Use of behavioural observations and faecal progesterone sampling to monitor reproductive cyclicity and pregnancy in captive South China tigers, with regard to breeding and rewilding

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Abstract

The objective of this study is to determine specific behaviours associated with different phases of the reproductive cycle in captive South China tigresses (*P. t. amoyensis)* and to measure progestagen concentrations in faeces of these females to determine the success of mating. This was aimed to assist a breeding and rewilding programme for South China tigers. Behavioural observations were performed twice daily over a period of 67 days and faecal samples were collected at least once weekly for 102 days. Data about behaviours such as general activity, spraying and social interaction, differed between individuals. Significant (*p*-value < 0.05) changes were associated with different phases of the reproductive cycle. Data from one tigress indicated an increase in activity during proestrus of almost 42%, but a decrease during estrus of almost 72%. Spraying frequency was higher during anestrus, 40.9 ± 7.9 times per active hour, but dropped by almost 59% during proestrus to 16.8 ± 3.3 times per active hour and was even lower during estrus, 6.2 ± 2.1 times per active hour. The positive social interaction frequency increased when the tigress entered proestrus and, simultaneously with the negative interaction frequency, increased further during estrus. Alterations in these specific behavioural patterns may therefore be useful parameters to determine estrous cycle stage in tigresses. Faecal samples were analysed for progesterone metabolites using a radioimmunoassay. Stress might have influenced the results slightly, but if measures are taken (double and fresh sampling) accurate data can be collected in future research. Progesterone concentrations were elevated up to parturition and were therefore useful for (pseudo)pregnancy determination.

Introduction

Tiger numbers are declining. Since 1940, three subspecies of tigers have become extinct. The Bali tiger (*P. t. balica*), the Javan tiger (*P. t. sondaica*) and the Caspian tiger (*P. t. virgata*) have disappeared in the 1940s, 1980s and 1970s respectively. The remaining six species are listed as critically endangered by the IUCN; the Amur tiger (*P. t. altaica*), the Indochinese tiger (*P. t. corbetti*), the Malayan tiger (*P. t. Jackson*), the Sumatran tiger (*P. t. sumatrae*), the Bengal tiger (*P. t. tigris*) and the South China tiger (*P. t. amoyensis*). In 1900 the total free-ranging tiger population was probably around 100000 animals; this has dropped to only 7000 by the year 2000. If current national tiger number estimates are summed a total of 3062 – 5066 tigers is estimated as the global tiger population. This suggests a decline of approximately 41% over the past decade (Chundawat et al. 2010, Big Cat Rescue 2011, Luo et al. 2004).

Of the extant subspecies, *P. t. amoyensis* (South China tiger) is listed as critically endangered by the IUCN, and is believed to be extinct in the wild. In the 1950's only approximately 4000 South

China tigers were left and in 1982 only 150 - 200 were estimated to remain in the wild. The IUCN states that no current verifiable evidence is available on the number of wild South China tigers, although sightings have been reported. Several studies support this statement (Tilson et al. 2004, Luo et al. 2008). In 2007 the number of captive South China tigers was estimated globally at 72 individuals (Chundawat et al. 2010) and by December 2009 the number had increased to 92 captive individuals (Zhang et al. 2011). The increase of tiger numbers are of importance to the survival of this species and can now only be achieved realistically by targetted breeding programmes.

Decreasing Numbers

The decrease in tiger numbers has been caused primarily by poaching for trade (for instance for Chinese Medicine), extensive habitat loss, prey depletion and killing of tigers as prosecution for loss of livestock. During Mao Zedong's reign tigers, wolves and other predators were declared and propagandized 'enemies of man' and persecuted by the army (Tilson et al. 2004, Xu et al. 2007, Charlesworth and Charlesworth 1987). According to the IUCN, 93% of the historical tiger range has been lost. The locations where tigers remain have been depicted by the IUCN on a map, as Tiger Conservation Landscapes, or TCLs. These are areas where the presence of tigers has been confirmed and where the habitat is sufficient to support at least five tigers. A total of 67 TCLs have been reported of which most (80%) are smaller than 10000 km^2. However, many TCLs also contain areas which are non-tiger habitat, such that the actual range of the tigers to live in is even smaller than the total TCL size. Fragments, areas where tigers occur but are considered too small to sustain a long-term population, have also been mapped; 543 have been mapped. The most common tiger habitat type is tropical or subtropical moist broadleaf forest, although the major requirement for a suitable habitat is an adequate prey base. Habitat loss has been established by conversion of forests to agricultural land or human settlements. The prey base has also suffered from land conversion, but is also affected by competition with domestic livestock. The size of a tiger's home range is partly dependent on the prey density. Home ranges of 20 km^2 are found where prey density is high, and over 450 km^2 where prey density is low (Chundawat et al. 2010).

Inbreeding

Land conversion leads to habitat fragmentation, such that tiger populations have become isolated, increasing the risk of inbreeding (Chundawat et al. 2010). Reduction in gene flow, genetic drift and human range contraction have all led to genetic partitions into different species and populations (Luo et al. 2004, Charlesworth and Charlesworth 1987). The problem of inbreeding is faced not only by free-ranging tigers, but also by those kept in captivity. In the 1990's, inbreeding became evident via a low reproductive rate (approximately 35.3%) and a high juvenile mortality rate (approximately 50%). Lethal factors mentioned were cold shock, accidental injury, stillbirth, foetal malformation, maternal rejection, inadequate lactation, disease and general weakness. In 2004 the highest inbreeding coefficient found was 0.59, and in 2007 the inbreeding coefficient was reported to range between 0 and 0.5. (Xu et al. 2007) even claimed that, according to studbook data, further elevations of inbreeding coefficients would result, for all possible parental pairing combinations. 58% of those combinations would result in offspring with an inbreeding coefficient of more than 0.25. The captive South China population are all progeny of only two males and four females, and is divided into a Shanghai line and a Guiyang line. The latter has become extinct after four generations, and the former, the more popular line for breeding, is likely to have suffered introgression of other subspecies. A phylogenetic study found evidence of zoo tigers, claimed to be *P. t. amoyensis*, to have indistinguishable lineages to *P. t. corbetti*. All factors mentioned above make conservation of the South China Tiger a great challenge (Zhang et al. 2011, Xu et al. 2007).

Breeding and Rewilding

For some tiger species, conservation management is used to ensure survival of the species in the wild. Unfortunately, this is not enough on its own, and breeding and reintroduction programs therefore have to be taken into consideration (Luo et al. 2004). Successful management of tigers depends on exchanging genes between populations, thereby maintaining the genetic diversity of the population. Reproduction is controlled by hormones, therefore information about the reproductive endocrinology of a species can be used to maximize breeding success. This information can be used to develop ovulation or pregnancy tests, indicate time of parturition and assist implementation of other reproductive techniques such as to supplement natural breeding. Assisted reproduction may be preferred or necessary if genetically valuable pairs won't mate due to behavioural incompatibility, or to prevent animal transportation, which carries significant risks. The ultimate is to preserve maximal genetic diversity (Brown et al. 1994).

Save China's Tiger is an organisation working on breeding and rewilding the South China Tiger. A couple of young tigers, male and female, were retrieved from Zoos in China and taken to a large reserve, Laohu Valley, in South Africa. A couple of years later, another two young animals were brought to Laohu Valley. The animals have been released into a 'wild and natural environment', and taught to hunt for themselves. They are also used as breeding pairs. The cubs are raised by their mother such that hunting skills can be passed on from parent to offspring. The goal is to send healthy offspring, which have proven to be capable of hunting successfully, back to China. There they will undergo a second phase of 'rewilding' and will be released into their natural habitat. Since putting tigers back into unprotected wild habitat is not an option, the organisation is working with the Chinese government to secure a protected reserve in China with natural habitat for the tigers to roam freely without human interference.

Relevance

Research on tiger reproductive activity is important for tiger breeding projects; in this case for the South China tiger. The most practical method of determining (the phase of) the estrous cycle is by measuring reproductive hormone concentrations in blood samples. However, collection of blood samples presents a logistical problem; while blood can be recovered from rewilded tigers, it is not sensible to anesthetize and immobilize the animals to collect blood on a regular basis. Creating a situation, in which it is possible to get close enough to the animal to collect diagnostic samples, is likely to affect the rewilding program negatively (Keeley et al. 2011). Sedation of the animal on a regular basis, is uncomfortable for the animal and can have affect the results. Stress related to such procedures could affect hormone levels, cyclicity and welfare. Furthermore the collection of the samples can be dangerous, complete safety of the collector cannot be guaranteed. Alternative, more practical methods for monitoring reproductive cyclicity are therefore required.

In this study two alternatives were studied; non-invasive collection of faecal samples suitable for hormone analysis, and behavioural observations to investigate whether certain behaviours, or changes in behaviour, can be used to indicate cycle stage. Faecal hormone analysis is already validated for several other mammalian, bird, reptile, amphibian, and fish species; both wild-ranging and captive, domestic and laboratory animals. The set-up of the current research, using captive wildlife, makes it possible to collect samples frequently and therefore validate the techniques. If techniques could be validated it may then be possible to extrapolate to a larger study to monitor reproductive cyclicity of wild or 'rewilded' tigers. Direct extrapolation of research on other species to the tiger is not strictly valid since there are species-specific differences, even in closely related species. For example, the reproductive steroid hormones, progesterone and estradiol, are metabolized differently in different species, before being excreted into the faeces or urine. The primary metabolites found in excreta therefore differ among species and can even differ within species. An example of this divergence was reported in a study on reproductive function of four rhinoceros species, which showed very different major faecal hormone metabolites and great differences in reproductive cycle length. Reproductive cycle patterns have been reported for less than 50% of felid species. This underlines the importance of developing species-specific faecal hormone assays (Schwarzenberger 2007, Brown 2010).

Research Question

The objective of this study was to determine whether it was possible to monitor the reproductive cycle of captive female South China tigers, in terms of the occurrence of ovulation and establishment of pregnancy or pseudo-pregnancy, using behavioural observations and faecal progestagen sampling and analysis. The keeper of the tigers wanted to be able to predict the phase of the cycle in the female tiger by monitoring specific behavioural changes, and be able to adapt management practices on that basis.

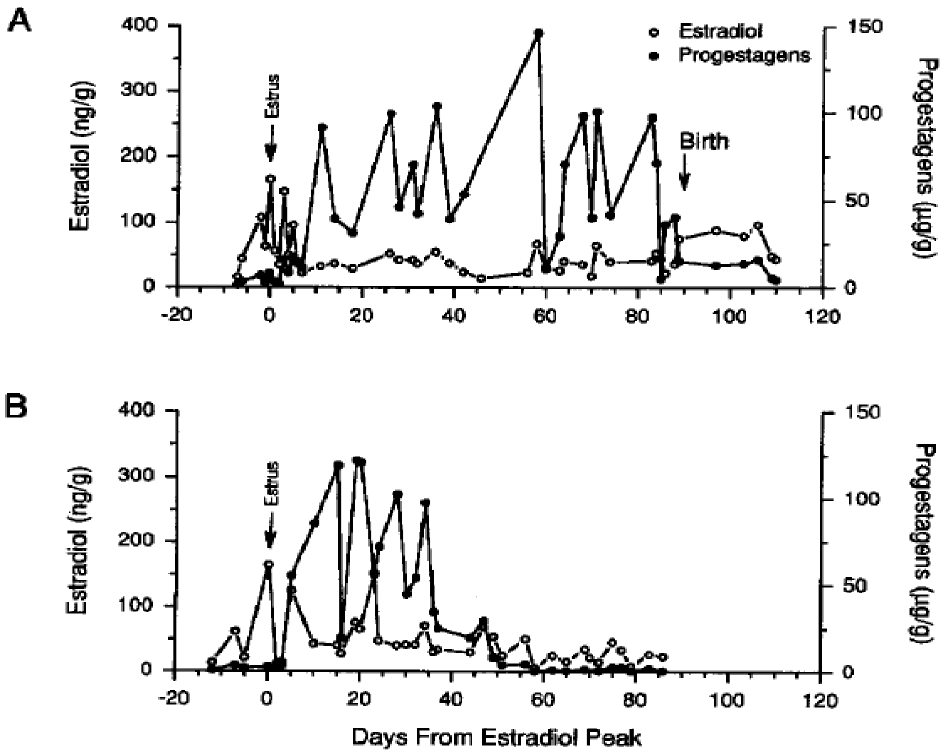


Figure 1: This graph from Brown (2010) *shows the faecal estradiol and progestagen concentrations in a clouded leopard during pregnancy (A) and pseudo-pregnancy (B) after natural mating; the pregnancy yielded four cubs. The data were aligned to the estradiol peak (Day 0).*

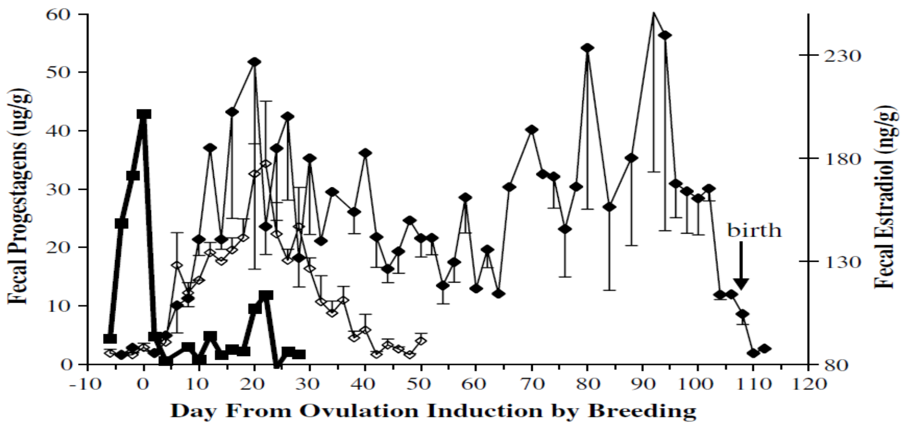


Figure 2: This graph from Graham et al. (2006) *shows the faecal estradiol concentrations (■) during estrus and breeding (n=8) and faecal progestagen concentrations during the subsequent non-pregnant luteal phase (n=4) (◊) or pregnancy (♦) (n=4) in tigers. Day 0 is the estimated day of breeding that induced ovulation based on faecal progestagen concentrations.*

The other objective was to use the faecal progestagen concentrations to a) confirm successful mating b) distinguish between pseudo-pregnancy and pregnancy and c) predict the date of parturition.

The working hypothesis was that behavioural changes would indicate specific phases of the estrous cycle, and that faecal progestagen concentrations would be a good indicator of successful mating, help distinguish pregnancy and pseudo-pregnancy, estrus and anestrus and predict the date of parturition.

Cyclicity

Before steroid hormones are excreted in the urine or through bile into the faeces they are metabolized in the liver. These metabolites can be re-absorbed via the enterohepatic circulation during intestinal passage. The re-absorption creates a time-lag between steroid circulation in the blood and their appearance in the faeces; this time-lag correlates with the time for passage from bile to rectum. Consequently, faecal steroid concentrations indicate 'average' endocrine activity over the previous hours with less interference from diurnal fluctuations (Schwarzenberger 2007). In domestic cats, 95% of reproductive steroid metabolites are excreted in the faeces. Seal et al. (1985) similarly reported that a very small proportion of steroid metabolites enter the urine of Siberian tigers.

Domestic cats are seasonally polyestrus, long-day breeders; other felids follow a similar general pattern, some more closely than others, and while some are short-day breeders (Kutzler 2007, Pelican et al. 2008, Michel 1993). Four phases of the estrous cycle are described for felids: proestrus, estrus, diestrus and anestrus (or interestrus) (Brown 2010, Adachi et al. 2010). Proestrus is the phase of follicular growth, associated with a rise of estradiol in the blood, estrus is the phase of expression of mating behaviour possibly ending with ovulation, diestrus is the phase in which the animal is pregnant or pseudo-pregnant, and anestrus is the dormant phase between cycles.

The length of anestrus differs between tiger sub-species. Graham et al. (2006) reported anestrus durations ranging from 50 to 148 days. During proestrus and estrus, the expression of mating behaviour is stimulated under the influence of rising estradiol concentrates in the blood, derived from the growing follicles (Meyerson 1964). Estradiol is secreted by ovarian follicles under the influence of follicle stimulated hormone (FSH) produced by the anterior pituitary gland, which is in turn stimulated by gonadotropin releasing hormone (GnRH) from the medial basal hypothalamus (Brown 2010). During proestrus, the female will look for a mate, and *vice versa*. In the wild, female felids spray more when they are cyclic (Mellen 1993). Pheromones in the urine may, for example, indicate to potential mates that a female is coming into estrus (Brown 2010, Schaller 1972). Graham et al. (2006) suggested that the onset of estrus is indicated by faecal estrogen concentrations being elevated above baseline for at least 2 days with no increase in faecal progestagen. Brown (2010) proposed that the onset of estrus is indicated when faecal oestradiol exceeds baseline values by more than 50%. Certainly, elevated faecal estrogen concentrations is associated with behavioural estrus. The duration of estrus in tigers is approximately 3.2 days, although a 5.3 day estrus has been reported in Siberian tigers (Seal et al. 1985) Baseline faecal estrogen concentrations have been reported as 65.8 ng/g, with peaks reaching 167.4 ng/g. Female tigers that are housed in the company of a male show higher peaks, approximately 262.3 ng/g. The presence of a male may enhance follicular estradiol production, caused by male pheromonal cues in excreta. This 'male effect' on reproductive function has also been reported for other species (Graham et al. 2006, Michel 1993, Hawken et al. 2009). During estrus, a 3-fold increase in faecal estrogen concentrations have been observed in cheetahs, which were highest in the periovulatory interval (Brown 2010) and a 5-fold increase in clouded leopard. During estrus, behaviours like vocalization, rolling and rubbing are more frequent (Brown 2010).

The baseline faecal progestagen concentrations has been reported as approximately 2.1 µg/g. Tigers, like all felids, are induced ovulators, meaning that ovulation and the rise in faecal progestagens only occurs after mating (Brown 2010, Tsutsui et al. 2009). In the absence of ovulation, time between estradiol peaks differs among individuals and varies from 6 to 40 days with a mean of 18 days (Graham et al. 2006) or 24.9 days (Seal et al. 1985). Ovulation is presumed to have occurred if faecal progestagen concentrations are elevated for at least one week. In natural breeding tigers, progestagen levels rise about 4 days after mating while faecal estrogen drops to baseline within 2 days. To put this into context, in the domestic cat, ovulation occurs 24 - 48 hours after mating, and there is a time-lag of 24 - 48 hours before blood steroid hormone peak concentration is detected in the faeces (Graham et al. 2006). Brown (2010) reported a rise in faecal progestagens within two weeks after ovulation in cheetahs, within 5 days for leopard cats, and within 6-7 days for snow leopards. This latter study also reported similar baseline faecal progestagen concentrations among these three species, and are therefore assumed comparable with the tiger. In the absence of pregnancy, the luteal phase lasts approximately 34.5 days, compared to 104 - 108 days before parturition in pregnant animals (Seal et al. 1985; Graham et al. 2006). Brown (2010) reported that cheetahs display an elevation in faecal progestagen concentrations up to day 30 of gestation and thereafter gradually declines back to baseline by the time of parturition (97 days), compared to a decline to baseline by day 60 in the case of pseudo-pregnancy. For tigers, it is considered that continued elevation in faecal progestagen beyond 35 days after mating indicates pregnancy (Graham et al. 2006). Differences in progestagen profiles between non-mated, pseudo-pregnant and pregnant animals are shown for the clouded leopard (Figure 1) and Amur and Sumatran tigers (Figure 2).

Wild lionesses generally conceive again about 20 months after parturition, but litter-interval tends to be shorter for captive animals because of removal of the cubs from the mother for hand-rearing or because of reduced pressure of predation, prey and social stress. This is likely to be the same for tigers, where females will occasionally conceive within a month after parturition (Bertschinger et al. 2008).

Follicular growth in felids can be stimulated by administration of equine chorionic gonadotropin (eCG), and ovulation can be induced using human chorionic gonadotropin (hCG), after which artificial insemination (AI) can be performed (Graham et al. 2006, Kutzler 2007, Pelican et al. 2008, Brown et al. 1995). Artificial induction of follicle growth and ovulation results in higher faecal progestagen and estrogen concentrations that persist for a longer period than after natural mating, indicating ovarian hyperactivity. Unfortunately, AI is not very successful in tigers. Graham et al. (2006) reported a success rate of < 5%, and suggested that oviductal embryo transport may be disrupted by the supra-physiological estradiol concentrations. Brown (2010) showed a 9-fold increase in faecal estrogen concentrations after gonadotropin treatment and AI in two female snow leopards, followed by a second faecal estrogen peak one week later. Both treatments resulted in a pseudo-pregnancy. Tigers, stimulated with gonadotropin, show different endocrine patterns in faeces than those who are naturally mated. Especially the estrogen concentrations were higher and remained higher after administration of gonadotropin. As a result of the poor success rates, AI is not commonly used in tiger breeding projects.

Material and Methods

Due to the time schedule, not all of the results of the faecal progesterone sampling can be shown in this report. However, this subject was reviewed in detail in the antecedent text. The initial faecal steroid hormone results will be discussed.

Study Sites and Camps

The study was conducted at the Laohu Valley Reserve in Philippolis, Free State, South Africa. The reserve is 33000 hectares in size with 300 hectares dedicated to the breeding and rewilding of the South China tiger (Figure 3). The area dedicated to breeding and rewilding is divided into several camps of different size. In Appendix A an overview of the main tiger camps, the temporary accessory camps and their

characteristics can be found (Pitsko 2003). The two biggest camps, 100 hectares and 40 hectares, are used for the actual rewilding. In these camps the tigers are able to hunt free-ranging prey.
All the camps are fenced with electric fences working on solar power. The inner fence is approximately 50 cm high, the outer fence is approximately 3 m high and the two are approximately 40 cm apart. The electricity is generated by solar energy, with spare batteries available in case of bad weather. This way there is almost always an electrical current on the fence. Voltages can differ due to shortages, caused by vegetation touching the wire, or animals (usually bugs, incidentally tortoises), or due to lack of sunlight, causing depletion of the batteries. At full power, the fence runs a current of 8500 volts. On contact this will give a painful shock, but without damaging the tiger and will primarily scare the tiger off. The gates can also conduct an electrical current, but are usually left uncharged. Most camps can be entered at various gates. Gates used to let tigers from one camp to another are approximately 5 meters wide, 3.5 meters high, run on rail, and can be operated by a rod from outside the camp.
Some of the camps have a stream running through, which provides water all year round. Other camps, with less reliable or no natural permanent water source, have at least one water trough, which is checked every morning and afternoon and refilled if necessary. Water is pumped from the ground by a solar powered pump and stored in a water tank, from which pipes lead to the troughs.

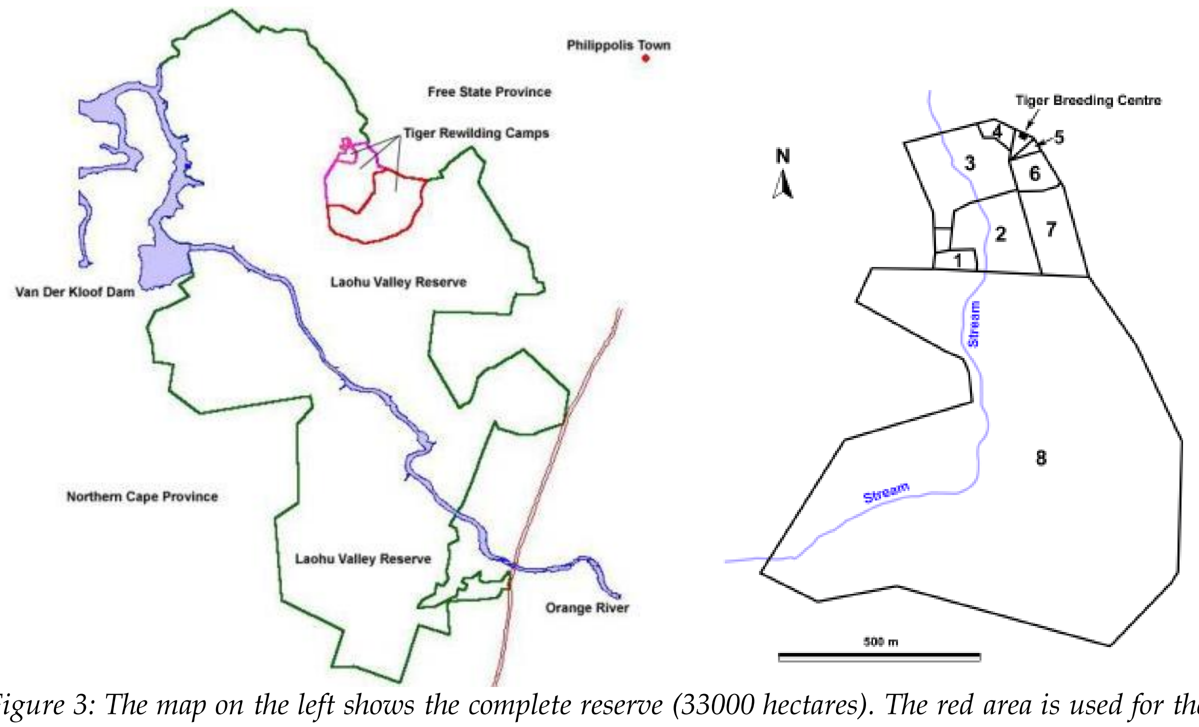


Figure 3: The map on the left shows the complete reserve (33000 hectares). The red area is used for the tiger breeding and rewilding project. The map on the right is an enlargement of the tiger camps. Different camps are indicated with different numbers. Number 8 actually consists of two camps, and camp 4 and the Tiger Breeding Centre are the camps that were used primarily for the tigers in this study.

Female Tiger Subjects

At the start of the study there were nine adult tigers present at the reserve, six males and three females. One of the females was the offspring of one of the subject females, and was not included in this study. This female was also too young to be included in the current study. General information about the other two females, the study subjects, can be found in Table 1, together with previous breeding information. Preceding this study, Cathay was last observed in estrus from 17th – 19th October 2010.

General Information Female Tiger Subjects

Name	Age	Origin	Date Arrival
Madonna	7 years	Shanghai Zoo, China	29 Oct 2004

Date Mated	Mated to	Nr of Pregnancies	Nr of Pseudo-pregnancies	Nr of Litters / abortions	Litter Sizes	Live Cubs per Litter	Dead Born Cubs per Litter	Rejected Cubs	Cubs Dead < 1 yr old
Jan 2008	TigerWoods	2	?	2/0	2;2	1;2	1;0	0;0	2;0
May 2008	TigerWoods								

Name	Age	Origin	Date Arrival
Cathay	8 years	Shanghai Zoo, China	2 Sep 2003

Date Mated	Mated to	Nr of Pregnancies	Nr of Pseudo-pregnancies	Nr of Litters / abortions	Litter Sizes	Live Cubs per Litter	Dead Born Cubs per Litter	Rejected Cubs	Cubs Dead < 1 yr old
Aug 2007	TigerWoods	5	?	5/0	1;2;1;1;2	1;2;1;1;2	0;0;0;0;0	0;0;0;1;0	0;0;1;0;0
Dec 2007	TigerWoods								
Sep 2009	327								
Oct 2010	327								
Apr 2011	327								

Table 1: General data for the two female tigers, Cathay and Madonna, including reproductive data as far as known, e.g. observed matings (resulting in successful pregnancies), with which male, occurrence of abortion, litter sizes and cub survival.

Natural matings with male 327 were observed, and on January 31st she gave birth to a single female cub, Huwaa. Unfortunately, Cathay rejected Huwaa, and the cub was subsequently hand reared in Lori Park Zoo, Johannesburg. Huwaa arrived back on May 14th and was reintroduced to her mother during the final week of this study. Madonna was last seen in estrus during October 2th - 5th 2010. During this estrus, Madonna mated with the males 327 and TigerWoods. During the study period, Madonna was in estrus on 4th March 2011; natural matings with TigerWoods were observed. This suggests that the previous estrus did not result in pregnancy. During the study she has not produced any litters. Both tigers were considered to be in good breeding condition, and representative information about cyclicity was therefore expected.

Behavioural Observations and Ethogram

Behavioural observations were conducted to identify possible associations between changes in the behaviour and hormonal status. Particular emphasis was placed on estrus, pregnancy and pseudo-pregnancy. Observations were conducted by *focal sampling* (Lehner 1998) every day from 14th March to 20th May 2011, at least one hour after sunrise and one hour before sunset for each female tiger; i.e. at least four hours a day. Observations at night are difficult and were considered unlikely to generate information vastly different to that from observing early in the morning and just before sunset, when the tigers are presumed to be most active (Schaller 1972). During the rest of the day tigers are known to exhibit only limited activity. To verify this presumption, observations of (almost) the whole day were conducted. During apparent estrus, elongated observation periods were used to see whether the activity of the female tigers changed.

Observations were recorded via a customized ethogram. The ethogram had to meet certain criteria; it had to include all behaviours, so analysis of aspects other than those focussed on in this study could be performed at a later stage. The ethogram included seven different behavioural groups; (1) 'Exploring/hunting behaviour', (2) 'Resting/common behaviour', (3) 'Homeostatic-related behaviour', (4) 'Communication behaviour', (5) 'Mating behaviour', (6) 'Parental behaviour' and (7) 'Stereotypic or other behaviour'; each containing behaviours that can be readily observed and distinguished [1]. For all behaviours, a detailed description was made to avoid bias or subjectivity of the observer[2] (Keeley et al. 2011, Mellen 1993, Pitsko 2003, Sveberg et al. 2011, Mega and Mellender de Araújo 2010). The notation of an observed behaviour on the ethogram is different to a standard ethogram. In a standard ethogram, the number of various behaviours displayed are summarized. In this study, the time at which a behaviour was observed was noted (a sort of *continuous recording*; according to Lehner 1998); from this the total number of occurrences and the duration of the behaviour could be calculated. The general circumstances of the observation were noted (who performed the observation, weather conditions, other tigers present in the same camp or in the surrounding camps and human influences). Special occurrences, that need further clarification, were recorded in a special section. This included time and amounts of feeding, explanation of interactions between tigers (Mega and Mellender de Araújo 2010), samples collected, and so forth.

Behavioural Analysis

The ethogram sheets were digitalized in Excel©. Data were reported as means ± SD. Certain behaviours were examined as frequencies and analysed for their duration; e.g. 'playing' was examined for its frequency and duration. The duration of a behaviour was determined by putting the behaviours in order and calculating how much time was dedicate to a given behaviour. For this, only behaviours that were examined for their durations were taken into account; such as 'walking', 'stalking', 'slow chase', 'fast chase', 'swim', 'rest belly', 'rest side',

[1] The ethogram sheets can be found in Appendix B.

[2] The list of descriptions of the behaviours can be found in Appendix C.

'rest back', 'laying in water', 'grooming', 'sitting', 'standing', 'eating food', 'plucking food', 'playing', 'play-fight', 'fight', 'feeding cubs', 'grooming cubs', 'pacing' and 'lethargic'. The sum of the durations of these behaviours accounted for the total observation time. If, for example an animal paces and sprayed whilst pacing, the spray was included in the pacing time when observing the order of the behaviours. If an animal was spraying whilst standing, the time for the spraying was included in the time standing. The duration of a spray or head rub to an object was not considered to be of extra interest.

The behaviours examined for their duration were arranged in order of expected energy costs, in Table 2. According to this order, they were given a fictional nonparametric value, ranging from 1 'inactive' to 7 'very active'. The higher the number, the higher the expected energy costs. Based on these values, a mean relative activity level was calculated for every minute of the whole-day-observation. The mean activity level during the morning (07.00 – 11.00), afternoon (11.00 – 15.00) and evening (15.00-18.00) were compared (taking 13.00 as the hottest part of the day). A nonparametric equivalent of a repeated-measures analysis of variance, the Friedman's test, was used to evaluate the differences in activity over the day (Bashaw et al. 2007, Petrie and Watson 2006). Furthermore, differences between subjects and between different phases of the estrous cycle were compared.

Relative Activity Level											
Activity	Lethargic	Rest back	Rest side	Rest belly	Lay in water	Sit	Stand	Grooming	Pluck food	Eat food	Grooming cubs
Activity Value	*1*	*1*	*1*	*1*	*1*	*2*	*2*	*2*	*3*	*3*	*3*
Activity	Feeding cubs	Walk	Swim	Stalk	Slow chase	Pacing	Play	Play-fight	Fast chase	Fight	
Activity Value	*3*	*4*	*4*	*4*	*5*	*5*	*6*	*6*	*7*	*7*	

Table 2: Estimated energy cost per activity, expressed with a fictional nonparametric value from 1 (low energy cost) to 7 (high energy cost).

On some occasions, such as sudden extreme rainfall, it was not possible to observe for exactly an hour, and sometimes an observation period lasted more than an hour. To amend for this, the frequencies calculated from the observations were converted from 'times per observation period' to 'times per hour'. Although observations were conducted in the morning and evening to enhance the likelihood of observing an active animal, long periods of inactivity were still observed sometimes. Observations lasting less than half an hour, and with less than 15 minutes of activity (25% of the observation time), were excluded (scenario 1). Additionally, spraying frequencies were converted from 'times per observed hour' to 'times per active hour'; active time included the total time a tiger spent walking, stalking, chasing, swimming, standing and pacing. Activities like resting, grooming, sitting and eating were not regarded as 'active', because no behaviours of interest (e.g. spraying or head rubbing) take place during such activities Subsequently, frequencies for the morning and evening observations were combined to an average. Differences in frequencies during different phases of the cycle (proestrus, estrus, diestrus (pseudo-)pregnancy and anestrus) were then compared.

During observations, the impression arose that certain occurrences influenced the behaviour of the animals, examples were 'being locked in a smaller enclosure to allow faecal sample collection', 'being fed' or 'more people present than the animal is used to (threshold set at five people)'. These events were therefore noted in the ethogram. Observations in which such an event (noted in the section 'happenings') occurred, persisting for more than ½ of the observation time, were excluded (scenario 2). Changes in behaviour after feeding of a big meal were also observed. Regression analysis was calculated for the spraying-frequency and the days after feeding to conclude this (Sveberg et al. 2011). Observations that occurred < 0.5 hour after feeding of a big meal (more than a quarter prey) were also excluded (scenario 3). The associated graphs can be found in the results.

In the wild, the amount of spraying gives information about a female tigers estrous cycle stage; therefore, extra attention was paid to this specific behaviour. In the wild, female tigers spray more frequent when in proestrus and estrus. The mean spraying frequency was calculated for proestrus, estrus, diestrus and anestrus for Cathay, who passed through all phases of the cycle during the study. The frequencies in the various cycle stages were then compared with each other. An overview of other scent-marking activities was also made.
Interaction frequencies with other individuals were plotted in a graph, with positive interactions given as times/hour and negative interactions as (times/hour)*-1.

Faecal Sample Collection

Faecal samples, to be analysed for progestagen concentrations, were collected from both adult females at least once a week during anestrus. The collection of faecal samples started on 20th December 2010, and will continue exceeding this study period. Samples collected up to 1st April were used for this study. Considering the feeding schedule, Thursday was set as the day of faecal sample collection. During apparent estrus, extra faecal samples were collected for additional analysis for estrogen concentration, although the results were not available for this report. The underlying intention of the project was to breed tigers to increase their numbers; therefore disturbances were to be minimized to avoid influencing matings and compromising breeding success. Interruption of the mating process, by for example separation of the male and the female to allow faecal sample collections, could prevent matings or reduces the number of matings and therefore lower the likelihood of ovulation, given that tigers are induced ovulators and multiple matings are needed to ensure ovulation (Tsutsui et al. 2009).
One of the females, Madonna, was introduced to different males during the first period of the behavioural observations to find a good mating partner. This strategy, introducing different males to a female in turns, is not well researched and there was therefore interest in the amount of stress associated with this procedure. Therefore, additional faecal samples were collected from this female to analyse cortisol levels. Those results were also not available for this report.

The number of samples was dictated by the opportunity for collection. This was complicated, since it was not always known or observed when a tiger defecated. She could be out of sight at the moment of defecation or the observer may not have been present (during the night for example). Observations indicated that the female tigers defecated 0.17 ± 0.29 and 0.09 ± 0.16 times an hour, i.e. once every 5.9 and once every 11.1 hour. The chances of observing defecation during two one hour observation periods a day was therefore limited. Measures were therefore taken to improve the likelihood of observing defecation; e.g. the feeding schedules were adjusted. The tigers were fed a small portion of food (i.e. a quarter or half a prey, instead of a whole one) the evening before sample collections to stimulate the digestive tracts. Both females were also locked into a smaller part of the camp to facilitate observation of defecation. The following morning the behavioural observations were performed. If no defecation had taken place, extra food was offered to stimulate the digestive track again. The tiger was then observed for the rest of the day. If defecation did not take place or was not expected, and there was faeces present from the preceding night, a sample of this material was collected.

The time of faecal collection was noted on the ethogram sheets. The tiger which defecated, the time of defecation (if known), the time of collection, whether it was fresh, probably fresh or an overnight sample, what it would be analysed for, and any abnormalities of the faeces were noted. On the collection bottle, were noted which tiger the sample was from, the date, the time of defecation and whether it was a fresh or an overnight sample. The faecal samples were then frozen immediately at -20 °C.

Faecal Sample Analysis

Faecal samples were transported to Onderstepoort Veterinary School, the University at Pretoria, for analysis of hormone metabolite concentrations. The faecal samples were prepared as described by Bertschinger et al. (2008), i.e. lyophilisation, pulverization, and extraction. First, 0.2 g of faecal powder was boiled with 5 ml of 90% ethanol:distilled water for 20 minutes. After centrifuging at 500 x g for 10 minutes, the pellet was resuspended and boiled again in 5 ml of 90% ethanol:distilled water. The supernatants were combined and dried, before being redissolved in 1ml methanol. The extracted samples were then vortexed for 1 minute, placed in a glass cleaner for 30 seconds to free particles adhering to the vessel wall, and then vortexed again for 15 seconds. The samples were then diluted in PBS (0.01 M PO_4, 0.14 M NaCl, 0.01% sodium azide; pH 7), after which analysis by radioimmunoassay (RIA) using the DSL RIA kit was conducted. This is a validated method for faecal hormone analysis in lions and other felid species, and has proven to be accurate and precise (Brown et al. 1994, Keeley et al. 2011, Graham et al. 2006, Bertschinger et al. 2008, Kubasik et al. 1984).

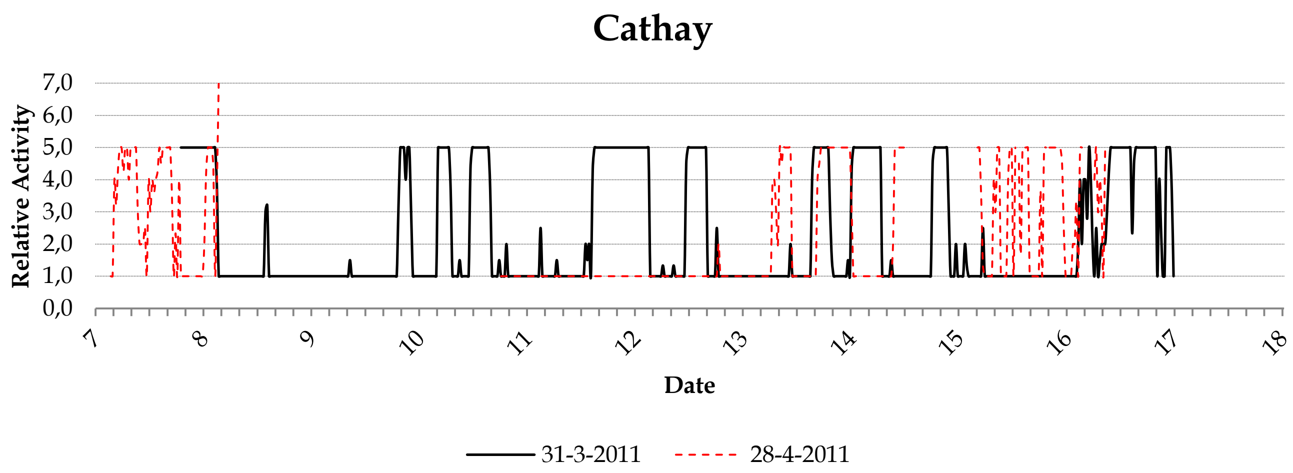


Figure 4: The relative activity of Cathay during two whole day observations, relative activity levels are plotted for against the time of the day. The - - line shows more activity in the morning and late afternoon.

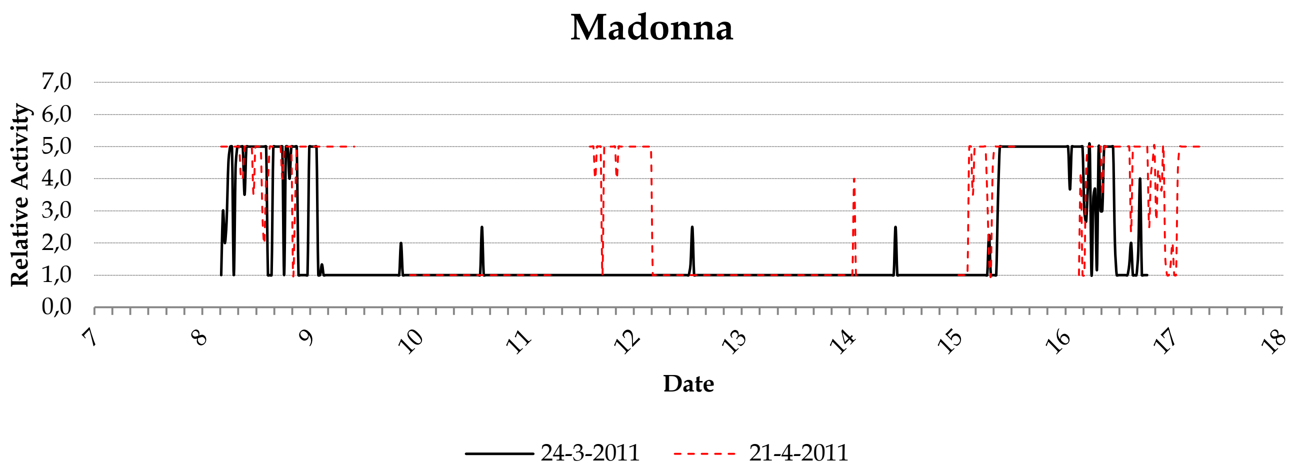


Figure 5: Relative activity for two whole day observations for Madonna, relative activity levels are plotted against the time of the day. Peak activity was observed in the morning and afternoon.

Results

Before faecal sample collection had started, Cathay and Madonna had been observed in estrus. For Madonna this resulted in nothing, but Cathay gave birth to one female cub on 31st January 2011. Cathay's samples would therefore include a diestrus (pregnancy) and an anestrus. On 4th March 2011, Madonna was in estrus but was only mated a few times before being separated from the male. No ovulation was expected, and no rise in progestagens was observed. Madonna's hormone data are expected to show anestrus, proestrus and estrus, but not diestrus. During the behavioural observations Cathay was observed to be in estrus during 7th – 10th April 2011. This led to a pregnancy, that resulted in two male cubs. Her behavioural data therefore spanned anestrus, proestrus, estrus and diestrus (pregnancy). Madonna was in estrus just before the start of behavioural observations, but no ovulation was expected because of a low number of matings. No estrus was observed during her observation period, so only information about anestrus was expected from her.

Cathay									
Day 1					Day 2				
Time of Day	*Observed Minutes*	*Mean*	*Variance*	*Standard Deviation*	*Time of Day*	*Observed Minutes*	*Mean*	*Variance*	*Standard Deviation*
Morning	193	**2,00**	2,81	1,45	Morning	76	**2,75**	3,17	1,66
Afternoon	240	**2,38**	3,42	1,73	Afternoon	210	**1,66**	2,08	1,08
Evening	120	**2,28**	2,93	1,54	Evening	65	**3,11**	3,24	1,69

Statistics							
Day 1				Day 2			
Test	*Time of Day*	*F for Anova / T for Paired T-test*	*P-value (two-tailed)*	*Test*	*Section of Day*	*F for Anova / T for Paired T-test*	*P-value (two-tailed)*
Anova		2,53	**0,08**	Anova		27,01	**0,00**
Paired T-test	Morning vs Afternoon	-2,22	**0,03**	Paired T-test	Morning vs Afternoon	4,78	**0,00**
	Afternoon vs Evening	0,49	**0,62**		Afternoon vs Evening	-5,91	**0,00**
	Evening vs Morning	-1,42	**0,16**		Evening vs Morning	-1,18	**0,24**

Table 3a: A summary of Cathay's activity levels during two whole day observations. A Friedmann's test indicated no significant differences in activity levels on day 1, but significant differences were indicated on day 2. Using paired T-tests, no significant differences were found between morning and evening relative activity.

Activity Level

Two whole day observations were conducted for each subject, 31st March and 28th April for Cathay and 24th March and 21st April for Madonna. Cathay's mean relative activity on 28th April was clearly higher in the morning and evening compared to the afternoon, respectively 1.65 and 1.87 times higher. On 3rd March no significant difference was visible between morning, afternoon and evening (Figure 4). Madonna showed an increased activity during the morning and evening compared to the afternoon for 24th March (respectively 1.91 and 3.26 times higher), and on 21st April (1.66 and 2.26 times higher: Figure 5). Statistical analysis indicated a significant difference (p-value < 0.05) in means activity on 3 of the 4 days (Tables 3a,b). In addition, a paired T-test indicated a significant difference for one of the days (second whole day observation for Madonna): relative activity in the evening was 1.36 times higher than in the morning. The first day showed 1.7 times more activity than in the morning, but this difference was not significant (p-value = 0.16).

Madonna

Day 1					Day 2				
Time of Day	*Observed Minutes*	*Mean*	*Variance*	*Standard Deviation*	*Time of Day*	*Observed Minutes*	*Mean*	*Variance*	*Standard Deviation*
Morning	193	**2,00**	2,81	1,39	Morning	140	**3,02**	3,81	1,92
Afternoon	170	**1,94**	2,72	0,03	Afternoon	167	**1,82**	2,57	1,30
Evening	106	**3,30**	3,46	1,77	Evening	100	**4,11**	2,25	1,22

Statistics

Day 1				Day 2			
Test	*Time of Day*	*F for Anova / T for Paired T-test*	*P-value (two-tailed)*	*Test*	*Time of Day*	*F for Anova / T for Paired T-test*	*P-value (two-tailed)*
Anova		24,87	**0,00**	Anova		57,94	**0,00**
Paired T-test	Morning vs Afternoon	0,35	**0,73**	Paired T-test	Morning vs Afternoon	5,79	**0,00**
	Afternoon vs Evening	-5,99	**0,00**		Afternoon vs Evening	-11,76	**0,00**
	Evening vs Morning	-1,42	**0,16**		Evening vs Morning	-4,90	**0,00**

Table 3b: A summary of Madonna's activity levels during two whole day observations. A Friedmann's test indicated significant differences at different times of day on both occasions. Paired T-tests indicated a significant difference between morning and evening on the second day.

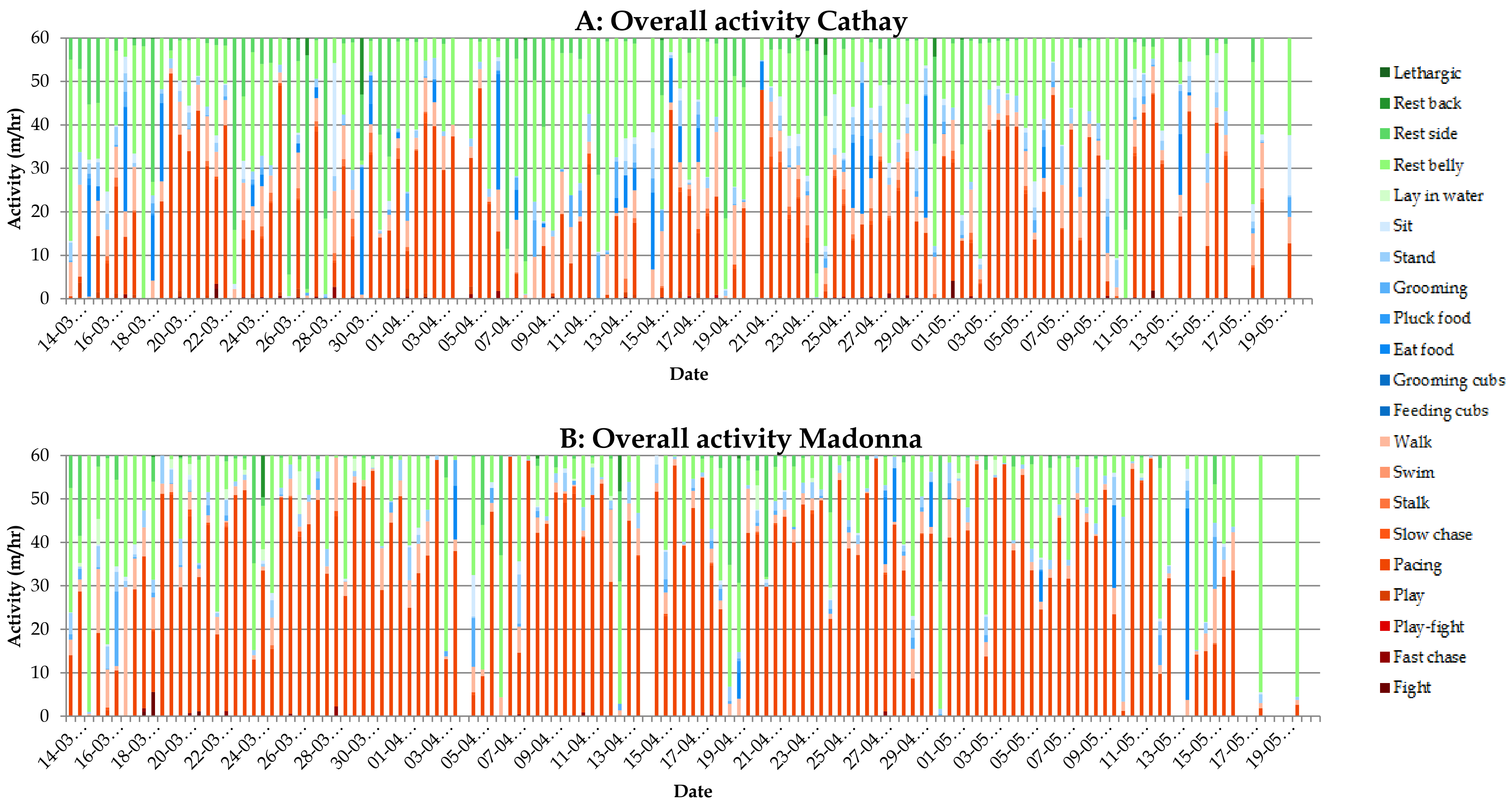


Figure 6: Overall activity plotted against time. The more red, the higher the energy costs; the more green the lower the energy costs of the activity (displayed behaviour). Blue behaviours are considered homeostatic behaviours. White lines refer to excluded or missing data. Cathay has more blue and green areas than Madonna, who has more red.

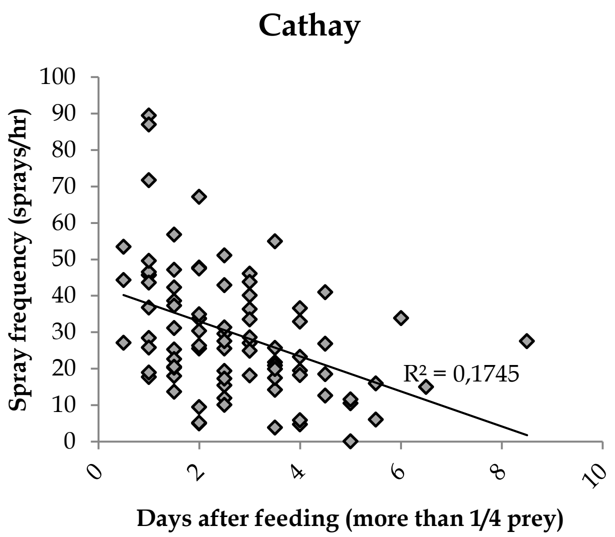
Only Cathay displayed estrus during the observation period and, therefore, only her data could analysed for effects of different phases of the cycle. Results are shown in Table 4. Values for diestrus and anestrus did not differ much, but values for proestrus and estrus did. In proestrus, the time spent resting declined by almost 36%, and the amount of pacing increased by almost 42%, compared to anestrus. On the other hand, during estrus the amount of resting increased by 60%, and the amount of pacing greatly decreased by 72%. The difference between proestrus and estrus was therefore particularly prominent. Continuous behaviours grouped into four categories (active, resting, feeding and pacing) were averaged over the whole observation period and compared between the two subjects, Cathay and Madonna. Cathay spent 9.2 min/hour active, 27.9 min/hour resting, 2.8 min/hour feeding and 20 min/hour pacing. Madonna spent 6 min/hour active, 18 min/hour resting, 1 min/hour feeding and 35.1 min/hour pacing. Madonna spent half as much time as Cathay being active, resting and feeding, but paced approximately 1.76 times more (Figure 6). In figure 6, green indicates reduced activity (low energy costs) and red increased activity (high energy costs). The difference between Cathay and Madonna is depicted in the activity graphs. The activity drop during Cathay's estrus is seen as an increased amount of green in the figure.

	Activity (minutes/hour)			
	Proestrus	Estrus	Diestrus	Anestrus
Walk	4,44	7,27	5,25	5,44
Rest	17,16	42,94	25,55	26,79
Pacing	28,39	5,65	19,55	20,04

Table 4: Mean values of activity for different phases of the cycle, obtained from data collected from the female Cathay.

Scent Marking

The impression arouse that certain events and feeding of a big meal (more than a quarter prey) influenced behaviour. For this reason, spraying frequencies for ½, 1, 1 ½, 2 and > 2 days after being fed a big meal were compared by simple regression (Figure 7). Graphs of the spraying frequency showed some outliers, anticipated to be caused by the factors mentioned. Regression of the data for Cathay yielded a R^2 of 0.1745 and for Madonna a R^2 of 0.1404 (both with a p-value <0.005). Although the regressions were not strong, they were thus statistically significant. The graphs also show that the effect of feeding was different for Cathay than for Madonna.



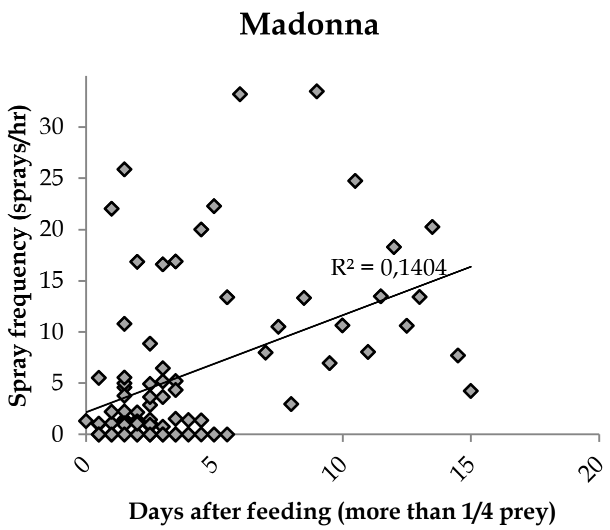


Figure 7: The dots depict the spraying frequency during an observation period after a given number of days (x-axis) after a large feed. A large feed was more than ¼ of a prey (blesbuck, springbuck, warthog or eland).

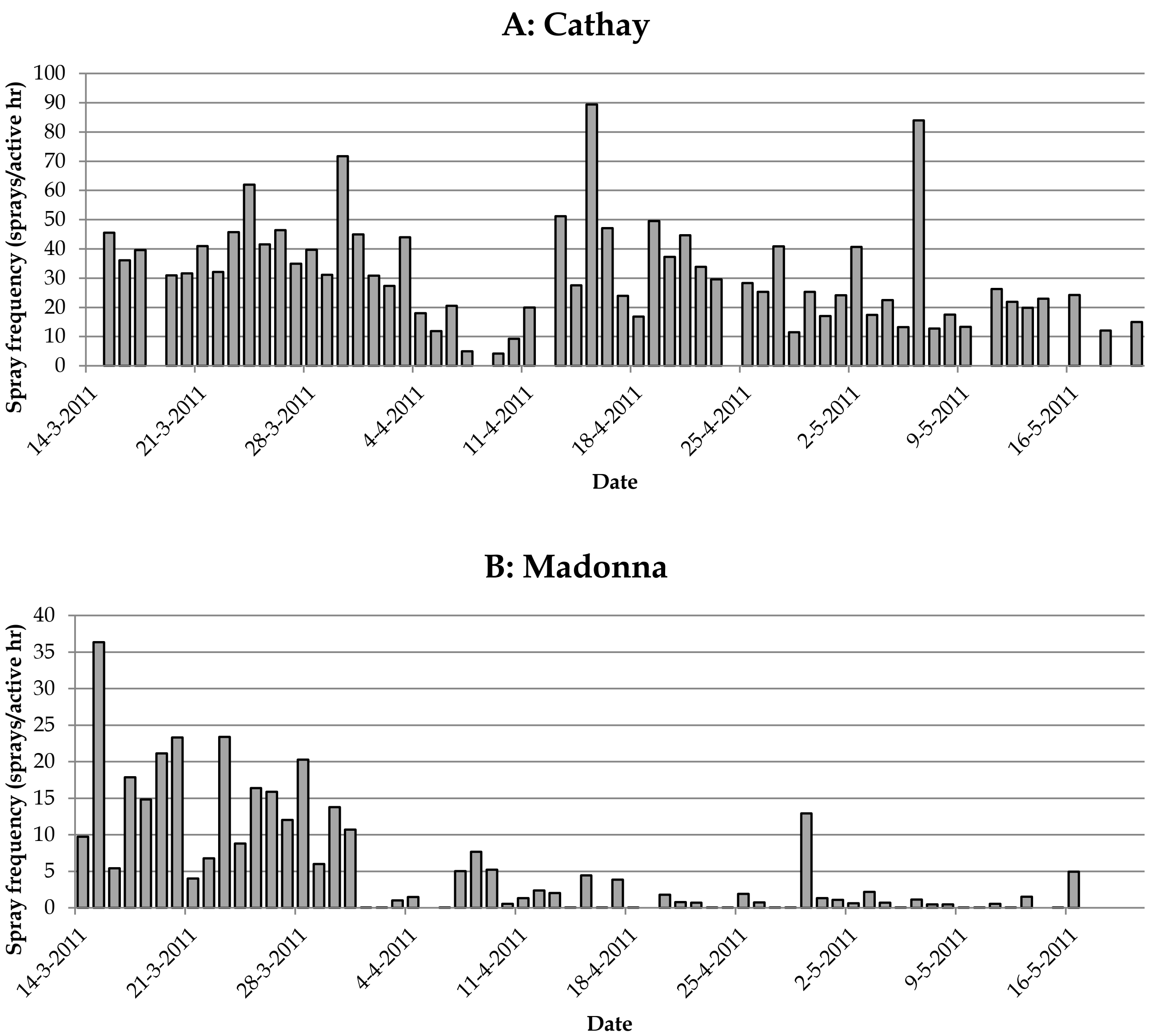


Figure 8: Spraying frequencies are plotted against date, in terms of the number of spraying events per active hour. (A) Cathay was in estrus from 7th – 10th April 2011. This resulted in pregnancy (diestrus). (B) Madonna was not observed in estrus during the observation period.

Data from scenario 1 plotted in a graph revealed some obvious outliers. It was possible that these outliers were caused by certain events or by the feeding schedule. Excluding the affected data points resulted in scenarios 2 and 3 (Appendix D). In scenario 3, the outliers were still present. Therefore further calculations were made by the use of data from scenario 1 (Figure 8).

Cathay was seen to be in estrus from 7th - 10th April 2011. During that time, her spraying frequency decreased significantly. During diestrus, it rose again, and then decreased slowly over time. Mean values for different phases of the estrous cycle are shown in Table 5. During anestrus, the mean amount of spraying was 40.91 ± 7.86 times per active hour, during proestrus this dropped by 58.9% to 16.81 ± 3.27 times per active hour and during estrus it was even lower at 6.15 ± 2.06 times per active hour. During the subsequent diestrus, the amount of spraying was lower than during anestrus at 29.63 ± 13.08 times per active hour, but higher than during proestrus and estrus.

Madonna's graph showed a high spray frequency during the earlier observations, 14.82 ± 6.24. From the beginning of April, her spraying frequency dropped to almost nothing for six days, 0.42 ± 0.55. Subsequently it increased and was fairly constant over the rest of the period, 1.75 ± 1.75.

Spraying Frequency (times/active hour)			
Proestrus	Estrus	Diestrus	Anestrus
14,79	6,15	28,59	40,08
ANOVA *p*-value		0,0029221	

Table 5: Spraying frequencies means for different phases of the cycle. Data were obtained from the female Cathay. An ANOVA indicated differences between the cycle stages ($p < 0.005$).

The lower spray frequencies during (pro)estrus for the other scent marking behaviours were not similar. Neither did Madonna's data show a drop in other scent marking behaviours in parallel with the decreased spray frequency. On the other hand, when frequencies of other scent marking behaviours peaked, so did the frequency of spraying, suggesting a relationship between the two. The lower frequencies of other scent marking behaviours make results of correlation test to spray frequencies inconclusive.

Social Interaction

Specific behaviours, related to social interaction with other individuals, are plotted in a graph (Figures 9a,b). Positive interactions were 'prusten', 'play', 'play-fight', 'head-rub' and 'chuffing';, while negative (antagonistic) interactions were 'growl', 'roar', 'fight' and 'kill'. Frequencies for negative interactions were given a negative value (times -1) and presented below the x-axis. During the first period of observations, Madonna was introduced to different males. She exhibited both positive and negative interactions with these males. In early April, they were moved away and there was no further interaction with other individuals. At the end of April, Madonna showed no further negative interactions. No interaction was visible during May. Cathay showed a lot of positive interactions, more particularly during estrus and early diestrus. During estrus she also showed a lot of negative interactions.

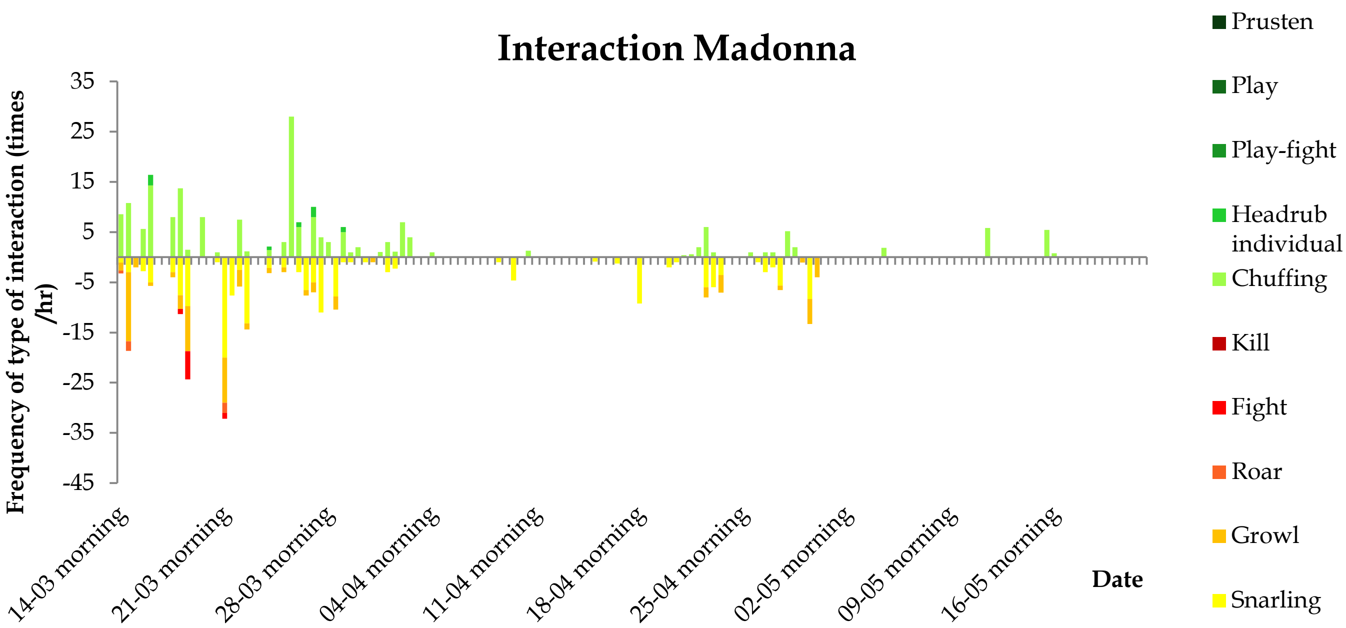


Figure 9a: Positive interaction frequencies are presented in green, negative in yellow to red. During the first period of the observations, up to earl April 2011, three different males were introduced to Madonna. From the 22nd April, daily tasks around the tigers were performed by a different person (male rather than female) for more than a week.

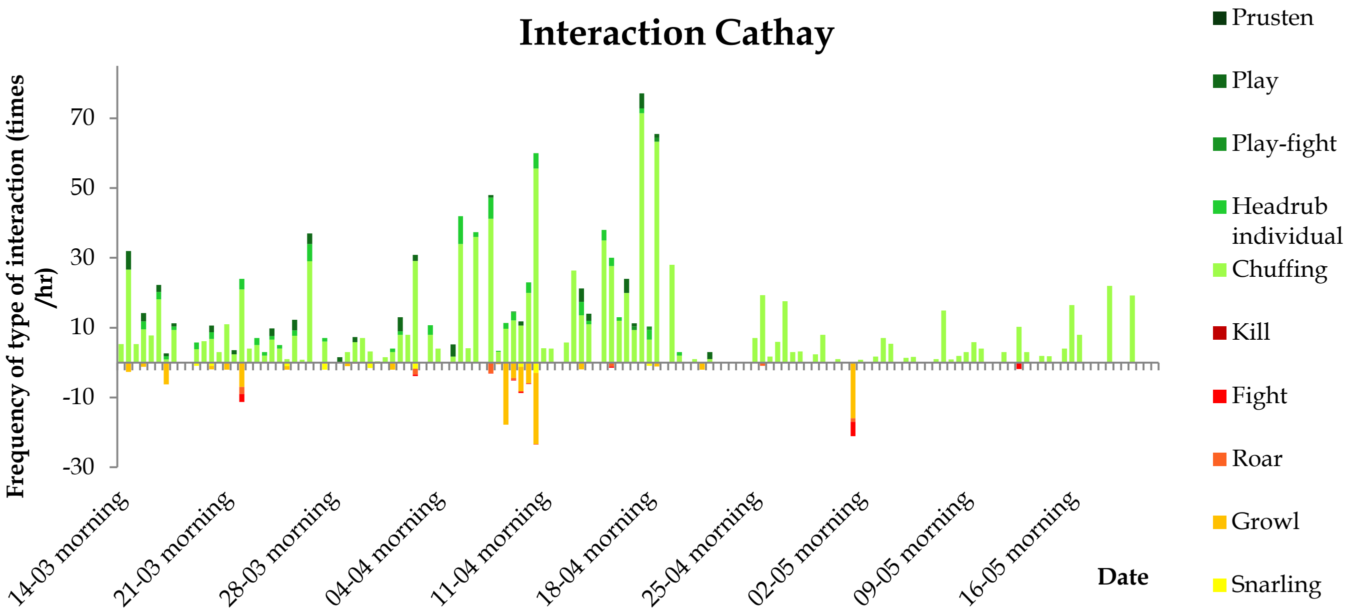


Figure 9b: Positive interaction frequencies are presented in green, negative in yellow to red. At the end of April 2010 the male in Cathay's enclosure was moved into the enclosure next to hers. From 7th to 10th April 2010, Cathay was in estrus.

Faecal Progesterone

The faecal samples collected were analysed for progesterone concentrations (Figures 10a,b). Graphs were normalized to the first day of the last mating before collection began (20th December 2010).

The arbitrary progestagen value for pregnancy confirmation was set at 300 ng/g dry faeces. The graphs show (expected) births and male introduction. Cathay showed a highly increased progestagen level during her pregnancy, and a rapid drop after birth.

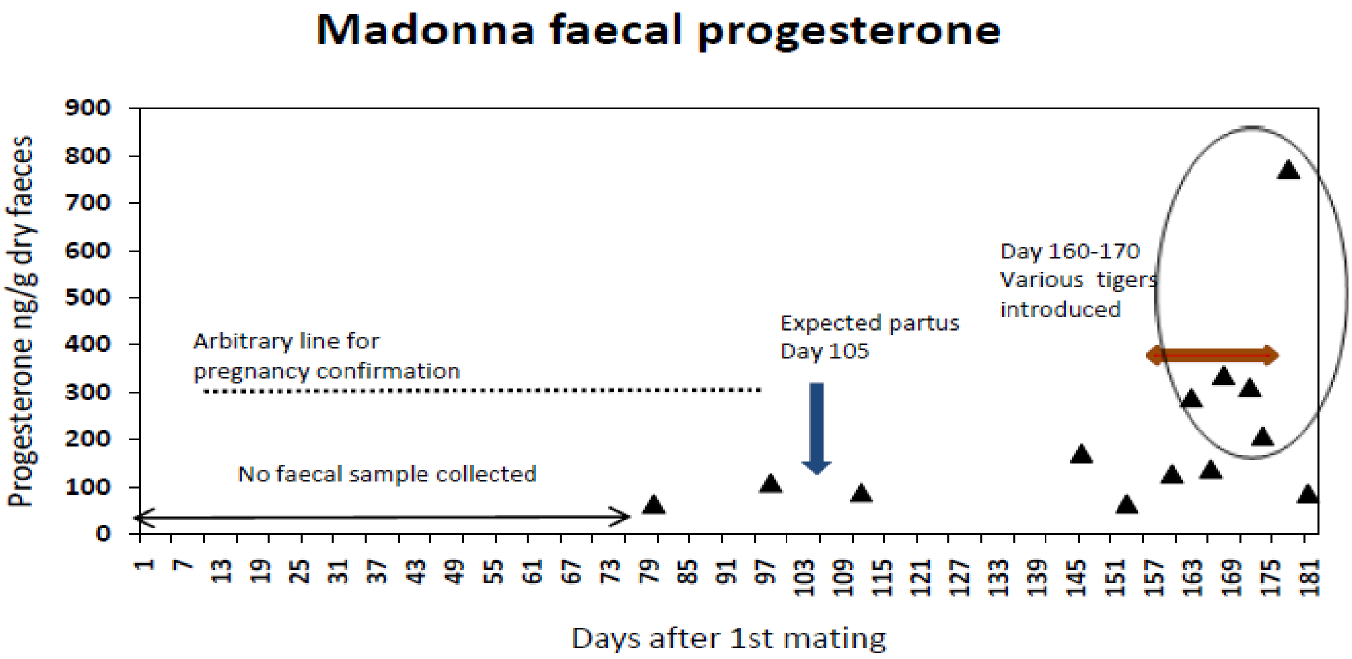


Figure 10a: Faecal progesterone concentrations (▲) during anestrus and the period in which the female was introduced to different males. Day 0 was the first day of observed matings, which did not lead to pregnancy.

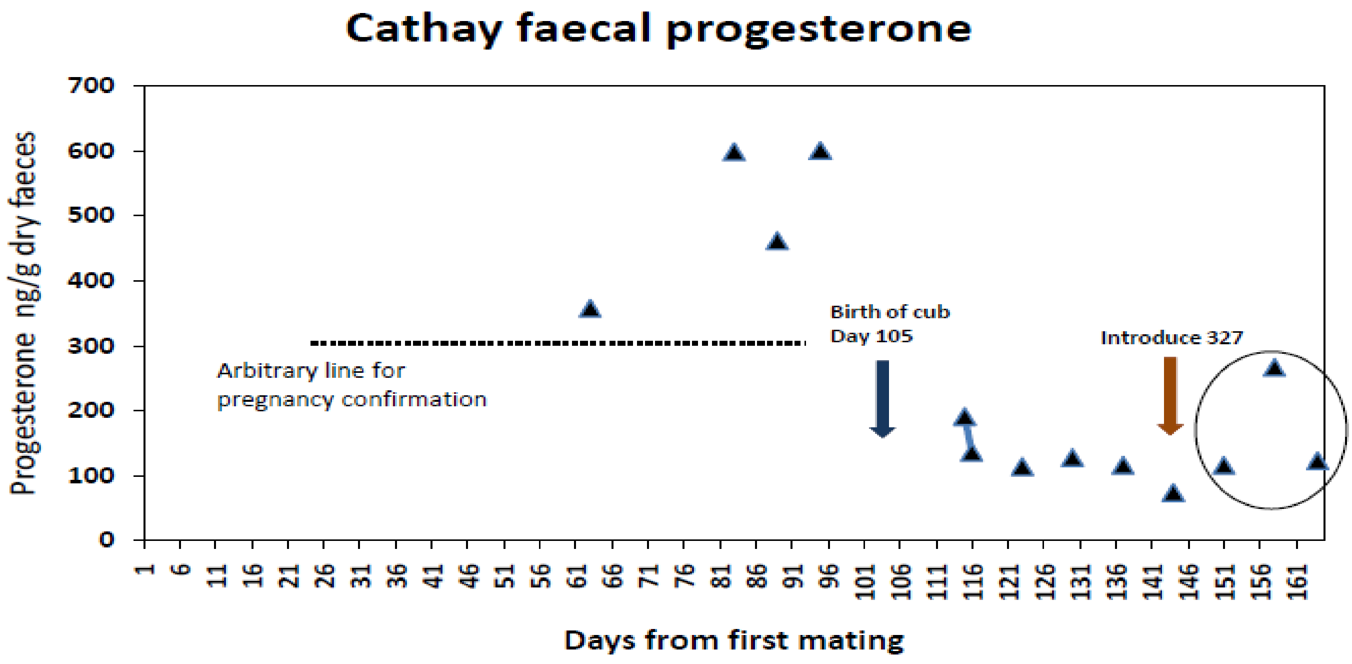


Figure 10b: Faecal progesterone concentrations (▲) during pregnancy and during the subsequent anestrus period for Cathay. Day 0 was the first day of observed matings that resulted in ovulation and pregnancy.

After introduction of the male 327 faecal progestagen concentrations peaked once, but did not exceed the 'pregnancy' line. Madonna's progestagen concentrations were not elevated after the first matings before collections, and these matings did not result in pregnancy. During the introductions to different males, progesterone concentrations were elevated , once rising above the 'pregnancy' line.

Conclusion and Discussion

Data were collected from only two female tigers during this study, which provides limited data. Even data from just these two individuals differed in some aspects. Data collection from more animals would be more representative for the species *P. t. amoyensis*. Secondly it would help identify true outliers or incorrect data. During this study, only one estrus was observed, which makes interpretation of the results difficult since there is no data for comparison.

Activity Level

On three of four whole day observations a significant increase ($p < 0.05$) in relative activity of at least 65% was observed. On one of these whole day observations, 31st March for Cathay, there was also a lot of activity during the day. Looking at the events of that day it was noted that it was a very hot day. The tiger was apparently looking for shade and went to lie down inside the breeding centre to which it had access. The observer also moved inside the breeding centre to allow observation of the animal and was thus only a few meters away from the tiger. The tiger looked up at the observer every now and then, and then went to walk outside and pace the fence. After a couple of minutes, the tiger would come back inside and lie down in the shade, again close to the observer. This sequence was repeated every couple of minutes. It is therefore believed that the close presence of the observer influenced the behaviour of the tiger, thus forcing her to move away, resulting in an increased activity. During the other whole day observations, the observer was much more distant from the tigers. Using information from the other three days, it was concluded that the tigers did indeed show more activity during the morning and evening than during the afternoon.

From Cathay's data, it appeared that her activity changed with the phase of the estrous cycle. Taking anestrus as the baseline, she was more active during proestrus (less resting, more pacing), but less active during estrus. The difference between proestrus and estrus was therefore very prominent. In proestrus, the female seemed restless, and she paced around a lot. Rise in blood estradiol concentrations, as a result of follicle growth, could have been the trigger for the animal to search for a mating partner, increasing her activity. When female tigers are in estrus, and a male is present, they mate often, interspersed by long periods of rest. During the mating period, wild tigers will not hunt and eat (Schaller 1972). In the Laohu breeding programme, wild conditions are simulated by not feeding the mating couple when the female is in estrus. Although it is conceivable that restraint from food would increase stress and therefore the amount of stereotypic behaviour, such as pacing, this was not the case. During estrus, activity between matings was much decreased, to predominantly lying.

For a breeding programme, data about the animal's activity can be helpful for indicating the female's phase of the cycle. On the other hand, comparing Cathay with Madonna showed that there are also significant differences in activity levels and patterns between individuals. It is therefore important to know the basic behaviour of a certain animal and base assumptions on individual norms. Besides individual differences, external factors can also influence behaviour of the animals. For example, Madonna's enclosure provided fewer places to hide from spectators, probably causing more stress to the animal and therefore possibly increasing her pacing activity (Bashaw et al. 2007). Effects from external factors, like influences from the type of enclosure, were not further examined in this study.

Scent Marking

The effect of feeding a large prey item on behaviour was plotted and a significant relationship was noted. However, the relationship from Madonna's data was the reverse of that from Cathay's. When Madonna was fed a large prey item she sprayed less the next day, while Cathay sprayed more. There is no obvious explanation for this difference in behaviour.

It was expected that certain events and the effect of large feeds would explain the outliers, but this was not the case. In scenario 3, the major outliers were still present. Although a significant regression is found, data from scenario 1 was used subsequently in this study (increase of n).

Theoretically spray frequency is expected to increase during proestrus. Our results showed different; the spray frequency decreased. In the wild, a female would want the male to know that she is about to come into estrus, and spray more often. In the captive situation, a known male (327) was introduced to Cathay about three-and-a-half week before she came into estrus. She has already mated with this male before. Because there was already a known and accepted male in her territory, it might not be necessary for her to signal the approach of estrus. So there would be no need for increased spraying, possible explaining the low frequencies recorded. During estrus, her activity was decreased a lot, possibly leading indirectly to reduced spraying. Smith et al. (1989) also found an increase in spraying before estrus and a decrease during estrus in wild female tigers (subspecies unknown) in India. In any case, a significant change in behaviour was observed which could be used as a parameter for determining a female's phase of the cycle.

Madonna's histogram showed a high spray frequency in the first period of the observations. During this period, she was introduces to different males to see if they would make good mating partners. The males introduced were old enough to achieve sexual maturity and it was hoped that introducing them to a female would stimulate maturity. The presence of a lot of different males might have stimulated the female to spray more to make it clear that it was her territory.

In early April, a great decrease in spraying frequency was noted to a mean of 0.42 times per active hour. During this time several things happened: Cathay (who is not too far away from Madonna) was in proestrus and about to come into estrus. In some species, it is known that females in a group synchronize their cycles or are influenced by other animals living in close proximity (Michel 1993, Sveberg et al. 2011, Roelofs et al. 2005). In some felid species, spontaneous ovulation is observed for female cospecifics living in close proximity, indicating an influence of each other's presence (Kutzler 2007). In October 2010, both females were in estrus, Madonna first followed by Cathay about two weeks later. For Cathay, the spraying frequency dropped during proestrus, so it is likely that Madonna was about to come into estrus as well (preceding Cathay again). To verify this, data on estrogen concentrations are required. During proestrus a rise in estrogens would be expected. Unfortunately, the relevant data from this study is not yet available. Faecal samples have been analysed for progesterone, but without mating no ovulation will take place, so no rise in progesterone was expected. Progesterone data therefore cannot be used to confirm whether a female was really in estrus or not. Secondly, the males were moved away, diminishing the tigresses 'needs' to mark their territory by spraying. But the decline was already apparent two days before they were moved away, making it a less likely explanation. Lastly the grass in Madonna's camp was cut short. Before this, Madonna had specific preferred spraying places, for example a specific small bush and a patch of very tall grass. After the cutting, she no longer seemed interested in these places for spraying. Instead, she sprayed directly on the ground and on a specific tree. Again, the drop in spray frequency was apparent for 4 days before this happened, making this explanation less likely. Nonetheless, social and environmental factors should be taken into account when interpreting spray frequency as a parameter for cyclicity (Pitsko 2003, Bashaw et al. 2007). Mellen (1993) concluded that a single spray is not a good indicator, but the change in spraying rate over time (as well as some other behaviours) are good indicators of reproductive stage.

There was no obvious pattern to ways of scent marking other than spraying. Only in the last period of Madonna's observations were similar peaks in scent marking behaviour observed. When the environment in Madonna's enclosure changed she found different places to mark with her scent, and used different ways of scent marking. Different types of vegetation might invite different ways of scent marking. Wood is apparently more appealing to head-rub and scratch than tall grasses. For example, (Schaller (1972) mentioned scraping on the ground to be more frequent on wet ground, supported by Mellen (1993). While Smith et al. (1989) described other scent marking behaviours as not solely related to reproductive activity; scratching of objects, for example, was more frequent for males than females. Environmental factors might be an explanation for the combined peaks in marking and praying behaviour observed in Madonna's data.

Social Interaction

Madonna showed positive and negative interactions during the period in which several males were introduced to her. Specifications of the interactions (extracted from the ethograms sheets; 'happenings'-section) showed that interactions toward the males in other enclosures were mostly positive, but when they entered her camp she showed negative (warning) signals. Positive interactions towards the male in her camp were only sighted sporadically. From 22nd April, the (female) daily caretaker for the tigers was away for a week and tasks were performed by others (men). During this period, Madonna showed more negative interactions towards her caretakers. Since no male was present when Madonna was (most likely) in estrus during the observation period, no related interaction data was collected.

Madonna showed a lot of positive interaction towards male 327, whom she was already familiar with. During proestrus and estrus, the amount of positive interaction increased significantly, especially 'chuffing' and 'giving a head rub' were more frequent. Other species show similar increase in interaction when coming into estrus (Keeley et al. 2011, Sveberg et al. 2011). During estrus, there is also many negative interactions, probably related to the mating ritual. During mating, the male will bite the female on the neck, inducing a lot of growling by the female. When the male succeeds, the female will end the mating with a roar and in most cases whirl around to fight off the male, causing a high negative interaction frequency. This behaviour is also observed in wild and other captive felid species (Mellen 1993, Schaller 1972). Shortly after

estrus female tigers shows more positive interactions, which decrease over time.
The behaviour from the female towards other individuals is a very important tool for determining cycle stage. In proestrus the female showed heightened interest in males by more frequent chuffing and head rubbing, which corresponds with several behavioural studies on felid cyclicity (Brown 2010, Kutzler 2007, Schaller 1972). Vocalizations preceding estrus are observed in wild and some captive tigers, but were not observed in this study (Smith et al. 1989). In estrus, a female tiger will often present herself to the male, ready to mate; a behaviour which is not observed in other phases of the cycle. When not in estrus, the female showed less interest in and less positive interactions toward males. In addition, Madonna showed negative interactions during most of anestrus. In a lot of species, the behaviour of the animal is used as a parameter for hormone status. Dairy cows, for example, are monitored on behaviours like 'anogenital sniffing' and 'initiating of mounting' by the farmer as an indication of whether they are coming into estrus and are ready for AI (Sveberg et al. 2011). Sows also display a 'standing reflex' when pressure is applied to their back during estrus. In any other phase of the cycle, they will not perform this behaviour and will walk away from the pressure (Sterning et al. 1998). In this study, the interaction with and from the male was not examined, but could also be an important tool. By using the flehmen reaction the male picks up pheromones and other hormones and scents, which provide him with information about the females cycle stage. Males tend to exhibit flehmen more when sniffing at scent-marking spots of females that are (about to come) in estrus. The reaction of the male could therefore indicate the status of a female (Mellen 1993, Schaller 1972, Sveberg et al. 2011, Mega and Mellender de Araújo 2010).

Faecal Samples

Thursday was set as the day for faecal sample collection, and the feeding schedule was adjusted accordingly. Therefore, the two tigers sometimes had to wait an extra day before being fed, or got fed a day earlier. There are questions about whether dietary factors could be of influence on the hormone metabolite excretion pattern, for example due to longer intestinal storage and altered faeces production. A study of the faecal output of progesterone metabolites in ovariectomized domestic cows with intravaginal devices releasing progesterone and on differing feed intake levels, showed no relationship between feeding and alteration of faeces production (Schwarzenberger 2007). Adjusted feeding scheduling is therefore most likely not of influence on the results.
Preparation of the samples was performed according to Bertschinger et al. (2008). According to Brown et al. (1994) the extraction efficiency is increased when 90% ethanol:distilled water is used compared to 100% ethanol, probably because felid faeces contain a large proportion of conjugated steroids, which are soluble in aqueous solutions. They also reported that drying, pulverizing and thorough mixing before analysis give more accurate results than using wet samples separated into portions in advance. Although using wet samples results in a shorter preparation time, the variation in results is larger, and a sample could wrongly be classified as baseline or estrual.

In some cases, paired samples were collected; where one sample was fresh and the other collected overnight. Analysis and comparison of these samples showed that if the samples were not freshly collected, the progestagen concentrations increased, yielding unreliable results. Similar findings were also reported in various studies performed by (Brown 2010). Yamauchi et al. (1999) considered this to be caused by intestinal microorganisms which convert conjugated steroids to unconjugated forms. For both female tigers (Madonna and Cathay), the faecal progestagen concentrations increased after introduction of a male into their camps. Introduction of the male probably caused stress to the female. There are two possible explanations for the elevations; (1) stress led to an elevation of progestagen levels in the blood, and (2) the progesterone antibody in the radioimmunoassay cross-reacts with faecal cortisol metabolites. The first explanation seems to be the case in cheetahs and lionesses.

Elevations of progestagens can also be stimulated in the domestic dog by ACTH injection. The metabolic pathway for progestagen production is similar to that for cortisol. Presumably progestagens are produced as 'by-products' in the adrenal cortex. Further research on this subject is needed to verify this suggestion.

Interpretation of the elevations in faecal progesterone concentrations must be carried out carefully, given the discontinuous sampling. A single elevation after the 35th day of gestation obtained from a single non-fresh sample cannot be considered to give an accurate representation. Even if fresh paired samples were analysed, one elevation after the 35th day does not approve pregnancy.

Monitoring Reproductive Activity using Faecal Steroid Hormone Analysis

Routine methods for analysing the endocrine status of an animal are blood sample collection at regular intervals and analysis for reproductive steroid hormones. This is rather impractical for tigers; not only is there a risk for the collector of the samples, but health and welfare risks for such stress-susceptible and endangered species as well. Therefore, alternatives have to be considered and validated; these include monitoring reproductive steroid hormones metabolites in faeces and urine. This is non-invasive and therefore less prone to influences that may alter the results (Schwarzenberger 2007).

Such methods have proven effective in many felid genera, and other species including primates, ungulates and equids, even whales, elephants and mice, to indicate cycle stage and pregnancy (Brown et al. 1994). The method can also be used in other reproductive physiology studies, like characterization of spontaneous versus induced ovulation, estrus-cycle length, lactational anovulation, age of onset of puberty, seasonal patterns of reproduction. With regards to breeding programs of (endangered) wildlife the diagnosis of pregnancy, presence or absence of ovarian cyclicity, diagnosis of infertility, cycle manipulation techniques, contraceptive treatments, endocrine responses to ovulation induction treatments and estrus synchronization are important issues (Schwarzenberger 2007).

Faecal hormone metabolite analysis is not only useful for captive wildlife, but also for studies of free ranging wild animals. Collecting blood samples is even more impractical, or even impossible, in truly wild animals, although faecal sample collection can also be challenging in this situation. The problem lies in locating the faeces rapidly enough, although Schwarzenberger (2007) reports that this can be overcome using dogs trained to detect scat. Faecal hormone metabolite analysis can also be combined with faecal deoxyribonucleic acid (DNA) analysis to link hormonal status to a free ranging individual, although such studies can yield questionable results, due to analysis of low quantities of DNA for example (Schwarzenberger 2007).

One disadvantage of faecal steroid analysis is the differences in major metabolites, and the primary route of excretion, in even closely related species (Schwarzenberger 2007). Specifically, little native progesterone or cortisol/corticosterone enters the faeces, because most is completely metabolized. In a study of four extant species in the family Rhinocerotidae a great variety of excreted steroid metabolites were found, such that different endocrine tests had to be developed for each species separately (Schwarzenberger 2007). On the other hand, studies on the estradiol metabolism in domestic cats (*Felis catus*) showed that > 95% of estradiol and progesterone is excreted in the faeces. Studies on the domestic cat have shown that estrogen is excreted in both conjugated (aqueous phase) and unconjugated (organic phase) forms but progesterone mostly as conjugates. Brown et al. (1994) showed that estrogen is an excretory product in various felid genera, and suggested that estrogens should be quantifiable in faeces of nondomestic felids, including the tiger (*Panthera tigris)*. They also found that progesterone metabolism was conserved among different felid species, and that progesterone RIA could be used confidently across the felid species to evaluate luteal function, although baseline and peak values do differ among species. Concentrations found in this study differed from the concentrations reported in other studies.

Overall it can be concluded that specific behaviours like activity, spraying and social interaction differ among individuals, but changes when the animal enters a different phase of the estrous cycle. Relative activity increases during proestrus, but decreases during estrus; spraying frequency is high during anestrus, drops during proestrus and is even lower during estrus; and positive social interactions increase during proestrus (close contact interactions increase in particular); negative interactions increases during estrus (due to mating rituals). Therefore, alterations in these specific behaviours can be used as aids to determining estrous cycle status in the tigress.
Furthermore, progesterone concentrations can be monitored in the faeces. While stress may influence faecal progesterone concentrations, if appropriate measures are taken (e.g. double, fresh sampling) accurate data can be collected. Progesterone concentrations seem to be elevated up to parturition and are therefore useful for pregnancy determination.

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Appendix A

Main Tiger Camps			
Camp Name	**Camp Nr**	**Camp Size**	**Camp Description**
Small Camp	1	0,5 ha	This camp consists of terraced section. A line of shrubs runs through the middle of the camp. There are sparse shrubs in the rear and a pole wooden shelter.
Ravine Camp	2	2 ha	The camp is divided in 2 portions by a fence. One portion is to be used for feeding purposes. The other main section has a river running through it, characterized by dense bushes and tall trees.
Catkins Camp	3	7 ha	The main feature of this camp is the stream with dense reeds an thick bushes along the banks. The tall trees standing out above the other vegetation is distinctive as is the scattered clumps of shrubs.
Tree Camp	4	0,5 ha	This camp is almost shaped like a kidney, it has a huge pepper tree accompanied by different sized shrubs. The one section is slightly higher than the rest of the camp and also contains a pole wooden shelter.
Maternity Camp	5	0,5 ha	It is triangular in shape, grassy with no trees or shrubs. A pole wooden shelter is present.
Ripples Camp	6	1 ha	Characterized by trees clustered in the centre with outer-lying clumps of dense bushes and smaller shrubs. A ditch runs through the camp, but only has water for a short while after heavy rain.
Grass Camp	7	3 ha	Grassy with a few low shrubs and one or two trees.
40 ha Camp	8	40 ha	A vast stretch of open grassy area, scattered trees except for the area where the river passes through. That part has a distinctive dense bush and tall trees. This camp contains a resident population of game.
100 ha Camp	-	100 ha	An open grassy area with scattered trees and bushes. The area around the river consists of distinctive dense bush and tall trees, and a part which is less dense. In the middle is a hill with a lookout on top. This camp contains a resident population of game.
Quarantine Camp	-	0,5 ha	This triangular camp is connected to the Breeding Centre. The camp is grassy with no bushes or trees. There are some wooden logs in there, and a pole wooden shelter.

Table A1: An overview of the main camps in which the tigers are being kept in the area of Laohu Valley Reserve, dedicated to the breeding and rewilding program of the South China tiger.

Appendix A

Temporary and Accessory Camps			
Camp Name	**Camp Nr**	**Camp Size**	**Camp Description**
Tree-annex	A	100 m^2	This small area is the connection between Tree Camp and Catkins Camp and is the entrance to Catkins Camp. This area can be used to house tigers for a short time.
Quarantine-annex	B	100 m^2	This small area is the entrance to Quarantine Camp. This area is not used to house tigers, but from here different gates can be operated.
Breeding Centre	C (1, 2, 3, 4, 5 and 6)	6 x m^2	The Breeding Centre consists of 6 separate enclosures. Three are on the west side and three on are on the east side. The four in front are inside and the two at the back are fenced. The enclosures can be linked together by shafts. Each enclosure can be entered by its own gate.
Maternity-annex	D	100 m^2	This small area gives no entry to any of the camps. It is also not used to house tigers, but from here different gates can be operated.
40 and 100 ha - annex	E	100 m^2	This small area gives entry to the 40 and 100 ha camp. It is not used to house tigers.

Table A2: An overview of the temporary and accessorily camps next to the main tigers camps.

Appendix B

Ethogram: Captive Tiger Behaviour

Observer:		Tiger:		Date:		Arrive morning:		Departure:	
General Information and Circumstances during Observation						Arrive afternoon:		Departure:	
						Arrive evening:		Departure:	

	Camp	Cloud (8)	Wind(11)	Temp. (°C)	Rain fallen (mm)	Other tigers same camp	Tigers in adjacent camps	Amount of prey in camp	Human influences
Morning									
Afternoon									
Evening									

Ethogram

1. Exploring/hunting behaviour																												
Alert																												
Smell																												
Chew/lick/claw object																												
Dig																												
Walk																												
Stalk																												
Slow chase																												
Fast chase																												
Change prey																												
Catch																												
Kill																												
Swim																												
Jump																												

2. Resting/common behaviour																												
Rest on belly																												
Rest on side																												
Rest on back																												
Roll over																												
Lay in water																												
Stretch																												
Yawn																												
Grooming																												
Sit																												
Stand																												

Appendix B

3. Homeostatic-related behaviour																												
Drink																												
Eating grass																												
Eating food																												
Plucking food																												
Defecate																												
Urinate																												

4. Communication behaviour																												
Bodyscratch against object																												
Headrub object																												
Headrub tiger																												
Scraping ground																												
Scratching object																												
Spraying																												
Vocalization																												
Chuffing																												
Prusten																												
Snarl																												
Growl																												
Roar																												
Play																												
Play-fight																												
Fight																												
Flee																												

5. Mating behaviour																												
Flehmen																												
Investigate																												
Seduction (f)																												
Mount (m)																												
Be mount (f)																												
Mate																												

Appendix B

6. Parental behaviour																												
Dragging nest/den-material																												
Feeding cubs																												
Grooming cubs																												
Move cubs																												
Ignore cubs																												
Snarling at cubs																												
Kill cubs																												

7. Stereotypic or other behaviour																												
Pacing																												
Lethargic																												
Cannot see																												

Happenings information

Start	End	Whom involved	Description of happening

Appendix C

Behaviour descriptions	
Alert	Individual is being watchful for possible danger or being prepared for action. Lifting up of the head, eyes focussed on subject of intention, both ears turned that way. Usually interrupting the performed activity at that moment.
Smell	Investigating object/ground/air by sniffing at it. Uptake of odours through the intake of air by the nostrils.
Chew/lick/claw object	Investigating object by chewing, licking or clawing it. Holding it in mouth can also be observed.
Dig	Digging the ground with front paws.
Walk	Directional moving from one place to another.
Stalk	Moving, slightly crouched, towards an individual. Holding crouched position can be part of the stalk. Intention can be serious hunting or playful. If observed, note circumstances as 'happening' and the individual being stalked.
Slow chase	Decreasing distance towards individual at draff. Intention can be serious hunting or playful. If observed, note circumstances of situation as 'happening' and/or the individual being chased.
Fast chase	Decreasing distance towards individual at gallop. Intention can be serious hunting or playful. If observed, note circumstances of situation as 'happening' and/or the individual being chased.
Change prey	Changing from one prey to another during stalk or chase.
Catch	(Attempt to) catch individual or object, sometimes followed by holding it in its grasp. Intention can be serious hunting or playful. If observed, note circumstances of situation as 'happening' and/or the individual or object being caught.
Kill	Bite and subsequent killing of individual.
Swim	Moving around in deep water.
Jump	This can be noted as jumping in the air, or onto something/someone. Standing upright to object is also noted as 'jump'.
Rest on belly	Eyes are closed or open, ears and head are sometimes moved. Hind legs can be underneath the body or laying flat, front legs are underneath the body.
Rest on side	Eyes are closed or open, ears and head are sometimes moved. Laying on lateral side, both hind and front legs on lateral side, shoulder touches the ground.
Rest on back	Eyes are closed or open, ears and head are sometimes moved towards objects of attention. Laying on dorsal side. Cranial part of the body is sometimes in a more lateral position.
Roll over	Rolling over dorsal side, from one lateral side to the other lateral side.
Lay in water	Laying in the water, not moving around.
Stretch	Stretching its legs and neck.
Yawn	-
Grooming	Licking or scratching of own body, more than 3 times (not a simple reaction to flies).
Sit	Caudal part of the body touches the ground. Hind legs are bend is sitting position, front legs stretched under torso, not bend.
Stand	Not to confuse with temporary interruption of activity due to an 'alert'.
Drink	-
Eating grass	-
Eating food	-
Plucking food	Removing the hairs/feather/skin of food without really eating.
Defecate	-
Urinate	Not to confuse with spraying.
Bodyscratch against object	Rubbing the body against an object.
Headrub object	Rubbing the head against an object.
Headrub tiger	Rubbing the head against another tiger. Note to which tiger this gesture is done.
Scraping ground	Scraping the ground with the hind feet.
Scratching object	Repeated clawing of object with both front paws. Scratching the nails over it.
Spraying	Not to confuse with urinating.
Vocalization	A soft friendly sound, mouth barely opened (huh-huh). Can be short, but also a long moaning sound.
Chuffing	A soft friendly sound, sounding nasal, but is actually created in the throat (thrtrtrtr). Note to which individual this gesture is done.
Prusten	A soft friendly sound, created in the nose, sort sneeze, sounds very alike chuffing (gththt). Note to which individual this gesture is done.

Appendix C

Snarl	Threatening hissing sound (hsss) or vocal bite. If observed, note circumstances of situation as 'happening' and/or the individual who is snarled at.
Growl	Soft threatening rumbling sound, created in throat (grrrr). If observed, note circumstances of situations as 'happening' and/or the individual who is being growled at.
Roar	Loud threatening rumbling sound, created in throat (graurgr). If observed, note circumstances of situation as 'happening' and/or the individual who is being roared at.
Play	Can be with object or other tiger or on its own. If observed, note circumstances of situation as 'happening' and/or individual who is played with.
Play-fight	Biting and/or clawing softly, without damaging each other. If observed, note circumstances of situation as 'happening' and/or individual who is interacted with.
Fight	Biting and/or clawing ferocious, possibly damaging each other. If observed, note circumstances of situation as 'happening' and/or the individual fought with.
Flee	Increasing distance between itself and object of fear, backing off or showing submission (ears flat, body in low position and occasionally looking behind).
Flehmen	Opening the mouth, lips raised, to take in pheromones. Small part of the tongue is usually hanging out of the mouth.
Investigate	Male sniffs underneath the tail of the female. Or the female is being investigated by the male.
Seduction (f)	Female rolls over on back and shows herself to the male, inviting him.
Mount (m)	Male mounts the female to mate.
Be mount (f)	Female being mounted by the male to mate.
Mate	Accepting mounting and subsequent mating. Characterized by the male biting the neck of the female, moaning and roaring, and the female growling.
Dragging nest/den-material	Dragging materials to make a den or nest to give birth in.
Feeding cubs	Bringing food to the cubs.
Grooming cubs	Licking the cub. If observed, note circumstances of situation as 'happening' and/or the cub being groomed.
Move cubs	Moving cubs (in mouth or walking along) towards other place. If observed, note circumstances of situation as 'happening' and/or cub being moved.
Ignore cubs	No interest in cubs, and ignoring their attempts for attention. If observed, note circumstances of situation as 'happening' and/or the cub being ignored.
Snarling at cubs	Snarling at the cubs. If observed, note circumstances of situation as 'happening' and/or the cub being snarled at.
Kill cubs	Bite and subsequent killing of cubs. If observed, note circumstances of situation as 'happening' and/or the cub being killed.
Pacing	Walking up and down same path continuously, intention less.
Lethargic	No reaction on surrounding stimuli, only to pain stimuli.
Cannot see	Tiger is out of vision of the observer.

Appendix D

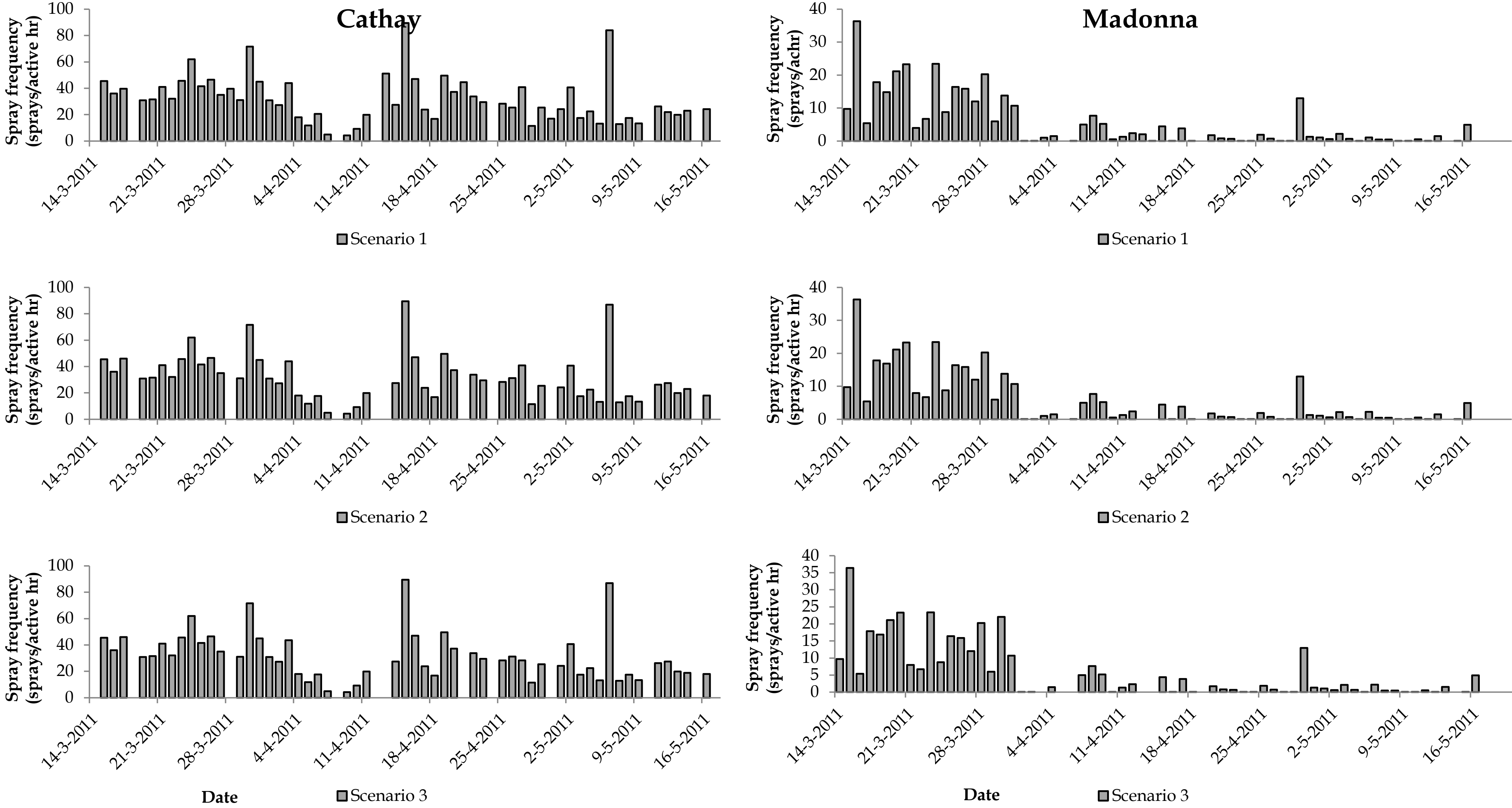

Figure D1: Histograms of the spraying frequencies from Cathay and Madonna. On the top scenario 1 is illustrated, for which data are used only from observations which last > 30 minutes and in which the animal is active > 15 minutes. In the middle scenario 2 is illustrated, for which data from observations during which an event occurred are excluded. On the bottom scenario 3 is illustrated, for which data from observations ½ a day after feeding of a big feed are excluded. In scenario 3 outliers from scenario 1 are still present.

Appendix E

A: Cathay

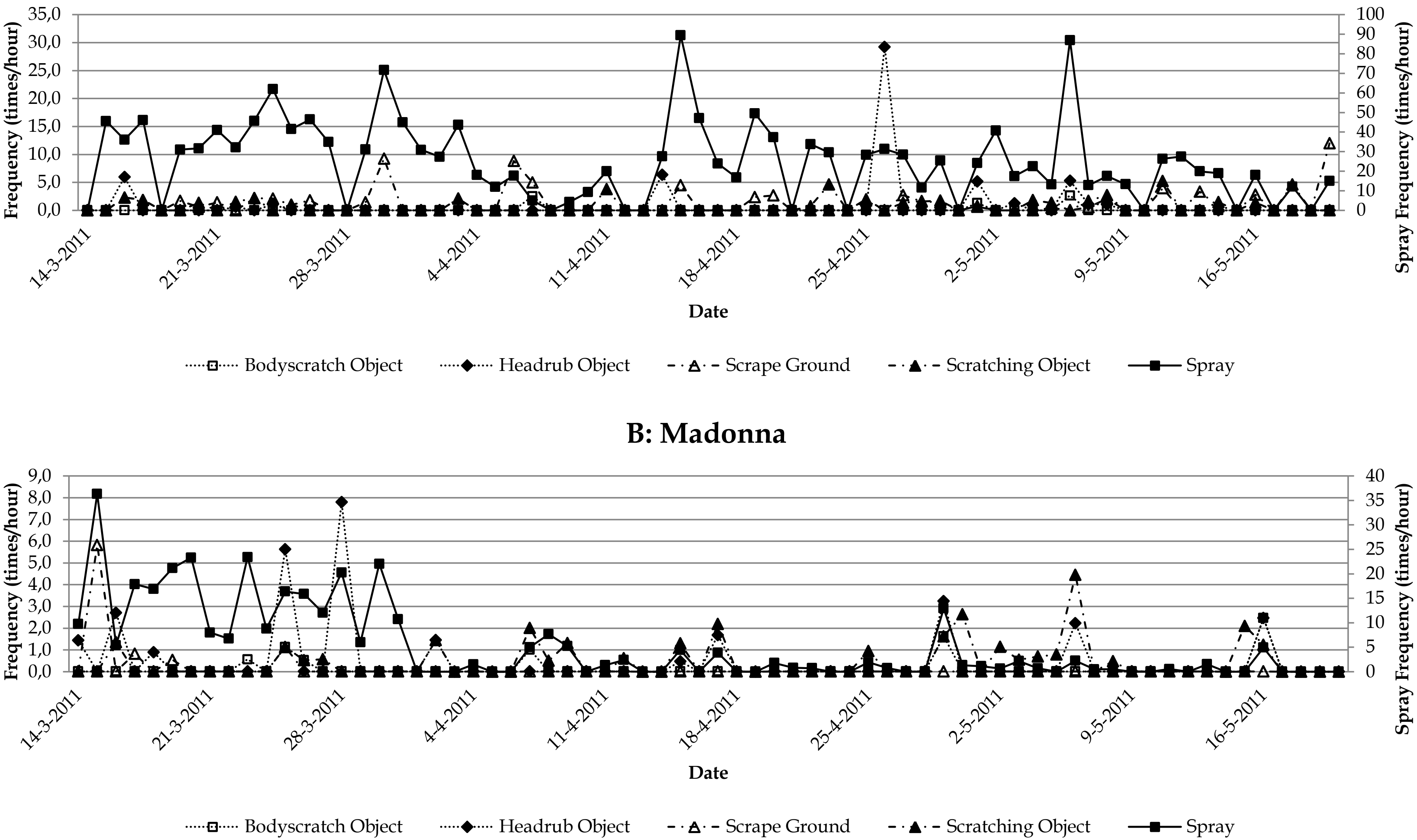

Figure E1: Frequencies of different scent marking behaviours are plotted against date. For the spraying frequency the y-axis is set on the right, for the others the on the left. (A) Cathay displayed different scent marking behaviours, but they were not related. The observed decrease in spraying frequency is not visible for the other scent marking behaviours. (B) Madonna also displayed different scent marking behaviours. Peaks seemed to be at the same time as peaks of the spraying frequency, particularly visible in the last part of the observation period. No correlation is calculated, because of the low frequencies of other scent marking behaviours.