Reproductive Science and Integrated Conservation

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CAMBRIDGE UNIVERSITY PRESS
Cambridge, New York, Melbourne, Madrid, Cape Town, Singapore, São Paulo

Cambridge University Press
The Edinburgh Building, Cambridge CB2 2RU, United Kingdom

Published in the United States of America by Cambridge University Press, New York

www.cambridge.org

Information on this title: www.cambridge.org/9780521812153

First published in print format 2002

ISBN-13 978-0-511-06459-3 eBook (NetLibrary)
ISBN-10 0-511-06459-4 eBook (NetLibrary)

ISBN-13 978-0-521-81215-3 hardback
ISBN-10 0-521-81215-1 hardback

ISBN-13 978-0-521-01110-5 paperback
ISBN-10 0-521-01110-8 paperback

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Reproductive and welfare monitoring for the management of *ex situ* populations

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INTRODUCTION

Non-invasive reproductive hormone monitoring methods originated many years ago. Urine-based pregnancy diagnosis methods for humans and livestock species were developed in the 1920s (Cowie, 1948), to measure either gonadotrophins or oestrogens. However, these techniques were non-invasive only for the subject of study and relied on the assessment of physiological changes in other species (usually mice) following injection of the subject's urine. Chemical urinary pregnancy tests followed approximately 10 years later, but at that time were not considered reliable or accurate enough to be routinely used (Cowie, 1948). The advent of specific, immunological tests for identifying and quantifying hormones (Yalow & Berson, 1959) provided new opportunities for reproductive monitoring, allowing the development of the non-invasive techniques used today.

Quantifying hormone concentrations non-invasively has gained popularity within the conservation community over the last 15 to 20 years, as the practical and welfare implications of collecting blood samples from intractable and endangered animals have been recognised. Commonly, steroid hormone metabolite concentrations are measured in excreta, such as urine, faeces, saliva and sweat, by radioimmunoassay or more recently, enzyme immunoassay. In isolation, knowledge of the reproductive status of wild animals in captivity is a useful indicator of individual well-being. However, when integrated with other data it becomes a powerful management tool. As such, predictions about, for example, the outcome of an *ex situ* conservation programme, the success of an assisted reproduction programme, the point when available resources for housing animals will become

limiting, or the impact of the environment on an animal's physiology can be made.

One drawback of the wealth of information published about non-invasive hormone monitoring is the diversity of analytical methods adopted

Table 9.1 *Summary of the available literature describing non-invasive hormone monitoring in Rhinocerotidae*

Species	Sample	Metabolites measured	Source of information
White rhinoceros	Urine	20α-hydroxyprogesterone	Hindle & Hodges (1990); Hindle *et al.* (1992)
		Oestradiol-17β	
		Oestrone conjugates	Ramsay *et al.* (1994)
		Pregnanediol-3-glucuronide	
		C19/C21 progesterone metabolites	
	Faeces	20α-hydroxyprogesterone	Strike (1999)
		20-oxo-pregnanes	Schwarzenberger & Walzer (1995); Schwarzenberger *et al.* (1998)
		Progesterone metabolites	Radcliffe *et al.* (1997)
		11α-pregnanedione	Patton *et al.* (1999)
Black rhinoceros	Urine	20α-hydroxyprogesterone	Hindle *et al.* (1992)
		Oestrone	
		Oestrone conjugates	Ramsay *et al.* (1987, 1994)
		Pregnanediol-3-glucuronide	
		C19/C21 progesterone metabolites	
	Saliva	20α-hydroxyprogesterone	Czekala & Callison (1996); Thorne *et al.* (1998)
		Oestradiol	
	Faeces	20α-hydroxyprogesterone	Schwarzenberger *et al.* (1993)
		Pregnandiol-3-glucuronide	
		20-keto-progestagens	
		20-oxo-progestagens	Schwarzenberger *et al.* (1996b)
		Oestrogen metabolites	Berkeley *et al.* (1997)
		Progesterone metabolites	
Sumatran rhinoceros	Urine	Oestradiol	Heistermann *et al.* (1998)
	Faeces	Pregnanediol	Heistermann *et al.* (1998)
		5α-pregnane-3α-ol-20-one	
Indian rhinoceros	Urine	Total immunoreactive oestrogen	Kassam & Lasley (1981)
		Oestrone sulphate	Kasman *et al.* (1986)
		Pregnanediol-3-glucuronide	

by different laboratories. Each publication contributes to our knowledge of wildlife physiology, but frequently information produced by different organisations is difficult to compare. By way of example, Table 9.1 summarises the available literature relating to non-invasive analysis of reproductive hormones in rhinoceros species in captivity. Descriptions of progesterone metabolite analyses alone refer to the quantification of eight different progestagen groups, some of which have been measured by more than one author, but not necessarily using the same antibody or methodology. In many cases, the specific compound being measured by a particular antibody remains uncharacterised. While much can be gained from comparing relative differences, rather than absolute changes in sample concentration, strength will always be added to the conclusions that can be drawn from the data if like can be compared with like. For many conservation programmes financial resources are often limited. Therefore, an internationally coordinated approach to hormone monitoring, with an effort to ensure accurate use of steroid nomenclature and full antibody validation, would minimise duplication of effort and maximise the relevance and significance of each laboratory's output.

The practicalities of non-invasive monitoring procedures and the species to which they have been applied have been reviewed extensively by other authors (see e.g. Heistermann *et al.*, 1995; Schwarzenberger *et al.*, 1996a), and will therefore not be covered in detail in this chapter. There have been a number of integrated investigations, which have used non-invasive monitoring to help understand or explain other observations and enhance our efforts in conservation biology. Examples of these will be given. However, it should be recognised that data from non-invasive monitoring studies may be unable to resolve physiological problems. Nevertheless, the data provide a valuable diagnostic tool to aid our comprehension of biological events. The purpose of this review is to examine how the ability to monitor reproduction and stress non-invasively has contributed to the success of *ex situ* conservation programmes. The methods we use today are still far from ideal. Therefore, this chapter will also address some of their shortcomings and possible ways to overcome these in the future.

STATE OF THE ART

Genetic management

The benefits of applying assisted reproduction techniques in conservation programmes have long been recognised. However, the ease of application

and success of these techniques varies greatly among species. As such, net economic considerations should be taken into account when planning a programme of assisted reproduction. Often skilled personnel and specialised equipment are required, which may not be locally available. The cost of bringing these together should be judged against the overall likelihood of success.

Since 1995, the Institute of Zoology (Zoological Society of London) and the Estación Experimental de Zonas Áridas (Consejo Superior de Investigaciones Científicas) have collaborated to develop assisted reproduction and genetic resource bank technologies to support the conservation of the Mohor gazelle (*Gazella dama mhorr*). This species became extinct in the wild in 1968, but has survived in captivity since 1971 when remnant populations were brought together for *ex situ* conservation. Despite originating from just 12 founder individuals, this species has bred well in captivity, and provides a useful model population for investigating approaches to the conservation of endangered ungulates. The success of this *ex situ* programme has resulted in distribution of the population to different geographical locations, i.e. various zoos, where these subpopulations are likely to suffer genetic drift and further inbreeding. Artificial insemination and the establishment of a genetic resource bank were proposed to minimise these inbreeding effects (see Holt *et al.*, Chapter 17).

No data on the reproductive physiology of this or any closely related species were available at the outset of the programme, thus limiting the logical development of artificial insemination protocols. Initial attempts at artificial insemination of Mohor females, without investigations of endocrine physiology, had a limited degree of success (Holt *et al.*, 1996), resulting in 30% conception rates. Frozen-thawed semen was used, and the oestrous cycles of the females synchronised using exogenous progesterone. Therefore, several possibilities were available to increase the success of the manipulations. We undertook to characterise the endocrine attributes of the oestrous cycle by closely monitoring cyclic females in the presence of a vasectomised male, and conception and pregnancy after oestrous synchronisation and natural insemination (Pickard *et al.*, 2001a). Non-invasive faecal hormone analysis was used for this purpose to avoid stressing the animals.

Conception rates using natural mating were similar to those achieved by artificial insemination. The faecal progestagen analyses showed no evidence that the females had conceived and suffered subsequent early pregnancy loss. Investigation of each animal's life history demonstrated that under natural breeding conditions the fertility of the study group was also

approximately 30%. Therefore, we can surmise that some form of ovulation or fertilisation failure may occur in this species. Further detailed hormone analyses suggest that successful conception is related to periovulatory steroid hormone excretion. The ratio of faecal oestrogen: progestagen is positively correlated with the mean time to conception ($R^2 = 0.58$; $P = 0.046$) (Figure 9.1).

Using this information we now have the opportunity to revise predictions of future population growth, and the economic impact of undertaking an assisted reproduction programme for this species can now be assessed relative to a known fertility rate. While further investigations may find a way to improve the reproductive output of this endangered species, it is possible that this may only be achieved by undertaking invasive assessments of ovarian function and oocyte competence. These would currently be considered inappropriate.

Behaviour

Ex situ conservation programmes inevitably result in animals being housed in relatively unnatural conditions, which can adversely affect individual behaviour. Hand-reared individuals, or those raised in isolation, may relate better to humans than their conspecifics and may not develop the appropriate social skills to recognise and breed successfully with potential mates. Breeding pairs or groups may also be unable to exhibit their full repertoire of social behaviours due to restrictions in enclosure space. Mating attempts in zoos can be compromised, and in some cases (such as large felids), individuals are at risk of injury if not death owing to mate aggression. In contrast, in the wild, they would normally be able to escape.

In such circumstances, non-invasive hormone assessments can provide an indication of the best time to introduce individuals (e.g. during female sexual receptivity), to minimise risk of injury. Similarly, predicting when a female is likely to give birth allows separation of the father or isolation from the group, in cases where there is a high risk of infanticide. Preparation for intervention, where females have a history of failing to rear their offspring successfully can also be made, improving the likelihood of rearing a subsequent generation.

Behavioural observations are themselves a form of non-invasive monitoring which may have practical use when circumstances preclude the collection and analysis of endocrine samples. To be economically effective, batch sample analysis is usually undertaken, resulting in a retrospective,

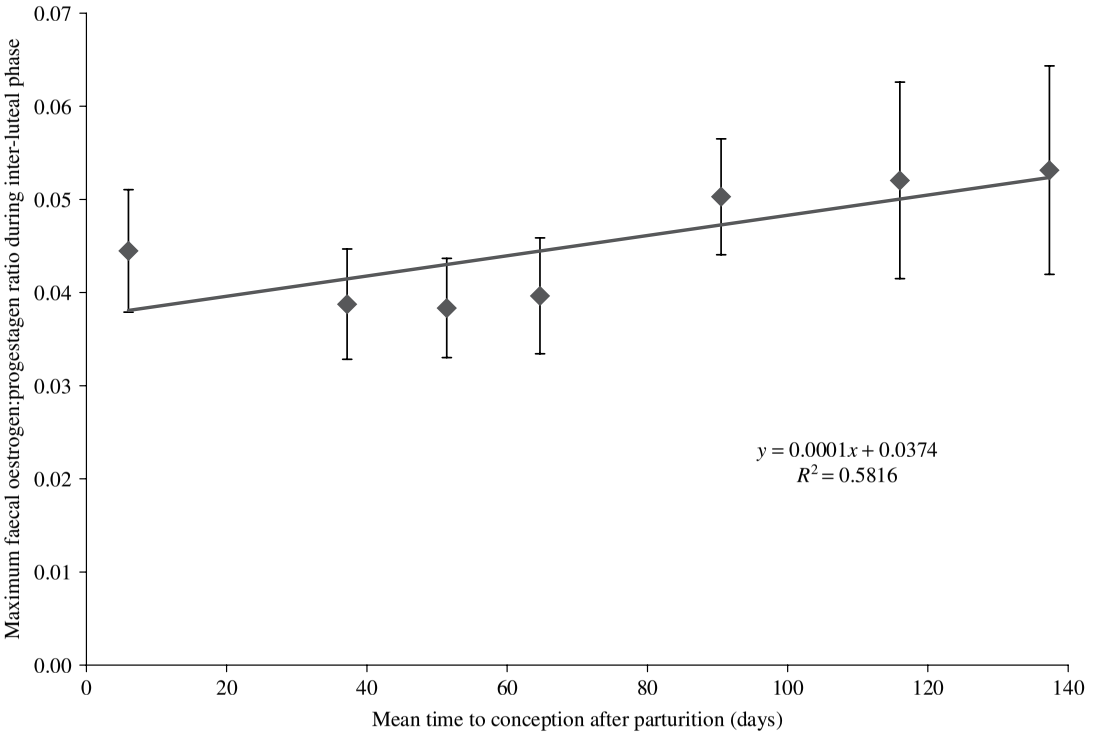

Figure 9.1 Correlation between maximum inter-luteal phase oestrogen:progestagen ratio and mean time to conception for seven female Mohor gazelles. Data are taken from life-history characteristics and represent 26 inter-gestational periods. (From Pickard *et al.*, 2001a.)

rather than real-time indication of an animal's physiological condition. In practical terms, the net benefits of reproductive monitoring are therefore reduced. With this in mind, as part of the investigations of Mohor gazelle physiology described above, we undertook a detailed analysis of the relationship between faecal hormone excretion and the behavioural interactions between cycling female gazelles and a vasectomised male. Our objectives were to quantitatively examine specific reproduction-associated behaviours to ascertain whether they could be used as predictors of reproductive success. Reproduction-associated behaviours were grouped into three categories: exploratory or testing behaviours, courtship behaviours and mounting behaviours.

Our results indicated that the frequency of mounting and copulation is positively correlated with peri-ovulatory faecal oestrogen concentrations ($R = 0.546$; $P = 0.01$) and the frequency of pre-copulatory, exploratory behaviours ($R = 0.744$; $P < 0.0001$) (Pickard *et al.*, 2001b). Since steroid hormone excretion at this time is related to reproductive success, observing the intensity of courtship interactions may provide a useful alternative to faecal hormone assessments for monitoring an individual's fertility.

Inbreeding

The effects of inbreeding are considered to be a major contributing factor to the accelerated decline of small, isolated populations (see Taylor, Chapter 5 and Koeninger Ryan, Chapter 6). Few studies have directly highlighted effects on reproduction in endangered species, but these effects are potentially exacerbated in captivity, where populations can only usually be kept in modest numbers and transfer of genetic material between populations is difficult because of geographical or physical barriers, including behavioural incompatibility. The relationship between inbreeding and population fitness varies considerably among species. A wide variety of factors such as mating strategy, generation time and extrinsically induced variations in population demography also impact on reproductive fitness.

Experimental manipulation of wild, domestic and laboratory species has, however, demonstrated effects of inbreeding on reproductive success. For example, territorial scent-marking behaviour in inbred male Mongolian gerbils is increased when compared with founder generations (Turner & Carbonell, 1984). Although this effect was found to be androgen-independent, the consequences for the reproductive success of outbred individuals might be significant if they were unable to maintain a territory.

Other studies have also shown that mechanisms of steroid biochemistry can vary in inbred versus outbred strains of rabbits (Schwab & Johnson, 1985), and that pituitary function differs in squirrel species which adopt different mating strategies to avoid inbreeding (Boonstra & McColl, 2000). In inbred mice, differences in oocyte maturation have been attributed to differences in the dynamics of ovarian steroid production (Krzanowska & Szoltys, 1996). It is possible, therefore, that the consequences of inbreeding may be detected through the use of comparative non-invasive hormone analysis in some cases.

Environment

Commonly, in *ex situ* conservation programmes the animals are substantially removed from their natural environment. Suitable foodstuffs may be unavailable locally, patterns of daylight and seasonality may be significantly altered and these, together with confinement, may all serve to affect breeding potential adversely. Nevertheless, physiological compensation usually occurs, and most individuals continue to reproduce, although it is difficult to determine whether optimal reproductive rates are achieved.

The negative effects of translocation to captivity may or may not be obvious immediately. For example, large proportions of wild-born Southern white rhinoceros (*Ceratotherium simum simum*) have failed to breed *ex situ*. Furthermore, captive-bred females also suffer from low birth rates (Göltenboth & Ochs, 1997), directly affecting the viability of the *ex situ* population. Rhinoceros managers predict a demographic crisis in coming years (Patton *et al.*, 1999), but the cause of this breeding failure has yet to be determined. However, the ability to compare physiological parameters between animals in their natural environment, with captive populations which have high rates of breeding success and those which suffer substantial reproductive failure, may provide an indication of the source of the problem. Schwarzenberger *et al.* (2000) have undertaken the first such investigation for the Southern white rhinoceros, and found that fertility in the wild is not compromised as it is in captivity. Nutrition, for example, could be a major factor limiting rhinoceros reproduction *ex situ*. Nutritional imbalances may compromise steroidogenesis and ovarian or testicular function and foetal development (see McEvoy & Robinson, Chapter 3). Locally available foodstuffs or synthetic substitutes may contain compounds such as phyto-oestrogens or tannins, which may mildly or severely compromise reproduction in some species. Such effects may be detected or monitored

by close examination of data generated through non-invasive reproductive assessments.

Contraception

Most frequently, reproductive monitoring is used to evaluate the success of captive breeding programmes or to diagnose infertility in specified individuals. Nevertheless, some captive breeding programmes are over-successful, resulting in more individuals than the zoo community can reasonably accommodate. Limiting breeding opportunities must then be considered, to prevent the problem being exacerbated. However, merely separating males and females presents its own problems, just as interfering with the social organisation of a breeding group can cause behavioural problems, as described above. Sterilisation represents one alternative, although this is normally considered irreversible and therefore not suitable for many endangered species breeding programmes. As discussed elsewhere in this book (see Rodger, Chapter 18 and Mate & Hinds, Chapter 19), contraception offers a potentially reversible method for limiting breeding.

A non-invasive, urinary assay for the contraceptive agent levonorgestrel was reported by Munro *et al.* (1996) for monitoring breeding in contracepted non-human primates, and Bettinger *et al.* (1997) demonstrated that the use of Norplant implants (which contain levonorgestrel) in chimpanzees did not adversely affect reproductive physiology or oestrogen excretion. However, the majority of studies that have used non-invasive hormone analyses to study the effects of contraception in captive wildlife are those performed within the scope of medical research. Few if any studies have been reported within the conservation literature.

Welfare monitoring in *ex situ* conservation programmes

The preceding discussion has related almost exclusively to the use of non-invasive monitoring techniques for assessing reproduction in threatened species in captivity. Significant attention in the literature and the zoo community has, however, recently focused on the measurement of glucocorticoid hormones for welfare assessment. A recent publication by Wasser *et al.* (2000), reports the use of a non-specific glucocorticoid radioimmunoassay capable of monitoring the excretion of cortisol metabolites in a wide range of mammalian and non-mammalian species, which provides new opportunities to monitor stress in individuals under different management regimes.

The principles of non-invasive glucocorticoid analysis are the same as those for monitoring reproductive hormones. There are significant advantages in monitoring stress by means of non-invasively collected samples. The samples can be collected relatively easily with minimal disturbance to the animals, and provide an integrated measure of the individual's stress physiology over the preceding hours or days, rather than a 'point-in-time' measure as would be generated from a blood sample. Furthermore, measuring glucocorticoid concentrations provides an indication of the sustained stress response to a stimulus, as opposed to the instantaneous response of the sympathetic nervous system, which releases catecholamines very rapidly following stimulation. However, caution should be used when interpreting the data generated from non-invasive glucocorticoid analyses, particularly if it is to be used for welfare assessments and to influence management practice.

Determining whether an individual is exhibiting a stress response is impossible without significant baseline data from each animal. It must be ascertained that the metabolite concentrations measured exceed the normal physiological range for that particular animal. For example, Jurke *et al.* (1997) reported three categories of baseline faecal cortisol metabolite concentrations in unstimulated female cheetahs, and individual sample concentrations approximately 10 times greater than the peak value recorded for an experimentally stressed male. In this example, the two females categorised as 'high in cortisol' also demonstrated impaired reproductive function, providing a reasonable indication that their biology was compromised. Nevertheless, other animals housed under the same conditions had significantly lower faecal cortisol concentrations and reproduced without intervention. In such cases, it would be difficult to advise on the suitability of the management techniques used for the group as a whole.

Glucocorticoid analyses are further complicated by the difference in physiological response to acute versus chronic stress. For example, acute glucocorticoid stimulation (e.g. by injection) can increase locomotor activity, whereas chronic glucocorticoid release (e.g. from a hormone implant) suppresses it. This can be relevant, for example, when studying competitive mating systems where males compete for access to females, and high circulating concentrations of glucocorticoid may, in this case, be positively correlated with reproductive success. Furthermore, the response to repeated stressors differs from that to novel stressful stimuli as the individual begins to exhibit a 'learned response' based on its ability to judge the physiological consequences of the stimulus. It is therefore vitally important to consider the circumstances under which glucocorticoid data are generated before

drawing conclusions about an individual's welfare. Researchers undertaking these studies would be best advised to consider their findings in conjunction with other objective physiological measures (such as conception rate or other endocrine measures) when interpreting their observations.

PRIORITIES FOR THE FUTURE

Many Western nations now have facilities for undertaking non-invasive hormone analyses. Improved international communications allow rapid shipment of samples and/or reagents without compromising their integrity, and the available laboratory technologies can provide the accurate, reliable data required by animal managers. However, the laboratories undertaking these analyses are often dedicated to scientific research and method development, and routine sample analysis using established assays is outside the scope of their remit. Unfortunately, there are limited resources to offer what essentially becomes a commercial service once techniques have been established and fully validated for a particular species, at a price that can be afforded by the conservation community. Therefore, there is significant scope for improving the efficiency of sample analysis, either in terms of economics, time or sample throughput, which would greatly increase the availability of this technology to conservation biologists.

Hormone analyses inevitably provide a retrospective indicator of an individual's physiological status. While this information is doubtless useful, in many cases more benefit would be gained from building up an ongoing profile of each animal's endocrine characteristics. The ability for the analysis to be conducted locally, without the need for specific technical expertise or complicated laboratory equipment, would provide significant new opportunities in this field. The need for import/export permits to transfer samples across borders, and the associated courier charges, would be removed, reducing some of the administrative and financial burden of generating the data. Conservation organisations would be in a position to make judgements about the need for, or frequency of, sample analysis depending on the results generated for a particular individual on a long-term basis and their specific data requirements at that time. Where, for example, assisted reproduction or infertility treatments are being attempted, a rapid indication of the efficacy of a protocol greatly improves the quality and relevance of the data resulting from it, and can make the difference between a successful intervention and a 'useful experience'.

The concept of a rapid, cheap 'stick-test' for generating data about endocrine parameters is very appealing to the conservation community.

However, to date little progress has been realised in achieving this aim. Our experiences with non-invasive methods have demonstrated that quantitative assessments of changing hormone concentrations are generally required for a full evaluation of species physiology, and that a simple colour-change, like that of human home pregnancy diagnostic kits, is inadequate. Nevertheless, recent developments in biosensor technology and the miniaturisation of circuits and electronic components may provide novel opportunities to develop equipment to meet our requirements. Preliminary trials (A. R. Pickard & T. T. Mottram, unpublished observations) indicate that biosensors being developed for the in-line analysis of progesterone in the milk of dairy cows (Pemberton *et al.*, 1998) will prove useful for monitoring reproductive status in endangered species. Extracts of faecal material from Mohor gazelles, when analysed by standard ELISA techniques and by prototype biosensor methodology, provide comparable longitudinal profiles, which indicate the future promise of developments in this area. Currently, prototype equipment is still at the bench-test stage, and further developments are required to standardise and automate sample preparation procedures in such a way that they can be undertaken in the zoo or field environment in a reliable and repeatable manner. Nevertheless, the prospect of portable endocrine monitoring equipment is more realistic than it was two years ago.

It should be recognised, however, that the development of biosensor or other real-time monitoring equipment will not necessarily provide an immediate generic system for non-invasive monitoring. Interspecies variability may mean that initial systems are more suited to some species than to others, in the same way that no single immunoassay system can be applied across all species. Furthermore, expert analysis of the data generated will continue to be required to maximise its practical use and value. This presents enhanced opportunities to improve our understanding of biological systems. With modern Internet communications, data could be collated centrally, and databases similar to those used in the genome mapping projects developed. By making this information available to the wider conservation community, new opportunities would arise: for example, to evaluate the positive and negative aspects of different animal husbandry systems, the effect of the environment on productivity or welfare, and to make revised predictions about population viability.

Clearly, current technology in non-invasive monitoring has opened new possibilities for the management of wild animals *ex situ*, but the role of scientists in this field is to press forward with the development of improved analytical techniques and methods for data interpretation. Conservation

scientists and reproductive biologists need to remain aware of technological developments in seemingly unrelated fields to be able to take advantage of new technologies as they arise. For example, advances in thermal image capture and analysis have already proved useful as a tool for diagnosing pregnancy in circumstances where non-invasive endocrinology is not readily available (Hilsberg 1998; A. R. Pickard, A. Ferguson & A. Tucker, unpublished observations). Collaboration with scientists in other disciplines will facilitate this and allow the potential of non-invasive monitoring to be fully realised within integrated conservation programmes.

ACKNOWLEDGEMENTS

Sincere thanks go to Drs Teresa Abaigar, Mar Cano and Toby Mottram for ongoing collaborations which contribute to much of the research highlighted in this text, and to Dr Bill Holt for having the inspiration and dedication to bring many conservation disciplines together.

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