/or genetic drift then it cannot be regained except by mutation. It is, therefore, vitally important to appropriately sample the wild population for founders, and to effectively manage the population to limit the loss of genetic diversity as has been previously discussed in detail.

Beyond this, there is still the problem of individuals and populations adapting to life in captivity, reducing their chances of survival back in a wild habitat. This occurs through the 'selection' of animals most suited for life in captivity: those with more stable temperaments, amenable to keeper interactions and other interventions. The

classic example of this ‘domestication’ process is that of the 1959 Farm-Fox experiment (Belyaev and Trut 1975). Generations of silver foxes were selected for tameness over a 40 year period, resulting in a large population of ‘tame’ foxes, with the the emergence of physical and behavioural characteristics associated with domestic dogs (Trut 1999), proposed to be the result of reduced thyroid function during embryonic development (Crockford 2002) . Although this is an extreme example, it illustrates how repeated selection for a single behavioural trait can result in fundamental changes in physiological and anatomical form.

The best ways to reduce this adaptation process is to provide living conditions as similar to the wild as possible, and to limit the number of generations the species is kept for before release back into the wild (Frankham, Ballou et al. 2002). The greatest risk of adaptation is with larger populations, but smaller populations are at greater risk of inbreeding and loss of genetic diversity, as described previously. This poses a problem which can be overcome either by holding some interim number of animals, or managing a larger number as a series of smaller metapopulations (Frankham 2008).

Research

In the last 10-20 years, the contribution zoos and animal parks make to conservation efforts have moved beyond the realms solely of captive breeding to their integration with *in-situ* projects and to the wider activities of research and education (Ryder and Feistner 1995).

Zoo-based research is increasing in quantity and quality, and is a legal requirement of zoos in the European region as set out in the EU Zoo Directive (Council Directive

1999/22/EC). It can contribute to understanding the fundamental aspects of species biology that would be difficult or impossible in a wild animal or population, and is especially useful in assessing the requirements of threatened species about which little may otherwise be known. The EAZA Research Strategy (Reid, Macdonald et al. 2008) sets out the many ways in which animal collections can be used to further research, from the smallest financial contributions, to the largest multi-institutional projects.

Education

Education is a fundamental element of the modern zoo, and is a key requirement for institutions within the regional associations of zoos and aquaria, as outlined in the World Zoo and Aquarium Conservation Strategy, WZACS (WAZA 2005). The combined visitor numbers to zoos and aquaria is huge and the number of zoo visits worldwide was estimated in the early 1990s at around 619 million per year (IUDZG/CBSG 1993), and draws from a wide demographic of society (Hosey, Melfi et al. 2009). As such, they are ideally placed to deliver conservation messages to a diverse audience, and to suggest ways in which they can contribute to it.

It is only through education that we can change attitudes and show how our actions impact on the world beyond our own back door. Zoos and animal parks can lead the way in promoting key conservation messages, and can inspire their visitors by being sustainable in their own activities and by showing people that they care about animals and their welfare, not only those within their collections but also those hanging on to survival in their increasingly threatened wild habitats.

The influence of fashions and finance

As visitor attractions and businesses, zoo collections can be influenced by many different factors that could potential limit their ability to support conservation breeding.

As with any type of collection, there are changing fashions with regard the species people want to see in zoos, and rarely seen species can bring in greater visitor numbers. For example, the giant panda (*Ailuropoda melanoleuca)* is only found in three zoos in Europe, making them a huge draw for the public and a significant status symbol for the zoos who have them. But are European zoos able to contribute to the conservation breeding of this species? Well, in this particular case the animals are only on loan from China, with any youngsters to return there. The numbers involved are far too small to contribute significantly to the breeding population, and the commitment is very short-term. It could be argued that sending giant pandas 'on loan' to European zoos plays a greater role in East-West relations and zoo popularity than it does in conservation as the risks involved in intercontinental travel perhaps outweigh any benefit to the species.

A different kind of example is of the meerkat (*Suricata suricatta*). They are a favourite for the public due to their engaging and playful nature yet they are cited as 'Least concern' in the IUCN Red List of Threatened Species™. Is there a justification for keeping such a species in a zoological collection, taking up space that could be used for a species actually under threat in its home range?

Some of these biases in individual animal collections can be balanced to some extent within the regional zoo associations, where Regional Collection Plans (RCPs) are

used to prioritise species held in the region. There are numerous criteria, including: how much space is available; what is the species' status in the wild; what is the educational value of the species; how much husbandry expertise is there; and what are the other regions holding (www.eaza.net, visited 10/02/2010). However, the animal species held in zoological collections are still often at the whim of zoo managers who have particular interests, regardless of the bigger conservation picture or the Regional Collection Plan.

Commercial implications may also limit conservation breeding. Some of the threatened large megafauna e.g. rhino and elephant are very costly to maintain as they need large enclosure spaces and huge amounts of food. It has been estimated that maintaining a single elephant in captivity for only a year can cost up to $100,000. With additional huge costs in developing suitable enclosures the total can run into millions of dollars (www.pawsweb.org, visited 10/02/10). Is this really a good use of resources that could fund large conservation projects *in situ*? Smaller more easily managed species such as rodents or amphibians, would be preferable in terms their space and food requirements, so would free up valuable resources (Balmford, Mace et al. 1996), but they may not have the same public appeal.

Overall, there is a need to balance what the public want to see with what is best managed for conservation purposes, taking into account the financial and other implications i.e. what it costs to keep a species v money generated by visitors who want to see that species. RCPs should at least help prioritise the species that zoos and animal parks choose to keep, but there will always be some independence in each collection, with individual preferences, financial restrictions, visitor interests

and availability contributing to the uniqueness of each institution. This will be regardless of whether the collection is run by an individual, by a local authority, a charity or a business, and is what will make zoos interesting places where people can see different species and learn about the threats they face in their wild habitat.

APPENDICES

Appendix 1: Full definitions for each risk category and each criterion in the IUCN Red List, 2008 from 'Guidelines for Using the IUCN Red List Categories and Criteria, Version 7.0.

Table 2.1. Summary of the five criteria (A-E) used to evaluate if a taxon belongs in a threatened category (Critically Endangered, Endangered or Vulnerable).

Use any of the criteria A-E	Critically Endangered	Endangered	Vulnerable
A. Population reduction	Declines measured over the longer of 10 years or 3 generations		
A1	> 90%	> 70%	> 50%
A2, A3 & A4	> 80%	> 50%	> 30%

A1. Population reduction observed, estimated, inferred, or suspected in the past where the causes of the reduction are clearly reversible AND understood AND ceased based on and specifying any of the following:

(a) direct observation
(b) an index of abundance appropriate to the taxon
(c) a decline in area of occupancy (AOO), extent of occurrence (EOO) and/or habitat quality
(d) actual or potential levels of exploitation
(e) effects of introduced taxa, hybridisation, pathogens, pollutants, competitors or parasites.

A2. Population reduction observed, estimated, inferred, or suspected in the past where the causes of reduction may not have ceased OR may not be understood OR may not be reversible, based on any of (a) to (e) under A1

A3. Population reduction projected or suspected to be met in the future (up to a maximum of 100 years) based on any of (b) to (e) under A1.

A4. An observed, estimated, inferred, projected or suspected population reduction (up to a maximum of 100 years) where the time period must include both the past and the future, and where the causes of reduction may not have ceased OR may not be understood OR may not be reversible, based on any of (a) to (e) under A1.

B. Geographic range in the form of either B1 (extent of occurrence) OR B2 (area of occupancy)			
B1. Either extent of occurrence	< 100 km^2	< 5,000 km^2	< 20,000 km^2
B2. or area of occupancy	< 10 km^2	< 500 km^2	< 2,000 km^2
and 2 of the following 3:			
(a) severely fragmented or # locations	= 1	≤ 5	≤ 10

(b) continuing decline in (i) extent of occurrence (ii) area of occupancy, (iii) area, extent and/or quality of habitat, (iv) number of locations or subpopulations and (v) number of mature individuals.

(c) extreme fluctuations in any of (i) extent of occurrence, (ii) area of occupancy, (iii) number of locations or subpopulations and (iv) number of mature individuals.

C. Small population size and decline			
Number of mature individuals	< 250	< 2,500	< 10,000
and either **C1** or **C2**:			
C1. An estimated continuing decline of at least up to a maximum of 100 years	25% in 3 years or 1 generation	20% in 5 years or 2 generations	10% in 10 years or 3 generations
C2. A continuing decline and (a) and/or (b)			
(a i) # mature individuals in largest subpopulation	< 50	< 250	< 1,000
(a ii) or % mature individuals in one subpopulation =	90-100%	95-100%	100%
(b) extreme fluctuations in the number of mature individuals			

D. Very small or restricted population			
Either (1) number of mature individuals	< 50	< 250	< 1,000
or (2) restricted area of occupancy	na	na	typically: AOO < 20km^2 or # locations ≤5

E. Quantitative Analysis			
Indicating the probability of extinction in the wild to be at least	50% in 10 years or 3 generations (100 years max)	20% in 20 years or 5 generations (100 years max)	10% in 100 years

Appendix 2: Genotype data for Surabaya samples (from the CRC, Antwerp)

Stud#	Sample#	Marker									
		S0215		SW72		SW632		SW951		SW936	
353	1	147	153	120	130	196	196	134	148	88	90
324	2	147	163	120	130	182	196	134	148	?	?
325	3	153	163	130	130	196	196	134	148	?	?
345	4	147	147	130	130	196	196	134	148	?	?
359	5	153	163	120	126	182	196	144	148	?	?
327	6	147	163	120	130	196	196	144	148	88	90
365	7	163	163	120	130	182	196	134	140	?	?
355	8	147	153	120	130	196	196	134	148	88	90
347	9	147	153	120	120	182	196	134	148	82	90
333	10	163	163	122	122	182	182	140	148	?	?
354	11	153	163	126	130	182	196	140	148	88	88
350	12	147	163	122	122	182	196	134	148	88	90
356	13	147	153	122	122	196	196	148	148	?	?
352	14	147	163	130	130	182	196	140	148	88	90
348	15	153	163	130	130	182	196	134	140	88	88
329	24	147	153	120	130	196	196	134	148	88	88
340	25	163	163	130	130	196	196	134	140	88	88
341	26	153	163	130	130	196	196	?	?	88	90
351	27	147	153	120	120	182	196	148	148	88	90
361	28	163	163	120	130	182	196	140	148	90	90

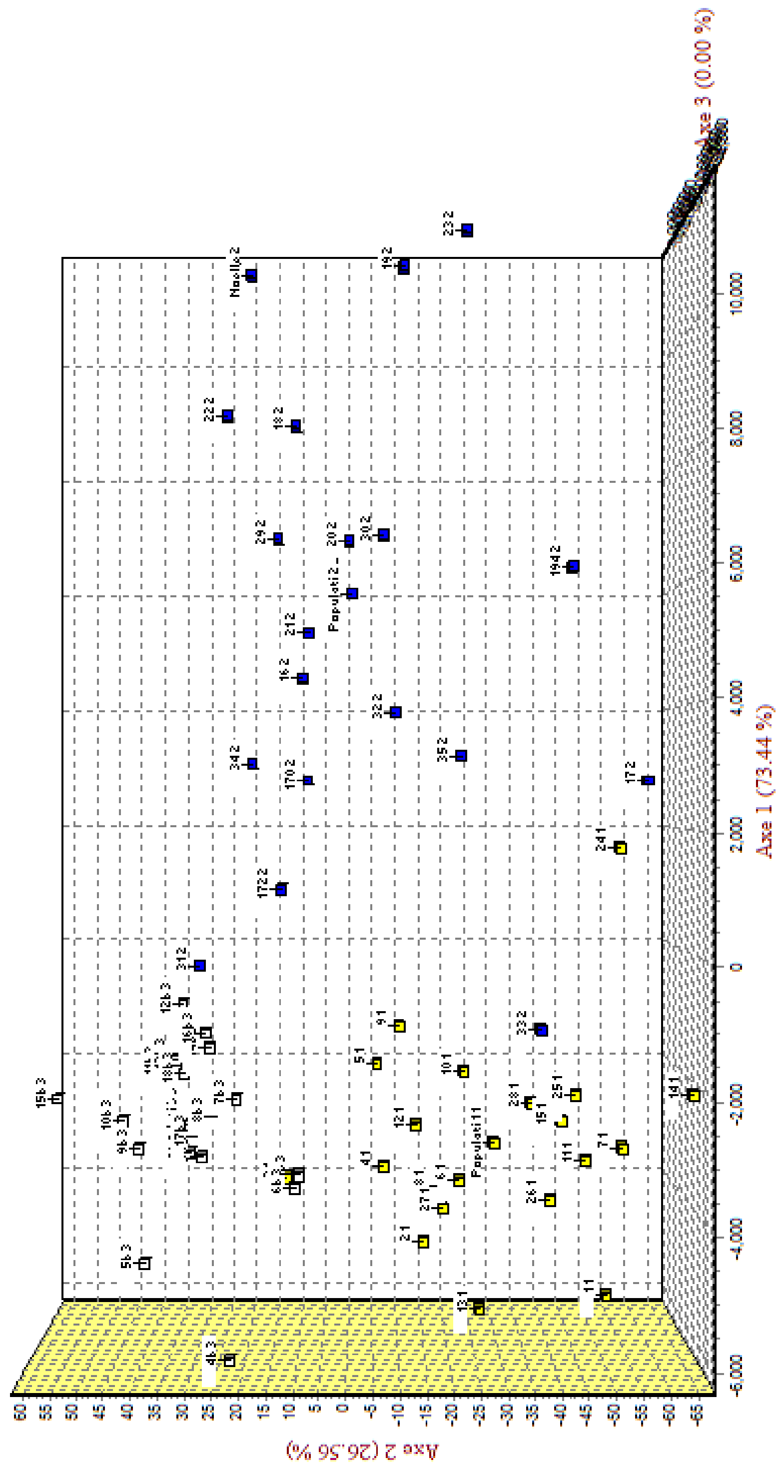
Stud#	Sample#	Marker									
		S0214		S0386		S0026		S0149		S0228	
353	1	125	129	158	168	106	108	321	343	316	320
324	2	125	129	158	168	106	106	321	343	312	316
325	3	129	129	158	164	106	106	331	343	298	316
345	4	129	129	164	164	106	106	321	341	312	316
359	5	125	129	164	170	106	108	315	343	264	316
327	6	125	129	158	168	106	108	315	321	312	320
365	7	129	129	?	?	106	106	?	?	?	?
355	8	125	129	158	164	106	108	315	343	316	320
347	9	125	129	162?	164?	106	108	321	343	298	312
333	10	129	129	164	164	106	108	331	343	312	316
354	11	125	129	158	170	106	106	315	331?	312?	318?
350	12	129	129	164?	168?	106	108	343	343	316	316
356	13	125	125	158	162	106	106	321	343	312	320
352	14	125	129	162	170	106	106	315	333	264	318
348	15	129	129	?	?	106	106	315	343	312	316
329	24	129	129	162	162	106	108	341	341	312	312
340	25	129	129	158?	162?	106	106	333	343	312	316
341	26	129	129	158	164	106	106	333	343	312	318
351	27	125	129	164	164	106	108	315	321	312	318
361	28	125	129	164	168	108	108	315	343	312	316

Appendix 3: Genotype data for EEP samples (from the CRC, Antwerp)

Stud#	Sample#	Marker									
		S0215		SW72		SW632		SW951		SW936	
61	16	147	163	126	126	182	182	148	154	90	90
131	17	163	163	120	120	186	196	134	148	?	?
47	18	147	163	126	126	182	196	148	154	88	90
160	19	147	163	120	120	186	196	134	154	88	88
122	20	163	163	120	120	186	196	148	154	?	?
294	21	163	163	120	130	186	196	134	148	88	88
183	22	163	163	126	126	182	196	148	154	88	88
206	23	147	163	120	120	182	186	154	154	?	?
430	29	147	147	120	120	182	196	148	154	88	88
4	30	147	163	126	126	182	186	134	144	88	90
19	31	147	163	126	126	182	196	?	?	88	90
146	32	153	153	120	130	186	196	148	148	82	88
230	33	163	163	120	134	196	196	134	140	82	90
147	34	153	163	120	120	186	196	144	148	88	90
151	35	163	163	134	134	196	196	134	134	82	88
170	170	147	163	120	120	182	196	148	154	88	88
172	172	147	163	?	?	182	196	?	?	88	90
194	194	147	153	120	130	196	196	154	154	?	?
50	Lotty	147	147	120	126	182	182	154	154	88	90
66	Noelle	147	147	120	126	182	182	154	154	88	90

Stud#	Sample#	Marker									
		S0214		S0386		S0026		S0149		S0228	
61	16	129	131	158	162	106	106	315	315	298	312
131	17	129	129	164	168	90	106	341	341	316	316
47	18	127	131	158	162	106	108	315	339	298	312
160	19	129	129	162	162	90	90	?	?	264	264
122	20	129	129	162	170	90	106	343	343	264	312
294	21	129	129	170	170	90	106	?	?	264	312
183	22	127	127	158	158	108	108	315	339	298	312
206	23	129	129	158	162	90	108	315	341	264	312
430	29	129	129	158	170	90	108	331	349	312	312
4	30	125	129	158	162	90	108	331	341	312	324
19	31	129	129	158	164	106	108	331	343	264	312
146	32	129	129	158	164	90	90	343	343	312	316
230	33	125	129	164	170	106	108	?	?	312	316
147	34	129	129	164	164	90	90	343?	343?	298	316
151	35	129	129	162	164	90	106	?	?	312	312
170	170	129	129	?	?	106	108	?	?	312	312
172	172	129	129	?	?	106	108	315	315	298	312
194	194	129	131	?	?	106	108	333	343	?	?
50	Lotty	127	131	158	158	108	108	315	349	298	312
66	Noelle	127	131	158	158	108	108	315	349	298	312

Appendix 4: PCA to show population differentiation based on a sample of individuals from Singapore, Surabaya and the EEP



Appendix 5: Table showing basic reproductive parameters of various pig species

Species	Common name	Age at puberty (months)	Length of cycle (days)	Average no. of corpora lutea	Gestation time (days)	Litter size	Average litter size
Sus scrofa	**European Wild Pig**	?	21-23 [c]	5.3 [b]	114[a] 115[c]	4-7 [a] 2-6 [b] 1-13 [c]	4.6 [b] 5.3 [c]
	Asian Wild Pig	?	21-23 [c]	?	114 [a] 115[c]	3-9 [b] 1-13 [c]	5.4 [b] 5.3 [c]
	Domestic Pig	3[dM] ~6[dLW]	21 [c] 19.1[dM] 19.8[dLW]	14.1[dM] 18.5[dLW]	114 [c]	1-20 [c]	12 [c] 13-15[dM] 10-11[dLW]
Sus salvanius	**Pygmy Hog**	?	21 [c]	?	110-120 [c]	2-6 [a,b] 1-7[c]	3.0 [b] 4.7 [c]
Sus verrucosus	**Javan Warty Pig**	?	?	?	?	3-9 [a] 3-8[b]	5.0 [b]
Sus barbatus	**Bearded Pig**	10 [a]	?	?	90-120 [a,c]	3-11[a,b] 3-12[c]	7 [a,c]
Sus cebifrons	**Visayan Warty Pig**	?	?	?	140 [c]	5-8 [a] 1-3[c]	1-2 [c]
Sus philippensis	**Phillipine Warty Pig**	?	?	?	?	5-8 [a] 3-8[c]	?
Sus celebensis	**Sulawesi Warty Pig**	?	?	?	?	2-8 [a,b] 1-8[c]	5 [c]
Potamochoerus larvatus	**Bushpig**	?	?	?	120 [a,c]	1-6 [a] 1-7[c]	3 [a] 3.2 [c]
Potamochoerus porcus	**Red River Hog**	?	~30 [c]	?	120 [a,c] 130[b]	1-6 [a,c] 2-7[b]	3 [a] 3-4[b] 3.4 [c]
Hylochoerus meinertzhageni	**Forest Hog**	18 [a]	?	?	151 [a,b,c]	2-4 [a,c]	4 [b] 3 [c]
Phacocoerus aethiopicus	**Desert Warthog**	?	?	3.6 [b]	172 [a] 171-175[b]	1-7 [a] 1-8[b]	3 [a,b]
Phacocoerus africanus	**Common Warthog**	?	?	?	172 [a,c]	1-7 [a] 1-8[c]	3 [a,c]
Babyrousa babyrussa	**Babirusa**	5-10 [a]	28-42 [a,c]	?	155-160 [a,b] 158[c]	1-3 [a,b,c]	1.8 [b,c]

[a] From (Macdonald 1993)
[b] From (Macdonald, Kneepens et al. 1984)
[c] From (Macdonald 2000)
[d] From (Fuller, Ford et al. 2001). M = Meishan pig and LW = Large White pig

Appendix 6: Raw data from assessment of extraction efficiency

	A: shaken and spun		B: dried		C: reconstituted		Overall extraction efficiency (mean±SD)
Sample	cpm	% extraction efficiency	cpm	% extraction efficiency	cpm	% extraction efficiency	
1310a	1523	79.16	1534	79.73	1563	81.24	**80.04±1.07**
	1524	79.21	1516	78.79	1412	73.39	**77.13±3.25**
Mean±SD		**79.18±0.04**		**79.26±0.66**		**77.31±5.55**	**78.59±1.10**
1310b	1433	74.48	1448	75.26	1385	71.99	**73.91±1.71**
	1470	76.40	1387	72.09	1416	73.60	**74.03±2.19**
	1502	78.07	1425	74.06	1435	74.58	**75.57±2.18**
Mean±SD		**76.32±1.79**		**73.80±1.60**		**73.39±1.31**	**74.50±1.58**
1310c	1762	91.58	1729	89.86	1560	81.08	**87.51±5.63**
	1630	84.72	1479	76.87	1512	78.59	**80.06±4.13**
	1569	81.55	1520	79.00	1535	79.78	**80.11±1.30**
Mean±SD		**85.95±5.13**		**81.91±6.97**		**79.82±1.25**	**82.56±3.12**
1315a	1518	78.90	1482	77.03	1586	82.43	**79.45±2.75**
	1853	96.31	1795	93.30	1695	88.10	**92.57±4.15**
Mean±SD		**87.60±12.31**		**85.16±11.50**		**85.27±4.01**	**86.01±1.38***
1315b	1537	79.89	1545	80.30	1602	83.26	**81.15±1.84**
	1653	85.91	1540	80.04	1530	79.52	**81.83±3.55**
	1639	85.19	1613	83.84	1566	81.39	**83.47±1.92**
Mean±SD		**83.66±3.29**		**81.39±2.12**		**81.39±1.87**	**82.15±1.31**
1315c	1683	87.47	1552	80.67	1647	85.60	**84.58±3.52**
	1767	91.84	1686	87.63	1764	91.68	**90.38±2.39**
	1751	91.01	1709	88.83	1718	89.29	**89.71±1.15**
Mean±SD		**90.11±2.32**		**85.71±4.41**		**88.86±3.06**	**88.22±2.27**

Notes: Sample in red showing excessive extraction efficiency, probably due to error adding tracer (too much added).
*calculation includes the values in red

Appendix 7: Raw data from assessment of intra-assay variation

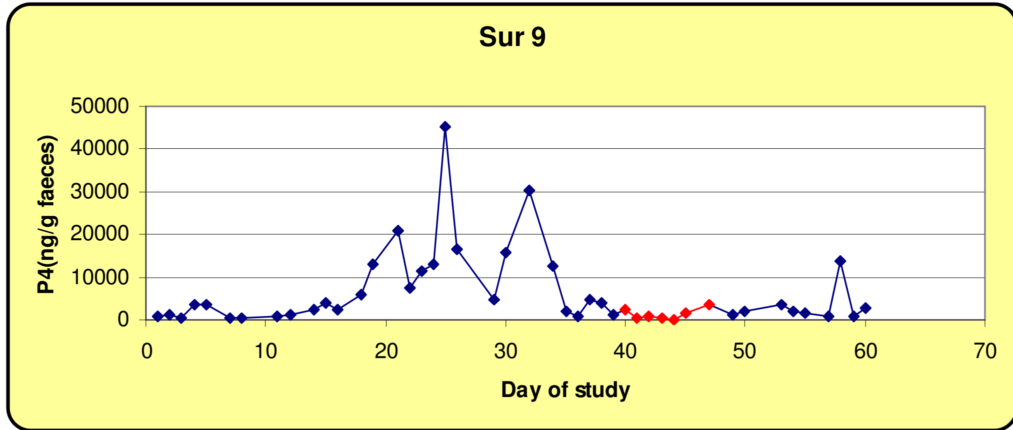
Data set	Mean duplicate variance	# samples
Sur 53	8.34%	32
Sur 66	11.51%	48
Sur 9	13.42%	45
Sur 20	19.24%	48
Sur 3	11.26%	55
Sur 81	11.32%	55
Sur 86	16.13%	48
Sur 59	13.35%	48
Sur 1	10.45%	54
Kota	15.88%	175
Majene	18.72%	279
Muhiba	19.84%	18
All	**14.12%**	**905**

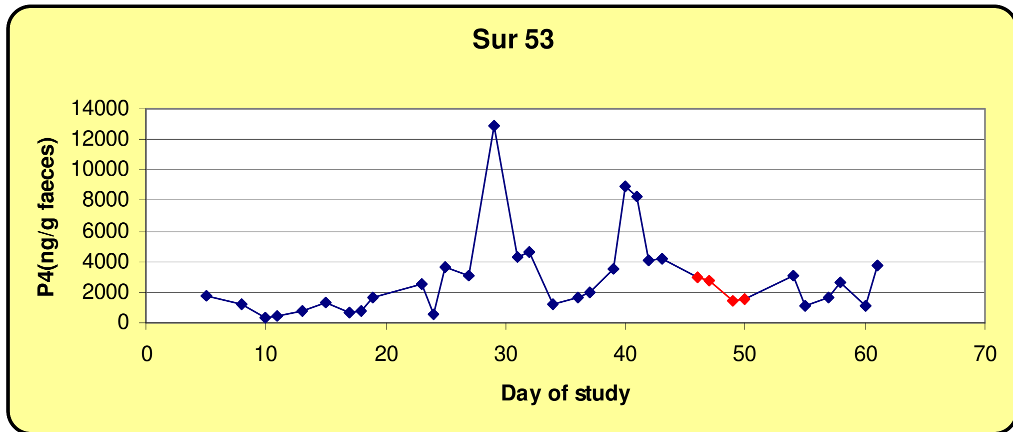
Appendix 8: Raw data from assessment of inter-assay variation

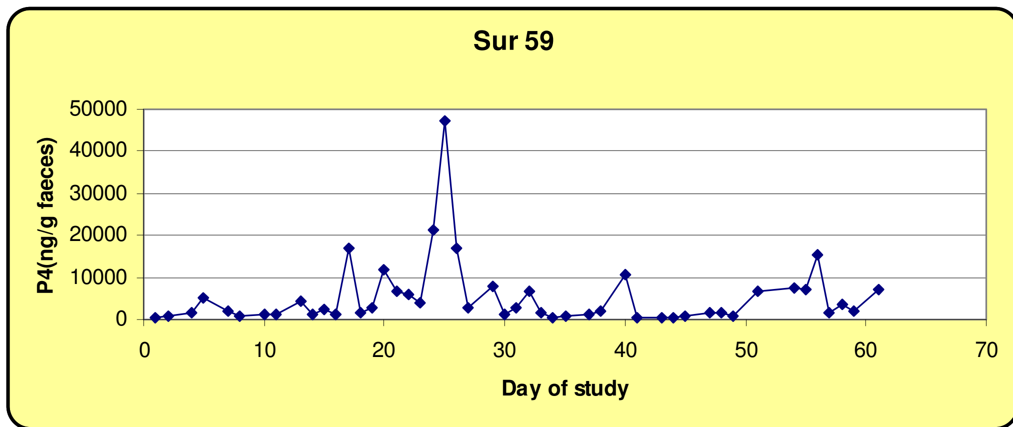
RIA	TC	%bind	NSB	ED20	ED50	ED80	QC1	QC2	QC3	QC4
1	11712	57.6	1.8	294.8	74.0	12.0	36.82	75.90	502.72	560.04
2*	14273	50.4	1.0	294.8	72.6	12.8	24.88	29.55	317.2	446.32
3	11830	50.4	0.8	304.6	94.2	28.3	28.54	38.25	291.43	338.36
4	11852	48.2	0.6	260.0	90.4	33.0	34.86	41.93	304.97	293.62
5	11523	53.3	0.8	296.7	94.2	29.0	36.59	45.20	290.14	356.0
6	12049	49.7	1.1	347.7	102.5	33.0	42.17	42.38	342.29	391.62
7	10577	58.3	1.2	304.6	89.8	22.4	40.53	53.79	340.94	431.75
8*	15729	52.0	0.8	281.4	99.9	34.0	-	-	-	-
9	14767	49.5	0.7	338.6	100.8	28.3	47.68	65.15	423.86	469.91
10	15456	38.4	0.7	329.8	91.1	21.7	36.98	59.84	453.28	427.87
11**	10096	33.4	1.2	249.9	59.0	14.6	31.54	51.61	569.41	512.05
Mean		49.2	1.0	300.3	88.0	24.5	36.1	50.4	383.6	422.8
Stdev		7.4	0.3	30.4	13.7	8.3	6.6	13.8	98.1	80.9
± %		**15.1**	**35.3**	**10.1**	**15.6**	**33.9**	**18.4**	**27.4**	**25.6**	**19.1**

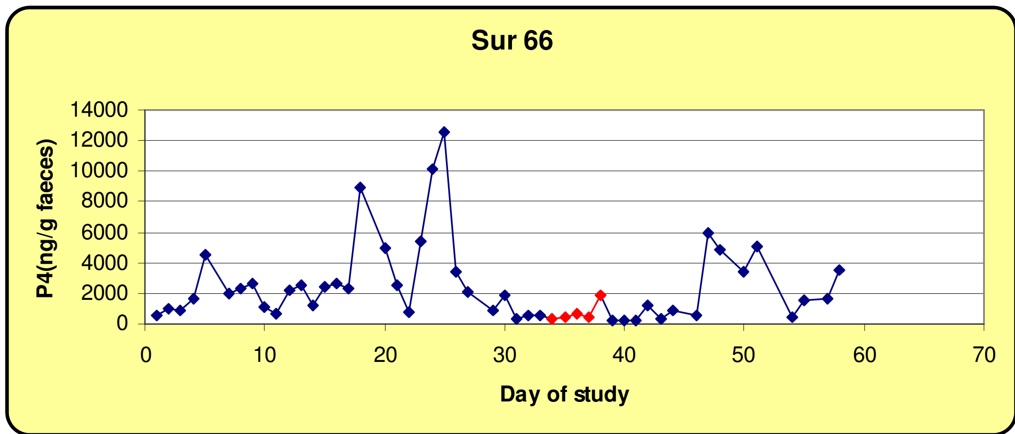
Notes: *Curve was poor so standard count values were back-calculated and adjusted
**Tried unsuccessfully to modify a poor standard curve

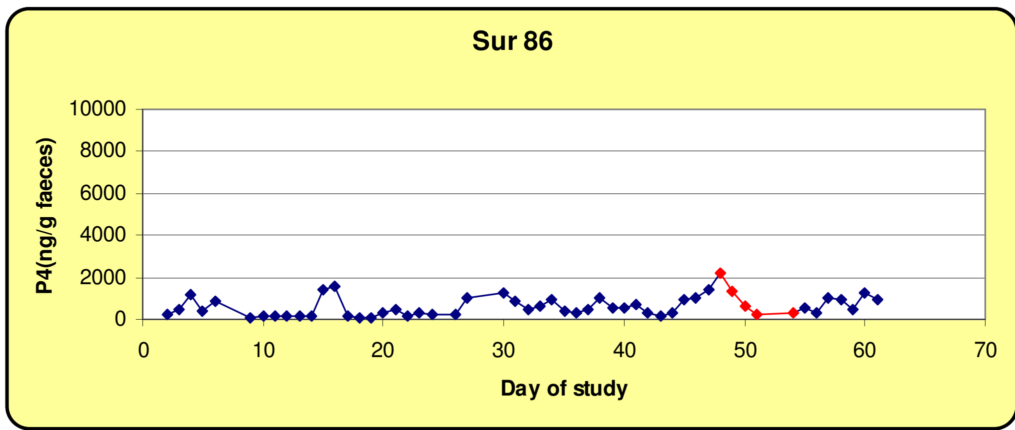
Appendix 9: Faecal progestagen traces for all animals included in **Chapter 4**

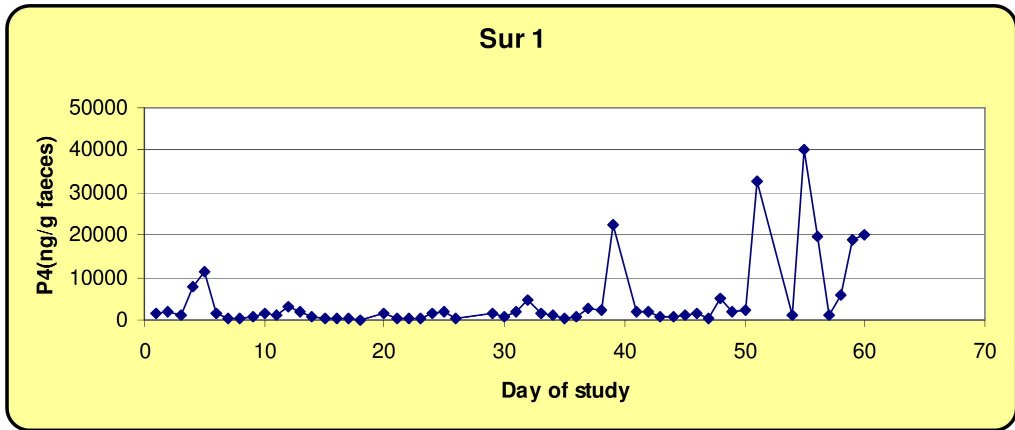


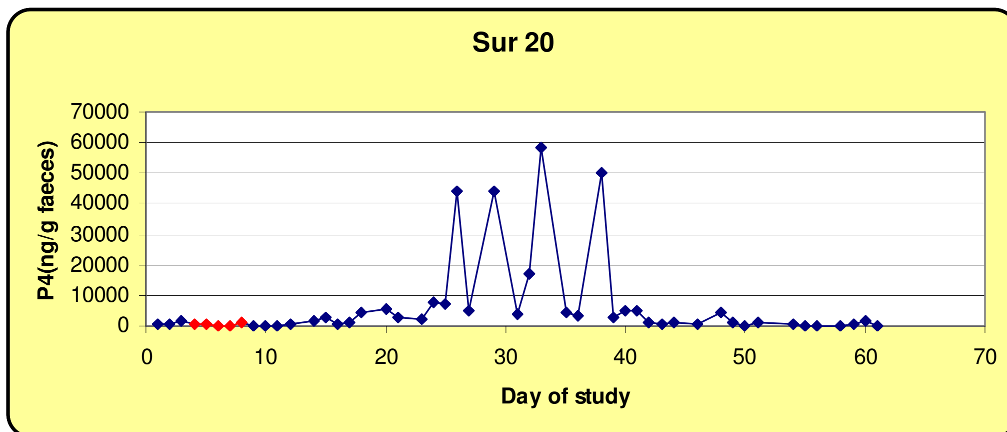


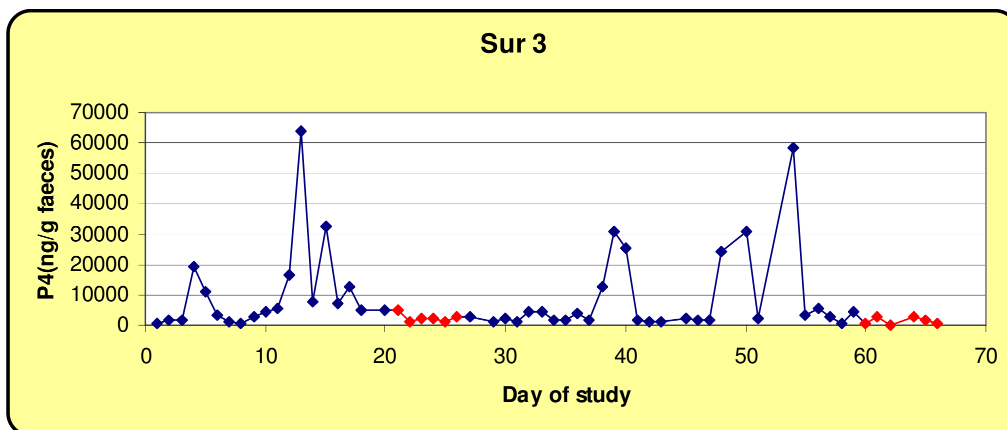


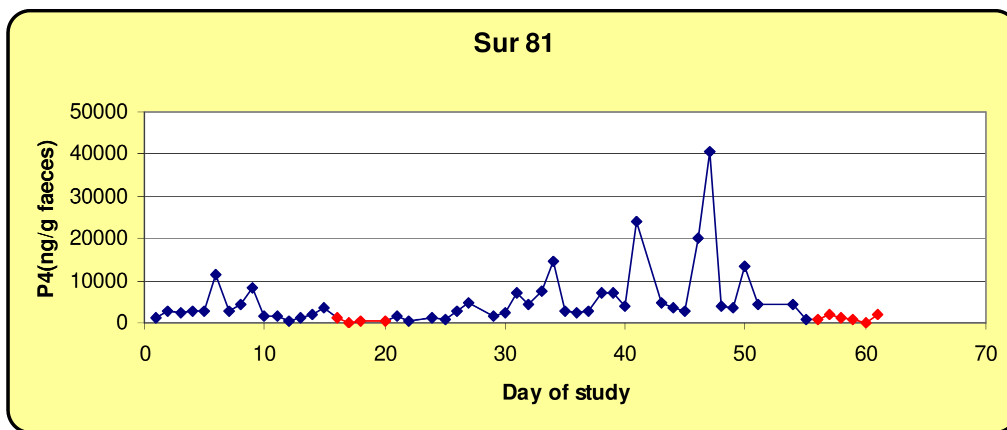

Sur 66
P4(ng/g faeces)
14000
12000
10000
8000
6000
4000
2000
0
0
10
20
30
40
50
60
70
Day of study

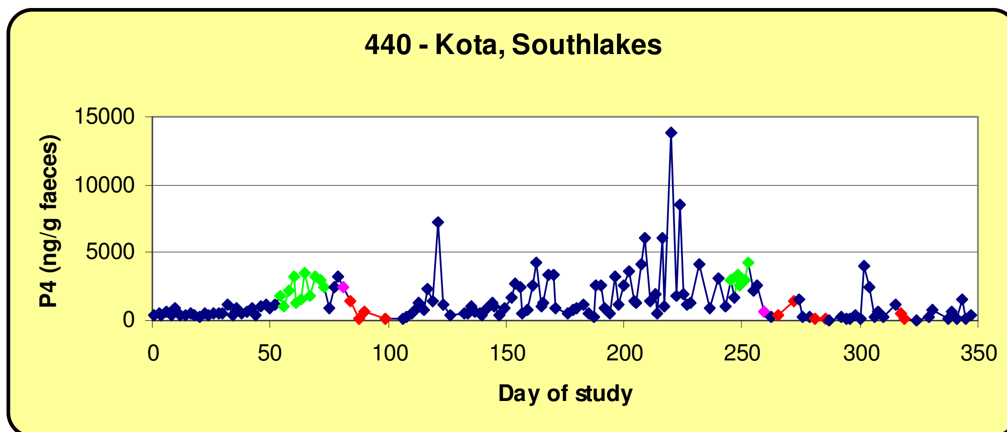

Sur 86
P4(ng/g faeces)
10000
8000
6000
4000
2000
0
0
10
20
30
40
50
60
70
Day of study

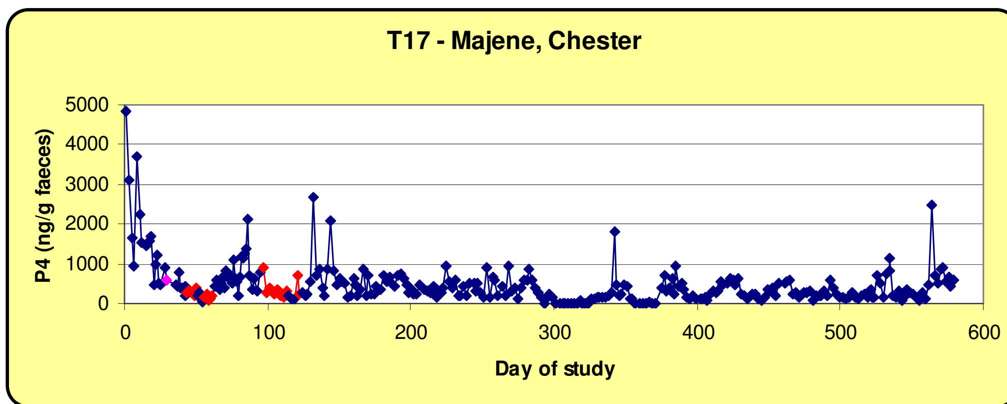

Sur 1
P4(ng/g faeces)
50000
40000
30000
20000
10000
0
0
10
20
30
40
50
60
70
Day of study

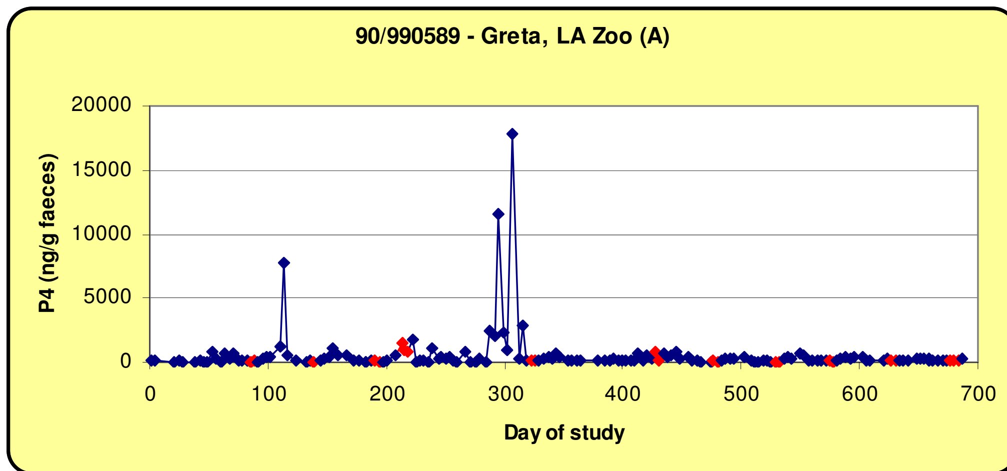

Sur 20
P4(ng/g faeces)
70000
60000
50000
40000
30000
20000
10000
0
0
10
20
30
40
50
60
70
Day of study

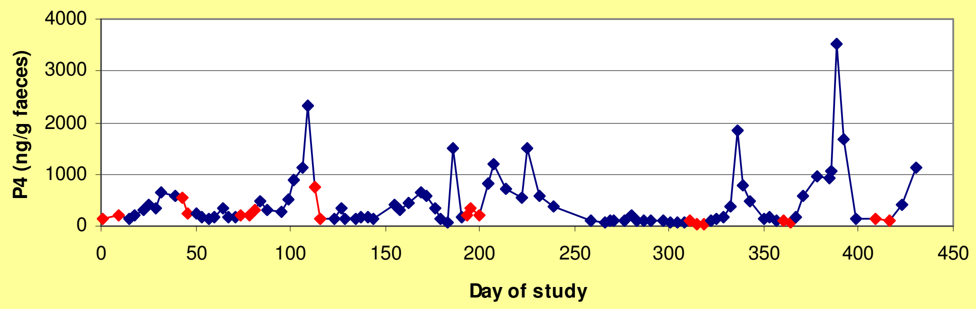

Sur 3
P4(ng/g faeces)
70000
60000
50000
40000
30000
20000
10000
0
0
10
20
30
40
50
60
70
Day of study

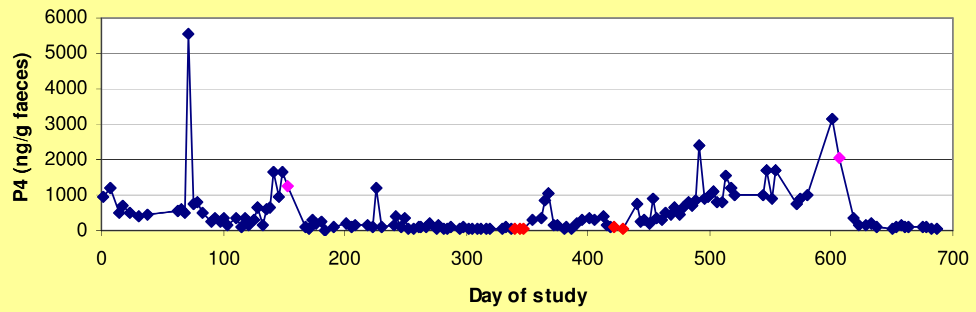

Sur 81
P4(ng/g faeces)
50000
40000
30000
20000
10000
0
0
10
20
30
40
50
60
70
Day of study


440 - Kota, Southlakes
P4 (ng/g faeces)
15000
10000
5000
0
0
50
100
150
200
250
300
350
Day of study


T17 - Majene, Chester
P4 (ng/g faeces)
5000
4000
3000
2000
1000
0
0
100
200
300
400
500
600
Day of study


90/990589 - Greta, LA Zoo (A)
P4 (ng/g faeces)
20000
15000
10000
5000
0
0
100
200
300
400
500
600
700
Day of study


90/990589 - Greta, LA Zoo (B)
P4 (ng/g faeces)
4000
3000
2000
1000
0
0
50
100
150
200
250
300
350
400
450
Day of study


104/991136 - Beret, LA Zoo
P4 (ng/g faeces)
6000
5000
4000
3000
2000
1000
0
0
100
200
300
400
500
600
700
Day of study

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PUBLICATIONS

1. The following chapter was published in 2008, a review of the biology of reproduction in pigs, peccaries and hippos, and is available here in full with permission from the publishers:

Ogle, S & A.A. Macdonald (2008). Die Fortpflanzungbiolgie im Überblick. In: Wilde Schweine und Flußpferde. Macdonald, AA & Ganslosser, U (eds.), Filander: 33-66.

2. This collaborative paper is currently in press:

Ziehmer, B., S. Ogle, et al. (2010). "Ovulation rate restricts litter size in the Babirusa (*Babyrousa celebensis*)." In Press: Theriogenology.