Faculty of Veterinary Sciences **University of Pretoria**

Monitoring and controlling aspiration fluid temperature during ovum pick-up in southern white rhinoceros (*Ceratotherium simum simum*)

By

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SUMMARY

Assisted reproductive technologies need to be developed to assist in the battle against the extinction of endangered and threatened species. Temperature control during oocyte recovery and transportation is essential for ensuring *in vitro* embryo development. In this study, we examined the temperature pathway of oocyte aspiration fluid during ovum pick-up (OPU) in the southern white rhino (*Ceratotherium simum simum*) in South Africa, and its role in oocyte maturation success. The large aspiration instrument used was made from PVC plastic. As a poor heat conductor, it needed to be pre-heated for several hours before the OPU procedure commenced to avoid cooling of the oocytes. Fewer temperature fluctuations were recorded when oocytes were transported in a portable transport heating/cooling incubator than in an Equitainer. A greater number of oocytes were harvested per aspirated follicle during the suggested predominant breeding season (November – April), after adjusting for the duration of the OPU procedure. Oocytes harvested from rhino cows given a warm-water enema prior to the OPU procedure were 2.31 times more likely to mature *in vitro* than those harvested from cows receiving a cold-water enema, after adjusting for the individual effect of the rhino and the negative effect of follicular wave stimulation on the oocyte maturation success. This study indicates that to ensure the successful maturation of oocytes in southern white rhino, oocyte hypothermia during aspiration and transportation is a major concern. The results and conclusions of this study may inform future research with respect to temperature control of oocytes during recovery and transportation for other endangered wildlife species.

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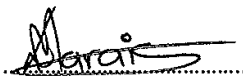
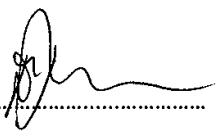
Signature of student: ..... 

Signature of supervisor: ..... 

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LIST OF ABBREVIATIONS

ART	Assisted reproductive technology/technique
CO_2	Carbon dioxide
cm	Centimetre
°C	Degrees Celsius
DMEM	Dulbecco's modified eagle medium
EP	Embryo Plus
EGF	Epididymal growth factor
EQ	Equitainer
FBS	Foetal bovine serum
FSH	Follicle stimulating hormone
g	Gram
IRRC	International Rhino Reproductive Collaborative
IUCN	International Union for Conservation of Nature
IU / 2L	International units per two litres
ICSI	Intracytoplasmic sperm injection
IV	Intravenous
IVF	*In vitro* fertilisation
IVM	*In vitro* maturation
LH	Luteinising hormone
µg / mL	Microgram per millilitre
µL	Microlitre
mg	Milligram
mg / mL	Milligram per millilitre

mL	Millilitre
mm	Millimetre
mM	Millimolar
mU / mL	Milli units per millilitre
MEM NEAA	Minimum essential medium non-essential amino acids
ng / mL	Nanogram per millilitre
N	Nitrogen
OPU	Ovum pick-up
O_2	Oxygen
pO_2	Partial pressure of oxygen
SAWS	South African Weather Services
SD	Standard deviation
TOPS	Threatened or protected species
TI	Transport incubator
UP	University of Pretoria

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1. LITERATURE REVIEW

1.1 Introduction

The once diverse and widespread group known as Perissodactyla (odd-toed hoofed mammals) has today diminished to only a few species [1]. Within Perissodactyla, there are three families; Rhinocerotidae (hereafter referred to as rhino), Tapiridae (tapirs), and Equidae (horses, donkeys and zebras) [1–6].

All five extant rhino species [black (*Diceros bicornis*), white (*Ceratotherium simum*), Indian (*Rhinoceros unicornis*), Javan (*Rhinoceros sondaicus*) and Sumatran (*Dicerorhinus sumatrensis*)] currently have extremely low population estimates and appear on the red list of the International Union for Conservation of Nature (IUCN). The black, Javan and Sumatran rhino are classified as critically endangered [7] and on the brink of extinction, while the Indian rhino is designated as vulnerable, and the white rhino as near threatened [4,7]. Given its conservation status and being the most abundant species [7,8], the white rhino is used in this study.

1.2 Declining rhino populations

The harsh reality is that not only one, but several factors are contributing to the decline in rhino numbers, including ever-increasing human populations and poaching for the illegal trade in rhino horn. Furthermore, rhino habitat fragmentation and declining numbers, are impacting negatively on the genetic diversity of rhino species.

The current decline in species numbers is closely linked to anthropogenic pressures [8]. In the case of the rhino, human population growth [9] is associated with the clearing of natural habitats to develop settlements and agricultural land, confining

rhinos to fragmented patches of land and restricting their ability to roam freely [5]. The rise in human populations also increases the chances of human-rhino conflicts, and facilitates unsustainable hunting and poaching practices [5,10].

Along with species and ecosystem diversity, genetic diversity is an essential consideration in conservation and biodiversity enhancement practices [11]. It is important for the potential of a species to evolve and adapt to changes in the environment [11]. Low genetic diversity translates into low adaptive potential which means that a species is less able to adapt and survive in the face of rapid environmental changes. This can, in turn, lead to whole species being lost [12]. Another vital reason for maintaining or enhancing genetic diversity is that inbreeding can lead to a decline in reproductive fitness and thus population growth potential [11].

Gene flow between species populations is of critical importance for preventing a decrease in genetic diversity. Because the rhino is the second largest mammal in Africa [11] and requires large habitats with extensive home ranges, interaction between populations is not always possible. Anthropogenic development and disturbances, such as habitat fragmentation and destruction, lead to restricted inter-population movement and thereby a decrease in genetic diversity [5].

Of all anthropogenic activities, poaching is by far the biggest concern. Since 2008, there has been a surge in rhino poaching for the horn; the bulk of which goes to the Southeast Asian markets, which are considered to be the driving force behind the poaching plague. Rhino horn is used in the preparation of traditional Chinese medicine or as decoration [13]. In traditional African cultures, rhino horn and bone are also used in rituals and to indicate royal status [14]. Despite efforts to reduce and prevent these losses, poaching remains a threat to this magnificent species, and the loss of individual

animals continues to contribute to declining genetic diversity. Total population numbers are further affected by the number of males and females who can reproduce successfully within a population, thus the effective population [11]. The fact that rhino do not reproduce easily in captivity further reduces the effective reproductive population and thereby the genetic diversity of the species. Some authors are nonetheless of the opinion that establishing reproductively active populations in captivity is essential for the conservation and management of the rhino species [15–17].

1.3 Rhino reproduction

Considerable research has been conducted on the female reproductive physiology of the white rhino [18]. The total length of the genital tract of the white rhino is 100 cm or more, and the uterine horns can surpass 50 cm in length [19]. Ovarian size varies quite considerably between and within rhino species [19]. In cycling white rhino, ovarian size has been found to range between 34.1 (± 4.3) cm^3 and 29.2 (± 2.2) cm^3. In rhino with inactive ovaries, a size of 14.7 (± 1.3) cm^3 is reported [5,20]. The left and right ovaries are assumed to be similar in size [19]. White rhino are mono-ovulatory which means that dominant pre-ovulatory follicles suppress the development of other follicles in each follicular wave [9,21–23]. A premature follicle has been defined as a follicle < 30 mm in diameter, while a pre-ovulatory follicle is defined as one that has reached a size of 30 to 36 mm [21,22,24,25]. Among land mammals, the rhino has the second longest gestation period, ranging from 15 to 18 months [19] with an assumed mean of 16 months in black and white rhino [24,26].

White rhino that are not exposed to anthropogenic disturbances are thought to breed throughout the year, and are thus considered non-seasonal breeders [19,25,27–

32]. However, birthing peaks have been observed from April to July and November to January [28]. Another study observed a higher peak in the December through April period than in the April through December period [33]. Males reach sexual maturity between 7 and 9 years and females between 4 and 6 years [5]. Numerous articles report that animals in captivity may reach sexual maturity earlier or later than this [5]. Reproductive pathologies such as anoestrus females [4], sub-oestrus (also known as 'silent oestrus'), and susceptibility to disease [30] are all factors that highlight the need for assisted reproductive technologies (ARTs) to counteract the decline in genetic diversity and population decimation due to poaching [4].

1.4 Assisted reproductive technologies (ARTs)

In broad terms, ARTs are the technologies used to facilitate assisted reproduction in humans and animals. These include semen collection and cryopreservation; oestrus synchronisation and superovulation; artificial insemination; embryo flushing; *in vitro* fertilisation (IVF); *in vitro* maturation (IVM); intracytoplasmic sperm injection (ICSI); embryo cryopreservation; and embryo transfer. All these techniques have been mastered in humans and in most domestic animal species [19]. Applying this extensive knowledge to wildlife breeding could offer new possibilities for improved captive endangered species breeding [34]. Artificial insemination (AI) using either fresh or frozen-thawed semen has been highly successful in producing pregnancies and live births in white rhino [22,24,34,35].

The goal of the aspiration, maturation and fertilisation of oocytes is the *in vitro* production of a blastocyst that is cryopreserved for later transfer to a reproductively healthy recipient rhino cow [2]. Through the use of aspirated oocytes to produce viable

embryos for implantation, females which are not capable of delivering healthy offspring can contribute to the gene pool [9].

Rhino oocyte aspiration is performed by way of transrectal ovum pick-up (OPU) procedures guided by ultrasound and following chemical immobilisation of the animal [2,9]. It is caried out transrectally rather than transvaginally, as in horses (sister *taxa* of the rhino), because of the distance from the vulva to the ovaries and that it is impossible to palpate ovaries in a rhino and hold them through the rectal wall as done in mares [2,15,34]. The immense gut of the rhino makes gas insufflation for better visibility impossible and dangerous which precludes the use of the laparoscopic approach. Surgically incising the skin is invasive and can lead to postoperative complications such as infections and scar tissue formation, which can hamper future oocyte collections [15].

The risk of oocyte contamination by bacteria and faecal matter during a transrectal OPU procedure [4, 9] can be countered by cleaning the rectum with a distilled water and salt solution enema. The transrectal OPU procedure remains the safest, least invasive, most repeatable and advanced method of oocyte extraction in rhino [4,9]. As a planned procedure it produces better quality oocytes compared to those recovered from *post mortem* material [4]. However, it is essential that all exogenous factors to which oocytes are exposed during the procedure are properly controlled. One such factor is temperature [36].

1.5 Temperature control during ARTs

The core body temperature of mammals is kept within a specific range to ensure the maintenance of bodily functions, even under extreme environmental conditions.

The normal body temperature of rhino ranges between 34.5 and 37.8 °C [26], but appears to vary between rhino species. Ungulates use two main evaporative cooling mechanisms for thermoregulation, namely sweating and panting [37]. However, as a member of the order Perissodactyla (odd-toed ungulates), the rhino does not have sweat glands for thermoregulation [38–40], and unlike Artiodactyla (even-toed ungulates), it cannot use selective brain cooling to protect the brain against heat shock or to prevent excessive water loss [41] due to not having a carotid rete [37,41]. To prevent damage to vital organs, the rhino therefore thermoregulates by panting or behaviours such as mud bathing [37,41].

During ART procedures, oocytes should be maintained at a temperature similar to the animal's core body temperature or cooled slowly. Heat or cold stress can severely affect their development and functioning [36,42]. Such single cells are particularly sensitive to changes in temperature [43,44]. The detrimental effects of heat or cold stress on oocytes include meiotic spindle disruption; abnormal spindle formation; missing spindles; and an altered zona pellucida, cytoskeleton or nucleus, to name but a few [42–45]. While temperature fluctuations are bound to occur during an ART procedure, temperature control must be a priority [45].

Given the difficulty of regular ultrasound scanning to determine the developmental stage of a follicle, and by inference the developmental stage of oocytes in rhino, the maturation status of aspirated oocytes is often unknown, and even the slightest change in temperature can be detrimental. Oocytes from dominant follicles need to be maintained at core body temperature as they have already commenced meiosis prior to aspiration [46]. The ideal temperature for rhino oocyte transportation has not yet been established. Preis *et al.* (2004) report successfully impregnating mares using *post mortem* equine ovaries transported at temperatures of 19 and 25 °C [47].

In similar Sumatran and black rhino *post mortem* studies, with ovaries transported at 22 °C, none of the Sumatran rhino oocytes matured, while 87% of the black rhino oocytes did [16,17]. Maturation occurs when oocytes complete meiosis, presenting with a polar body, an essential developmental stage prior to fertilisation. While transporting ovaries at 22 °C may be viable, oocytes could prove to be more of a challenge. Single cells are known to be more vulnerable to environmental temperature fluctuations than multicellular organs such as ovaries [43]. Further investigations are needed to determine how to maintain a constant temperature during the oocyte recovery procedures and transportation.

1.6 Study rationale

Temperature control during oocyte recovery and transportation is essential for enhancing *in vitro* embryo development in rhino. In this study, we recorded the longitudinal temperature of oocytes in aspiration medium from aspiration to the point of arrival at the laboratory using strategically placed temperature loggers.

This study identified techniques for monitoring aspiration fluid temperature during OPU procedures and identified the optimal equipment needed for maintaining a constant temperature. The results and conclusions may inform future research with respect to temperature control during oocyte recovery and transportation in the case of other endangered wildlife.

2. PROJECT AIMS AND OBJECTIVES

The aim of this study was to minimise temperature fluctuations during the OPU procedure in rhino under field conditions. The objectives of the study were threefold: (1) to monitor temperatures at various points in the OPU aspiration line; (2) to determine the impact of temperature variations during the OPU procedure on the oocyte maturation success; and (3) to investigate ways in which the OPU procedure can be modified to minimise temperature fluctuations.

The hypotheses were as follows:

H_0(1): The temperature of collected aspiration fluid at different points in the OPU procedure cannot be accurately measured in rhino.

H_I(1): The temperature of collected aspiration fluid at different points in the OPU procedure can be accurately measured in rhino.

H_0(2): No temperature fluctuations occur along the OPU aspiration line in rhino.

H_I(2): Temperature fluctuations occur along the OPU aspiration line in rhino.

H_0(3): Temperature fluctuations during the OPU procedure have no impact on *in vitro* maturation of collected oocytes in rhino.

H_I(3): Temperature fluctuations during the OPU procedure have an impact on *in vitro* maturation of collected oocytes in rhino.

H_0(4): Significant temperature fluctuations that occur along the OPU aspiration line cannot be controlled.

H_I(4): Significant temperature fluctuations that occur along the OPU aspiration line can be controlled.

3. MATERIALS AND METHODS

3.1 Study site, species, and management of rhinos

The procedures were conducted at three privately owned properties (*Table 1*), the locations of which remain undisclosed to protect the rhinos from potential poaching risks. Consent forms were signed by the relevant owners, stipulating that data collected during the OPU procedures (n = 20) could be used in this study (*Appendix II*).

Table 1: A summary of the three study sites where the transrectal ovum pick-up procedures on southern white rhinoceros took place. Weather data acquired from the South African Weather Services (SAWS).

	Site 1	Site 2	Site 3
Total approximate number of rhinos housed at the facility	20	55	65
Approximate number of female rhinos housed at the facility	10	18	35
Approximate percentage (%) of breeding females	0%	40%	95%
Farming system	Intensive[1] (bomas)	Semi-intensive (bigger camps with shelters)	Extensive[2] (free roaming)
Nutrition	Hay (lucerne [*Medicago sativa]* based mixed with others)	Teff (*Eragrostis teff*) bales	Natural veld
Total number of OPU procedures conducted	8	10	2
Number of OPU procedures conducted with follicular wave stimulation	6	3	0
Approximate distance from laboratories (km)	150	100	180
Mean maximum daily temperature April - September (°C)	25.00	24.80	25.95
Mean maximum daily temperature October - March (°C)	30.67	29.82	31.38

Mean minimum daily temperature April - September (°C)	6.98	6.80	7.25
Mean minimum daily temperature October - March (°C)	17.13	15.92	18.23
Total precipitation per year (mm)	531	617	416

[1]Intensive farming system refers to a smaller farming unit, where animals are housed in bomas

[2]Extensive farming system refers to a larger farming unit in which animals roam free

Follicular wave stimulation protocols were conducted at two of the three sites (*Table 1*). This involved the use of GnRH, but the details of the follicular wave stimulation protocols were not included in this study [24].

The predominant breeding season was defined as 1 November to 30 April, and the non-predominant breeding season as 1 May to 31 October [33].

Harvested oocytes were taken to one of two IVF laboratories:

EP: The IVF laboratory at Embryo Plus, a privately-owned veterinary practice located in Brits Northwest Province, South Africa that specialises in assisted reproductive technologies in domestic ruminants and wildlife (www.embryoplus.com).

UP: The IVF laboratory in the Section of Reproduction, Department of Production Animal Studies, Faculty of Veterinary Science, University of Pretoria, Onderstepoort Campus, Gauteng Province, South Africa (www.up.ac.za/faculty-of-veterinary-sience). The EP laboratory served as the in-the-field laboratory for the recovery of oocytes after the OPU procedure.

3.2 Staff and responsibilities

All ultrasonographic reproductive examinations and OPU procedures were performed by registered veterinarian Dr Morné de la Rey and his team from Embryo Plus. The animals were anaesthetised by Dr Hendrik Johannes Hansen, a registered veterinarian with experience in wildlife immobilisation. After immobilisation rhinos were placed in lateral recumbency and were monitored throughout the procedure. Upon completion the immobilisation was reversed, and animals were returned to their housing facilities. All field laboratory work and laboratory work at the Embryo Plus premises was conducted by Ms Carla Herbst, embryologist at Embryo Plus and, the laboratory work at the Faculty of Veterinary Science Onderstepoort IVF laboratory, by embryologist Mr Mario Smuts.

3.3 Anaesthetic protocol

Topography, tree density and environmental temperature were assessed to establish the safety of a site for immobilisation. To determine their suitability for immobilisation and the OPU procedure, selected animals were visually examined in the field for age, size, body condition, health status, family status, and calf age (where applicable). These parameters were not included in the study's statistical analysis but were used by Dr Hansen to establish the best darting protocol. Animals deemed fit for the procedure were anaesthetised using a dart gun (model 389, Pneu-Dart Inc., Pennsylvania, United States) with darts (Motsumi 1.5 ml, 1.5^{n} needle, Pretoria, South Africa) containing 2 to 5 mg Etorphine and 30 to 60 mg Azaperone. Once immobilisation was achieved, a blindfold and earmuffs were placed on the animal to minimise external inputs.

A pulse oximeter (PalmSAT®2500 Series, Minneapolis, United States) was placed in the sub-conjunctival fossa of the right eye and secured. A needle (16-gauge, Jelco®, South Africa) was placed in the vein of the right ear. When necessary, Ringer's lactate was administered intravenously at an appropriate maintenance flow rate. Also, when necessary, an endotracheal tube was inserted into the right nostril to administer O_2 at a flow rate of three litres per minute. A capnometer was connected to the endotracheal tube to continuously monitor the CO_2 and respiration rate. Heartrate, partial pressure of oxygen (pO_2), respiration strength, temperature of both the environment and the animal, mucus membrane colour, blood colour, palpebral reflex, corneal reflex, ocular globe positioning, anal tonus, and auscultation were also monitored during the procedure. If temperature regulation was needed, the animal was sprayed with water. To maintain an acceptable level of anaesthesia for the duration of the OPU procedure, the following combination of chemicals was administered intravenously: Etorphine 9.80 mg / ml, Azaperone 100 mg / ml, Butorphanol 10 mg / ml, Ketamine 100 mg / ml, Medetomidine 20 mg / ml, Adrenaline, and Doxapram.

Upon completion of the procedure, anaesthesia was reversed by administering Antipamezole at 6 mg per every 1 mg alpha-2 used, and Naltrexone at 15 mg per every 1 mg Opioid used.

3.4 Procedures and observations

After immobilisation, the animal was placed in sternal recumbency to clean the rectum and conduct a reproductive examination. The rectum was first cleaned manually. This was followed by an enema consisting of 25 L double distilled water (Modulab technologies, United States) containing a buffered salt solution (NaCl, 205 g;

Na_2HPO_4, 18.1 g; KH_2PHO_4, 5.25 g). The solution was warmed to a maximum of 31 °C (higher than the mean ambient temperature during the OPU procedure). Once complete, a transrectal ultrasound examination to assess reproductive status and presence and size of follicles was conducted. If the rhino was found not to be pregnant, she was placed into lateral recumbency on her left side for the OPU procedure.

3.4.1 Oocyte harvesting

Oocyte harvesting consisted of two phases. During phase 1 oocytes were harvested by flushing the follicles with flushing medium while phase 2 took place in the in-the-field laboratory and involved the recovery of the oocytes from the aspiration fluid for further processing.

***Phase 1*:** The round aspiration instrument (145 cm length, 38.50 cm diameter) was designed by Dr de la Rey and 3D printed out of PVC plastic (*Figure 1*). The instrument comprised three double lumen needles (17 g, 30 cm long, inner diameter 1.40 mm, outer diameter 2.10 mm, Marcus Medical, South Africa) and an ultrasound transducer (Shenzhen Mindray Bio-medical electronics M7Vet, China). The instrument was guided through the rectum towards the left ovary, allowing the follicles to be viewed. The ovarian dimensions, number and size of each follicle were recorded. The three separate needles (angled at 51°, 59° or 68° depending on the location of the follicle) were guided through the rectal wall into each suitable follicle. Flushing medium (2 L, ABT 360, United States) containing 31350 IU / 2L Heparin (Sigma-Aldrich, United States) was flushed (or injected) into each pierced follicle through the outer needle channel using a 20 ml lock syringe (Swavet, South Africa), this was repeated three times. The aspiration fluid was then aspirated through the inner needle channel and placed in a sterile sealed 250 ml collection bottle (Lasec, South Africa). The

procedure was repeated on the right ovary whilst the rhino remained in left lateral recumbency.

Figure 1: The round aspiration instrument (145 cm length, 38.5 cm diameter), 3D printed out of PVC plastic, which is comprised of three double lumen needles and an ultrasound transducer, used to recover oocytes during transrectal ovum pick-up procedures in southern white rhinoceros.

***Phase 2*:** At the in-the-field laboratory, the aspiration fluid was filtered through an embryo filter (68 µm, Spitronics Micro Ignitions, South Africa) and the residue examined for oocytes using a stereomicroscope at X 200 magnification (Olympus SZX7, South Africa). The temperature of the aspiration fluid was kept constant by means of a heated working stage (Research Instruments, Germany). Oocytes were washed in holding medium in tissue culture plates (4-well tissue culture plates, AEC Amersham, South Africa) and maintained at a constant temperature of 37.5 °C with another heat stage (WTA Technologies, Germany). Washed oocytes destined for the UP-IVF laboratory was transferred to a 2 ml sterile Nalgene® cryogenic vial (Merck, Germany) containing holding medium, and transported in an Equitainer (Minitube, Tiefenbach, Germany) at 22 °C. The holding medium contained 4 mL M199 Hank's salt; 4 mL M199 Earl's salt; 0.0002g sodium pyruvate (0.20 mM / ml); 0.0033 µL lactic acid; 20% FBS (2 mL / 15 mL); and 50 µg / mL Gentamicin [48]. Oocytes destined for the EP IVF laboratory were transferred to a 2 mL sterile Nalgene® cryogenic vial with maturation

medium, not holding medium, and transported at 37.5 °C in a trimix gas portable IVF transport incubator (Incubadora Portati Labmix, WTA Technologies, Germany) which was gassed to maintain a constant atmosphere of 7% O_2, 7% CO_2, and balanced N_2. The maturation medium contained DMEM; FSH (5 mU / 9 mL); 10% FBS (1 mL / 9 mL); Gentamicin (50 µg / mL); sodium pyruvate (0.20 mM / mL); lactic acid (2.92 mM / mL); MEM NEAA (50 µL / 9 mL); LH (10 µg / mL); EGF (10 ng / mL); Somatropin (1 mU / mL); and Estradiol (1 µg / mL) [49].

3.4.2 Temperature monitoring

Different temperature control and monitoring methods were implemented in this study. Temperature loggers (iButtons, thermochron logger devices, ColdChain ThermoDynamics, Fairbridge, Cape Town, South Africa) were used to record temperatures every minute to an accuracy of 0.0625 °C (activated by the ColdChain ThermoDynamics program; Version 4. 9. 2013. 12. 06. 100). The temperature loggers were wrapped in 2 x 4 cm water-resistant parafilm tape and placed in strategic positions to monitor temperature changes at each stage of the OPU procedure, from start to completion.

An additional temperature logger was placed inside a small sealable plastic bag (Ziploc, Cape Town, South Africa) and attached, two metres above the ground, to the aluminium structure of the 3 x 3 m portable shade canvas used to cover the hind quarters of the rhino and aspiration lines. The ambient temperature was defined as the temperature recorded by this logger.

To record the temperature of the rhino, two temperature loggers were secured to the aspiration instrument to monitor presumed rectal temperature. Two loggers

were used to prevent data loss should one malfunction or become detached during the procedure. The temperature loggers were secured using porous Protofix tape (120 x 1000 cm, Electro Spyres Medical, South Africa), which allows the temperature measurement in the surrounding tissues and fluids (*Figure 2*). To monitor vaginal and oral temperature, one logger was placed in the caudal cervical orifice and another inside the mouth folds. A string was tied around each of the temperature loggers for ease of retrieval.

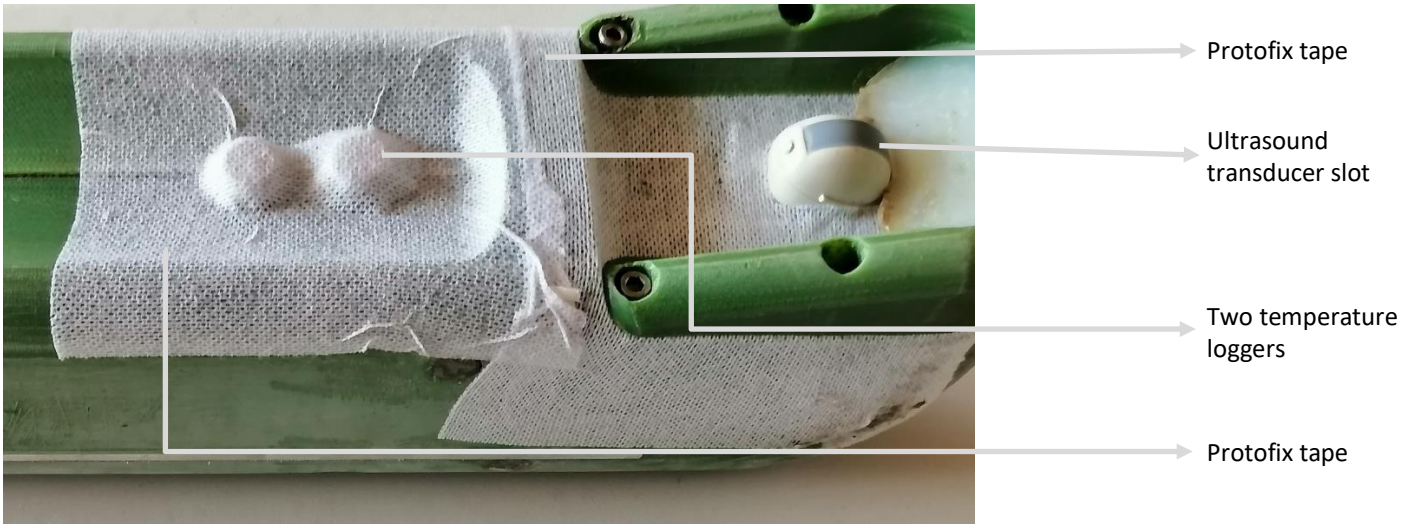

Figure 2: Temperature logger placement on the aspiration instrument, which was used to retrieve oocytes during the transrectal ovum pick-up procedures. Temperature loggers were secured with porous Protofix tape which allowed the temperature measurement of the surrounding tissues and fluids in the rectum of southern white rhinoceros.

To aseptically monitor the temperature of the flushing medium, two temperature loggers were secured to the outside of the flushing medium bag. Two heating methods to keep the flushing medium at a constant 37 °C, were tested. The first method involved suspending the flushing medium bag in a water bath filled with heated tap water prior to and during the OPU procedure. Care was taken to ensure

that the flushing medium sealed in the intravenous (IV) bag had reached the required temperature of the water bath before flushing commenced.

For the second method, a black plastic box (referred to as the EP Box) was used to hold the flushing medium bag. To ensure temperature control, the flushing medium bag was placed between a heating pad (Shenzhen Anpan Health Industry, China) and a heating blanket (Panamedic, South Africa).

The temperature of the aspiration fluid within the collection bottles could not be measured directly without risking contamination. Temperature loggers were therefore placed near the bottles, on the assumption that any difference between the inside and outside temperature of the collection bottles would be minimal. Depending on the heating method used, either two or three temperature loggers were used to record the temperature of the aspiration fluid in the collection bottles. Initially, six collection bottles were kept warm by means of pre-heated rice bags followed by a water bath, a dry water bath (Wasserbad, 10 L, Minitube, Australia) was later used. This dry water bath has a specially designed shelf with three levels (*Figure 3*).

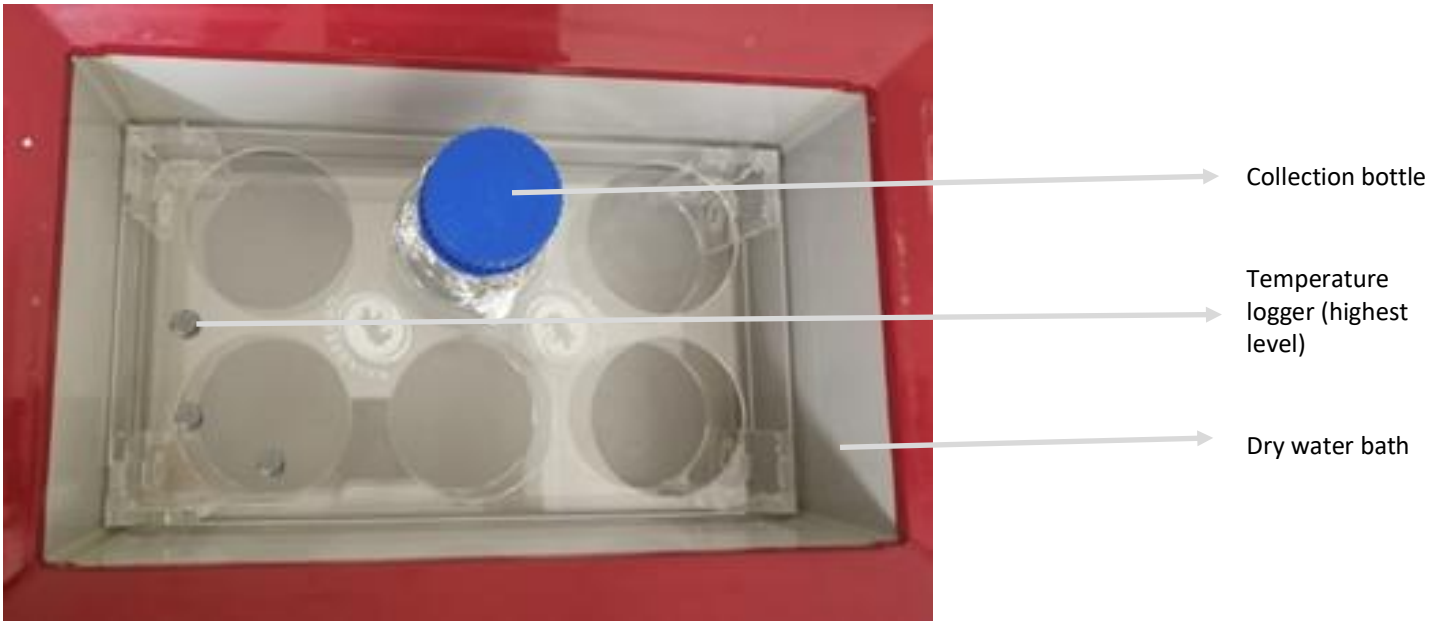

Figure 3: Temperature logger placement within the Wasserbad (dry water bath) housing six collection bottles into which aspiration fluid was aspirated during transrectal ovum pick-up in southern white rhinoceros.

To record the in-the-field laboratory ambient temperature, one temperature logger was secured to the inside of the laboratory; a second to the stereomicroscope heating stage; and a third to the separate laboratory heating stage. During transportation of the processed oocytes to the UP or EP IVF laboratory (see Phase 2 of the oocyte recovery procedure), two temperature loggers were placed inside the transportation device next to the vial containing the oocytes to record in-transit temperatures.

3.4.3 Oocyte maturation

Upon arrival at the laboratory, oocytes were removed from the transportation container, and the time of the end of transportation was recorded. The oocytes were washed three times in pre-equilibrated (equilibration was defined as > 3 hours) maturation medium, and then placed in 4-well culture dishes (AEC Amersham, South

Africa) with pre-equilibrated maturation medium, covered with 400 µl of mineral oil (Harrilabs, South Africa) to prevent the evaporation of medium within the incubator. Oocytes in the UP-IVF incubator (Thermo Forma Steri-cycle CO_2®, Labotec, South Africa) were matured at 38.5 °C in a 5.6% carbon dioxide (CO_2); 20% oxygen (O_2); and balanced nitrogen (N) atmosphere (Afrox, South Africa) for 40 to 44 hours. The oocytes in the EP IVF incubator (Cook Minc 1, Marcus Medical, South Africa) were matured at 37.5 °C in a 7% carbon dioxide (CO_2); 7% oxygen (O_2); and 86% nitrogen (N) atmosphere for 40 to 44 hours.

Following the maturation period, cumulus cells were removed by means of mechanical denuding. If cumulus cells were still present after mechanical denuding, the oocytes were treated with hyaluronidase (10µL, Sigma-Aldrich, United States) to facilitate the process. Each oocyte was then examined for the presence of a polar body as a sign that maturation had occurred. Matured oocytes (those presenting with a polar body) were fertilised by means of ICSI conducted by an experienced embryologist. Frozen semen collected through electro-ejaculation or epididymal flush was used for the ICSI procedure. Motile sperm with normal morphology were selected.

3.5 *In vitro* temperature simulation study

Due to the risk of contamination and the limited space, it was not possible to attach a temperature logger to the inside of the aspiration instrument during the OPU procedure. The instrument's temperature could only be measured by securing a temperature logger to the outside. Given that oocytes were exposed to the inside of the aspiration instrument during the flushing phase of the OPU procedure, it was essential to ascertain whether the measured outside temperature of the aspiration instrument was an accurate indication of the inside temperature. Furthermore, as PVC

plastic is a poor heat conductor, it was necessary to evaluate how long it took the inside of the aspiration instrument to equilibrate with the outside temperature, and by inference, the temperature of the rhino. To minimise the number of animals and OPU procedures needed to determine this, an *in vitro* temperature model simulating the body temperature of a rhino was developed to assess different controlled temperature scenarios.

To simulate the rhino core temperature, a 210 L plastic container (560 mm diameter, 975 mm height, Jojo, Pretoria, South Africa) was filled with water. Three 7.5 cm diameter holes were cut in the lid of the container to accommodate portable electric water heating elements (MIC Model, 2000 Watts, Krazeprice, Johannesburg, South Africa) for heating the water. A digital temperature thermostat (STC 1000, 2K / Watts, RA Smart Technologies, Johannesburg, South Africa) was included to maintain the water temperature between 37.5 °C and 38°C to simulate the core body temperature of rhinos.

To ensure consistent water temperature throughout the container, a fountain pump (Dragonfly 4P0006 pump fountain, Chamberlains, South Africa) was positioned at the bottom of the container to circulate colder water to the surface where the elements were situated. Ambient temperature was measured with a temperature logger positioned within 2m of and at the same height as the plastic container. Three temperature loggers wrapped in 2 x 4 cm water-resistant parafilm tape were secured at distinct levels inside the container namely, just below the water surface (high); ± 48.75 cm from the top (medium); and at the bottom of the container (low). To measure temperatures inside and outside the OPU instrument, two temperature loggers each were placed on the inside and outside, respectively. To simulate the rectal wall and cavity, it was covered in an elastic stocking which was saturated with silicone (*Figure*

4). The OPU instrument was passed through another 20 cm hole in the lid of the container and submerged once the water had reached 38 °C.

Figure 4: An in vitro temperature simulation study was conducted to ascertain whether the measured outside temperature of the aspiration instrument (used to recover oocytes during transrectal ovum pick-up in southern white rhinoceros) was an accurate indication of the inside temperature, the aspiration instrument was encased in a silicone-saturated elastic stocking to simulate the rhinoceros rectal membrane.

To determine the influence of seasonal temperatures the time taken for the aspiration instrument to reach 38 °C, two temperature scenarios were tested. For summer conditions, the aspiration instrument was left overnight at room temperature (22 °C), and inserted into the container for three hours once the water temperature had reached 38 °C. For winter conditions, the aspiration instrument was left in a 4 °C walk-in fridge overnight, and inserted into the container for three hours the next day, once the water temperature had reached 38 °C. This procedure was repeated six times; three for the summer and three for the winter scenarios, respectively.

3.6 Data analyses

The OPU procedures in which equipment issues were encountered (n = 1) were excluded. Cows that were pregnant (n = 1) or presented with no follicles (n = 1) were excluded from the study.

After each OPU procedure, the temperature logger data were downloaded using the ColdChain ThermoDynamics program (version 4.9.2013.12.06.100). The data were then exported into a Microsoft Excel® spreadsheet (with date, time, temperature reading, and temperature unit separated by means of the text-to-columns function) and investigated for anomalies. The start and completion times of each procedure were noted in the field. Data points before and after this recorded time were excluded. This was performed for all the temperature recordings, i.e., ambient, aspiration instrument, vaginal, oral, collection bottles with aspiration fluid, flushing medium, in-the-field laboratory ambient, stereomicroscope heating stage, and separate laboratory heating stage. Transportation temperature monitoring commenced at the end of the OPU procedure and concluded when the oocytes arrived at the respective laboratories. Data points before and after the recorded time were excluded. This monitoring also made it possible to determine if any adjustments to the equipment were needed.

After the removal of invalid data points, the means of the temperature recordings involving more than one temperature logger were obtained. Ambient, oral, vaginal and aspiration instrument temperatures, were used to construct a line graph of each OPU procedure. Then, after conducting a Shapiro-Wilk normality test, a Dunn's *post-hoc* test was performed to determine the differences between these three measured temperatures using statistical program PAST 4.03. After that, a Spearman's rank correlation coefficient test was conducted on ambient and aspiration instrument temperatures measured. Bonferroni corrected *p*-values were used to determine statistical significance ($p < 0.05$).

In addition to the temperature data, recordings made included the date; animal ID; aspiration number (a number was assigned to each OPU procedure, independent

of animal ID); study site (Site 1 / Site 2 / Site 3); laboratory (EP / UP / both); transportation medium (DMEM / holding); number of follicles aspirated left, right and in total; number of oocytes harvested; number of oocytes matured; follicular wave stimulation (stimulated / not stimulated); enema temperature (cold / warm); procedure duration; and finally the breeding season (predominant / non-predominant).

Using statistical program STATA® Version 14 (STATA, California, United States of America), a pairwise comparison of means was carried out to determine whether there were any differences between the three study sites (Sites 1, 2 and 3) with respect to number of follicles aspirated (left, right, and in total); mean ambient, oral, vaginal and aspiration instrument temperature; enema temperature; and procedure duration. In addition, a *t*-test was conducted to determine whether there was a significant difference ($p < 0.05$) between the number of follicles aspirated from the left and right ovaries.

Two mixed effect logistic regression models were constructed in STATA 14.0. The first was to model the odds of oocyte harvest per aspirated follicle. The second was to model the odds of maturation per oocyte harvested. The individual effect (animal ID) was used as a random variable, while the variables of study site (Site 1 / Site 2 / Site 3); laboratory (EP / UP / both); transportation medium (DMEM / holding); follicular wave treatment (stimulated / not stimulated); enema temperature (cold / warm); enema temperature (°C); mean ambient, oral, vaginal and aspiration instrument temperature (°C); OPU procedure duration (minutes); breeding season (predominant / non-predominant); flushing medium heating equipment (EP box / none / water bath); collection bottle heating equipment (rice bags / water bath / Wasserbad / none); microscope stage and in-the-field laboratory heating (yes / no);

transportation method (Transport incubator / Equitainer); and transportation duration (minutes) were used as potential predictor variables. Variables with the highest *p*-value were removed in a stepwise manner to identify the independent predictors of the respective outcomes ($p < 0.05$). To control for confounding, all variables were inserted back into the final model, one by one. If the odds ratio changed by > 15%, confounding was considered, and the variable was kept in the model.

For the temperature simulation study, temperature data were similarly downloaded from the temperature loggers using the ColdChain ThermoDynamics program (version 4.9.2013.12.06.100) and exported into a Microsoft Excel® spreadsheet. The data were cleaned by removing any data points recorded before and after placement of the aspiration instrument inside the water container, yielding a timeframe of 180 minutes. Where anomalies were observed between two or more temperature loggers placed in the same location, the data of the logger with irregular measurements were removed (n = 1). After the trial, both the ambient temperatures and the water temperatures over the six repetitions were averaged. For the inside and outside aspiration instrument temperatures, the data for each of the two temperature scenarios (winter: 4 °C and summer: 22 °C) were averaged, after which the ambient and water temperatures, and the temperatures measured inside and outside the aspiration instrument in each scenario were divided into 1-hour intervals, and the means compared using the Dunn's *post-hoc* test.

4. RESULTS

Temperature fluctuations during OPU procedures (n = 20) in southern white rhino (*Ceratotherium simum simum*), and the impact thereof on *in vitro* maturation outcomes were monitored. After anaesthesia cows were monitored upon waking and then reunited with their calf or family. No animals died or were euthanised during or following the procedures.

4.1 Temperature control equipment

The following observations were made regarding temperatures recorded at different points in the aspiration line, following adjustments made to rectify any temperature fluctuations:

1. An attempt was initially made to keep the temperature of the flushing medium at 37 °C by using a water bath (n = 2). However, upon further scrutiny, it was determined that this method might lead to contamination, as any perforation of the flushing medium bag could result in water from the water bath entering the flushing medium, which would compromise the entire contents of the bag. As an alternative, the flushing medium was not warmed during the procedure (n = 5), but it was eventually established that the cleanest and most manageable temperature control option was to use a black box containing a heating blanket and a heating pad (n = 13). The flushing medium warming method (EP black box / water bath / none) was therefore included as a potential predictor variable in the logistic regression model of maturation.
2. Collection bottles were initially heated by using rice bags (n = 1), but it transpired that the rice bags did not keep the temperature constant. To address this and

the instability of the bottles when empty (as well as the same contamination risk mentioned above) the use of a water bath (n = 1) was rejected. A Wasserbad (n = 17) was finally used and maintained a constant temperature throughout the procedure. There was one instance when none of the above-mentioned forms of heating equipment was available.

3. Heating stages of both the stereomicroscope and in-the-field laboratory maintained a constant temperature and required no adjustments. There were, however, three instances where they were not available due to being required elsewhere.
4. The temperatures recorded in the portable IVF transport incubator (n = 15) ranged between 34.88 and 38.02 °C, while those of the Equitainer (n = 4) ranged between 17.84 and 33.38 °C. The details of temperature fluctuations for each aspiration are given in *Table 2*. There were cases where no oocytes were collected and therefore no transporting took place (n = 2).

Table 2: Temperatures recorded during transport of southern white rhinoceros oocytes harvested after each ovum pick-up procedure. Oocytes were transported to the IVF laboratory at either Embryo Plus in a transport incubator (TI) or the University of Pretoria, Onderstepoort campus, in an equitainer (EQ). Aspirations 9 and 14 did not yield any oocytes and thus transport temperature was not recorded.

Aspiration number	Transport method[1]	Intended temperature (°C)	Duration of transport (minutes)	Temperature recorded every minute during transport (°C)			
				Mean	±SD	Min	Max
1	TI	37.5	241	36.63	0.37	35.92	37.20
2	EQ	22.0	421	22.81	1.30	20.14	24.62
3	TI	37.5	110	36.81	0.13	36.61	37.05
3	EQ	22.0	110	31.51	0.07	30.76	33.38
4	TI	37.5	421	36.63	0.22	36.19	37.03
5	TI	37.5	304	36.63	0.22	36.19	36.94
6	TI	37.5	246	36.78	0.12	36.57	37.03
7	TI	37.5	185	36.75	0.12	36.57	37.03
8	EQ	22.0	351	20.71	1.87	17.84	24.29
10	TI	37.5	301	35.47	0.35	34.89	36.07
11	EQ	22.0	181	21.73	0.21	21.36	22.48
12	TI	37.5	195	37.63	0.24	37.14	38.02
13	TI	37.5	60	37.89	0.05	37.80	38.02
15	TI	37.5	354	36.91	0.22	36.03	37.18
16	TI	37.5	371	36.91	0.32	36.46	37.43
17	TI	37.5	116	37.18	0.19	36.84	37.43
18	TI	37.5	455	36.25	0.15	35.87	36.52
19	TI	37.5	406	36.22	0.13	35.87	36.46
20	TI	37.5	36	35.82	0.03	35.78	35.87

[1]**TI = Transport incubator and EQ = Equitainer**

4.2 Temperatures of the rhinos and aspiration instrument

During all the OPU procedures (n = 20), the mean vaginal temperature recorded (n = 19) was higher than the mean oral and aspiration instrument temperatures. The mean oral temperature was also higher than the mean aspiration instrument temperature (vaginal: 36.55 ± 1.61, oral: 35.58 ± 2.10 and aspiration instrument: 35.02 ± 2.31 °C, $p < 0.01$). Temperature data of the animal (oral and vaginal) and the aspiration instrument, as well as the ambient temperature, were assembled into a line graph accompanied by a table outlining the specific details of each OPU procedure as well as the heating equipment (*Appendix IV*). When a Shapiro-Wilk normality test indicated that the data were non-parametric, a Dunn's *post-hoc* test was conducted to test for significant differences ($p < 0.05$) between the mean oral, vaginal and aspiration instrument temperatures during each individual OPU procedure. The three temperatures differed ($p < 0.05$) in all but five of the OPU procedures recorded (*Appendix IV*). Aspirations 6, 7 and 9, showed no differences between the mean oral and vaginal temperatures ($p = 0.15$, $p = 0.54$, $p = 0.58$, respectively). In Aspiration 11, there were no differences between the mean vaginal and aspiration instrument temperatures ($p = 0.13$), while in Aspiration 16, no difference was observed between the mean oral and aspiration instrument temperatures ($p = 0.08$). Additionally, the mean vaginal and aspiration instrument temperatures of rhinos which had undergone follicular wave stimulation by means of exogenous hormone administration was higher than those that did not (Vaginal: Stimulated 36.78 ± 1.20 vs not stimulated 36.00 ± 2.22; Aspiration instrument: Stimulated 35.51 ± 2.07 vs not stimulated 33.93 ± 2.52; $p < 0.01$). However, the mean oral temperatures were lower in rhinos which had undergone follicular wave stimulation than those that did not (Oral: Stimulated 34.99 ± 2.05 vs not stimulated 36.86 ± 1.71).

4.3 Ambient and aspiration instrument temperatures

There was a positive linear correlation between ambient temperature and aspiration instrument temperature (Spearman's rank correlation coefficient = 0.43, R^2 = 0.14, $p < 0.01$, Y = 0.23X + 26.57; *Figure 5*). The same positive linear correlation was observed when comparing mean ambient and aspiration instrument temperatures (Spearman's rank correlation coefficient = 0.37, R^2 = 0.55, $p < 0.01$, Y = 0.34X + 23.24) of each OPU procedure (*Figure 6*). This correlation was independent of enema water warming or not (indicated by a circle; *Figure 6*).

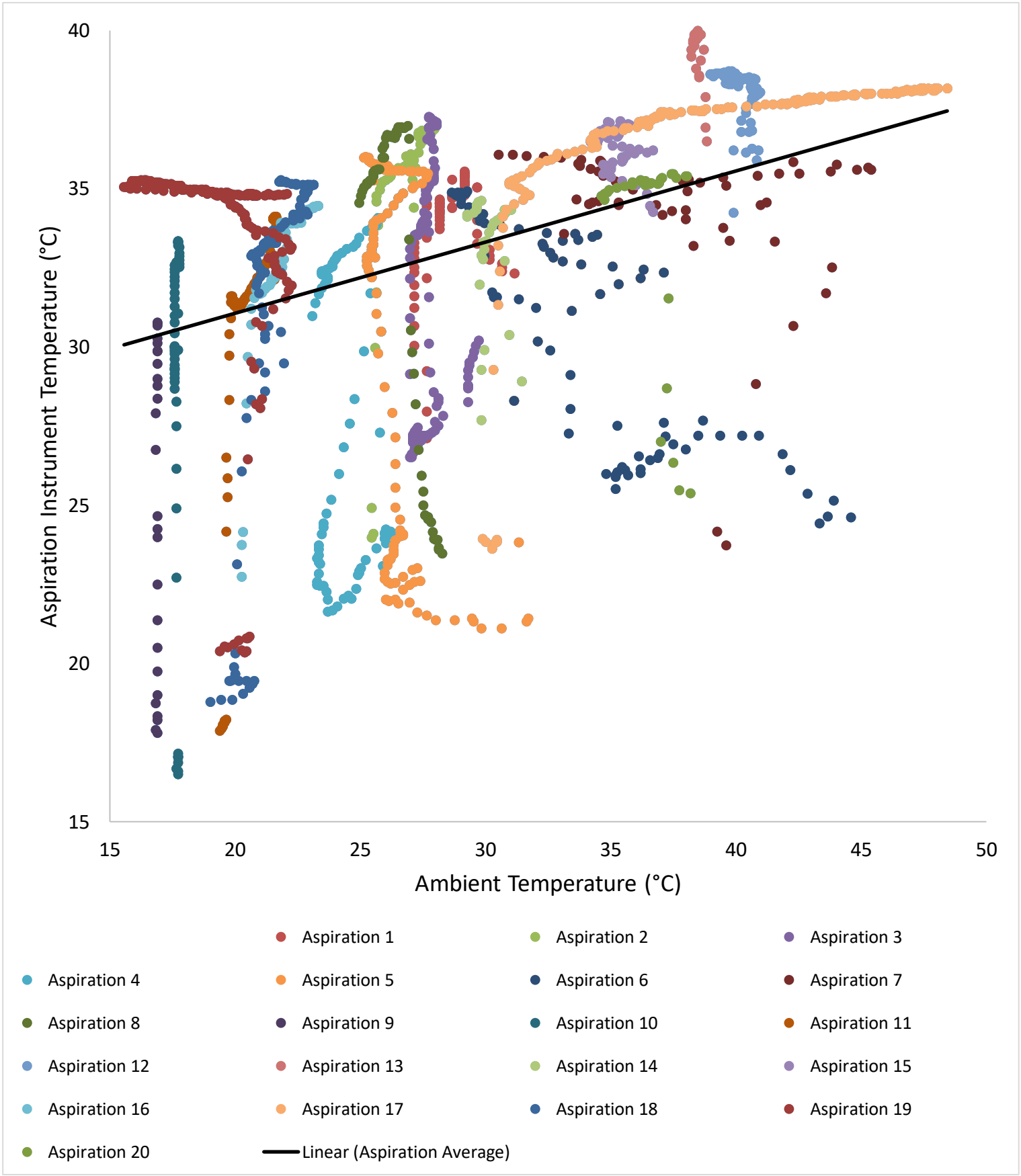

Figure 5: Scatterplot of the positive linear correlation between the ambient and aspiration instrument temperature (°C) per individual data collection point (Spearman's rank correlation coefficient = 0.43, R^2 = 0.14, $p < 0.01$, $Y = 0.23X + 26.57$) measured during ovum pick-up procedures on southern white rhinoceros.

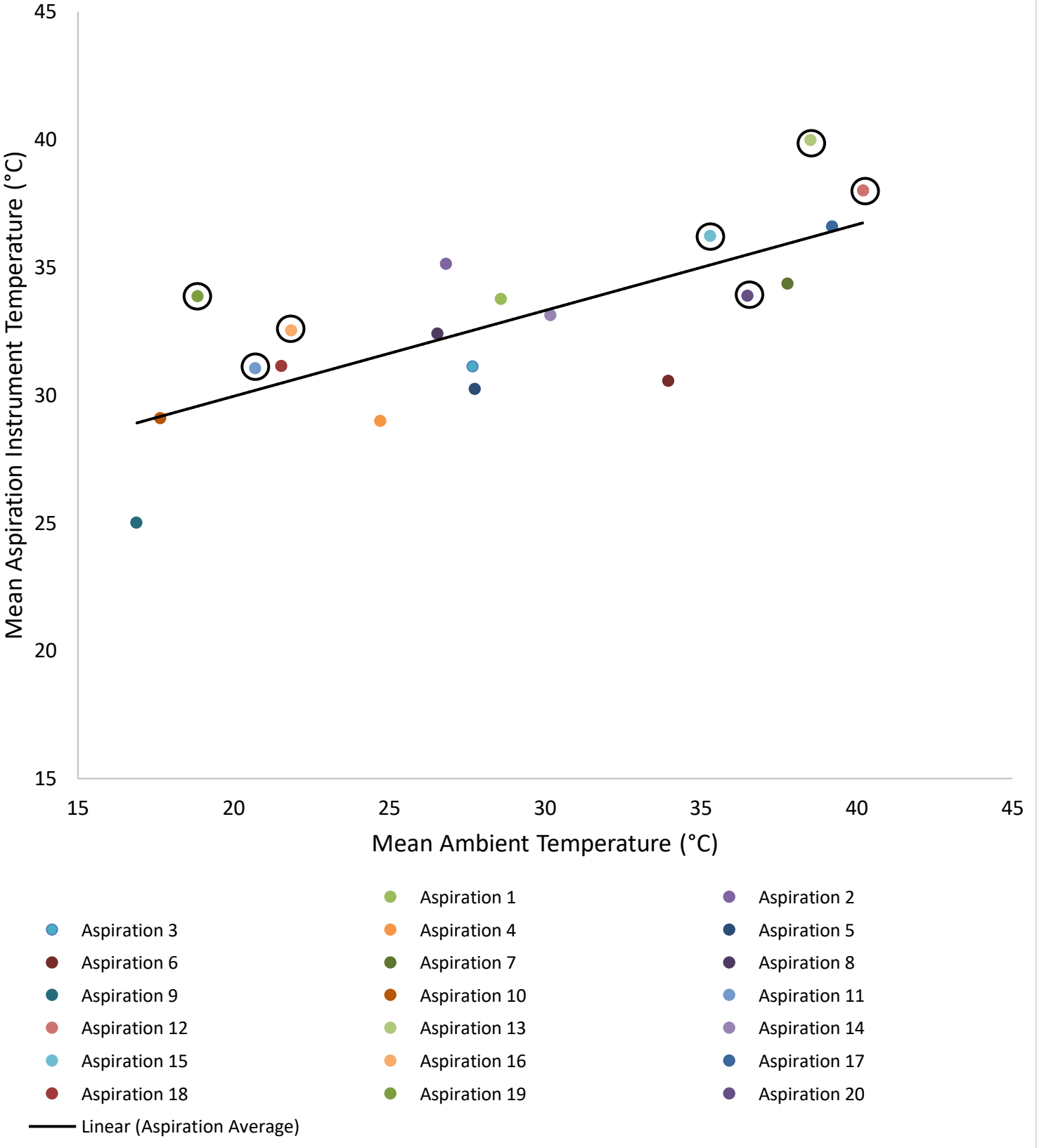

Figure 6: Scatterplot of the positive linear correlation between the mean ambient and aspiration instrument temperatures (°C) per ovum pick-up procedure on southern white rhinoceros (Spearman's rank correlation coefficient = 0.37, $R^2 = 0.55$, $p < 0.01$, $Y = 0.34X + 23.24$). The ◯ indicates the procedures where the enema water, with which the rectum was cleaned prior to the procedure, was warmed.

4.4 *In vitro* temperature simulation study

In the temperature simulation study, two scenarios were tested, summer conditions (22 °C) compared to winter conditions (4 °C). A Shapiro-Wilk normality test revealed that the data were not normally distributed, and a Dunn's *post-hoc* comparison test was therefore conducted. The means of the ambient temperature and the temperature of the water in the container (proxy for the rhino's core temperature) remained stable in all cases (n = 181; mean 25.99 ± 0.34 °C and 37.89 ± 0.06 °C, respectively).

In the summer temperature scenario (22 °C), the mean inside and outside temperatures of the aspiration instrument differed significantly (n = 181; mean 36.04 ± 1.69 °C and 37.22 ± 0.92 °C, respectively), and were both lower than the temperature of the simulated rhino core (n = 181; 37.90 ± 0.06 °C; $p < 0.05$) in all cases (*Figure 7*). Similarly, in the winter temperature scenario, the inside and outside temperatures of the aspiration instrument (n = 181; mean 33,23 ± 4.28 °C and 36.51 ± 2.13 °C, respectively) differed significantly, and neither the inside temperature nor the outside

temperature reached that of the simulated rhino core (n = 181; 37.90 ± 0.06 °C) in the 3-hour long trial (*Figure 7*).

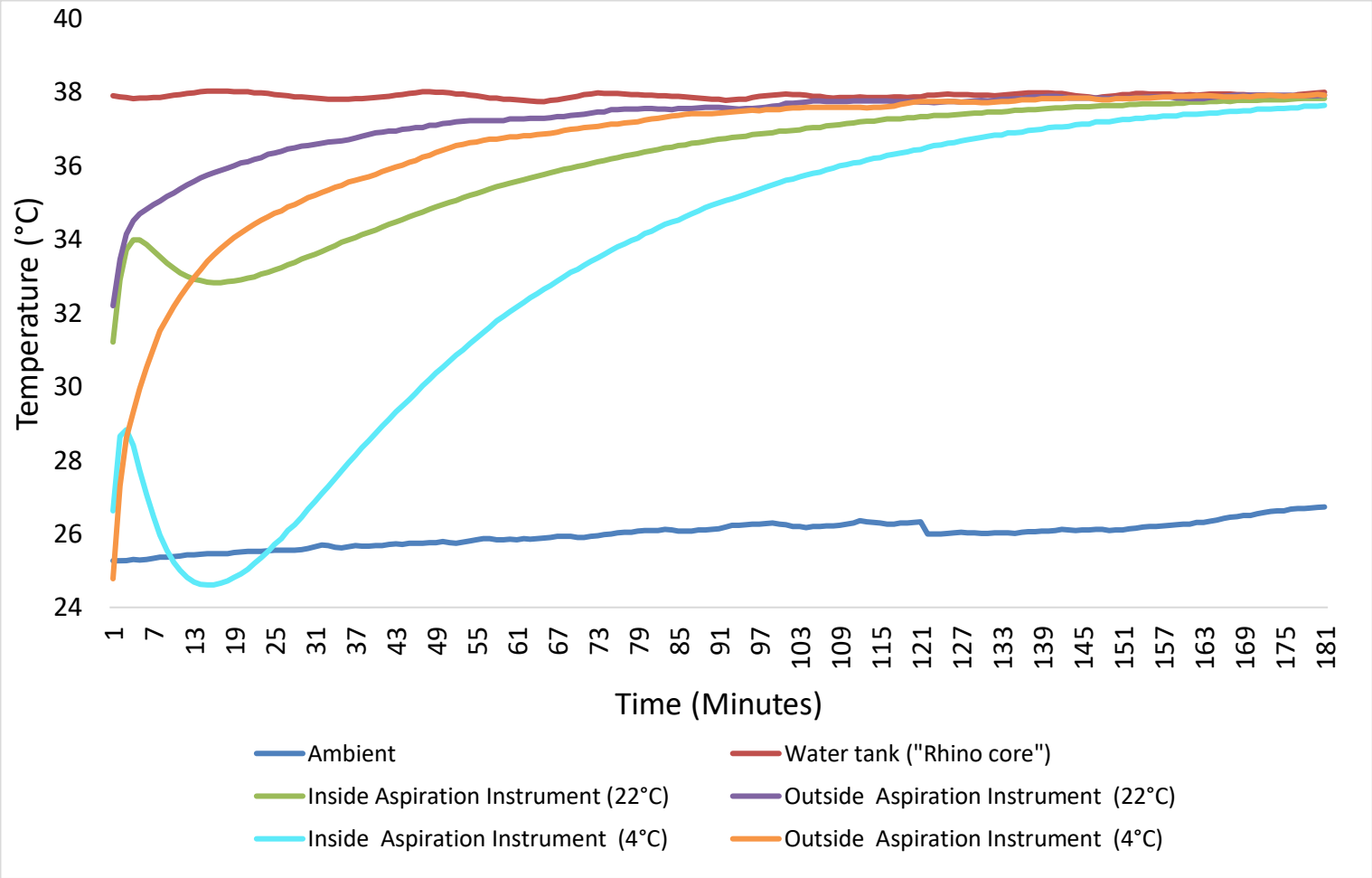

Figure 7: To ascertain whether the outside aspiration instrument temperature, used during ovum pick-up in southern white rhinoceros, was an accurate indication of the inside temperature, an in vitro simulation study was conducted. The mean ambient, water container, and aspiration instrument temperatures (inside and outside) were recorded over time in two temperature scenarios: 1) where aspiration instrument was at 22 °C (representing a summer day); and 2) where aspiration instrument was cooled to 4 °C (representing a winter day). In both scenarios, neither the inside nor the outside temperature of the aspiration instrument reached that of the simulated rhino core within 3 hours.

4.5 Effect of site on ovum pick-up procedures

The pairwise comparison of means revealed that there were no significant differences between Sites 1, 2 and 3 (see *Table 3*) in terms of the recorded number of follicles aspirated left, right and in total, and the number of oocytes harvested. However, the number of follicles aspirated from the right ovary at Site 3 tended to differ from Sites 1 and 2 (pairwise comparison test, $p < 0.10$). No significant differences were found between the sites in terms of ambient, oral, vaginal, aspiration instrument and enema temperatures, or OPU procedure duration. However, the mean vaginal temperatures tended to be higher at Site 3 (free roaming; 37.26 ± 0.96) compared to Sites 1 and 2 (35.64 ± 1.74 and 35.64 ± 0.40 respectively, $p = 0.10$).

Table 3: A summary of the means of known variables between the three study sites where ovum pick-up procedures took place on southern white rhinoceros.

	SITE 1	SITE 2	SITE 3
Number of procedures (n)	8	10	2
Stimulated (n)	6	3	0
Warm water enema (n)[1]	3	4	0
	Mean (± SD)	Mean (± SD)	Mean (± SD)
# Follicles aspirated left (n)	5^{a} (± 3)	5^{a} (± 4)	5^{a} (± 2)
# Follicles aspirated right (n)	4^{a} (± 2)	4^{a} (± 5)	3^{b} (± 2)
# Follicles aspirated in total (n)	10^{a} (± 4)	9^{a} (± 8)	7^{a} (± 0)
# Oocytes harvested (n)	4.75^{a} (± 4.83)	3.80^{a} (± 5.44)	1.00^{a} (± 0.01)
Aspiration rate (%)[2]	45.42^{a} (± 25.75)	29.11^{a} (± 27.99)	9.52^{a} (± 0.01)
# Oocytes matured (n)	2.13^{a} (± 1.46)	2.00^{a} (± 3.09)	0.01^{a}
Maturation rate (%)[3]	58.91^{a} (± 36.01)	38.78^{a} (± 36.99)	0.01^{a}
Enema temperature (°C)	30.50^{a} (± 4.12)	28.67^{a} (± 6.22)	-
Ambient temperature (°C)	32.41^{a} (± 7.45)	25.38^{a} (± 7.19)	26.45^{a} (± 0.73)
Oral temperature (°C)	36.00^{a} (± 2.58)	35.72^{a} (± 1.46)	38.83^{a} (± 0.11)
Vaginal temperature (°C)	37.26^{a} (± 0.96)	35.64^{b} (± 1.74)	38.15^{c} (± 0.40)
Aspiration instrument temperature (°C)	35.70^{a} (± 2.64)	33.51^{a} (± 2.11)	36.02^{a} (± 0.05)
Duration of OPU procedure (min)	61.13^{a} (± 39.56)	64.60^{a} (± 52.66)	41.50^{a} (± 9.19)

[1]When enema water temperature was higher than the mean ambient temperature during the OPU procedure.

[2] Oocytes harvested as a proportion of total follicles aspirated (mean).

[3] Oocytes matured as a proportion of oocytes cultured (mean).

[a, b, c] Mean values with different superscripts tended to differ from each other ($p < 0.10$)

4.6 Oocytes harvested

A mean of four oocytes were harvested per OPU procedure (3.90 ± 4.94 oocytes per OPU procedure). All follicles were aspirated, irrespective of size. In the mixed regression model of number of oocytes harvested per follicle aspirated, the random variable of individual effect (animal ID) did not have an effect (p = 1.00) and was therefore excluded. The likelihood of an oocyte being harvested from an aspirated follicle was influenced by the site of the procedure, with the likelihood being highest at Site 1, then Site 3, and finally at Site 2 (*Table 4*), after considering variables such as individual effect (animal ID) and whether the animal was stimulated or not. Season also affected the likelihood of recovery, which was higher during the predominant breeding season (1 November – 30 April), after adjusting for the other significant variables, including the duration (n = 20; mean 60.90 ± 44.06 minutes) of the OPU procedure (*Table 4*).

Table 4: Logistic regression model of the odds of harvesting an oocyte per follicle aspirated during ovum pick-up on southern white rhinoceros, after accounting for individual effect and whether the animal was hormonally stimulated.

Variable		Odds Ratio (95% CI)	p-value
Study site	Site 1	1.00 (0.00 - 0.00)	-
	Site 2	0.48 (0.24 - 0.99)	0.05
	Site 3	0.12 (0.02 - 0.61)	0.01
Duration of OPU procedure (min)		1.02 (1.01 - 1.02)	< 0.01
Season	Predominant	1.00 (0.00 - 0.00)	-
	Non-predominant	0.28 (0.13 - 0.64)	< 0.01
Constant		0.72 (0.36 - 1.44)	0.35

4.7 Oocytes matured

For the purposes of this study, maturation was regarded as a successful outcome since none of the oocytes, which were fertilised *in vitro* by means of ICSI, developed into embryos. In the mixed regression model of oocytes matured per oocytes harvested, the random variable of individual effect (animal ID) had no effect (p = 1.00) and was therefore excluded. The likelihood of a harvested oocyte's maturing was independent of follicular stimulation and enema water temperature (*Table 5*). Stimulation of cows did not increase the number of follicles (mean = 10.55 vs 7.11, p = 0.214). Oocytes harvested from unstimulated cows that received a warm enema were more likely to mature.

Table 5: Logistic regression model of oocytes matured per oocyte harvested (p < 0.10) during ovum pick-up in southern white rhinoceros, after accounting for the variable of individual effect.

Variable		Odds Ratio (95% CI)	p-value
Follicular wave treatment	Not Stimulated	1.00	-
	Stimulated	0.22 (0.05 – 0.93)	0.04
Enema temperature	Cold	1.00	-
	Warm[1]	2.31 (0.88 – 6.10)	0.09
Microscope and in-the-field laboratory stage	Not warmed	1.00	-
	Warmed	4.42 (0.50 – 38.89)	0.18
Constant		0.50 (0.09 – 2.73)	0.42

[1]When enema water temperature was higher than the mean ambient temperature during the OPU procedure.

5. DISCUSSION

The data support the hypothesis that the temperature of the aspiration fluid at different points in the OPU procedure can be accurately measured, and that temperature fluctuations do occur along the OPU procedure aspiration line. The data also showed that temperature fluctuations during the OPU procedure do have an impact on *in vitro* maturation of the collected oocytes and that these significant temperature fluctuations along the OPU procedure aspiration line can be controlled. These hypotheses helped to achieve the main aim of this study which was to minimise temperature fluctuations during the OPU procedure performed in rhino under field conditions.

5.1 Temperature control equipment

A few different temperature control methods were implemented in this study. Strategically placed temperature loggers assisted with the identification of the most suitable equipment and methods for maintaining the temperature required during OPU procedures in the three locations. However, it is important to note that the efficacy of the temperature control methods was influenced by the environment and corresponding site challenges. Furthermore, this is observational evidence, and while we were able to record the temperatures reached during OPU procedures, it must be noted that continuous modifications were made based on the sometimes-unanticipated prevailing field conditions. These modifications were mostly properly recorded which allowed them to be modelled into our statistical analyses. Using the analyses, we were able to determine the impact of the various warming methods implemented specifically under field conditions. Warming methods found to be

effective in this study may not be as effective in other study populations or environmental conditions.

During transportation of the oocytes in the portable IVF transport incubator the temperature ranged from 34.88 to 38.02 °C, whereas in the Equitainer the range was 17.84 to 33.38°C. The data thus indicate that the Equitainer is less suitable for maintaining the required temperature than the portable IVF transport incubator. It would therefore seem advisable to use portable heating/cooling incubators for the transportation of harvested oocytes.

Oocytes transported in the portable IVF transport incubator were placed in maturation medium and were gassed with trimix gas to maintain the pH, while those in the Equitainer were transported in holding medium. Limited information is available on methods of transporting oocytes harvested in the field from live rhino and the final outcomes. Comparative studies that investigated the effect of transport protocols on equine oocyte maturation and blastocyst rates, found that immature oocytes can be held in holding medium at room temperature (22 °C). The temperatures to which immature oocytes are exposed can gradually be decreased without any downstream effects on maturation rate or blastocyst development [2,46,50,51]. However, it would seem that oocytes harvested from dominant follicles should be maintained at body temperature , as meiosis will have commenced prior to aspiration [46]. Oocytes are extremely sensitive to variations in factors such as temperature [36]. It has been suggested that even a minor drop in temperature to 32 °C for only 1.5 minutes, following harvesting from a dominant follicle, can cause irreversible depolymerisation of the oocyte meiotic spindles [45,46]. Furthermore, placing oocytes from dominant follicles in holding medium at a temperature lower than body temperature can be detrimental, especially if they fluctuate. Temperature

fluctuations recorded in the Equitainer during transport are considered to be harmful to oocytes from both dominant and immature follicles [46,50]. In this study, the developmental stage of the oocytes was unknown, and the optimal temperature for their transportation has not been determined. Even though the two laboratories used different transportation methods, there were many variables between the two laboratories and for this reason it cannot be assumed that the different outcomes were solely due to the different transportation methods. Further investigations are therefore needed.

5.2 Temperatures of the rhinos and aspiration instrument

Although not validated, the normal body temperature of a rhino is said to range between 34.5 and 37.8 °C [26]. In this study, the mean vaginal temperatures were higher than those of the oral and aspiration instrument temperatures. Further investigations are needed to determine the reason/s for the differences. The initial assumption when designing this project was that the temperature of the aspiration instrument would closely reflect rectal temperatures. However, after further investigation this was found to not be the case. We can therefore not extrapolate with any certainty that the aspiration instrument temperatures represent the rectal temperatures, and we thus cannot conclude that the rectal temperatures are lower than the vaginal and oral temperatures.

The vaginal, oral and aspiration instrument temperatures measured in this study differed significantly from one another in all but five of the OPU rhinos. In Aspirations 6, 7 and 9, there was no difference between the mean oral and vaginal temperatures. In Aspiration 16, no significant difference was found between the mean oral and aspiration instrument temperatures. Interestingly, Aspiration 11 showed no significant

difference between the mean vaginal and aspiration instrument temperatures. Possibly this was an effect of the enema heated to 31 °C. However, the same did not apply to the six other aspirations, where the enema was heated to 29 °C or greater. However, contrary to our finding, a study performed in cattle found that vaginal temperature was on average lower than rectal temperature, while also noting that vaginal temperature was less variable and affected by fewer external factors, making it a more reliable reflection of core body temperature [52]. It is therefore reasonable to assume that the vaginal temperatures measured in this study, provide the most accurate indication of core body temperature.

In this study, oral temperature during the OPU procedure was measured by placing the temperature logger inside the mouth folds of the rhinos. Rhinos thermoregulate by panting, or behaviours such as mud bathing [37,41]. Furthermore, the administration of immobilisation drugs may have reduced the peripheral blood flow to the lips [53]. The possible decreased blood supply to the mouth and heat loss due to panting may have caused the mean oral temperatures to be lower than the vaginal temperatures. Future studies might consider measuring oesophageal rather than oral temperature, as it is less likely to be affected by respiration or changes in peripheral blood flow [23,54].

Additionally, the mean vaginal and aspiration instrument temperatures of rhinos where follicular wave stimulation had been used were significantly higher than those without stimulation. However, the opposite was true for mean oral temperatures, with significantly lower oral temperatures being measured in the stimulated animals. As the follicular wave stimulation protocol was not part of this study, there is no further speculation about the reasons for this, but the subject should be considered for future research.

It is important to note that none of the temperature measurement methods used in this study are flawless, and each has its own limitations. Vaginal temperatures might have been affected by logger placement or air movement due to the animal lying down [55]. In addition to daily circadian rhythms, the positioning of loggers, thermoregulatory responses, and mouth movement during the OPU procedure could have been responsible for the variable oral temperatures. Future studies could include measurement of these temperatures throughout the day and during normal behaviour to determine which method is most effective for determining the core body temperature of rhinos.

5.3 Ambient and aspiration instrument temperatures

Interestingly, there was a positive linear correlation between ambient temperature and aspiration instrument temperature in these data (*Figures 5* and 6). The correlation was weak, and only 14% of the variation in the aspiration instrument temperature being explained by variation in the ambient temperature.

Some variations could be explained by the temperatures of the rhinos. During rhino immobilisation, Morkel *et al.* (2012) found that for every 1 °C increase in ambient temperature, rectal and muscle temperatures increased by 0.09 °C. Temporal changes in body temperature during the course of a day as a result of circadian rhythm are observed in many mammalian species [52,54]. Thus, circadian rhythm may have played a minor role in this study. Irrespective of the possible reasons for variations in animal temperature, this might explain some of the variation observed in aspiration instrument temperatures. It is, however, important to mention that such variations may also be explained by other factors such as the insertion depth of the aspiration instrument, removal of dung, and temperature of the enema solution.

5.4 *In vitro* temperature simulation study

The aim of the temperature simulation study was to determine whether the temperature of the aspiration instrument reflects rectal temperature, and, if not, how long the aspiration instrument should be heated to rhino body temperature (assumed to be 37.5 °C) prior to the OPU procedure for the oocytes not to experience temperature fluctuations. Two scenarios were tested: summer (22 °C) and winter (4 °C). In both scenarios, after three hours of equilibration in the water container, the internal and external instrument temperatures were significantly lower than the simulated rhino body temperature (*Figure 7*). This implies that, despite being preheated for 180 min prior to the OPU procedure, the aspirated oocytes will be exposed to a temperature lower than rhino body temperature. In practice, the study suggests that the plastic aspiration instrument should be preheated for a minimum of 180 min, and longer in winter, before starting the OPU procedure. If not, the oocytes are likely to be exposed to suboptimal temperatures, which may affect maturation rate or blastocyst development [2,50,51]. It was initially presumed that the aspiration instrument would reach a temperature similar to rectal temperature quite rapidly, but the findings indicate that the aspiration instrument material is a poor conductor of heat. It is therefore suggested that the aspiration instrument should be made of material/metal that is a good heat conductor, and thus, the aspiration procedure could potentially begin shortly after insertion of the instrument into the rectum and without prior heating of the aspiration instrument.

5.5 Effect of site on ovum pick-up procedures

There were no significant differences between Sites 1, 2 and 3 in terms of the number of follicles aspirated in total, left ovary or right ovary, and total number of

oocytes harvested (*Table 3*). However, the number of follicles aspirated from the right ovary at Site 3 was marginally lower than at Sites 1 and 2. Similar follicle numbers being aspirated left and right may be related to the symmetry of ovarian size in rhinos. A study in Sumatran rhinos found that the right ovary was smaller than the left [56]. As Sumatran rhinos are smaller, the ovaries could be palpated subjectively transrectally; a method that is possibly not as accurate as measurements taken by means of transrectal ultrasound. Limited information exists on ovarian symmetry in rhinos, which is a suitable topic for further investigation.

There were no significant differences between sites in terms of ambient, oral, vaginal, aspiration instrument and enema water temperatures, as well as OPU procedure duration. However, the mean vaginal temperatures tended to differ between sites, with the highest temperatures being recorded at Site 3, followed by Site 1, and then Site 2. A similar pattern (although not significant) was observed with oral and aspiration instrument temperatures. Ambient temperature, was highest at Site 1, followed by Site 3 and then Site 2, but was not significantly different. Ambient temperature does therefore not explain the pattern observed in vaginal temperatures. Meyer *et al.* (2008) proposed that hyperthermia in captured impala was the result of a stress response rather than ambient temperature or physical exertion [57]. Impalas that were not habituated to humans showed a greater increase in body temperature due to a higher stress response. Furthermore, the impalas that were believed to be habituated to humans through being handled and housed in bomas presented with lower plasma cortisol concentrations than those that were not [57]. The habituation status of rhinos and consequently their stress response to darting might be a viable hypothesis for the site differences in vaginal temperatures measured in this study. The rhinos at Site 3 are free-roaming animals and the farming system is extensive (*Table*

1), with the result that they very seldom encounter humans unless darted for a procedure. The fact that they are unhabituated might lead to a greater stress response to the darting. Due to these animals being free roaming they have more space in which to run, once darted, which could explain the higher vaginal temperatures at this site. The farming system at Site 1 is intensive, and the animals are not able to move between bomas or engage in natural behaviour. During the course of the study, the rhinos at this site seemed more stressed than those at Site 2. For example, during one of the OPU procedures a male in an adjoining boma continuously mock-charged the boma where the procedures took place, and then sprayed urine in the direction of the researchers in territorial marking behaviour. Furthermore, prior to darting, females at Site 1 were often observed pacing alongside the boma fence once the researchers arrived. This was not observed at Site 2, where the rhinos paid little attention to the researchers until darting had occurred. Finally, the rhinos at Site 2 were semi-intensively managed. The bomas are large allowing the animals to move freely and interact with one another, while also accustomed to humans moving in and between the bomas. As a result of being the most habituated group, it is proposed that the rhinos at Site 2 were less stressed by the presence of humans prior to being darted. This may explain why the lowest vaginal temperatures were recorded at this site. Future studies could investigate the effect of stress levels on oocyte development potential in rhinos.

5.6 Oocytes harvested

In this study, a mean of four oocytes was harvested per OPU procedure, with maximum 18 oocytes in one female. In a black rhino study, between two and nine oocytes were harvested per OPU procedure [9]. In another study on white rhino,

between two and 11 were harvested [2]. At San Diego Zoo, between two and nine oocytes were harvested in 2020 and zero oocytes were harvested in 2022 from southern white rhino, respectively (Barbara S. Durrant, personal communications). The number of oocytes harvested in the current study is thus on par with or better than similar studies on rhino species.

Other studies have indicated that stimulation prior to the OPU procedure, increased the number of follicles available to be flushed [9]. This was not the case in the current study. On account of the small sample size, a type 2 error cannot, however, be excluded. Furthermore, it is possible that the follicular wave stimulation protocol used needs to be refined. The number of oocytes harvested per follicle aspirated may be affected by the number of flushes conducted. Further investigation is needed to determine the effect flushing more than three times has on the number of oocytes recovered. Although the likelihood of harvesting an oocyte was highest at Site 1; followed by Site 3 and then Site 2, the estimated proportion of breeding females was highest at Site 3 (95%) followed by Site 2 (40%), and no breeding females at Site 1 (0%) (*Table 1*). Other factors that may explain these differences, such as the number of breeding males present, age strata of females, were not recorded in this study.

Because they have oestrous cycles that have been observed throughout the year, it has been suggested that, neither wild nor captive white rhinos, are seasonal breeders [25,27–32]. In this study, the likelihood of retrieving oocytes during the presumed non-predominant breeding season (May – October) was lower than during the predominant breeding season (November – April). An increase in births from December to April has also been noted in long term studies [33]. If gestation is 16 months [24,26], conception would have occurred between September and January of the previous year. This may explain why seasonal differences in the number of oocytes

harvested in this study was observed. This study supports the evidence of a clear seasonal pattern resulting in more calves being born from December to April [33]. By contrast, Roth (2006) suggests that any seasonal trends observed in captive populations are most likely due to management practices [25]. Future studies are needed to investigate this.

5.7 Oocytes matured

The mixed regression model (*Table 5*) used to predict the number of oocytes matured per oocytes harvested revealed that oocyte maturation tended to differ based on follicular wave treatment (stimulated / not stimulated). After adjusting for other factors, oocytes harvested from non-stimulated animals were more likely to mature than stimulated animals. This was also observed by Hermes *et al.* 2009 [9]. The reasons for this need further investigation. The aim of stimulation is presumably to increase the number of follicles available for the transrectal OPU procedure and not to improve oocyte maturation rate [9]. However, the data from this study could not confirm that this was achieved. Although the small sample size may have resulted in a type 2 error in the results, we suggest that the follicular wave stimulation protocol needs further investigation.

The likelihood of an oocyte maturing following a warm-water enema was 2.31 times higher than when a cold-water enema was used. The probable reason for this was the warming effect of the warm enema on the aspiration instrument, which took long to warm up. Oocytes harvested from cows given a warm-water enema were therefore not exposed to a considerably lower temperature in the aspiration instrument. Interestingly, the aspiration instrument temperature did not influence this model, due to collinearity with the enema temperature. On the other hand, warming

the microscope and field laboratory stage influenced the logistic regression model as an independent predictor of the likelihood of oocyte maturation because it changed the odds ratio for follicular wave stimulation by 41%. Warming the microscope and field laboratory stage was consequently considered a potential confounding effect.

During capture and immobilisation, animals may experience hyperthermia, and sometimes capture myopathy or even death [57]. Meyer *et al.* (2008) found that the capture-induced increase in body temperature in impala (irrespective of capture method) was not related to physical exertion or a higher ambient temperature, and proposed instead that the increase was in response to the stress induced by capture [57]. In this study, emphasis was placed on cooling the rhinos during immobilisation, especially when the ambient temperatures were high. The data of this study indicates that rectal and aspiration instrument temperatures should receive greater consideration in terms of ensuring the success of ARTs. With the elevated risk of oocyte hypothermia during the OPU procedure used here, the use of a low temperature enema would seem to be contraindicated.

6. CONCLUSIONS

This study contributes to the long-term IRRC study which aims to develop innovative assisted reproductive technologies for sustainable rhino conservation breeding to protect the species from extinction. Furthermore, this study assisted in determining which procedural and temperature variables play a role in the success rate of oocyte harvesting and maturation.

1. Portable heating/cooling incubators are preferred for the transportation of rhino oocytes to the IVF laboratory as they are less prone to temperature fluctuations.
2. The plastic aspiration instrument used in this study is a poor conductor of heat and, as a result, took more than three hours to reach body temperature.
3. After adjusting for other factors, the likelihood of retrieving an oocyte during the presumed non-predominant breeding season (May – October) was lower than during the predominant breeding season (November – April).
4. The rate of oocyte harvesting was affected by the site at which the rhinos were kept, after adjusting for other factors.
5. The oocytes harvested from rhino cows that did not undergo follicular wave stimulation prior to the procedure were more likely to mature, after adjusting for other factors.
6. Oocytes harvested from rhinos which received a warm-water enema were 2.31 times more likely to mature than oocytes harvested from those which did not, after adjusting for other factors including temperature control of the field laboratory, individual effect, and stimulation protocol.

7. FUTURE RESEARCH QUESTIONS EMERGING FROM THIS STUDY

1. Which temperature measurement sites are most representative of the core body temperature of rhino?
2. What influence does circadian rhythms have on the body temperature of rhino?
3. Can heating the aspiration instrument used in this study take place under field conditions?
4. Can the aspiration instrument used in this study be redesigned to improve the thermal properties, thereby decreasing the impact of temperature fluctuations on the oocytes?
5. Can the rhino OPU procedure be refined to improve oocyte harvesting and development rates?
6. How do follicular wave stimulation protocols affect the success of oocyte development?

8. STUDY LIMITATIONS

As this was an observational study, it was impossible to control all variables. Furthermore, we could only determine which techniques were most effective and easy to implement under field conditions. Warming techniques found to be effective in this study might not be as effective in other environments or study populations.

Due to the limited available sample size the conclusions from this study cannot necessarily be generalised or applied to other study populations and/or environments.

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10. APPENDICES

10.1 Appendix I: Documentation

Section 20 Permit

agriculture,
forestry & fisheries

Department:
Agriculture, Forestry and Fisheries
REPUBLIC OF SOUTH AFRICA

Directorate Animal Health, Department of Agriculture, Forestry and Fisheries
Private Bag X138, Pretoria 0001

Enquiries: Mr Herry Gololo • Tel: +27 12 319 7532 • Fax: +27 12 319 7470 • E-mail: HerryG@daff.gov.za
Reference: 12/11/1/1/8

Dr Kate May
Department of Production Animal Studies
Faculty of Veterinary Science
Old Soutpan Road
Onderstepoort, 0110

Email: kate.may@up.ac.za; CC: Mario.smuts@up.ac.za

Dear Dr May,

RE: PERMISSION TO DO RESEARCH IN TERMS OF SECTION 20 OF THE ANIMAL DISEASES ACT, 1984 (ACT NO. 35 of 1984)

Your application, submitted on 29 March 2019, requesting permission under Section 20 of the Animal Disease Act, 1984 (Act No. 35 of 1984) to perform a research project or study, refers.

I am pleased to inform you that permission is hereby granted to perform the following research/study, "*Developing assisted reproductive technologies for the conservation of the rhinoceros (Cerathotherium simum simum)*" with the following conditions:

Conditions:

1. This study is approved as per the application form dated 18 March 2019 and the correspondence thereafter. Written permission from the Director: Animal Health must be obtained prior to any deviation from the conditions approved for this study under this Section 20 permit. Please apply in writing to HerryG@daff.gov.za
2. If required, an application for an extension must be made by the responsible researcher at least one month prior to the expiry of this Section 20 approval.

3. This permission does not relieve the researcher of any responsibility which may be placed on him by any other act of the Republic of South Africa;
4. All potentially infectious material utilised or collected during the study is to be destroyed at the completion of the study. Records must be kept for five years for audit purposes. A dispensation application may be made to the Director Animal Health in the event that any of the above is to be stored or distributed;
5. Samples to be transported must be packaged in compliance with the Regulations of the National Road Traffic Act, 1996 (Act No 93 of 1996) or IATA requirements;
6. The study may only start after Ethics approval was obtained;
7. Post-mortem tissue may be harvested from deceased rhinoceroses at Buffalo Dream Ranch;
8. Gametes and embryos may be stored in liquid nitrogen at the IVF Laboratory, Section of Reproduction and EmbryoPlus Laboratories;
9. The stored gametes and embryos may only be used for further research after having obtained new Section 20 approval;
10. The stored gametes and embryos may not be outsourced without prior written approval from DAFF;
11. This section 20 expires on 01 April 2024.

Title of research/study: "Developing assisted reproductive technologies for the conservation of the rhinoceros (*Cerathotherium simum simum*)."

Researcher (s): Dr Kate May

Institution: Department of Production Animal Studies, Faculty of Veterinary Science, Onderstepoort

Your Ref./ Project Number: 12/11/1/1/8

Our ref Number:

Kind regards,

Mmaja,

DR. MPHO MAJA
DIRECTOR OF ANIMAL HEALTH
Date: 2019 -04- 15

SUBJECT: Developing assisted reproductive technologies for the conservation of the rhinoceros (Cerathotherium simum simum).

Faculty of Veterinary Science Threatened or Protec ted Species (TOPS) Permit

STANDING PERMIT

(Issued in terms of the provisions of the National Environmental Management: Biodiversity Act 2004, Act 10 of 2004)

PERMIT NUMBER: S 02559

NAME OF ISSUING AUTHORITY	
NAME	DEPT OF ENVIRONMENTAL AFFAIRS
ADDRESS	PRIVATE BAG X 447 PRETORIA 0001
PROVINCE	NATIONAL DEPARTMENT

	PROVINCIAL DEPARTMENT	NATIONAL DEPARTMENT	
	PROTECTED AREA MANAGEMENT AUTHORITY	VETERINARIAN	
	REGISTERED CAPTIVE BREEDING OPERATION	REGISTERED SCIENTIFIC INSTITUTION	X
	REGISTERED SANCTUARY	REGISTERED REHABILITATION FACILITY	
	REGISTERED COMMERCIAL EXHIBITION FACILITY	REGISTERED GAME FARM	
	REGISTERED WILDLIFE TRADER	REGISTERED NURSERY	

UNIQUE REGISTRATION NUMBER N78/770713 5156 084

DETAILS OF PERMIT HOLDER			
NAME	University of Pretoria	ID NO.	770713 5156 084
SURNAME	C/o Vinny Naidoo	PASSPORT NO.	
	POSTAL ADDRESS	RESIDENTIAL ADDRESS	
ADDRESS	Faculty of Veterinary Science	Faculty of Veterinary Science	
ADDRESS	University of Pretoria	University of Pretoria	
ADDRESS	Private bag X 04	Old Soutpan Road	
TOWN	Onderstepoort	Onderstepoort	
POSTAL CODE	0110	0110	
PROVINCE	Gauteng	Gauteng	

PROPERTY WHERE RESTRICTED ACTIVITIES WILL BE CARRIED OUT	
NAME AND SURNAME OF APPLICANT	University of Pretoria
NAME AND SURNAME: RESPONSIBLE PERSON	Vinny Naidoo
NAME AND SURNAME OF AGENT	
PHYSICAL ADDRESS OF FACILITY	University of Pretoria
PHYSICAL ADDRESS OF FACILITY	Faculty of Veterinary Science
DISTRICT	Old Soutpan Road
PROVINCE	Gauteng
REGISTERED NAME AND NUMBER (in the case of game farm)	

DETAILS OF SPECIES INVOLVED				
SPECIES		SEX (if known)	QUANTITY	MARKING (if applicable)
COMMON NAME	SCIENTIFIC NAME			
All avian, raptile, mammalian and fish species listed as threatened or protected in terms of Section 56 of the National Environmental Management Biodiversity Act (Act No 10 of 2004)				

DETAILS OF RESTRICTED ACITIVIES INVOLVED
Collecting/gathering samples, receiving, transporting or moving, receiving as donation, giving, temporary possession, darting and capturing

PERMIT VALIDATION			
PERIOD OF VALIDITY	FROM: 12/02/2019	TO: 11/02/2022	
RECEIPT NUMBER			
SIGNATURE OF ISSUING OFFICER	DEPT. VAN OMGEWINGSAKE PRIVATE BAG X 447	SIGNATURE PERMIT HOLDER	Office of the Dean Faculty of Veterinary Science Private Bag X04 Onderstepoort 0110
DATE STAMP:	2019 -02- 12 PRETORIA 0001 DEPT. OF ENVIRONMENTAL AFFAIRS		

STANDARD PERMIT CONDITIONS

1. This permit is not transferable.
2. Any unauthorised alteration to this permit shall render it invalid.
3. This permit is subject to the provisions of any applicable law in force during the period of validity of the permit.
4. This permit is valid only within the province where it was issued.
5. The holder of this permit shall, at the request of a person authorised in terms of applicable legislation so to demand, forthwith produce such permit to such person.
6. This permit shall be invalid until such time that it is signed by the permit holder.
7. This permit shall be deemed invalid when it is lost or destroyed and no copy thereof shall be issued.
8. This permit may be withdrawn by an authorised person if the execution of any activity may be detrimental to the welfare of any wild animal or the safety of any person, provided that the permit holder is given notice of such intention and be granted the opportunity to appeal to such withdrawal.
9. The prescribed fees paid for the issuing of this permit shall not be refunded.
10. If the holder of this permit contravenes or fails to comply with any permit condition or requirement to which this permit is subject, he or she shall be guilty of an offence.
11. This permit shall be subject to any applicable norms and standards in existence at the time of issuance of this permit.

SPECIAL CONDITIONS

1. The issuing authoriy shall determine the species and restricted activities for which this permit will apply.
2. If this permit applies to a registered game farm, the holder of this permit must:
   a. Have a copy of this permit authorising the hunt, on his or her person during the hunt;
   b. Within 21 days after the hunt, furnish the issuing authority with a written return on the hunt stating:
      (i) the permit number and date of issuance if the permit;
      (ii) the species, sex and number of animals hunted; and
      (iii) the location where the hunt took place.
   c. Return all used copies of the game farm hunting permits within 3 weeks after the end of the calander year following the issuance of the game farm hunting permit, to the Issuing Authority.

Animal Ethics (REC053-19)

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Faculty of Veterinary Science
Animal Ethics Committee

22 May 2019

Conditionally Approval Certificate
New Application

AEC Reference No.: REC053-19
Title: Developing assisted reproductive technologies for the conservation of the rhinoceros
Researcher: Dr CE May
Student's Supervisor:

Dear Dr CE May,

The **New Application** as supported by documents received in April 2019 for your research, was approved by the Animal Ethics Committee on its quorate meeting of 6 May 2019.

Please note the following about your ethics approval:

1. The use of species is approved:

Species and Samples	Number
Rhino	50

2. Ethics Approval is valid for 1 year and needs to be renewed annually by March 2020.
3. Please remember to use your protocol number (REC053-19) on any documents or correspondence with the AEC regarding your research.
4. Please note that the AEC may ask further questions, seek additional information, require further modification, monitor the conduct of your research, or suspend or withdraw ethics approval.

Ethics approval is subject to the following:

- The ethics approval is conditional on the research being conducted as stipulated by the details of all documents submitted to the Committee. In the event that a further need arises to change who the investigators are, the methods or any other aspect, such changes must be submitted as an Amendment for approval by the Committee.

Conditions: Progress reports to be submitted quarterly and the researcher needs to indicate specifics on where and how the samples are collected. For collection from euthanased animals, the committee requires an SOP to be produced that allows for ethical sampling i.e. euthanasia must be properly justifiable.

We wish you the best with your research.
Yours sincerely

Prof V Naidoo
CHAIRMAN: UP-Animal Ethics Committee

Room 6-13, Arnold Theiler Building, Onderstepoort
Private Bag X04, Onderstepoort 0110, South Africa
Tel +27 12 529 8463
Fax +27 12 529 8321
Email aec@up.ac.za
www.up.ac.za

Fakulteit Veeartsenykunde
Lefapha la Diseanse tša Bongakadiruiwa

Addendum application (REC176-21):

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Faculty of Veterinary Science
Animal Ethics Committee

28 April 2022

Approval Certificate
New Application

AEC Reference No.:	REC176-21
Title:	Monitoring and regulating aspiration fluid temperature fluctuations during ovum pick-up in southern white rhinoceros (Ceratotherium simum)
Researcher:	Ms L Marais
Student's Supervisor:	Prof DE Holm

Dear Ms L Marais,

The **New Application** as supported by documents received between 2022-01-19 and 2022-03-28 for your research, was approved by the Animal Ethics Committee on its quorate meeting of 2022-03-28.

Please note the following about your ethics approval:

1. The use of species is approved:

Species	Number
Rhinoceros	34
Samples	Number
Temperature data (Samples from live animals)	34

2. Ethics Approval is valid for 1 year and needs to be renewed annually by 2023-04-28.
3. Please remember to use your protocol number (REC176-21) on any documents or correspondence with the AEC regarding your research.
4. Please note that the AEC may ask further questions, seek additional information, require further modification, monitor the conduct of your research, or suspend or withdraw ethics approval.
5. **All incidents** must be reported by the PI by email to Ms Marleze Rheeder (AEC Coordinator) within 3 days, and must be subsequently submitted electronically on the application system within 14 days.
6. The committee also requests that you record major procedures undertaken during your study for own-archiving, using any available digital recording system that captures in adequate quality, as it may be required if the committee needs to evaluate a complaint. However, if the committee has monitored the procedure previously or if it is generally can be considered routine, such recording will not be required.

Ethics approval is subject to the following:

- The ethics approval is conditional on the research being conducted as stipulated by the details of all documents submitted to the Committee. In the event that a further need arises to change who the investigators are, the methods or any other aspect, such changes must be submitted as an Amendment for approval by the Committee

Room 6-13, Arnold Theiler Building, Onderstepoort
Private Bag X04, Onderstepoort 0110, South Africa
Tel +27 12 529 8434
Fax +27 12 529 8321
Email: marleze.rheeder@up.ac.za

Fakulteit Veeartsenykunde
Lefapha la Diseanse tša Bongakadiruiwa

We wish you the best with your research.

Yours sincerely

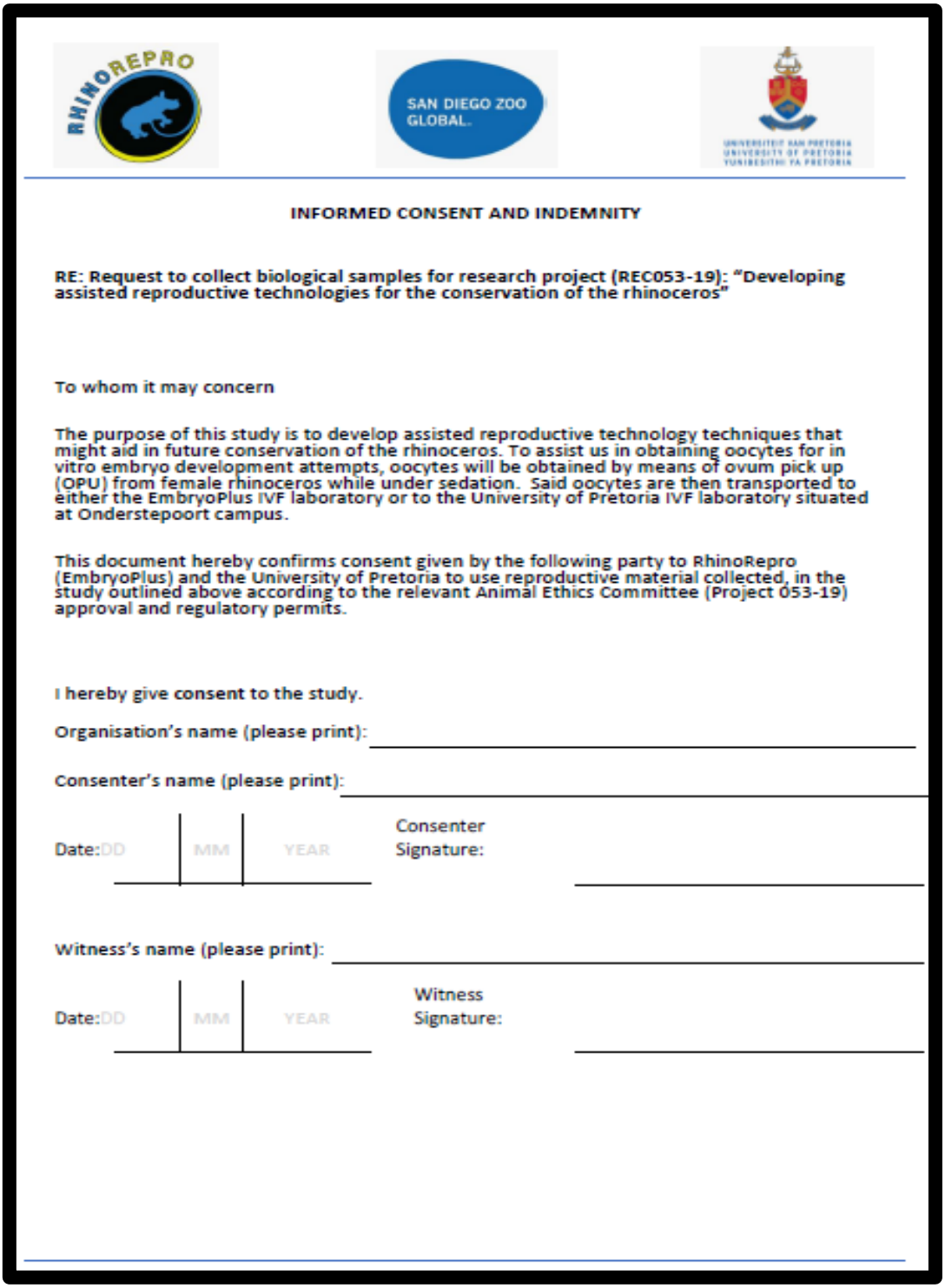
Prof A Tordiffe
DEPUTY CHAIRMAN: UP-Animal Ethics Committee

Room 6-13, Arnold Theiler Building, Onderstepoort
Private Bag X04, Onderstepoort 0110, South Africa
Tel +27 12 529 8434
Fax +27 12 529 8321
Email: marleze.rheeder@up.ac.za

Fakulteit Veeartsenykunde
Lefapha la Diseanse tša Bongakadiruiwa

10.2 Appendix II: Consent form

Consent form signed by study site owners allowing use of data for the purposes of this study and publications to follow.



INFORMED CONSENT AND INDEMNITY

RE: Request to collect biological samples for research project (REC053-19): "Developing assisted reproductive technologies for the conservation of the rhinoceros"

To whom it may concern

The purpose of this study is to develop assisted reproductive technology techniques that might aid in future conservation of the rhinoceros. To assist us in obtaining oocytes for in vitro embryo development attempts, oocytes will be obtained by means of ovum pick up (OPU) from female rhinoceros while under sedation. Said oocytes are then transported to either the EmbryoPlus IVF laboratory or to the University of Pretoria IVF laboratory situated at Onderstepoort campus.

This document hereby confirms consent given by the following party to RhinoRepro (EmbryoPlus) and the University of Pretoria to use reproductive material collected, in the study outlined above according to the relevant Animal Ethics Committee (Project 053-19) approval and regulatory permits.

I hereby give consent to the study.

Organisation's name (please print): _____

Consenter's name (please print): _____

Date: DD | MM | YEAR Consenter Signature: _____

Witness's name (please print): _____

Date: DD | MM | YEAR Witness Signature: _____

10.3 Appendix III: Graphs and commentary tables for every OPU procedure

Words indicated in red indicate the option used during that procedure

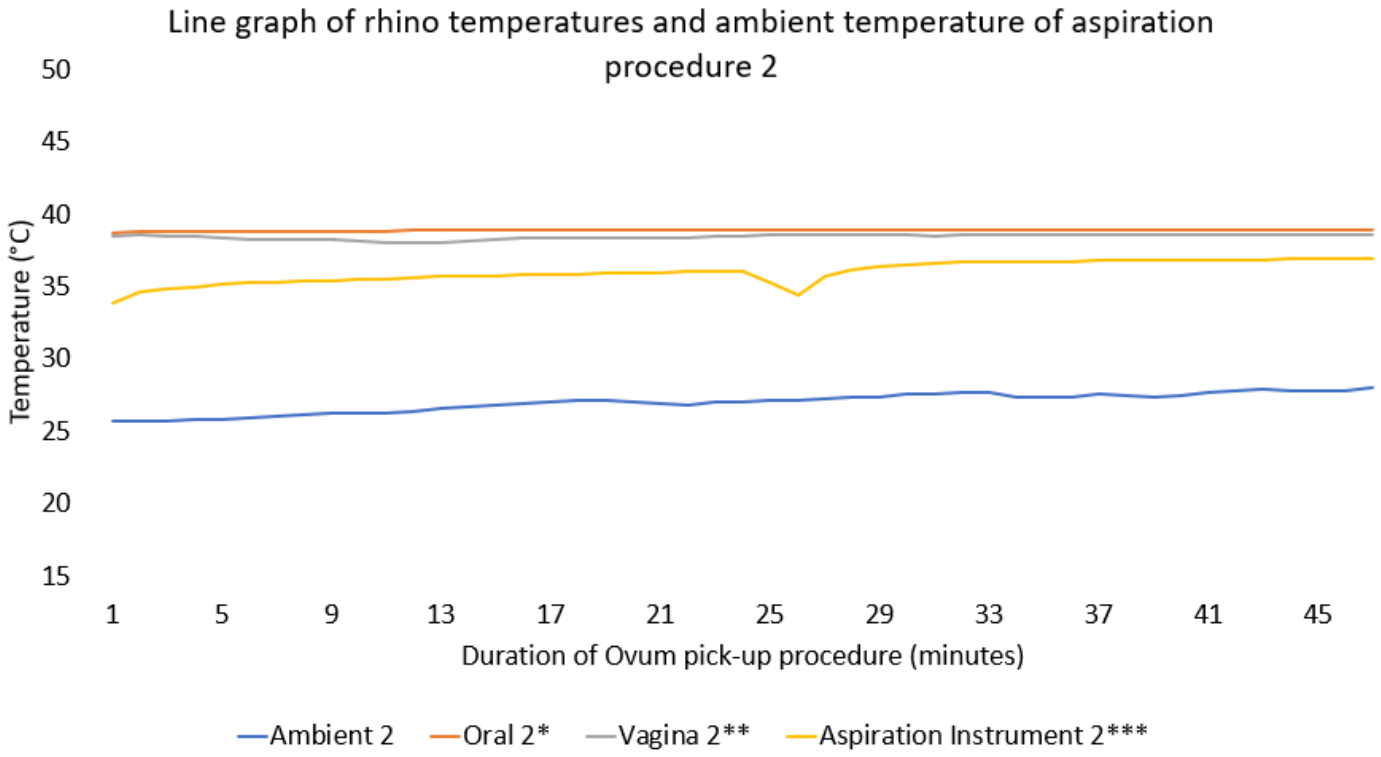
Y – Yes, this equipment was used

N – No, this equipment was not used

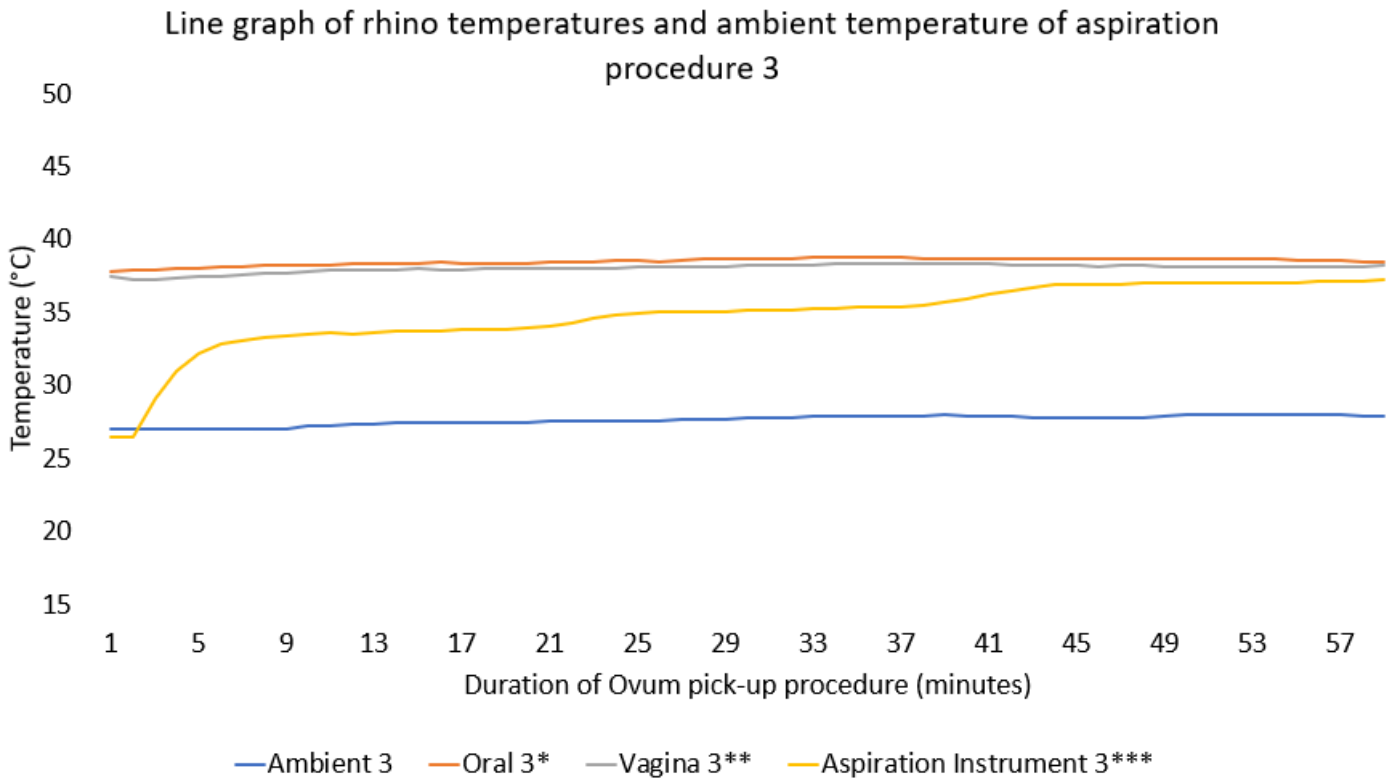
*No line graph for the first procedure due to equipment malfunction.

Aspiration #1		Temperature Data:	
Date:	23 February 2021	X̄ Ambient:	-
Animal ID:	Elsa	X̄ Oral	-
Laboratory:	UP / EP	X̄ Vaginal	-
Transport medium:	DMEM / HOLDING	X̄ Aspiration Instrument	-
Study site:	Site 1 / Site 2 / Site 3	Flushing Medium Equipment:	EPBox / Water Bath / N
OPU Duration (min):	-	Collection Bottle Equipment:	RiceBags / Water Bath / Wasserbad / N
#Oocytes:	4	Microscope Heating Stage:	Y / N
#Matured:	2	Laboratory Heating Stage:	Y / N
#Cleaved:	0	Transport Method:	Transport Incubator / Equitainer
Comments:			
No temperature loggers were available to collect the rhino temperatures, however, her procedure data is relevant.			

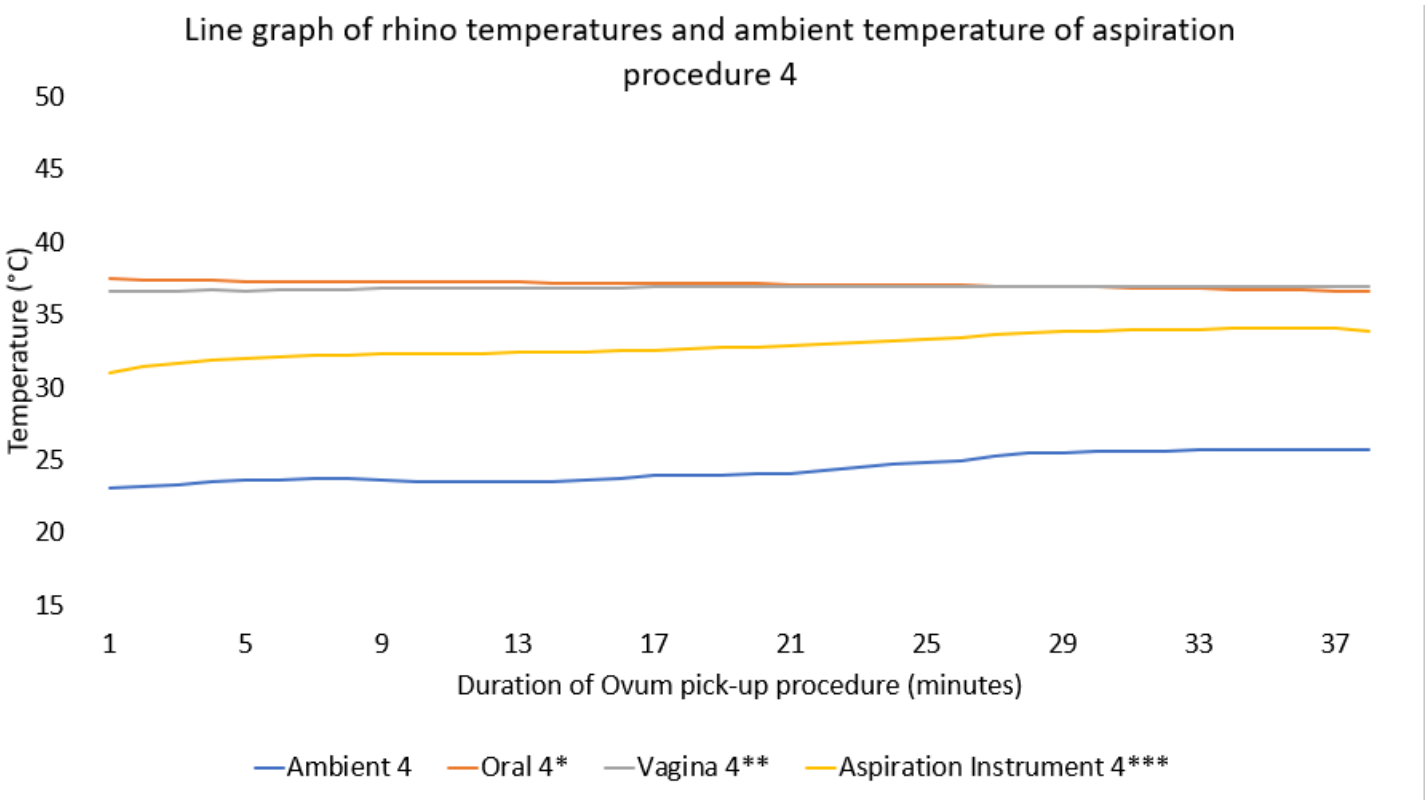
Aspiration #2		Temperature Data:	
Date:	2 March 2021	X̄ Ambient:	26,97
Animal ID:	33	X̄ Oral	38,90
Laboratory:	UP / EP	X̄ Vaginal	38,43
Transport medium:	DMEM / HOLDING	X̄ Aspiration Instrument	35,98
Study site:	Site 1 / Site 2 / Site 3	Flushing Medium Equipment:	EPBox / Water Bath / N
OPU Duration (min):	48	Collection Bottle Equipment:	RiceBags / Water Bath / Wasserbad / N
#Oocytes:	1	Microscope Heating Stage:	Y / N
#Matured:	0	Laboratory Heating Stage:	Y / N
#Cleaved:	0	Transport Method:	Transport Incubator / Equitainer
Comments:			
Water Bath is hard to manage & risk of contamination.			



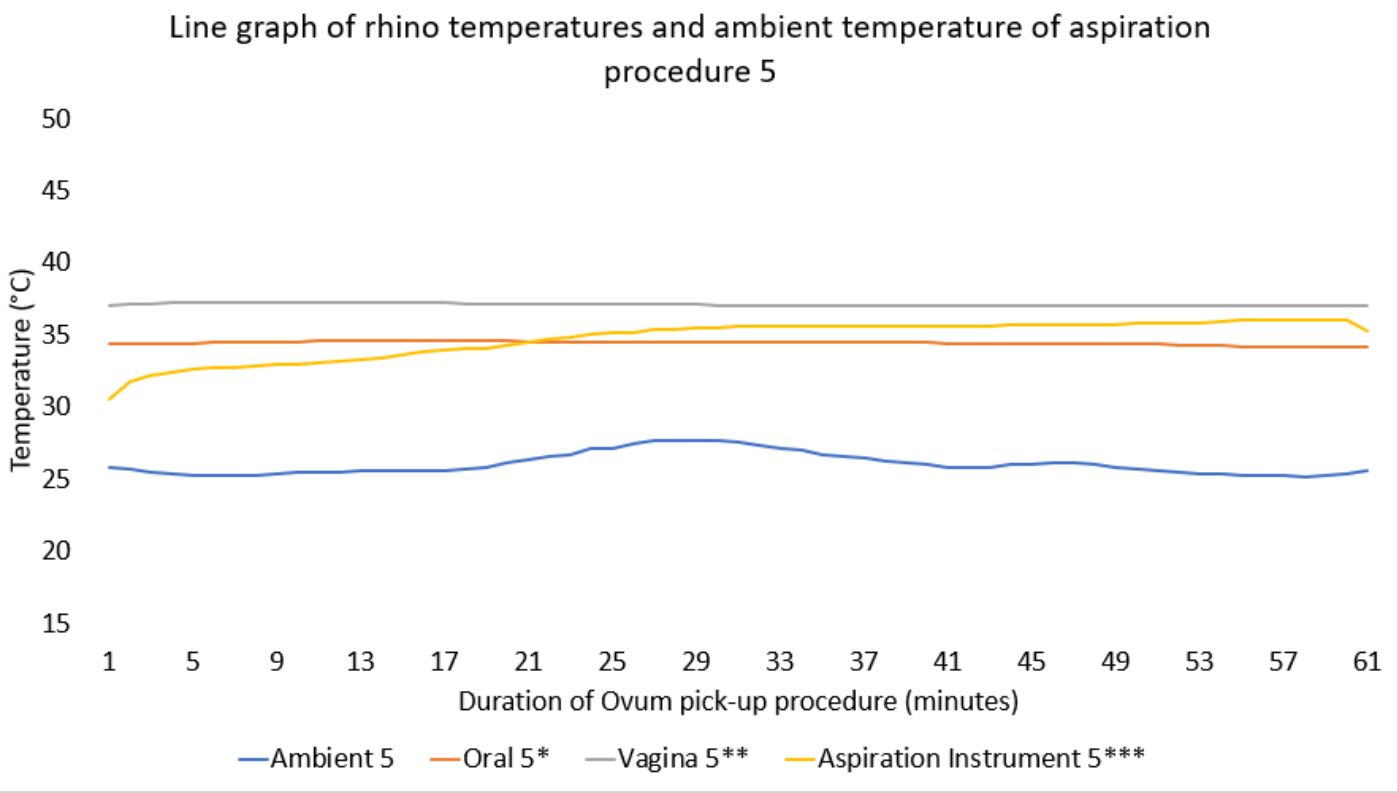
Aspiration #3		Temperature Data:	
Date:	16 March 2021	X̄ Ambient:	27,62
Animal ID:	Dezi	X̄ Oral	38,48
Laboratory:	UP / EP	X̄ Vaginal	38,03
Transport medium:	DMEM / HOLDING	X̄ Aspiration Instrument	34,78
Study site:	Site 1 / Site 2 / Site 3	Flushing Medium Equipment:	EPBox / Water Bath / N
OPU Duration (min):	59	Collection Bottle Equipment:	RiceBags / Water Bath / Wasserbad / N
#Oocytes:	7	Microscope Heating Stage:	Y / N
#Matured:	5	Laboratory Heating Stage:	Y / N
#Cleaved:	0	Transport Method:	Transport Incubator / Equitainer
Comments:			
The oocytes were split between HOLDING (3) and DMEM (4) Transport medium.			



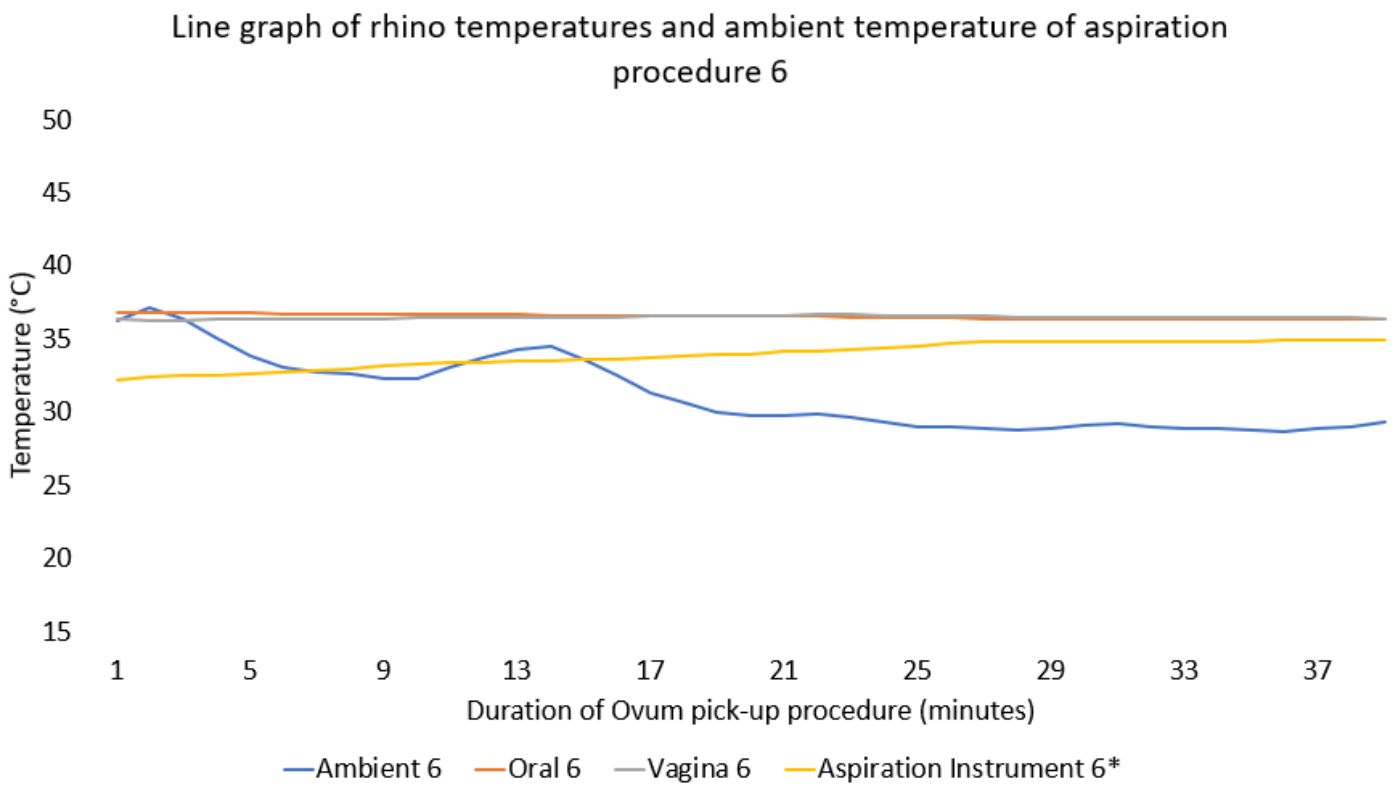
Aspiration #4		Temperature Data:	
Date:	25 March 2021	X̄ Ambient:	24,38
Animal ID:	3.1.O	X̄ Oral	37,07
Laboratory:	UP / EP	X̄ Vaginal	36,84
Transport medium:	DMEM / HOLDING	X̄ Aspiration Instrument	32,89
Study site:	Site 1/ Site 2 / Site 3	Flushing Medium Equipment:	EPBox / Water Bath / N
OPU Duration (min):	38	Collection Bottle Equipment:	RiceBags / Water Bath / Wasserbad / N
#Oocytes:	3	Microscope Heating Stage:	Y / N
#Matured:	2	Laboratory Heating Stage:	Y / N
#Cleaved:	0	Transport Method:	Transport Incubator / Equitainer
Comments: STIMULATED			



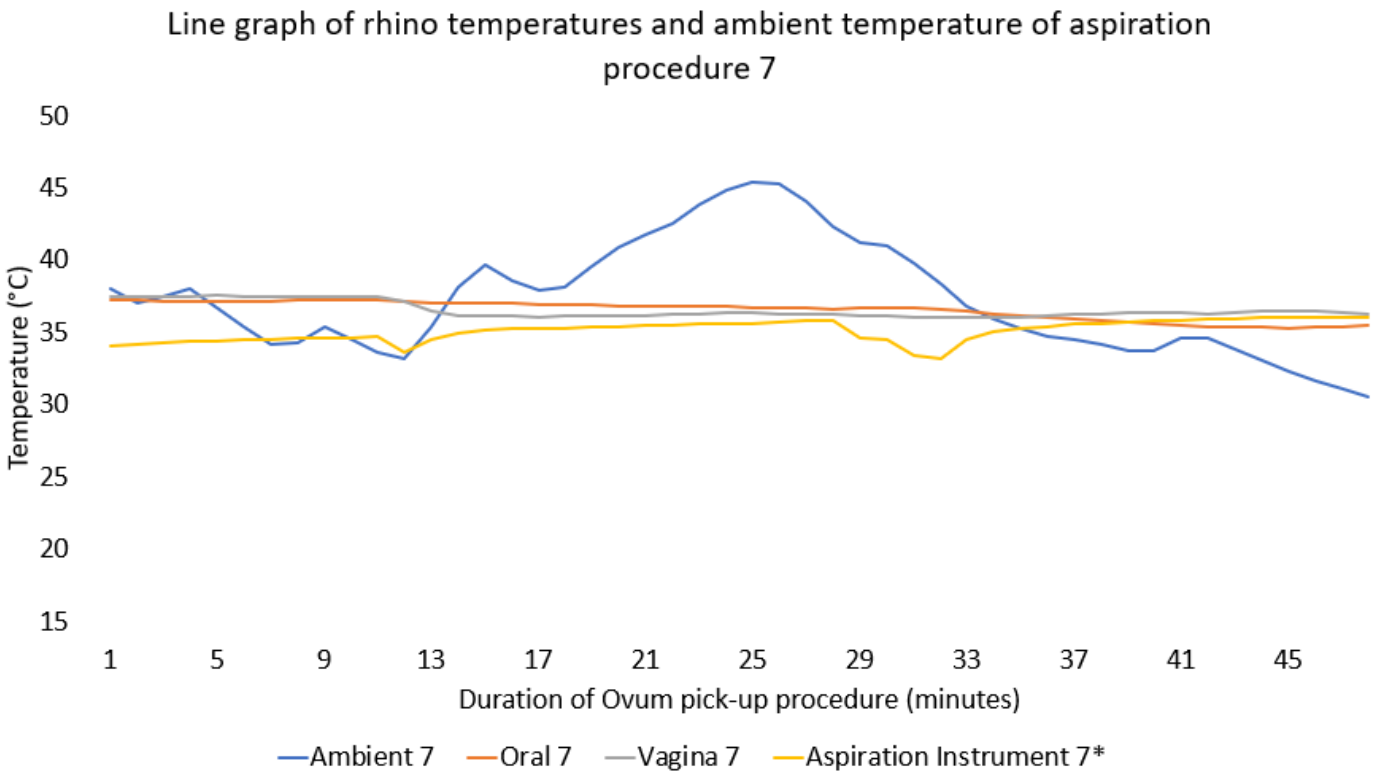
Aspiration #5		Temperature Data:	
Date:	25 March 2021	X̄ Ambient:	26,01
Animal ID:	1.6.K	X̄ Oral	34,42
Laboratory:	UP / EP	X̄ Vaginal	37,10
Transport medium:	DMEM / HOLDING	X̄ Aspiration Instrument	34,72
Study site:	Site 1 / Site 2 / Site 3	Flushing Medium Equipment:	EPBox / Water Bath / N
OPU Duration (min):	61	Collection Bottle Equipment:	RiceBags / Water Bath / Wasserbad / N
#Oocytes:	6	Microscope Heating Stage:	Y / N
#Matured:	2	Laboratory Heating Stage:	Y / N
#Cleaved:	0	Transport Method:	Transport Incubator / Equitainer
Comments: STIMULATED			



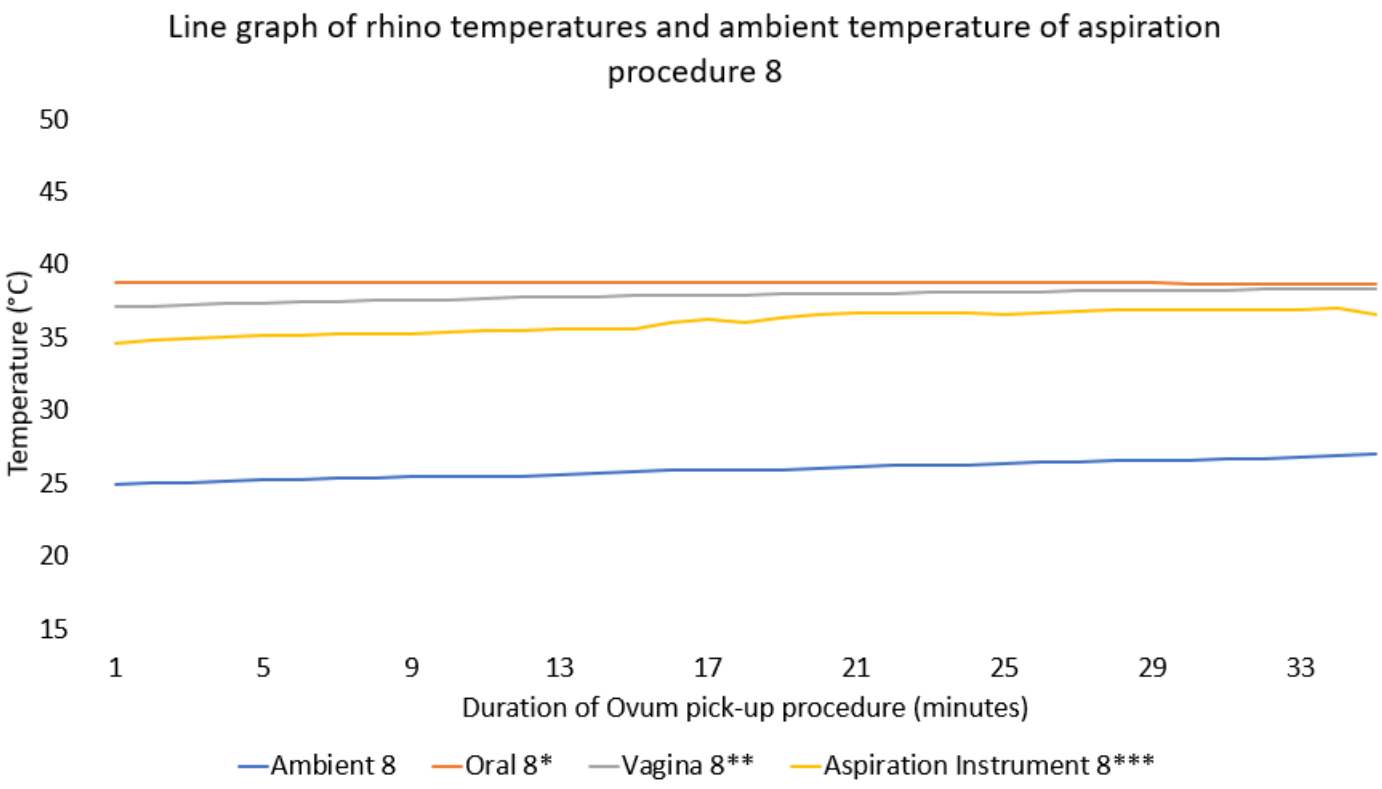
Aspiration #6		Temperature Data:	
Date:	25 March 2021	X̄ Ambient:	31,22
Animal ID:	9.7.O	X̄ Oral	36,53
Laboratory:	UP / EP	X̄ Vaginal	36,47
Transport medium:	DMEM / HOLDING	X̄ Aspiration Instrument	33,92
Study site:	Site 1 / Site 2 / Site 3	Flushing Medium Equipment:	EPBox / Water Bath / N
OPU Duration (min):	39	Collection Bottle Equipment:	RiceBags / Water Bath / Wasserbad / N
#Oocytes:	3	Microscope Heating Stage:	Y / N
#Matured:	3	Laboratory Heating Stage:	Y / N
#Cleaved:	0	Transport Method:	Transport Incubator / Equitainer
Comments: STIMULATED			



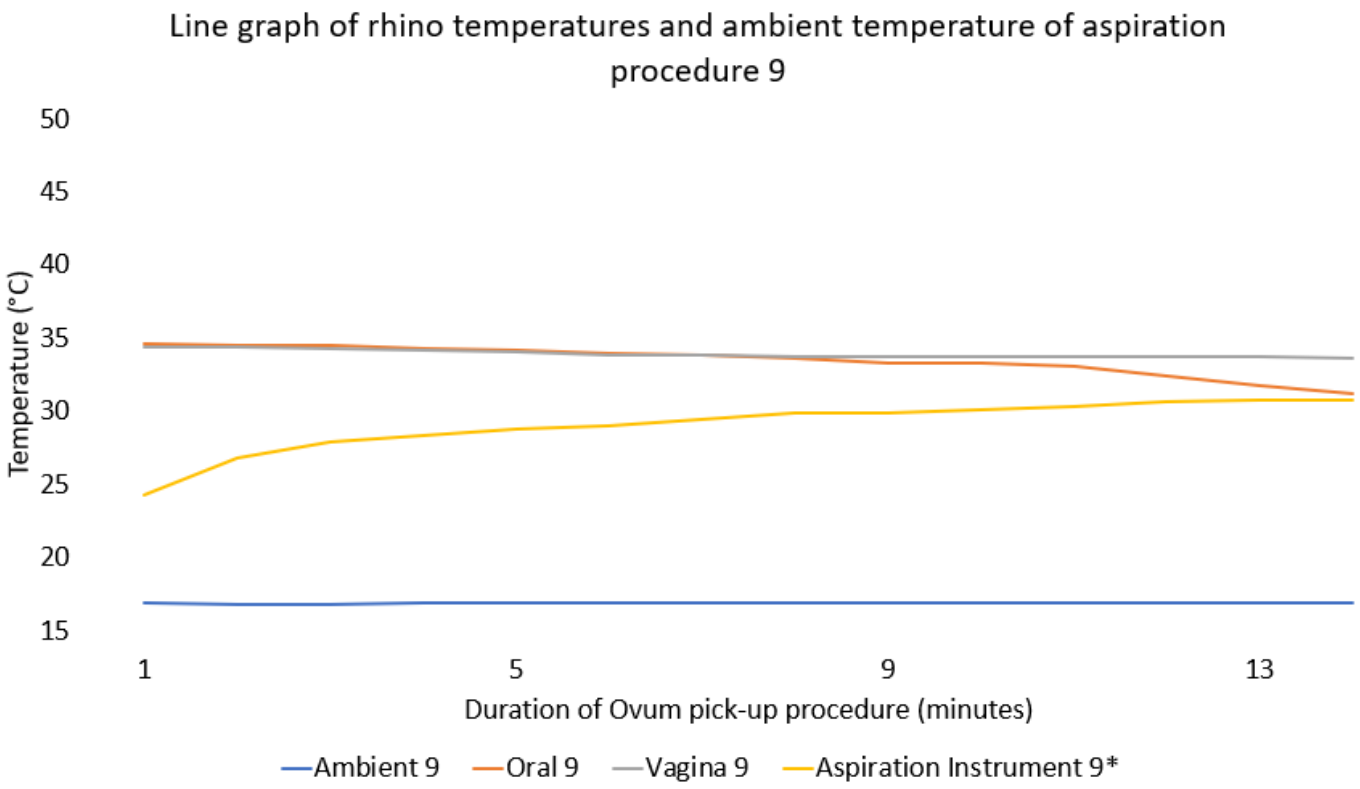
Aspiration #7		Temperature Data:	
Date:	25 March 2021	X̄ Ambient:	37,21
Animal ID:	1.5. O	X̄ Oral	36,51
Laboratory:	UP / EP	X̄ Vaginal	36,53
Transport medium:	DMEM / HOLDING	X̄ Aspiration Instrument	35,09
Study site:	Site 1 / Site 2 / Site 3	Flushing Medium Equipment:	EPBox / Water Bath / N
OPU Duration (min):	48	Collection Bottle Equipment:	RiceBags / Water Bath / Wasserbad / N
#Oocytes:	2	Microscope Heating Stage:	Y / N
#Matured:	0	Laboratory Heating Stage:	Y / N
#Cleaved:	0	Transport Method:	Transport Incubator / Equitainer
Comments:			
STIMULATED; This was the fourth procedure of the day and took place in direct sunlight. This could explain some of the variation in ambient temperature.			



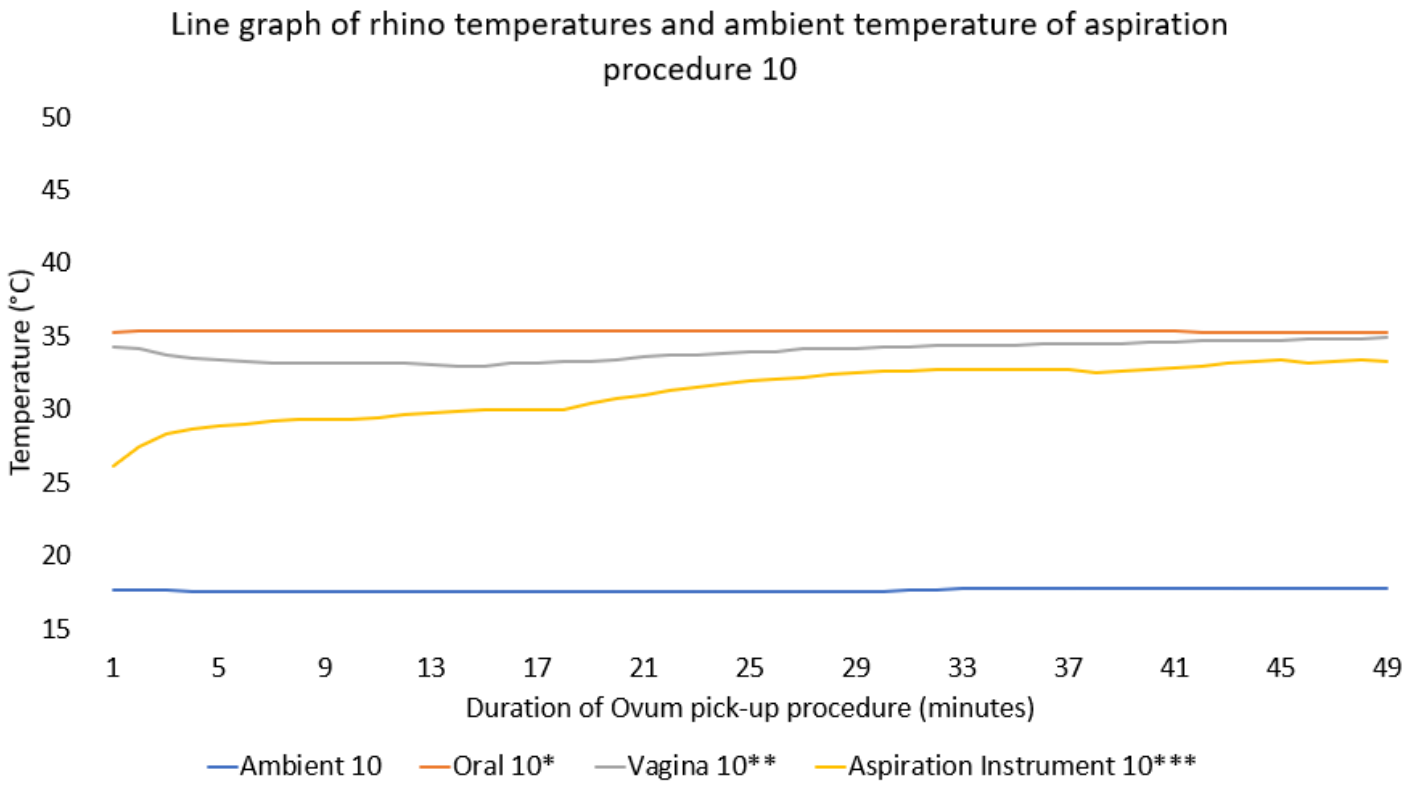
Aspiration #8		Temperature Data:	
Date:	15 April 2021	X̄ Ambient:	25,94
Animal ID:	10	X̄ Oral	38,75
Laboratory:	UP / EP	X̄ Vaginal	37,86
Transport medium:	DMEM / HOLDING	X̄ Aspiration Instrument	36,05
Study site:	Site 1 / Site 2 / Site 3	Flushing Medium Equipment:	EPBox / Water Bath / N
OPU Duration (min):	35	Collection Bottle Equipment:	RiceBags / Water Bath / Wasserbad / N
#Oocytes:	1	Microscope Heating Stage:	Y / N
#Matured:	0	Laboratory Heating Stage:	Y / N
#Cleaved:	0	Transport Method:	Transport Incubator / Equitainer
Comments: No heating equipment was available.			



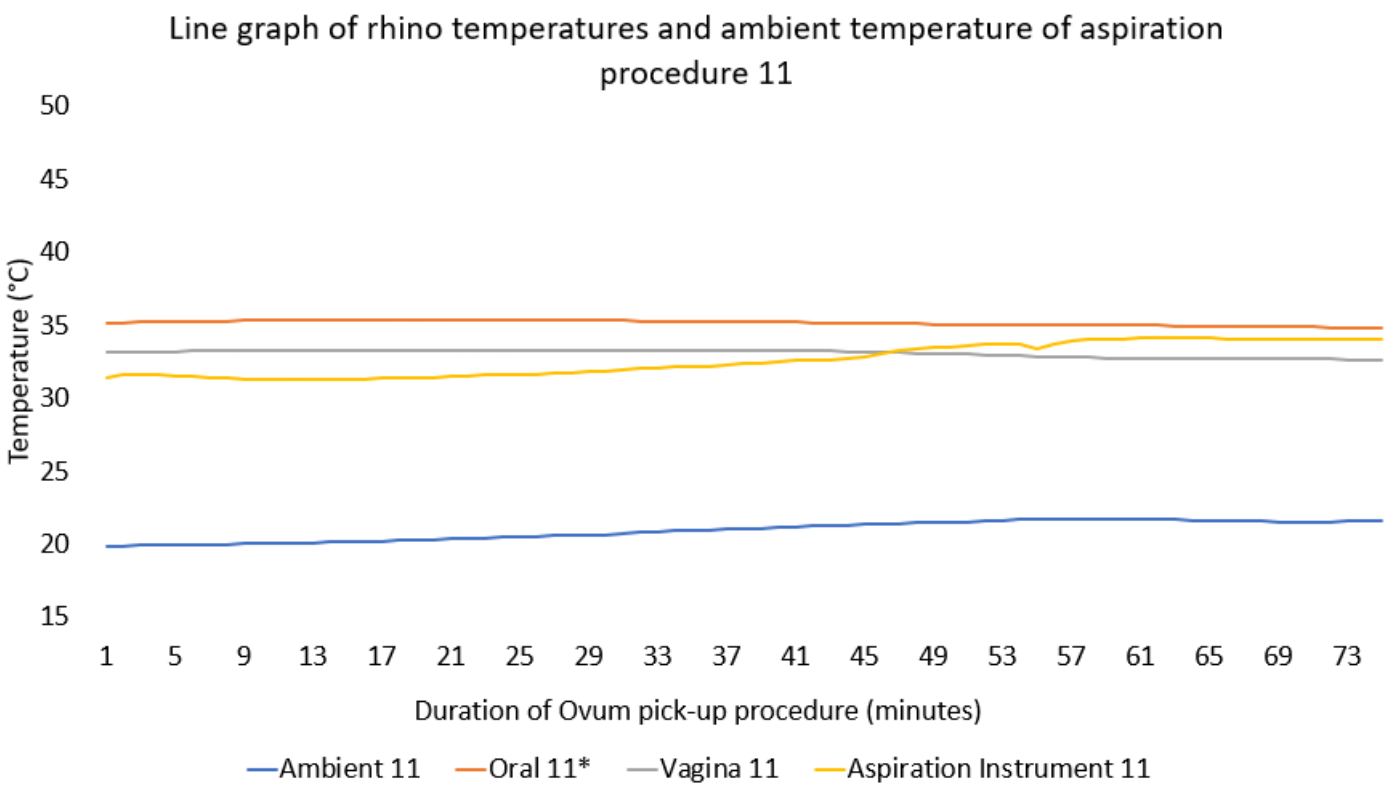
Aspiration #9		Temperature Data:	
Date:	8 June 2021	X̄ Ambient:	16,89
Animal ID:	Dezi	X̄ Oral	33,44
Laboratory:	UP / EP	X̄ Vaginal	33,91
Transport medium:	DMEM / HOLDING	X̄ Aspiration Instrument	29,06
Study site:	Site 1 / Site 2 / Site 3	Flushing Medium Equipment:	EPBox / Water Bath / N
OPU Duration (min):	14	Collection Bottle Equipment:	RiceBags / Water Bath / Wasserbad / N
#Oocytes:	0	Microscope Heating Stage:	Y / N
#Matured:	0	Laboratory Heating Stage:	Y / N
#Cleaved:	0	Transport Method:	Transport Incubator / Equitainer
Comments:			
Was not cycling properly, only had two small follicles.			



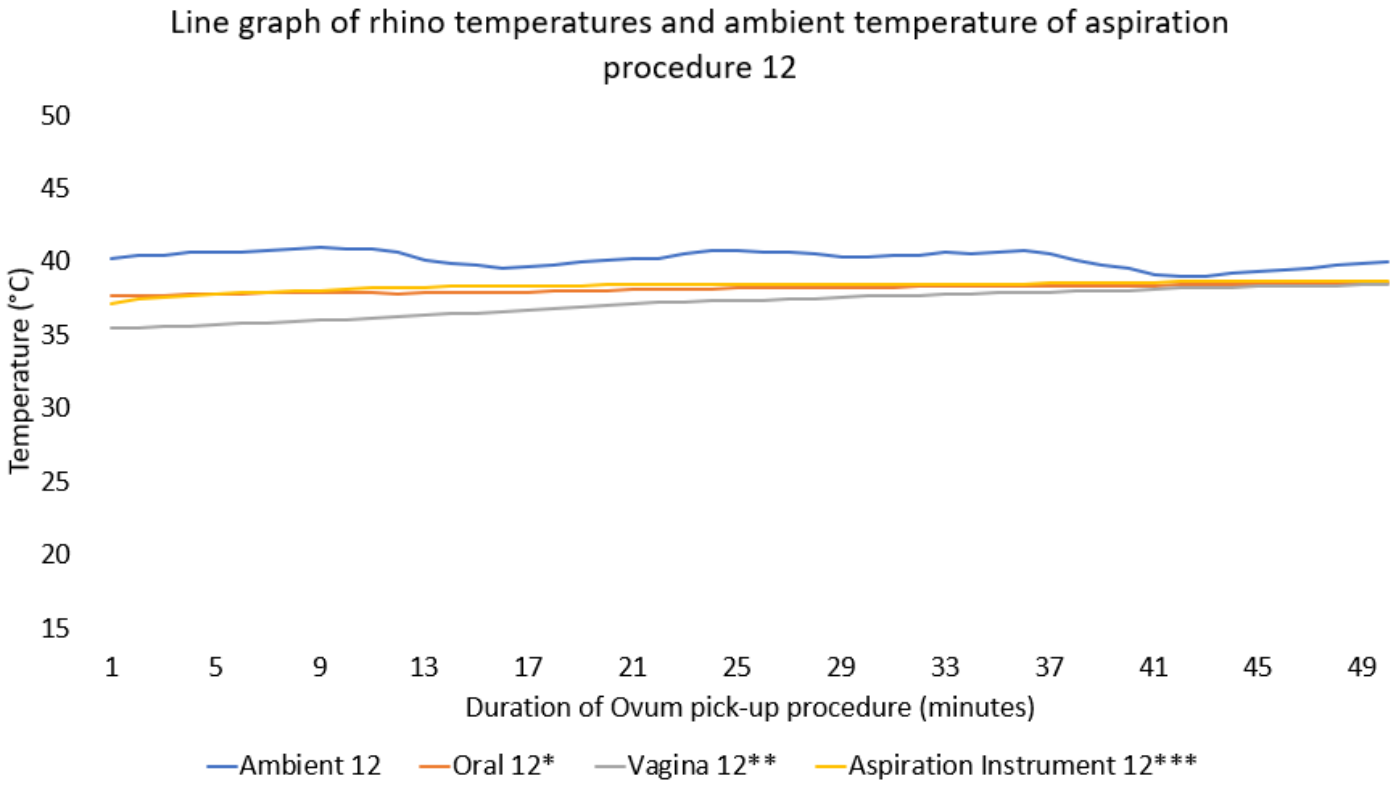
Aspiration #10		Temperature Data:	
Date:	8 June 2021	X̄ Ambient:	17,65
Animal ID:	May	X̄ Oral	35,34
Laboratory:	UP / EP	X̄ Vaginal	33,94
Transport medium:	DMEM / HOLDING	X̄ Aspiration Instrument	31,23
Study site:	Site 1 / Site 2 / Site 3	Flushing Medium Equipment:	EPBox / Water Bath / N
OPU Duration (min):	49	Collection Bottle Equipment:	RiceBags / Water Bath / Wasserbad / N
#Oocytes:	0	Microscope Heating Stage:	Y / N
#Matured:	0	Laboratory Heating Stage:	Y / N
#Cleaved:	0	Transport Method:	Transport Incubator / Equitainer
Comments:			



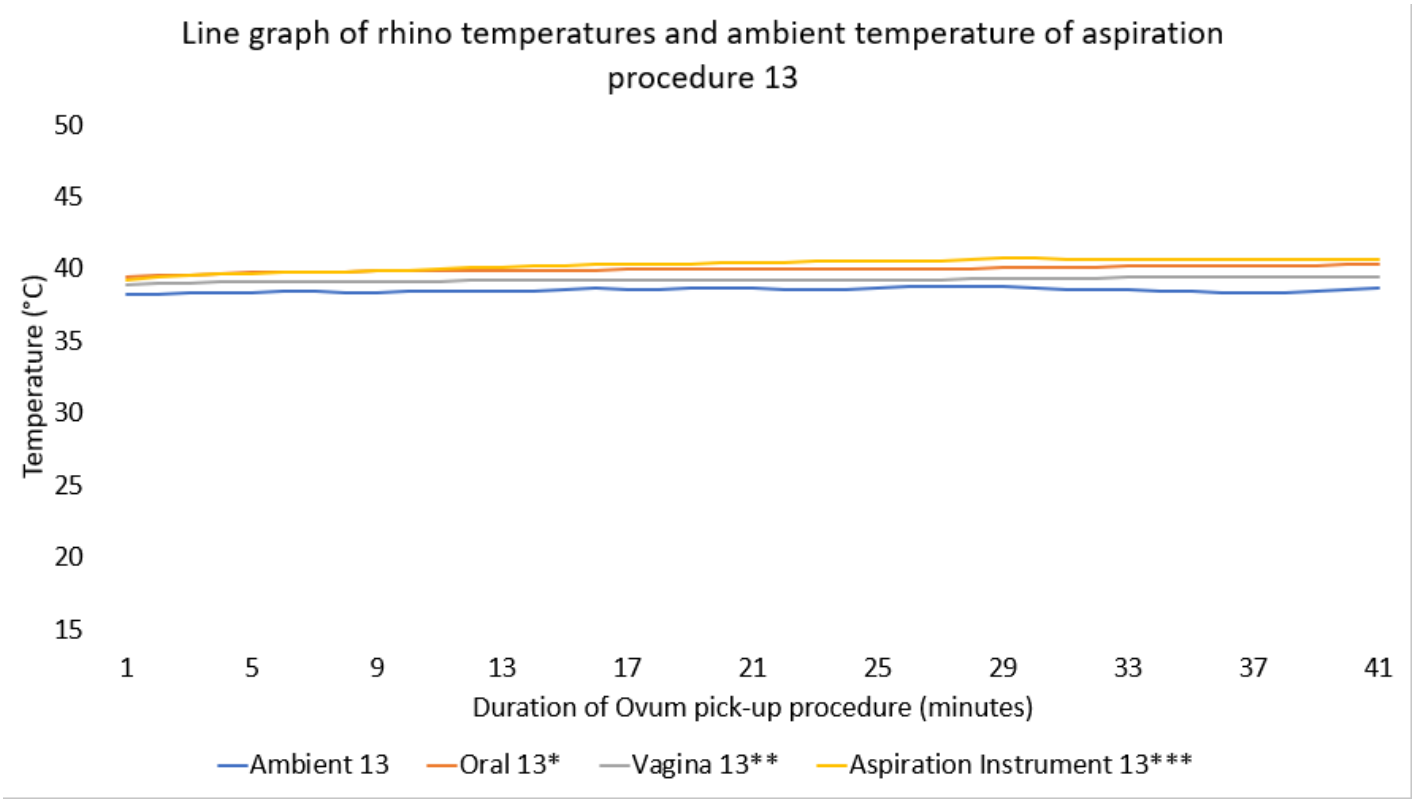
Aspiration #11		Temperature Data:	
Date:	24 August 2021	X̄ Ambient:	20,91
Animal ID:	Dezi	X̄ Oral	35,12
Laboratory:	UP / EP	X̄ Vaginal	33,04
Transport medium:	DMEM / HOLDING	X̄ Aspiration Instrument	32,59
Study site:	Site 1 / Site 2 / Site 3	Flushing Medium Equipment:	EPBox / Water Bath / N
OPU Duration (min):	75	Collection Bottle Equipment:	RiceBags / Water Bath / Wasserbad / N
#Oocytes:	4	Microscope Heating Stage:	Y / N
#Matured:	4	Laboratory Heating Stage:	Y / N
#Cleaved:	0	Transport Method:	Transport Incubator / Equitainer
Comments:			
Warmed enema water = 31°C			



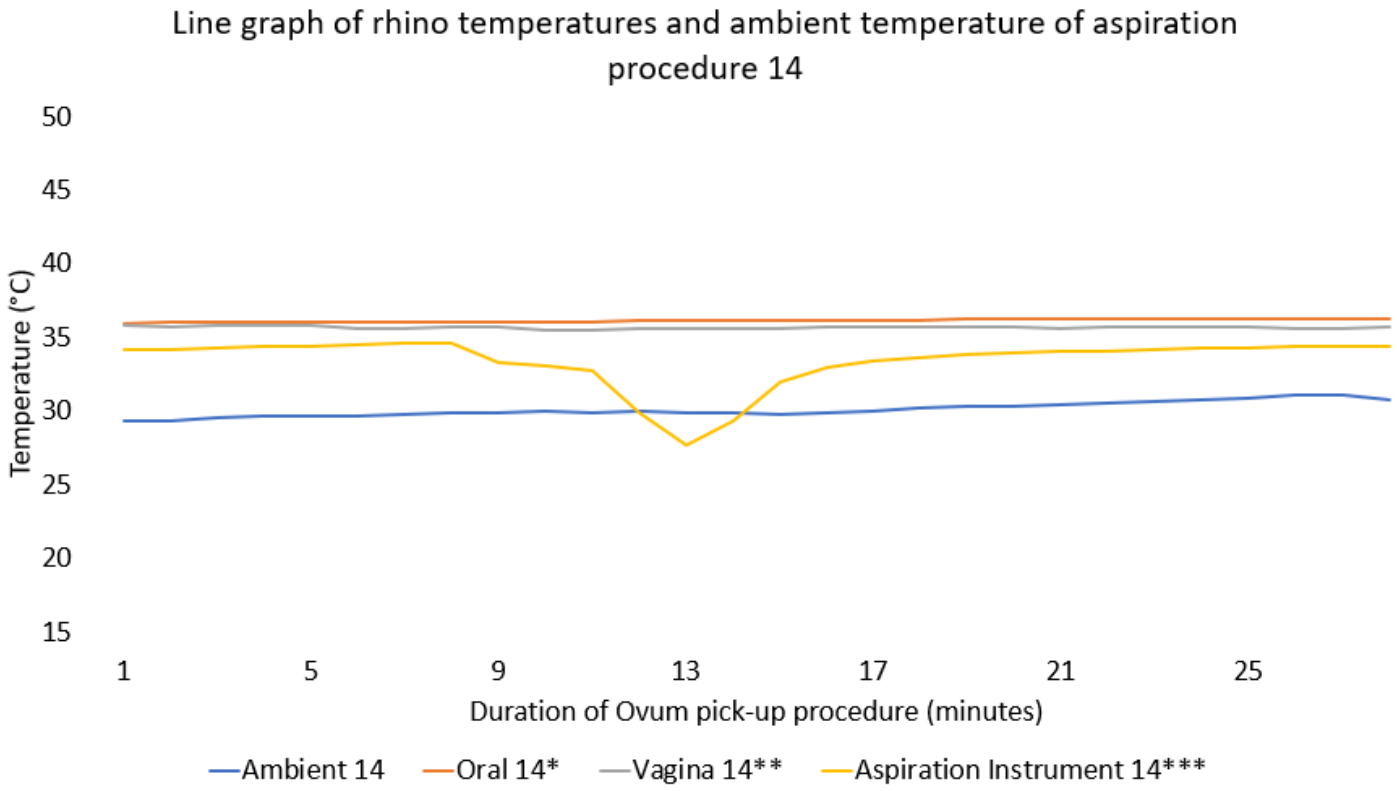
Aspiration #12		Temperature Data:	
Date:	28 September 2021	X̄ Ambient:	40,17
Animal ID:	14.11. O	X̄ Oral	38,14
Laboratory:	UP / EP	X̄ Vaginal	37,17
Transport medium:	DMEM / HOLDING	X̄ Aspiration Instrument	38,33
Study site:	Site 1 / Site 2 / Site 3	Flushing Medium Equipment:	EPBox / Water Bath / N
OPU Duration (min):	50	Collection Bottle Equipment:	RiceBags / Water Bath / Wasserbad / N
#Oocytes:	1	Microscope Heating Stage:	Y / N
#Matured:	1	Laboratory Heating Stage:	Y / N
#Cleaved:	0	Transport Method:	Transport Incubator / Equitainer
Comments: STIMULATED; Warmed enema water = 35°C			



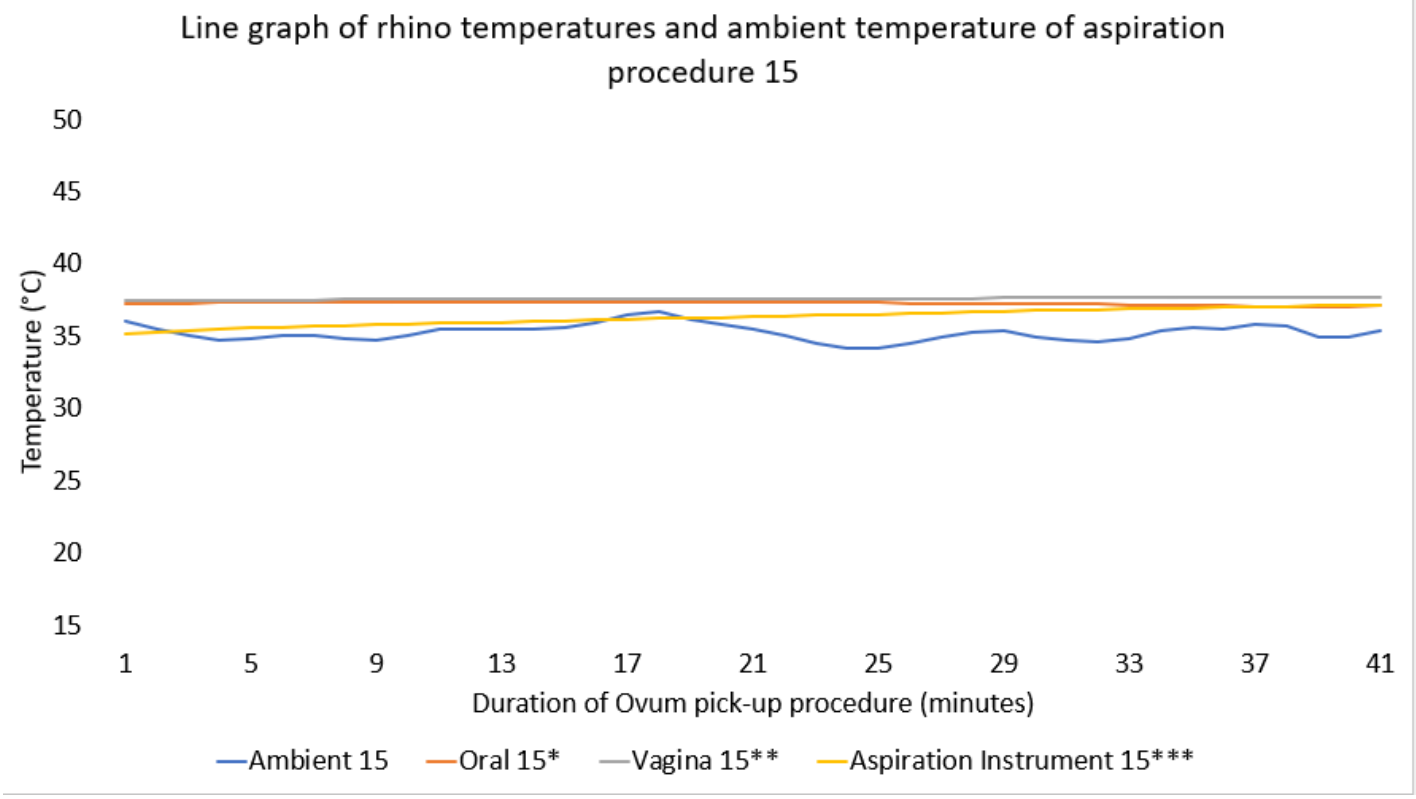
Aspiration #13		Temperature Data:	
Date:	28 September 2021	X̄ Ambient:	38,50
Animal ID:	14.12. G	X̄ Oral	39,96
Laboratory:	UP / EP	X̄ Vaginal	39,23
Transport medium:	DMEM / HOLDING	X̄ Aspiration Instrument	40,27
Study site:	Site 1 / Site 2 / Site 3	Flushing Medium Equipment:	EPBox / Water Bath / N
OPU Duration (min):	41	Collection Bottle Equipment:	RiceBags / Water Bath / Wasserbad / N
#Oocytes:	2	Microscope Heating Stage:	Y / N
#Matured:	2	Laboratory Heating Stage:	Y / N
#Cleaved:	0	Transport Method:	Transport Incubator / Equitainer
Comments: STIMULATED; Warmed enema water = 31°C			



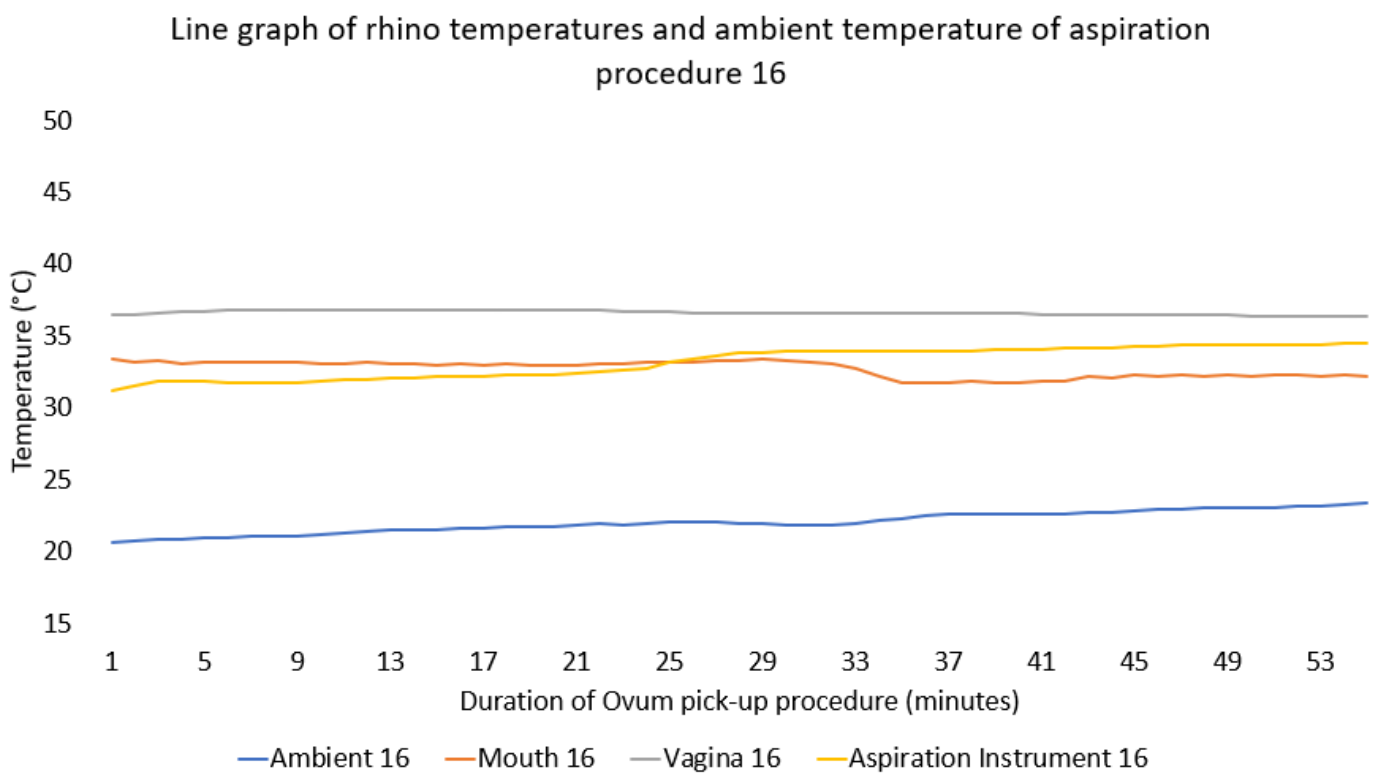
Aspiration #14		Temperature Data:	
Date:	6 October 2021	X̄ Ambient:	30,09
Animal ID:	Nola	X̄ Oral	36,12
Laboratory:	UP / EP	X̄ Vaginal	35,65
Transport medium:	DMEM / HOLDING	X̄ Aspiration Instrument	33,37
Study site:	Site 1/ Site 2/ Site 3	Flushing Medium Equipment:	EPBox / Water Bath / N
OPU Duration (min):	28	Collection Bottle Equipment:	RiceBags / Water Bath / Wasserbad / N
#Oocytes:	0	Microscope Heating Stage:	Y / N
#Matured:	0	Laboratory Heating Stage:	Y / N
#Cleaved:	0	Transport Method:	Transport Incubator / Equitainer
Comments: During the procedure, the aspiration instrument was taken out to clean and to let out air that was trapped in the rectum.			



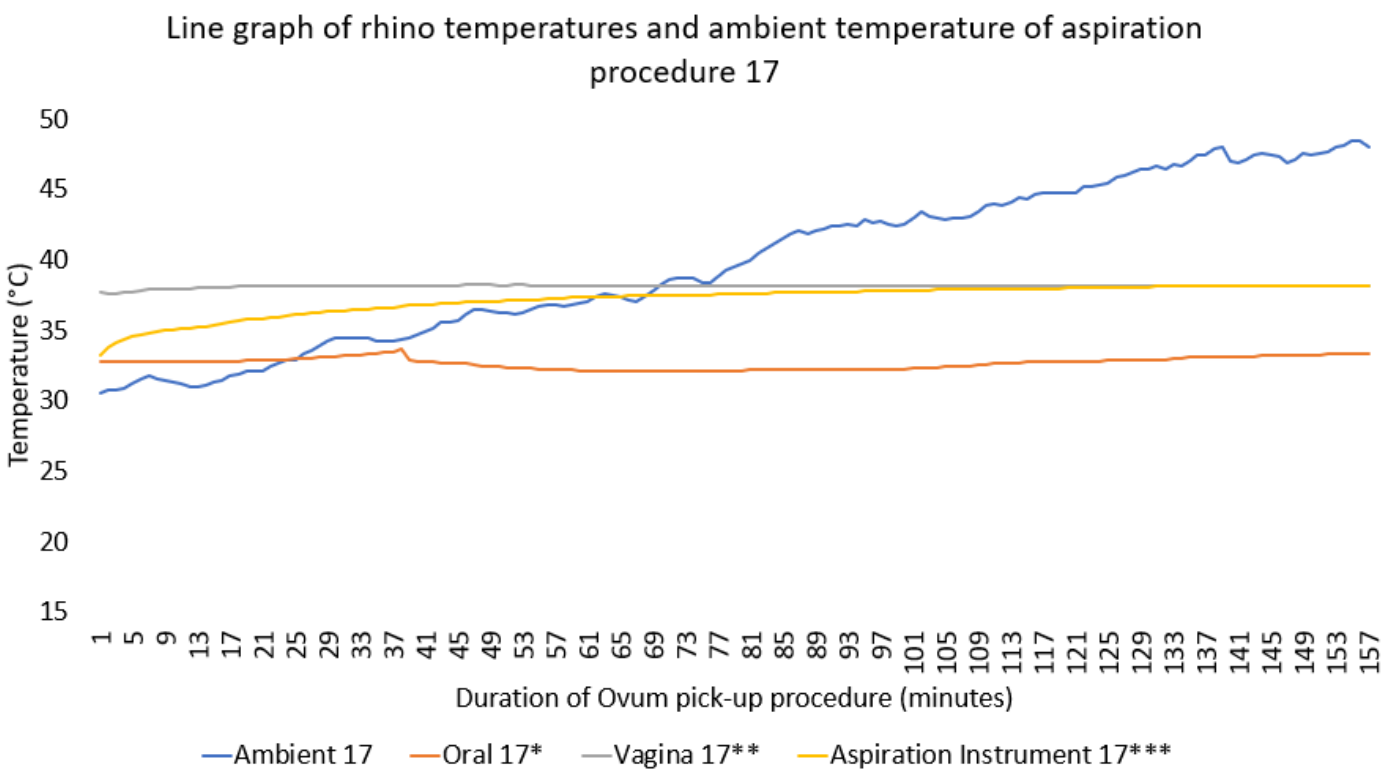
Aspiration #15		Temperature Data:	
Date:	6 October 2021	X̄ Ambient:	35,23
Animal ID:	Cherry	X̄ Oral	37,26
Laboratory:	UP / EP	X̄ Vaginal	37,58
Transport medium:	DMEM / HOLDING	X̄ Aspiration Instrument	36,30
Study site:	Site 1/ Site 2/ Site 3	Flushing Medium Equipment:	EPBox / Water Bath / N
OPU Duration (min):	41	Collection Bottle Equipment:	RiceBags / Water Bath / Wasserbad / N
#Oocytes:	1	Microscope Heating Stage:	Y / N
#Matured:	0	Laboratory Heating Stage:	Y / N
#Cleaved:	0	Transport Method:	Transport Incubator / Equitainer
Comments: Warmed enema water = 29°C			



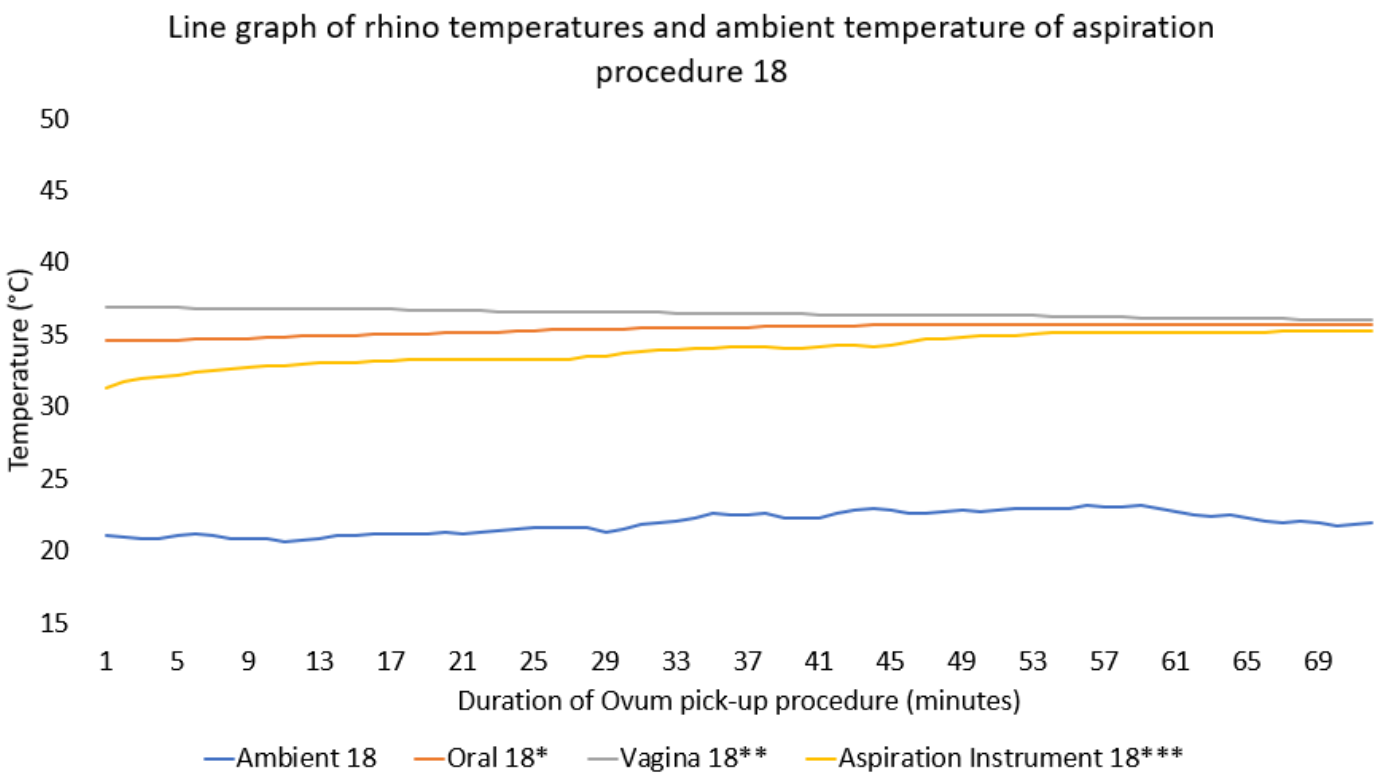
Aspiration #16		Temperature Data:	
Date:	12 October 2021	X̄ Ambient:	22,03
Animal ID:	5.13. O	X̄ Oral	32,69
Laboratory:	UP / EP	X̄ Vaginal	36,62
Transport medium:	DMEM / HOLDING	X̄ Aspiration Instrument	33,17
Study site:	Site 1/ Site 2/ Site 3	Flushing Medium Equipment:	EPBox / Water Bath / N
OPU Duration (min):	55	Collection Bottle Equipment:	RiceBags / Water Bath / Wasserbad / N
#Oocytes:	5	Microscope Heating Stage:	Y / N
#Matured:	2	Laboratory Heating Stage:	Y / N
#Cleaved:	0	Transport Method:	Transport Incubator / Equitainer
Comments: STIMULATED; Warmed enema water = 31°C			



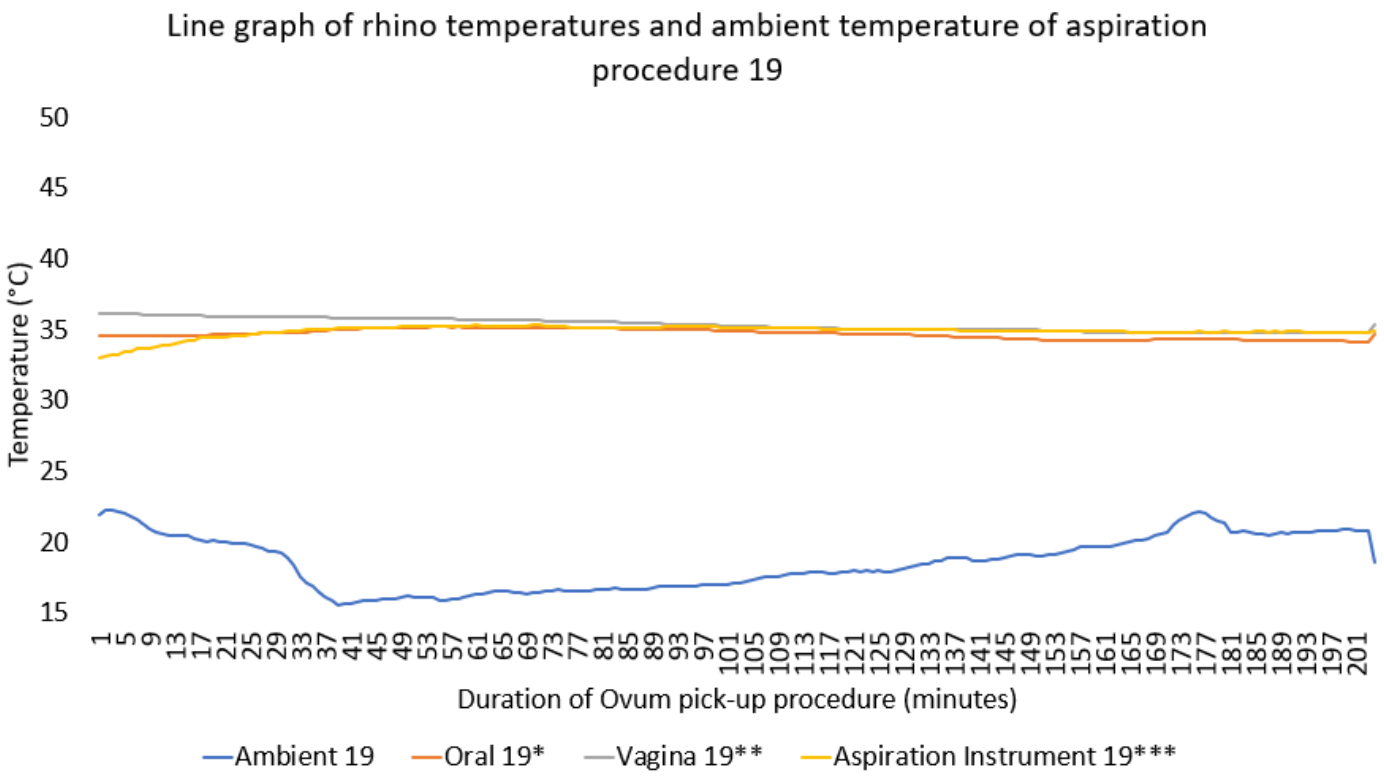
Aspiration #17		Temperature Data:	
Date:	12 October 2021	X̄ Ambient:	39,74
Animal ID:	3.14. O	X̄ Oral	32,64
Laboratory:	UP / EP	X̄ Vaginal	38,13
Transport medium:	DMEM / HOLDING	X̄ Aspiration Instrument	37,18
Study site:	Site 1/ Site 2/ Site 3	Flushing Medium Equipment:	EPBox / Water Bath / N
OPU Duration (min):	157	Collection Bottle Equipment:	RiceBags / Water Bath / Wasserbad / N
#Oocytes:	16	Microscope Heating Stage:	Y / N
#Matured:	5	Laboratory Heating Stage:	Y / N
#Cleaved:	0	Transport Method:	Transport Incubator / Equitainer
Comments: STIMULATED			



Aspiration #18		Temperature Data:	
Date:	19 October 2021	X̄ Ambient:	21,94
Animal ID:	Shiva	X̄ Oral	35,37
Laboratory:	UP / EP	X̄ Vaginal	36,48
Transport medium:	DMEM / HOLDING	X̄ Aspiration Instrument	33,96
Study site:	Site 1/ Site 2/ Site 3	Flushing Medium Equipment:	EPBox / Water Bath / N
OPU Duration (min):	72	Collection Bottle Equipment:	RiceBags / Water Bath / Wasserbad / N
#Oocytes:	4	Microscope Heating Stage:	Y / N
#Matured:	0	Laboratory Heating Stage:	Y / N
#Cleaved:	0	Transport Method:	Transport Incubator / Equitainer
Comments:			
STIMULATED; The Aspiration Instrument was removed momentarily to expel air.			



Aspiration #19		Temperature Data:	
Date:	19 October 2021	X̄ Ambient:	18,53
Animal ID:	Dezi	X̄ Oral	34,67
Laboratory:	UP / EP	X̄ Vaginal	35,32
Transport medium:	DMEM / HOLDING	X̄ Aspiration Instrument	34,87
Study site:	Site 1/ Site 2/ Site 3	Flushing Medium Equipment:	EPBox / Water Bath / N
OPU Duration (min):	203	Collection Bottle Equipment:	RiceBags / Water Bath / Wasserbad / N
#Oocytes:	18	Microscope Heating Stage:	Y / N
#Matured:	9	Laboratory Heating Stage:	Y / N
#Cleaved:	3	Transport Method:	Transport Incubator / Equitainer
Comments: STIMULATED; Warmed enema water = 28°C			



Aspiration #20		Temperature Data:	
Date:	30 November 2021	X̄ Ambient:	36,32
Animal ID:	Nola	X̄ Oral	35,64
Laboratory:	UP / EP	X̄ Vaginal	36,78
Transport medium:	DMEM / HOLDING	X̄ Aspiration Instrument	35,16
Study site:	Site 1/ Site 2/ Site 3	Flushing Medium Equipment:	EPBox / Water Bath / N
OPU Duration (min):	30	Collection Bottle Equipment:	RiceBags / Water Bath / Wasserbad / N
#Oocytes:	0	Microscope Heating Stage:	Y / N
#Matured:	0	Laboratory Heating Stage:	Y / N
#Cleaved:	0	Transport Method:	Transport Incubator / Equitainer
Comments: STIMULATED; Warmed enema water = 38.5 °C