Developing embryo technologies for the eland antelope (Taurotragus oryx)

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DEVELOPING EMBRYO TECHNOLOGIES FOR THE ELDAND ANTELOPE
(*TAUROTRAGUS ORYX*)

A Dissertation
Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
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Doctor of Philosophy

In

The Interdepartmental Program in
Veterinary Medical Sciences
through the Department of
Comparative Biomedical Sciences

by
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List of Tables

Table 2.1. List of mammalian species in which ART has been successful with offspring produced ........19
Table 2.2. Attempts to hybridize tragelaphine antelopes and the results ......................................27
Table 3.1. Design of Experiment 3.1 ...................................................................................................42
Table 3.2. Components and concentrations of modified KSOM, BM-3 and three groups of amino acids .................................................................43
Table 3.3. Development frequencies and cell count of in vitro-derived bovine embryos cultured in protein-free BM-3 or mKSOM supplemented with two groups of amino acids .........................46
Table 3.4. Blastocyst expansion and hatching frequencies of in vitro-derived bovine embryos cultured in protein-free BM-3 or mKSOM supplemented with two groups of amino acids ...............46
Table 3.5. Effects of osmotic pressure of modified BM-3 and supplements on the development of .................................................................48
Table 3.6. Effects of glucose, pyruvate, lactate and phosphate in high or low osmotic pressure modified BM-3 on the frequency of cleavage (day 2) and development to morula (day 7) and blastocyst (day 9) stages of in vitro-derived bovine embryos cultured in protein-free media ......49
Table 3.7. Effects of glucose, pyruvate, lactate and phosphate in high or low osmotic pressure modified BM-3 on the frequency (%) of blastocyst expansion (days 7 and 8) and hatching (day 9) in vitro-derived bovine embryos ..................................................................................50
Table 4.1. Mean interval (min) from conditioned sound cue to acceptance of handheld treats during morning and afternoon training sessions in elands ..............................................................................70
Table 4.2. Effect of sedative treatments on onset of sedation, frequency of recumbency or a need for second dosing of sedative agents in elands during handling and oocyte retrieval in a hydraulic chute ........................................................................................................71
Table 4.3. Some physiological parameters of female elands during sedation and handling in a hydraulic chute for oocyte retrieval ........................................................................................................75
Table 4.4. Some variations between elands with good (n = 5) or poor (n = 5) taming potential .........75
Table 5.1. Ovarian response and oocyte recovery rates in elands (n = 4) treated with Folltropin®-V given 2 (single) or 4 and 2 (double) days before ultrasound-guided oocyte retrieval ..................................................105
Table 5.2. Morphological distribution of cumulus oocyte complexes recovered from eland donors (n = 4) treated with Folltropin®-V for 2 (single) or 4 and 2 (double) days before oocyte retrieval ..........................................................................................................................105
Table 5.3. Effect of targeting the preovulatory or postovulatory follicular wave on ovarian response to FSH treatment and oocyte recovery rates in elands (n = 6). ........................................................................106
Table 5.4. Effect of three factors on the in vitro maturation (meiotic status) of eland oocytes recovered after Folltropin®-V-induced ovarian stimulation ..............................................................................112
Table 5.5. Development of eland oocytes subjected to three methods of *in vitro* production (IVP) of embryos.
List of Figures

Figure 3.1. Blastocyst development of *in vitro*-derived bovine embryos cultured in high osmotic pressure BM-3-20aa with different supplements. .................................................................51

Figure 3.2. Blastocyst development of *in vitro*-derived bovine embryos cultured in low osmotic pressure BM-3-20aa with different supplements. .................................................................52

Figure 3.3. Pathways of glucose metabolism & related factors influencing embryonic development 57

Figure 4.1. Sketch of the eland holding area and associated structures ........................................63

Figure 4.2. Blood glucose (mean ± SD) and hematocrit (mean or single sample values) in sedated elands handled in a hydraulic chute for oocyte collection (n = 18)..............................................73

Figure 4.3. Mean blood CPK level in sedated elands handled for oocyte retrieval (n = 18). ..........74

Figure 4.4. Heart rate (mean ± SD) profile during xylazine-butorphanol induced sedation in elands handled for oocyte retrieval (n = 25)........................................................................76

Figure 4.5. Blood oxygen saturation (mean ± SD) profile during xylazine-butorphanol-induced sedation in elands handled for oocyte retrieval (n = 23). ..............................................................77

Figure 4.6. Respiratory rate (mean ± SD) profile during xylazine-butorphanol induced sedation in elands handled for oocyte retrieval (n = 23)........................................................................78

Figure 4.7. Peripheral body temperature profile during xylazine-butorphanol induced sedation in elands handled for oocyte retrieval (n = 13). .................................................................79

Figure 5.1. Estrous synchronization and ovarian stimulation treatment schedule before ultrasound-guided oocyte collection in elands in Experiment 5.1..........................................................92

Figure 5.2. Treatment schedules to target preovulatory or postovulatory follicular waves in elands before conducting ultrasound-guided oocyte retrieval in Experiment 5.2a..........................94

Figure 5.3. Ultrasound transducer and a needle guide enclosed in the custom-made adaptor...........96

Figure 5.4. Effect of animal on follicles observed and oocytes recovered from eland donors. .......107

Figure 5.5. Mean number of follicles observed and oocytes recovered from eland donors during each month of the year. ........................................................................................................108

Figure 5.6. Correlation between the number of follicles observed and oocytes recovered from eland donors. ..................................................................................................................109

Figure 5.7. Morphological appearance of eland oocytes recovered by ultrasound-guided transvaginal follicular aspiration .............................................................................................111

Figure 5.8. (A-J) Status of eland oocytes after applying IVM, IVF (A-H) or SCNT with giant eland somatic cells (I, J)...................................................................................................................113

Figure 5.9. Day 7 blastocyst (400x) that developed after SCNT into an eland oocyte using a cumulus cell from the same oocyte donor.................................................................114
Abstract

Assisted reproductive technologies developed in domestic cattle serve as a starting point in similar studies on nondomestic bovids. The common eland is a useful model species for studies on rare tragelaphine antelopes. In Chapter 3 of the present study, effects of components/attributes of protein-free embryo culture media on the *in vitro* development of *in vitro*-derived bovine embryos were evaluated. A 2 x 2 factorial study comparing effects of groups of amino acids (20aa or 11aa) in two base media (modified KSOM or BM-3) demonstrated that amino acids and base medium affected embryonic development. A subsequent 7 x 2 factorial experiment to evaluate effects of osmotic pressure and supplement type in BM-3-20aa showed that embryonic development was largely affected by supplements and identified glucose (0.2 mM) as a crucial supplement.

In Chapter 4, the use of behavioral training and handling of elands in a hydraulic chute to perform transvaginal ultrasound-guided oocyte retrieval without inducing general anesthesia were evaluated. Nine of 10 females associated specific sound cues with food treats. Females varied in their response interval to audio cues and to training for voluntary entry into the chute. Handling elands for oocyte retrieval required sedation and increased blood glucose levels.

In Chapter 5, type of estrous synchronization or ovarian stimulation protocol did not affect ovarian response. Animals, but not month of the year, affected ovarian response. In 37 oocyte retrieval procedures using seven females, an average of 12.8 follicles yielded 9.8 oocytes, of which up to 73% matured to metaphase II. *In vitro* fertilization, intracytoplasmic sperm injection and nuclear transfer resulted in embryonic development. In conclusion, the bovine embryo culture study suggests that the beneficial effects of amino acids are influenced by the base medium and glucose plays more important roles in non-ATP producing pathways. Behavioral training and handling of sedated females in a hydraulic chute is a reliable method for collecting eland oocytes, which can undergo *in vitro* maturation and some *in vitro* embryonic development.
Chapter 1. Introduction

Many of the fauna and flora of the earth are under increasing risk of extinction, mainly due to loss of habitat because of human activities. According to the International Union for Conservation of Nature and Natural Resources (IUCN), one-fourth of the approximately 5,000 species of mammals are at risk of extinction. Extinction of mammals continues to be recorded as recently as the year 2000. For example, the last Pyrenean ibex (*Capra pyrenaica pyrenaica*) died in January 2000. In spite of improved awareness about the need for protection of biodiversity by some sectors of the society, the number of threatened species has continued to increase since 1996, when IUCN started an organized collection of data. For mammals, the number of threatened species increased from 1,096 to 1,137 between 1996 and 2002 (IUCN 2002). With the ever-increasing human population and its subsequent effect on the environment, more and more species are likely to suffer the imminent threat of extinction.

General measures involving the protection of habitat are key to the conservation of biodiversity; additionally, captive breeding is an important tool in the conservation of specific species. Not only can captive breeding be used to re-establish wild populations of rare and endangered species, it can also serve as “insurance” against extinction in case of natural disasters (Sillero-Zubiri *et al.* 1997; Holt and Pickard 1999; Hildebrandt *et al.* 2002). However, several factors could hinder captive breeding by natural mating (Lasley *et al.* 1994), including behavioral and genetic incompatibility of mating pairs.

Assisted reproductive technologies (ART) offer viable alternatives for alleviating some limitations encountered with natural breeding, including increased possibility for out crossing different individuals beyond behavioral, spatial (geographical) and temporal restrictions (Ptak *et al.* 2002). In spite of the huge potential of ART in the propagation of threatened species and also the
maintenance of genetic diversity, the use of such technologies has not been investigated for most of the species.

The limited application of ART can be attributed to a number of factors. First, except for artificial insemination, the efficiency of most ART is low even in domestic animals, and results are even lower when applying the technologies to nondomestic animals, for which basic information on reproductive physiology is lacking or limited. Second, the numbers of endangered nondomestic animals in captivity are scant; therefore, biological samples used to develop or apply ART are scarce. Third, the behavioural predisposition of most large nondomestic mammals requires inducing general anaesthesia, even for minor procedures. However, it is stressful to animals and technically not feasible to induce general anaesthesia at short/frequent intervals, as required for ART procedures.

Thus, for ART to have a positive impact on the conservation of biodiversity, available techniques in domestic animals should be improved. This will subsequently improve the prospect of success when the techniques are applied to nondomestic species, although similar efficiencies should not be expected. Furthermore, to effectively use nondomestic animals available in captivity, methods should be developed that minimize the stress of handling while performing ART procedures.

The common eland (*Taurotragus oryx*), one of the two largest African antelopes, is closely related to eight species of spiral-horned antelopes belonging to two genera: *Tragelaphus* and *Taurotragus*. Both genera contain at least 18 subspecies, of which three are endangered. Even the common eland itself may be threatened with extinction in the near future as many experts have expressed concern that the population in their natural habitat is declining rapidly. Nevertheless, compared with other spiral-horned antelopes, the common eland is well represented in captivity.

Moreover, baseline information is available to demonstrate the feasibility of developing ART in this species for application in other spiral-horned antelopes. The eland has been shown to be capable of maintaining pregnancy and producing offspring following interspecies transfer of bongo embryos (Dresser *et al.* 1985) and may be able to serve as a recipient of embryos from the
endangered giant eland. The reproductive patterns of tragelaphine antelopes also seem to be similar, as demonstrated by inter-specific breeding and subsequent live births of hybrid offspring involving at least five species (Benirschke et al. 1980).

Thus, the common eland can serve as a useful model species in which to study ART in tragelaphine antelopes. Additionally, the domestic bovid is a valuable general model species for development of ART such as in vitro embryo production in nondomestic bovids because economic impetus and animal availability has allowed the technology to advance rather quickly. It is hoped that eventually, ART developed in bovine can be applied successfully to the eland, and then to rare tragelaphine antelopes. Accordingly, the present study consists of two primary sections. In the first section (Chapter 3), domestic bovid embryos were used to optimize simple (defined) culture media for their in vitro development. In the second part (Chapters 4 and 5), investigations were carried to develop a minimally stressful and repeatable method of gamete (oocyte) collection in the eland. In addition, aspects of eland behavior and the in vitro developmental potential of eland oocytes were evaluated.
Chapter 2. Literature Review

2.1. The Role of Reproductive Technologies in Biodiversity Conservation

The definition and scope of ART varies among species. For example, the Centers for Disease Control and Prevention define human ART as “all fertility treatments in which both egg and sperm are handled”, including in vitro fertilization (IVF), gamete intra-fallopian transfer, zygote intrafallopian transfer and intracytoplasmic sperm injection (ICSI). However, artificial insemination (AI) and the use of superovulation treatments without a concomitant plan for retrieval of oocytes are not defined as ART (SART-CDC 2003). In nondomestic animals and species of agricultural importance, the term ART is used in a broader sense. For example, in the first two series of international symposia on “ART and Genetic Management of Wildlife” that met in 2001 and 2002 (Omaha, Nebraska, USA), frequent reference to ART was made when referring to artificial insemination, simple embryo transfer, in vitro maturation of oocytes or culture and preservation of somatic cells for potential use in cloning. This disparity in the definition of ART in the human and animals is a reflection of the different purposes served by the technology and the legal implications of human ART.

Human interest in assisting the breeding of plants and animals is probably as old as the history of domestication. However, the use and development of modern age assisted reproductive technologies emerged subsequent to the invention of the microscope that enabled visualization of gametes and embryos. Artificial insemination and embryo transfer are the first generation of such technologies.

Spallanzani documented the first artificial insemination using birds and dogs in the 1780s (Heape 1897; Foote 2002). Embryo transfer was first reported in 1890 in the rabbit (Heape 1890). Subsequently, these two technologies have been successfully applied in a wide range of species. The development of methods for cryopreservation of spermatozoa and, subsequently, of embryos and
oocytes starting in the 1940s has also enhanced the potential application of these technologies (see (Betteridge 2003); and Table 2.1).

The applications and benefits of ART in domestic or nondomestic mammals are numerous. Some examples are outlined below:

1. ability to salvage and propagate genetic material from postmortem animals and those with physical or physiological conditions/problems, including individuals that are geriatric, prepubertal, pregnant, incompatible with mate, seasonally anestrus, physically disabled, or have reproductive tract pathologies;

2. capability of transporting gametes or embryos across national or international boundaries for alleviating genetic depression and repopulating the natural habitat;

3. more efficient use of i) spermatozoa, including low quality samples, by using IVF or micro-assisted fertilization (ICSI, zona drilling) and ii) oocytes from genetically valuable females by in vivo collection and rescue of oocytes from the pool undergoing follicular atresia;

4. to reduce risk of disease transmission, that could occur during natural breeding;

5. ability to control sex ratio of offspring; and

6. to improve knowledge of the biology of gametes/early embryos.

For nondomestic mammals, regulations restricting the shipment of live animals across international borders make it difficult to maintain genetically viable populations of rare species at facilities carrying out captive breeding. Even when legal shipment of live animals is possible, the transport causes stress and deaths occur during transit. Behavioral and genetic incompatibility can also preclude safe mating. The use of ART, such as artificial insemination and embryo transfer, combined with cryopreservation of spermatozoa and embryos, “eliminates the problems of distance
and time as major obstacles in captive breeding programs” (Lasley et al. 1994; Rott 1996; Hildebrandt et al. 2002).

In addition, when combined with in vitro production (IVP) of embryos, ART would maximize the use of the large pool of male and female gametes that, otherwise, are “wasted” during a lifespan of natural breeding. Thus, ART would economize the use of gametes for production of offspring from endangered species. For example, theoretically, hundreds of calves can be produced from a single domestic cow by using a combination of technologies such as ultrasound-guided oocyte retrieval and in vitro maturation (IVM), IVF, embryo culture and embryo transfer (Hansel, 2003). Similarly, an enormous number of offspring could be produced from a single individual using somatic cell nuclear transfer (SCNT) and embryo transfer.

Early stage antral follicles (<1 mm) can be cultured in vitro to yield mature oocytes capable of undergoing fertilization and producing offspring [see (Miyano 2003)]. Activation of primordial follicles and understanding the mechanisms responsible for the atresia (apoptosis) of most precursor germ cells [see (Alonso-Pozos et al. 2003)] may also enable use of the large pool of female germ cells for ART. The possibility of in vitro production of both male and female germ cells/gametes from embryonic stem cells (Hubner et al. 2003; Toyooka et al. 2003) also suggests that additional tools for the propagation of rare species may become available.

2.2. Gamete Retrieval and In Vitro Embryo Production in Farm and Nondomestic Mammals

2.2.1. Methods of Gamete (Oocyte) Retrieval

Studies on in vitro gamete biology and the development of ART for mammalian species are dependent on the availability of both male and female gametes. Gametes (spermatozoa and oocytes) can be colleted postmortem or ante-mortem. The methods used in cattle have been reviewed (Gordon 1994). For domestic animals such as cattle, sheep, goats and pigs, slaughterhouse ovaries and testes are readily available and this resource has been used extensively in the development of ART. In
addition, gonads from small companion animals, especially cats and dogs, are easily obtained from local veterinary clinics and some animal shelters.

Semen can be collected from males of large domestic and nondomestic animals by using an artificial vagina, masturbation or automasturbation, electroejaculation, transrectal massage of the ampullae and accessory sex glands, collection during or after mating from the vagina or via a fistula made in the region of the penile urethra (Crump and Crump 1994; Pope et al. 1997; Schmitt and Hildebrandt 1998; Skidmore et al. 2001). Semen samples are also commonly collected from the epididymides and vasa deferentia of postmortem or castrated animals. Generally, spermatozoa are more readily collected than oocytes in many mammalian species. This accessibility has facilitated the propagation of selected paternal genotypes in economically important species, such as cattle.

Due to the visceral location of the ovaries, the in vivo recovery of follicular stage oocytes requires approaches that allow for visualization of follicles. Thus, invasive methods such as laparotomy, needle puncturing of ovaries that are positioned in the flank area by rectal manipulation, or transvaginally by surgical approaches (colpotomy) have been used [reviewed in (Gordon 1994)]. In smaller animals, laparoscopic methods of collecting oocytes in situ may be the most efficient method.

In large mammals, recent development of methods for real-time visualization of ovarian follicles (and other structures) using ultrasonography has led to a relatively non-invasive approach for collecting oocytes from live animals. Ultrasound-guided oocyte retrieval in domestic animals (cattle) was first reported in the 1980s (Callesen et al. 1987; Pieterse et al. 1988). The methods were based on similar developments in human reproductive technology (Gleicher et al. 1983). The feasibility of applying transvaginal ultrasound guided aspiration to recover oocytes has subsequently been demonstrated in other domestic species, including horses (Brück et al. 1992) and goats (Graff et al. 2000).
The potential of ultrasound-guided oocyte retrieval, when combined with IVP, for propagating large mammals can be demonstrated by the story of a cow from Auburn University:

“A 13-year-old Limousin cow ...was admitted to the IVF program in 1996. Her last calf had been born 4-years prior to her arrival. Over a 2-year period, 284 eggs were collected in 53 attempts...(5.3 eggs per collection). ...IVF techniques using semen from 13 different bulls...produced 81 transferable embryos ....resulting in 24 pregnancies. Twenty-one calves were born over the 2-year-period.” (http://www.vetmed.auburn.edu/art/Description_IVF_Text.htm, accessed October 31, 2003).

This is in contrast to a maximum of two calves that can be produced from a healthy and fertile domestic cow during two years by using AI or natural mating methods.

Transvaginal ultrasound-guided oocyte collection has also been applied in several nondomestic large mammals. The list includes the addax, mountain bongo, African buffalo, common eland, gaur, lowland gorilla, red hartebeest, llama, red deer, sable, tsessebe, wapiti, and Burchell’s and Hartmann’s zebra (Armstrong et al. 1995; Loskutoff et al. 1995; Meintjes et al. 1997; Pope et al. 1998b; Asa et al. 1998; Brogliatti et al. 2000; Wirtu et al. 2002a; Berg and Asher 2003; Loskutoff et al. 2004). This method has particular advantages as a method of in vivo oocyte recovery. It is rapid, simple, accurate, relatively non-invasive and can be repeated frequently (Brück et al. 1992; Pieterse et al. 1991). Using an ultrasound-guided approach, oocytes can also be recovered from pregnant females (Meintjes et al. 1995; Cochran et al. 1998) or postpartum animals as early as day 5 after parturition (Perez 2003). Moreover, it may be useful to ease restrictions on the import/export of embryos derived from IVF, as the donors can be tested for diseases over an extended period of gamete/embryo importation (Loskutoff et al. 1995). Other applications of ultrasound examination in ART include early pregnancy diagnosis, in vivo intra-follicular gamete transfer and determination of the physiological status of gonads and reproductive tracts.

The utility of ultrasound-guided method as a practical method for recovery of oocytes has been demonstrated in several nondomestic artiodactyls (see above). However, only in the gaur and wapiti have pregnancies been established and offspring born after IVF and transfer of embryos to
recipient females (Armstrong et al. 1995; Hammer et al. 2001; Berg and Asher 2003). While a few other nondomestic embryos been produced in vitro from oocytes recovered by ultrasound-guided approach, the efficiency is reduced, as compared with that seen in more, well-studied species, because of the lack of information about in vitro requirements for oocyte maturation/embryo culture and cryopreservation/processing of spermatozoa. Only improving the availability of nondomestic animal gametes and embryos for basic and applied studies can narrow the information gap. Greater accessibility to increased numbers of oocytes of nondomestic species is essential for accelerating progress in this area.

While ultrasound-guided oocyte retrieval does have several advantages over other methods of oocyte recovery in nondomestic artiodactyls, the ability to do multiple retrievals has been limited because of the necessity to use general anesthesia. Not only is there an inherent risk to general anesthesia, there is additional risk of physical injury during the induction. Furthermore, stress associated with the induction of general anesthesia (additional handling, pen shifting, restricting space) can compromise many aspects of animal physiology including reproduction (Haigh 2001). Therefore, alternative approaches that allow for multiple oocyte collections with minimal stress and reduced risk to animals and personnel need to be developed to allow more effective use of captive nondomestic artiodactyls for developing methods for in vitro production of embryos.

2.2.2. Approaches to In Vitro Embryo Production

Subfertility due to reproductive tract pathologies can occur in animals with normal gonadal function. Although artificial insemination and/or embryo transfer can be applied to treat some subfertility conditions, other problems may be managed using the more recently developed embryo technologies.

Moreover, during natural breeding, the male ejaculate contains millions of spermatozoa, of which only one is required to fertilize each ovulated oocyte. In the absence of mating, spermatozoa in
the testis and accessory glands are lysed and phagocytized or lost in urine. Similarly, the ovaries of fetal cattle contain about 3 million germ cells. Most of these undergo atresia, with about 200,000 (7%) remaining in the newborn calf. Still less than 1% of the germ cells present at birth are ovulated [(Erickson 1966); see also (Gordon 1994; Hansel 2003)]. Even among the gametes that are released/ovulated, only a fraction participates in fertilization and subsequent development.

Thus, the application of *in vivo* gamete recovery methods in combination with *in vitro* embryo production techniques and/or cryopreservation would theoretically maximize the efficiency of gamete use from an individual animal of interest. This is of special interest for basic and applied studies of ART in rare and endangered species, in which the supply of gametes is scarce.

### 2.2.2.1. *In Vitro* Fertilization

*In vitro* fertilization is a simple form of IVP, in which male and female gametes are co-incubated under conditions that would allow for sperm penetration and subsequent activation of the oocyte, formation of pronuclei, syngamy and subsequent cleavage. Recorded attempts at *in vitro* fertilization date back to 1878. However, M.C. Chang (Chang 1959) was the first to unequivocally demonstrate successful *in vitro* fertilization followed by the birth of live offspring [see (Bavister 2002a)]. Since then, similar successes have been reported in a number of species. *In vitro* fertilization, with subsequent production of offspring after the transfer of resulting embryos to recipient females has been reported in at least 30 domestic and nondomestic mammalian species (Table 2.1).

Although offspring have yet to be produced, the potential of *in vitro* fertilization has also been demonstrated in many other species. These include artiodactyls such as greater kudu (Loskutoff *et al.* 1995), mountain bongo (Pope *et al.* 1998b), addax (Hall-Woods *et al.* 1999), klipspringer (Raphael *et al.* 1991) and common eland (Wirtu *et al.* 2002a), and non-artiodactylids such as leopard, puma, cheetah, clouded leopard, jaguarondi, lion, black footed cat, snow leopard, jaguar, jungle cat
and zebra (Goodrowe *et al.* 1989; Miller *et al.* 1990; Meintjes *et al.* 1997; Pope 2000). However, low frequencies of fertilization in nondomestic species using standard domestic animal IVF protocols [e.g., (Winger *et al.* 1997; Kidson *et al.* 2000; Wirtu *et al.* 2002a)], indicates the critical need for further investigations and the refinement of these technologies.

### 2.2.2.2. Intracytoplasmic Sperm Injection (ICSI)

Various approaches have been adopted to maximize fertilization rates *in vitro*. For example, when quality or quantity of spermatozoa are low, micro-assisted fertilization by intracytoplasmic sperm injection (ICSI) is often used. Offspring have been produced after transferring ICSI-produced embryos in several species including cattle, sheep, horse, pig and cat (Goto *et al.* 1990; Catt 1996; Cochran *et al.* 1998; Pope *et al.* 1998a; Martin 2000). This approach circumvents the inability of spermatozoa to fertilize oocytes due to low sperm numbers or poor motility (Goto *et al.* 1990; Pope *et al.* 1998a; Lacham-Kaplan *et al.* 2003). Moreover, complete structural integrity of the spermatozoon is not required to achieve fertilization after ICSI (Ward *et al.* 1999).

Given that spermatozoa from most nondomestic artiodactylids have low viability after cryopreservation and that large numbers of ejaculates are required to develop reliable cryopreservation methods, ICSI appears to have higher potential than conventional IVF protocols for propagation of rare artiodactylids. The success of IVF also depends on the ability to induce sperm capacitation, which is considered a limiting factor for successful IVF in some species (example, horses). However, sperm capacitation is not an absolute requirement to achieve successful fertilization after ICSI (Galli *et al.* 2003b). ICSI could also be used to circumvent polyspermic fertilization seen after IVF in species such as pigs (Kren *et al.* 2003).

Regardless of the potential, the application of ICSI in nondomestic mammals has so far been limited, partly, due to the technical skill and expensive micromanipulation equipment that is required. Nevertheless, three normal rhesus monkey offspring have been produced after ICSI (Chan
et al. 2000). Also, transferable jaguarundi (*Herpailurus yaguarondi*) embryos have been produced using ICSI (Pope et al. 1998a).

### 2.2.2.3. Cloning

The basis for the current nuclear transfer developments in various mammalian species can be traced back to the basic scientific questions raised by early investigators in the mid-1800s and early 1900s. The German zoologist, August Weismann (1834-1914) is credited for proposing the simple and testable hypothesis that in vertebrates “the zygote contains all the genetic determinants to form a complete individual”, and also “that genetic determinants of the zygote are divided when the egg divides” (McKinnel 1985). Subsequently, other German scientists including Wilhelm Roux (1850-1924) and Adolph Eduard Driesch (1867-1941) performed experiments to further test Weismann’s hypothesis.

However, it was Hans Spemann (1869-1941) who conclusively demonstrated for the first time that two blastomeres from a single vertebrate (salamander) embryo are capable of developing into two complete larvae. Spemann constricted a zygote using a loop of hair such that one side of the loop contained anuclear ooplasm and the other side contained nucleated ooplasm. After the nucleated ooplasm developed to a 16-cell stage embryo, he loosened the loop and allowed a single blastomere to pass to the anucleated side. Subsequently twin whole organisms developed. Thus, Weismann’s theory that the genetic determinants for forming a complete individual are divided during subsequent divisions of the zygote was disproved. Rather, totipotency was not only an attribute of the zygote but is also a characteristic of individual blastomeres; nevertheless, Weismann’s hypothesis served as a basis for the subsequent experiments.

With subsequent development of micromanipulation equipment, it became possible to introduce a single cell (nucleus) into an oocyte (nuclear transfer). This led to the first cloning of a higher vertebrate species (the frog) from embryonic cells (Briggs and King 1952). That individual
embryonic cells (blastomeres) are capable of developing into a whole organism has subsequently been demonstrated in various species including lower vertebrates and mammals [reviewed in (Di Berardino 1997) (see also Table 2.1 for a list of mammals cloned from embryonic cells)].

Further studies on totipotency using somatic cells have also led to the demonstration that differentiated (adult) cells can be reprogrammed to direct embryonic development. Since the groundbreaking report by the Roslin Institute (University of Edinburgh) group on the birth of lambs after SCNT using cells from adult and fetal (day 26 old) cell lines (Wilmut et al. 1997), the totipotency of mammalian somatic cells has been demonstrated in several species of domestic and laboratory animals. The list, which is still growing, includes cattle, goat, pig, horse, mule, cat, rabbit, mice and rat (see Table 2.1). It is interesting to note that some of the pioneers working in the area of nuclear transfer believed in the “lack of totipotency of nuclei from adult organisms” until 1997 (McKinnel 1985; Di Berardino 1997). Nevertheless, in less than 10 years of the demonstration of the totipotency of somatic cells, SCNT is already affecting many aspects of agriculture and biomedical research.

Potential applications of SCNT in the propagation of endangered species are also emerging, as demonstrated by offspring production in four nondomestic mammalian species: gaur, mouflon sheep, banteng and African wildcat [(Lanza et al. 2000a; Ptak et al. 2002; Holden 2003) (http://www.auduboninstitute.org/rcenter/res_cloning.htm, accessed February 22, 2004)]. In each of these studies, a closely related domestic species served as the source of oocytes used for nuclear transfer (interspecies somatic cell nuclear transfer, iSCNT) and as recipients of the resulting embryos (interspecies embryo transfer, iET). These approaches are an extension of studies on the possibility of embryonic development after iSCNT (Dominko et al. 1999) and of pregnancy and live births after iET (Table 2.1). Some hope that iSCNT and iET will be utilized not only for the propagation of rare species but also for reviving extinct species (http://www.chl.ca/cnewsscience0001/10_clone2.html, accessed February 22, 2004).
However, the low success rates after SCNT, <4% live birth for each micromanipulated oocyte (Wilmut et al. 2002), or after iET (Loskutoff 1999) appear to be somewhat discouraging for using these approaches in the propagation and genetic management of rare mammalian species. The problem with iET could partly be due to maternal immune response to the embryo from the other species (Kraemer 1983; Loskutoff 1999). Factors negatively affecting the success of SCNT include failure of complete genomic reprogramming of the somatic cell or lack of its synchrony with the cell cycle stage of the cytoplast ooplasm, and abnormal placentation and pregnancies after the transfer of nuclear transfer embryos to recipient females (Wilmut et al. 2002). The presence of heterogeneous cytoplasm and organelles from the cytoplast and the somatic cell further complicates interspecies SCNT; however, steady improvements in the success rate of SCNT are being recorded.

Another form of cloning is embryo bisection. As described earlier, blastomere(s) of vertebrate embryos are totipotent. This developmental capability has been applied to produce two or more genetically identical siblings from a single embryo in several species including mice, sheep, cattle, horse and monkeys [see (McKinnel 1985; Di Berardino 1997)]. In mammals, the first evidence for the developmental potential of isolated blastomeres was provided in the rat (Nicholas and Hall 1942). Among nondomestic artiodactylids, embryo bisection has been successful in the common eland (Gelwicks et al. 1989). The latter investigators recovered seven embryos from a single female and non-surgically transferred bisected embryo-pairs to five female eland recipients, one of which delivered a live calf. However, the application of this technology has been limited, in both domestic and nondomestic mammals, mainly due to the less than expected number of offspring resulting following the transfer of manipulated embryos to recipient females.

2.2.2.4. Gamete Transfer

Besides natural mating and artificial insemination, transferring oocytes into the oviduct and subsequently inseminating the female (oocyte transfer), or transferring both gametes into the oviduct
gamete intrafallopian transfer, GIFT) facilitates embryo production. Oocyte transfer and gamete intrafallopian transfer have been successful in horses [see (Squires et al. 2003)] and pigs (Rath et al. 1994).

Direct deposition of both gametes into follicles before ovulation (intrafollicular gametes transfer) or spermatozoa into dominant follicles have also led to the birth of human babies (Werner-von der et al. 1993; Nuojua-Huttunen et al. 1995). Intrafollicular oocyte transfer can be done in cattle (Bergfelt et al. 1998) and has led to the establishment of pregnancies in the horse [see (Hinrichs 1998)].

Thus, gamete transfer technologies have potential applications in propagation and genetic management of rare mammalian species; however, to date, this potential has not yet been investigated in nondomestic species.

2.2.2.5. Embryo Culture

Zygotes or early embryos produced by standard IVF or techniques involving micromanipulation (ICSI, nuclear transfer) require a period of in vitro culture before transfer to a recipient female for continued development. In most ungulates in which embryo transfer has been successful, non-surgical uterine transfer of embryos is feasible and can be accomplished during simple physical restraint. For successful uterine transfer, IVP embryos should be cultured to the morula or blastocyst stage. Thus, in vitro culture (IVC) of embryos is an integral component of ART.

In domestic cattle, in vitro-cultured embryos display marked morphological, biochemical and functional deviations when compared with those produced in vivo (Massip et al. 1995; Holm and Callesen 1998; Niemann and Wrenzycki 2000; Thompson 2000). These anomalies are due, partly, to inappropriate in vitro gamete/embryo handling and culture systems (Massip et al. 1995; Abe et al. 2002). Embryos cultured in media containing complex components, such as serum, display severe abnormalities (Massip et al. 1995; Niemann and Wrenzycki 2000; Jacobsen et al. 2000; van
Wagtendonk-de Leeuw et al. 2000; Cho et al. 2002). Thus, many investigators are advocating the development and use of simple and defined or semi-defined embryo culture media.

Many aspects of IVP require improvements. The longest phase of IVP is the culture of embryos after IVF or SCNT, usually five to seven days. This long duration of IVC presents a primary opportunity for improvements of the IVP system. Moreover, in addition to cell cleavage, key events take place during IVC, including the maternal-embryonic transition of genetic control of development, compaction and blastocyst differentiation. Each of these processes can easily be disrupted in vitro.

Aspects of IVC that influence embryonic development include the lack of proper nutrients and/or presence of potentially toxic products of external or embryonic origin (Bavister 1995; Thompson 1996; Thompson 2000). Different laboratories use different protocols, even when working on the same species, leading to confusion about what methods to use for IVC (Yang et al. 1993; Bavister 1995; Gardner et al. 2000). The choice of a culture method(s) is often made empirically rather than being based on scientific evidence. Evaluation of different culture systems under the same conditions could provide additional information on requirements (or non-requirements) of the embryo and on the method(s) to use when working on a new species for which information is not available.

Undefined media containing serum or bovine serum albumin (BSA) as external sources of protein are still widely used even though similar embryo development is seen in some species with protein-free media containing amino acids (Bavister 1995; Biggers et al. 2000). Although it is well established that serum and BSA can provide stimulatory factors that promote embryo development in vitro, there are negative aspects to their use, including pathogen risk, lot-to-lot variation (Kane 1983; Batt and Miller 1988; McKiernan and Bavister 1992), inhibition of oocyte maturation (Goodrowe et al. 1991) or early cleavage (Pinyopummintr and Bavister 1991; Bredbacka and Bredbacka 1995) and fetal/neonatal abnormalities (Young et al. 1998). In addition, the risk of pathogens of agricultural
and public health importance is minimized when using defined media than those containing undefined components such as serum albumin or serum. Another important advantage to the use of a defined medium is that the effect of each component on embryo metabolism and development can be assessed. Then that information can be used to design media that will improve embryos developmental potential.

Thus, there is a growing interest among many laboratories to use simple/defined media for IVP in different species, including rodents (Whitten and Biggers 1968; Kane and Bavister 1988; Goh et al. 2000), rabbits (Carney and Foote 1991), sheep (Ledda et al. 1992), goats (Keskintepe et al. 1997), primates (Schramm and Bavister 1996; Ali et al. 2000; Zheng et al. 2001), pigs (Yoshioka et al. 2002) and cattle (Lonergan et al. 1994; Liu and Foote 1997; Caamano et al. 1998; Olson and Seidel, Jr. 2000; Wrenzycki et al. 2001; Hernandez-Fonseca et al. 2002; Holm et al. 2002).

Amino acids have beneficial effects during in vitro culture of embryos of various mammalian species. They play important roles as energy substrates, osmolytes, regulators of pH and enzyme activity, scavengers of free radicals, chelators of heavy metals, precursors of macromolecules such as proteins and nucleic acids, and mediators of transport across the cell membrane (Bavister 1995; Liu and Foote 1995a; Lee and Fukui 1996). However, the beneficial effects may be restricted to certain amino acids (Bavister 1995; Steeves and Gardner 1999) and may be affected by the base medium (Van Winkle and Campione 1996). For example, Eagle’s essential amino acids inhibit the development of early-stage cattle embryos (Keskintepe and Brackett 1996), possibly due to toxic effects of ammonia production (Lane et al. 2001). Nevertheless, most laboratories use the mixture of 20 amino acids as originally developed for somatic cell culture. The total concentration in this system is less than 6 mM, whereas free amino acids constitute ~32 and 44 mM in bovine oviductal and uterine fluids, respectively (Elhassan et al. 2001).

Some studies have evaluated the effects of individual or groups of few amino acids during in vitro culture [(see (Bavister 1995; Gardner et al. 2000; Thompson et al. 2000)]. It would be difficult
to conclusively identify the role(s) of each of the 20 amino acids and the effect of their interactions and different concentrations on \textit{in vitro} development of bovine embryos. A logical step would be to evaluate the feasibility of protocols already developed in laboratory animals. For example, after extensive evaluations of the effects of all amino acids during \textit{in vitro} culture of hamster embryos in chemically defined system, 11 beneficial amino acids were identified (McKiernan \textit{et al.} 1995).

Osmotic pressure and/or high NaCl concentration of a medium can affect the development of embryos \textit{in vitro}. Lower osmotic pressure and a concomitant reduction in NaCl concentration is partially responsible for the improved development of mouse embryos in the potassium simplex optimized medium, KSOM (Summers \textit{et al.} 1995). In most instances, increased osmotic pressure of culture media is synonymous with a high concentration of NaCl. However, extracellular Na+ can affect intracellular pH via its effect on the Na+/H+ anti-porter (Lane and Bavister 1999). Reducing extracellular NaCl also alters gene expression (Ho \textit{et al.} 1994) and stimulates protein synthesis (Liu and Foote 1997). Osmotic stress could also activate the polyol pathway, thereby inducing apoptosis (Galvez \textit{et al.} 2003).

Culturing bovine embryos in media with >270 mOsmol was detrimental (Lim \textit{et al.} 1994; Liu and Foote 1996). Thus, base medium influences a number of embryonic attributes as well as the effects of supplements. Moreover, the mechanisms by which cattle embryos produce energy at each stage of preimplantation development could well be affected by the base medium.

\textbf{2.3. Assisted Reproductive Technologies in the Common Eland}

\textbf{2.3.1. Overview of Tragelaphine Antelopes}

Antelopes are a diverse group of species in the bovidae family. The term “antelope” does not have taxonomic significance; however, about 100 of some 160 species of bovidae are traditionally known by this name. Tragelaphine (spiral-horned) antelopes consist of two genera with nine species
Table 2.1. List of mammalian species in which ART has been successful with offspring produced

<table>
<thead>
<tr>
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<th>ART type</th>
<th>References</th>
<th>Comments</th>
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<td>1966</td>
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<td>Fernandez-Baca &amp; Calderon [see (Pugh and Montes 1994)]</td>
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<td></td>
<td>1974</td>
<td>ET</td>
<td>Sumar and Franco [see (Del Campo et al. 1995)]</td>
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<td>2000</td>
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<td>(Taylor et al. 2001)</td>
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<td></td>
<td>2000</td>
<td>iET</td>
<td>Wiesner et al. (<a href="http://www.medicine.ucsd.edu/cpa/indexfs.html">www.medicine.ucsd.edu/cpa/indexfs.html</a>)</td>
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</tr>
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<td>1983</td>
<td>iET</td>
<td>Lanza et al. [see (Holden 2003)]</td>
<td>Domestic cow recipient</td>
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<td>1988</td>
<td>AI</td>
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<td>(Dorn 1995)</td>
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<td>Sipko et al. [see (Rott 1995)]</td>
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<td>(Drost et al. 1983)</td>
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<td>(Dresser et al. 1985)</td>
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<td>Pig</td>
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<td>Kvasnitski [see (Kvasnitski 2001)]</td>
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<td>Hess et al. [see (Curry 2000)]</td>
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*a AI = artificial insemination; ET = embryo transfer; iET = interspecies ET; CP = cryopreserved; CPs = CP spermatozoa; CPe = CP embryo; CPo = CP oocyte; ECNT = embryonic cell nuclear transfer; SCNT = somatic cell nuclear transfer; iSCNT = interspecies SCNT; IVF = in vitro fertilization;  
b Attempts were made to cite the first successful report for each species.  
c AI in most farm animals was initiated in the late 1800 or early 1900’s [see (Foote 2002)].

25
Tragelaphus (bongo, bushbuck, greater kudu, lesser kudu, lowland nyala, mountain nyala, sitatunga) and Taurotragus (common eland, giant eland). Each species has at least two subspecies or races; thus, there are at least 18 species and subspecies.

Tragelaphine antelopes first appeared about 6 million years ago in sub-Saharan Africa, which is also their current native habitat (Pappas 2002). They are “medium to large antelopes with deep bodies, long necks and legs, narrow heads with big ears and twisted or spiral horns” (Kingdon 1982) and have “variously developed face and body pattern of white spots and stripes” (Nowak 1999). There is clear sexual dimorphism, males being larger than females and having horns in all species; however, only female elands (common and giant) and bongo have horns, while females of the remaining species are polled. All tragelaphine antelopes are non-territorial. They are also gregarious, with the exception of bushbuck and sitatunga, which are solitary (Nowak 1999). Average body weight of adult females ranges from 30 kg in the bushbuck to 450 kg in eland (Appendix A).

The beauty of tragelaphine antelopes has impressed many writers. Crandall (1964) described male kudus as “the handsomest of antelopes” and bongos “among the most beautiful” of antelopes, while Nowak (1999) asserted bongo as “the most beautiful of bovids”. The giant eland has been given royal status as “regal and delighting to the eye” [see (East 1999)]. The common eland has been hailed as the “apotheosis of antelope evolution” [see (Spinage 1986)]. Besides ecological importance, there is increasing interest in the economic use of tragelaphine antelopes through game farming or domestication (Treus and Lobanov 1971; Madzingira et al. 2002). In a recent study by the latter authors (Madzingira et al. 2002), four tragelaphine antelope species (greater kudu, common eland, bushbuck and lowland nyala) were among the seventeen species of antelopes kept by farmers practicing mixed cattle: antelope farming.

The IUCN classifies the threat to extinction of eight tragelaphine species as “lower risk”; but specifically three species (bongo, giant eland and sitatunga) are “near threatened” and the remaining five species are assigned “conservation dependent” status. Mountain nyala (T. buxtoni) and two
subspecies, the western giant eland (*T. derbianus derbianus*) and mountain bongo (*T. eurycerus isaaci*) are “endangered” [see (East 1999; IUCN 2002)].

With the exception of the mountain nyala, all species are represented in captivity in zoos. The only record of keeping the mountain nyala in captivity ended when a pair at the Zoological Garden of Berlin was killed during an air raid in 1944 (Crandall 1964). Additional descriptions of the general characteristics and biological aspects of the tribe and specific species are described elsewhere (Ralls 1978; Kingdon 1982; Spinage 1986; Nowak 1999).

The closeness of tragelaphine antelopes to each other is exemplified by the “unusual readiness to hybridize in captivity” (Kingdon 1982). To date, six hybrid pregnancies have been reported (Table 2.2), including a fertile bongo sitatunga hybrid (Koulischer *et al.* 1973) and a bushbuck sitatunga hybrid that survived for years (Kingdon 1982).

Tragelaphine antelopes are peculiar among mammals in having normal translocation of their Y-chromosomes to autosomes. Karyotype studies are not available for giant eland and mountain nyala; however, among the seven species, all except sitatunga and lesser kudu have the unusual translocation of the Y-chromosome (see Appendix A). The similarity among the species has also been demonstrated by analyzing loci of mitochondrial DNA (Essop *et al.* 1997). Accordingly, there is a tendency to classify all the species in the *Tragelaphus* genus (East 1999; IUCN 2002).

### Table 2.2. Attempts to hybridize tragelaphine antelopes and the results

<table>
<thead>
<tr>
<th>Male</th>
<th>Female</th>
<th>Offspring</th>
<th>Comment</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bongo</td>
<td>Sitatunga</td>
<td>Female, “Bongsi”</td>
<td>Fertile</td>
<td>(Koulischer <em>et al.</em> 1973)</td>
</tr>
<tr>
<td>Bushbuck</td>
<td>Sitatunga</td>
<td>Aborted</td>
<td></td>
<td>See (Benirschke <em>et al.</em> 1980)</td>
</tr>
<tr>
<td>Eland</td>
<td>Greater kudu</td>
<td>Males</td>
<td>Sterile</td>
<td>See (Benirschke <em>et al.</em> 1980)</td>
</tr>
<tr>
<td>Eland</td>
<td>Sitatunga</td>
<td>Females</td>
<td>Died</td>
<td>See (Benirschke <em>et al.</em> 1980)</td>
</tr>
<tr>
<td>Lesser kudu</td>
<td>Bushbuck</td>
<td>?</td>
<td>?</td>
<td>(Kingdon 1982)</td>
</tr>
<tr>
<td>Lesser kudu</td>
<td>Sitatunga</td>
<td>Male</td>
<td>Died</td>
<td>See (Benirschke <em>et al.</em> 1980)</td>
</tr>
</tbody>
</table>
The possibility of hybridization suggests that interspecies embryo transfer may be successful among several tragelaphine species. This has already been demonstrated by the successful transfer of bongo embryos to the common eland (Dresser et al. 1985). Hybrid females (e.g., mule) can support pregnancy after the transfer of embryos from either of the parental species (Davies et al. 1985). Similarly, hybrid female tragelaphine antelopes could probably support embryos from either parent. Immune rejection (Loskutoff 1999) would possibly be reduced in the hybrid surrogates in comparison to regular interspecies embryo transfer recipients.

2.3.2. Common Eland

The common eland is the largest African antelope. Three subspecies of the common eland are: *T. o. pattersonianus* (East African race), *T. o. livingstonii* (Zambezi race) and *T. o. oryx* (Cape or South African race). Skin color and the number of lateral body stripes are the most frequently mentioned criteria for differentiating the three subspecies. Stripes are more numerous and marked in the East African subspecies and may be absent in southern species (Crandall 1964; Spinage 1986; Pappas 2002). Body weights of up to 595 kg (females) and 942 kg (males) have been recorded (Crandall 1964; Kingdon 1982). At the Askanya-Nova Zoo, Ukraine, the average longevity of 23 females was 11 (maximum of 23) years (Treus and Lobanov 1971). In the United States, longevity of up to 25 years has been recorded (Crandall 1964).

The “ox-like appearance” of the eland appears to be the primary source of interest for the domestication of this species. Documented interest in eland domestication dates back to 1591 [see (Spinage 1986)]. The most successful effort to produce a fully domesticated herd of eland, leading to females that could be hand-milked, was carried out at the Askanya-Nova Zoo, Ukraine (Treus and Lobanov 1971). Domestication attempts have also been made in Kenya, Zimbabwe, Britain, France and Brazil (Kingdon 1982).
Advantages of domestication of the eland include their high tolerance to water shortage. The eland can reduce sweating and hence conserve water by raising its body temperature from 39°C to 42°C during increased environmental temperatures (Kyle 1972). Elands also consume more diverse forage species and are less competitive with conventional livestock (Abdullahi 1981). As a result, they have been recommended as a more viable alternative species (better than domestic cattle) for introduction and meat production in the Southwestern United States where invasive woody trees are a problem (Humphries, Jr. 1972). They are also more efficient in feed conversion of the diverse forage consumed. For example, eland gained 0.33 kg in body weight per day as compared with 0.14 kg in cattle managed under similar conditions (Kyle 1972). Also, they are resistant to diseases such as trypanosomiasis and theileriosis (Kingdon 1982) that hinder livestock production in many parts of Africa. Moreover, eland cows produce high quality meat and milk (Spinage 1986). With calving intervals as short as 10 months (Kingdon 1982), reproductive performance in elands is more efficient than most tropical cattle breeds.

Although the requirements by eland for large area and high quality feed that may not be fulfilled by regular grazing have been discouraging in many domestication attempts (Kingdon 1982), the eland could offer opportunities for agricultural diversification in such areas where trypanosomiasis and East Coast Fever (theileriosis) are prevalent. Large numbers of elands are kept in game ranches in southern Africa (East 1999; Madzingira et al. 2002). For example, eight of 11 farms practicing cattle-antelope mixed farming near Harare, Zimbabwe, kept an average of 33 elands per farm (Madzingira et al. 2002). Since economics is the primary driving force behind most technologies, ART included, the promotion of eland domestication could help advance antelope ART.
2.3.3. Reproduction and ART in Tragelaphine Antelopes

Ovarian cyclicity in tragelaphine antelopes occurs throughout the year. However, in their natural habitat, breeding and calving may be seasonally dependent on feed availability. Among tragelaphines, only the greater kudu shows marked seasonality in calving pattern, both in the wild and in captivity (Skinner *et al.* 2002). The estrous cycle length (Appendix A) is similar to that of domestic bovids. Like the mare, tragelaphine females show a fertile postpartum estrus (Kingdon 1982). Reported gestation lengths vary between 6 to 9 months (Appendix A). Members of the tribe reproduce well, both in captivity and in the wild, with calving intervals of a year or less (Crandall 1964; Kingdon 1982). However, detailed information on reproductive performance is available only for the eland.

Like other tragelaphines, common eland cows cycle throughout the year [(Treus and Lobanov 1971) and references cited there]. The estrous cycle ranges between 21-26 days (Atkinson *et al.* 1999; Nowak 1999) and estrus lasts 1-3 days (Nowak 1999). Age at puberty ranges from 1-3 yr in the female (Dittrich 1972), but may be delayed in the male (Nowak 1999). In North America, the median age at first calving of captive eland was about 40 months (Lydon 2001). The mean age at first calving was 36.4 months (n = 42) at the Askanya-Nova Zoo, Ukraine (Treus and Lobanov 1971). However, female elands have given birth as early as 16 months of age (Lydon 2001), suggesting that ovarian cyclicity can start before 10 months of age. Calves are produced after gestation length of 254 to 284 days (Dittrich 1972; Pope and Loskutoff 1999). Of 1,214 eland deliveries, a single calf was born in 99% of the calvings and the remaining 1% delivered twins (Lydon 2001). Domestic eland cows at the Askanya-Nova Zoo produced an average of eight calves (maximum 16) during their lifetime (Treus and Lobanov 1971).

Assisted reproduction studies in tragelaphine antelopes are scant; however, the common eland is the most studied. Treus and Lobanov (1971) described unsuccessful experiments of artificial insemination of domestic cows using eland semen. Although details are not readily available, Treus’
group may have been the first to collect eland semen and perform artificial insemination. The cryopreservation of eland spermatozoa was first reported in 1978 (Merilan et al. 1978).

Merilan and colleagues collected semen using electroejaculation from an eland bull under general anesthesia. They cooled the sample to 4°C and froze it in 0.5-mL straws in liquid nitrogen vapor. Post-thaw motility was 35% (fresh sample = 65%) after freezing semen in egg yolk-citrate-extender and 6% glycerol (Merilan et al. 1978). There are few additional studies evaluating cryopreservation of epididymal or ejaculated eland spermatozoa (Bartels et al. 2001; Herrick et al. 2002; Wirtu et al. 2002b). One study reported the birth of an eland calf after artificial insemination using frozen-thawed epididymal spermatozoa (Bartels et al. 2001). In that report, the semen was diluted in Triladyl® extender, cooled to 4°C and loaded into 0.25-mL straws and frozen in liquid nitrogen vapor. One of four eland cows inseminated with the frozen-thawed sample (55% motility) delivered a calf after a gestation period of 11 months. However, the reported gestation period is at least two months longer than the average for the eland (255 - 284 days, Appendix A).

In greater kudu, among seven cryoprotectant extenders that were evaluated (Schiewe et al. 1991a), EQ and BF5F supported the highest post-thaw motility of 51% and 40%, respectively. In another study (Schmid et al. 1997), cryopreservation of greater kudu epididymal spermatozoa in five extenders were evaluated, along with three thawing temperatures. Although results varied between bulls, samples frozen in Tris-citrate-based extender containing 20% egg yolk and 7% glycerol had the highest post-thaw motility (up to 65%), with slightly higher motility after thawing at room temperature. In four sitatunga bulls, the post-thaw motility of ejaculated spermatozoa frozen in Triladyl® extender ranged between 22% and 42% of the initial motility of 40 to 80% (Perez-Garnelo et al. 2000). In the bongo antelope, the post thaw motility of spermatozoa frozen in TEST-yolk buffer containing 6% glycerol was less than 40% (Dr. Earle Pope, personal communication). Similarly, ejaculated giant eland spermatozoa frozen at White Oak Conservation Center, Florida, had post-thaw motility of 30% or less after freezing in BF5F extender (personal observation). Thus, with the
exception of the two studies (Schmid et al. 1997; Bartels et al. 2001), the survival of tragelaphine spermatozoa frozen using standard bovine semen freezing protocols was unsatisfactory.

Successful ART involving tragelaphine embryos are available for the common eland and bongo. The transfer of fresh (unfrozen) embryos recovered after natural mating of superovulated eland and bongo females has resulted in normal offspring in both species. Dresser and colleagues recovered seven embryos from a superovulated bongo female 8 days after mating and transferred single embryos to four eland and a bongo at ~12 hr after embryo collection. The bongo and one eland recipient delivered normal bongo calves (Dresser et al. 1985). The same group reported the first tragelaphine pregnancies carried to term after the transfer of frozen-thawed eland embryos to the eland (Dresser et al. 1984; Pope and Loskutoff 1999). The transfer of bisected embryos to eland cows also resulted in a normal eland calf (Gelwicks et al. 1989). To date, four eland and two bongo calves have been produced after the transfer of in vivo produced embryos to recipient females (Pope and Loskutoff 1999).

The above pioneering tragelaphine ART studies demonstrated the feasibility of estrous cycle synchronization, ovarian stimulation and non-surgical embryo recovery and transfer in the two species. Non-surgical embryo recovery has also been reported in the greater kudu (Schiewe et al. 1991b). In the latter study, two embryos were recovered after uterine flushing of two females subjected to ovarian stimulation treatments and natural mating. However, details were not given on the quality/stage of the embryos.

In vitro embryo production, using standard IVF or iSCNT, has been reported in the mountain bongo, greater kudu, common eland and giant eland. Pope and colleagues (Pope et al. 1998b) collected 76 oocytes (average = 5 per animal) from bongo antelope using transvaginal ultrasound-guided follicular aspiration after ovarian stimulation treatments. IVF with frozen-thawed spermatozoa led to cleavage frequency of 35% (14 of 40 evaluated oocytes), with one embryo developing to the blastocyst stage. A similar frequency of cleavage (35%, 117/330 oocytes) after
IVM-IVF-IVC was observed in greater kudu oocytes recovered from ovaries collected postmortem (Loskutoff et al. 1995). Fresh or cooled epididymal spermatozoa were used for IVF. Four (1%) kudu blastocysts developed.

As part of the current study, our group reported the use of transvaginal ultrasound-guided follicular aspiration to recover common eland oocytes for IVP. We recovered an average of nine oocytes per female after ovarian stimulation treatments. Two of 26 metaphase II stage oocytes cleaved after IVF using frozen-thawed eland spermatozoa (Wirtu et al. 2002a). Obviously, IVF has been rather inefficient in the three tragelaphine antelopes (bongo, greater kudu and common eland) studied to date.

After interspecies somatic cell nuclear transfer to enucleated bovine oocytes, somatic cell nuclei of mountain bongo (Lee et al. 2003) and giant eland (Damiani et al. 2003) can support development to blastocysts. The average blastocyst development ranged from 19 - 27% (giant eland) and 11 - 24% (bongo) of activated SCNT couplets. Another group (Matshikiza et al. 2004) also reported the development of a blastocyst (2%) after “hand-made” nuclear transfer of common eland somatic cells into bovine oocytes. Enucleated common eland oocytes have also supported embryonic development up to the 8-cell stage after iSCNT with giant eland cells (Damiani et al. 2003). Therefore, development of blastocysts has been reported after iSCNT of bongo, giant eland and common eland somatic cells into bovine oocytes.

In applying or developing ART in a new species, the use of information developed in a closely related domestic and nondomestic species as a starting point is recommended (Loskutoff et al. 1995; Comizzoli et al. 2000; Pope 2000; Watson and Holt 2001; Leibo and Songsasen 2002). Considering the close relationship of common eland to other tragelaphine antelopes (discussed above), its availability and prolificacy in captivity, this species can serve as a sound model species to study tragelaphine ART. Interest in the use of the common eland for game ranching and
domestication could also provide additional economic incentive for developing and applying ART in the species.

2.3.4. Role of Behavioral Training and Advanced Handling Systems to Facilitate ART Procedures

As discussed previously, obtaining gametes is critical to the development of ART, especially, in rare and endangered species. Ultrasound-guided oocyte retrieval provides a safe and relatively atraumatic approach for collection of oocytes from live animals. In cattle, the procedure can be repeated on a single animal as frequently as twice per week without adverse effects (Goodhand et al. 1999; Chastant-Maillard et al. 2003; Petyim et al. 2003). Oocytes obtained by this method are capable of developing into viable embryos after IVP.

Oocytes have been collected using the ultrasound-guided method in several nondomestic species (reviewed in section 2.2). In most cases, general anesthesia was induced to facilitate oocyte collection. However, ART procedures require frequent handling to synchronize the estrous cycle, administer ovarian stimulation treatments, perform oocyte retrieval and transfer embryos.

The standard ovarian stimulation protocol in domestic cattle involves twice-daily injections of FSH for 4 or 5 days. Applying such a regimen in nondomestic ungulates such as the eland (Schiewe et al. 1991b) and bison (Dorn 1995) is stressful. Moreover, as with other remotely delivered medications, it is difficult to ensure complete delivery of the required medications, some of which are very expensive (e.g., FSH).

Immobilization or general anesthesia also poses multiple risks, such as cardiac and respiratory arrest or even death. In a study on four species of immobilized antelopes (oryx, bongo, kudu and common eland), assessment of blood cortisol levels indicated handling and immobilization procedures were most stressful to elands. Also, maintenance of general anesthesia was more difficult in the elands (Schiewe et al. 1991b). Individual variations to ovarian stimulation treatments among the four species of antelopes were attributed to handling-related stress. Handling-induced stress may
have a negative effect on in vitro and in vivo development of oocytes and embryos, and can affect the lifetime reproductive performance of the animal. Moreover, it is technically difficult to incorporate immobilization procedures in studies (for example, reproductive endocrinology) requiring frequent blood sampling or examinations.

Thus, because of the immediate and long-term risks associated with conventional nondomestic animal handling/immobilization practices and the fact that such practices are technically difficult to apply in routine ART procedures, there is a need to develop alternative methods of animal handling. Studies on domestic cattle indicate that dissolving FSH in carrier solutions, such as polyvinyl pyrrolidone (PVP), instead of saline, can prolong hormone action. This approach has been used to reduce the number of injections of gonadotropins without affecting ovarian response (Yamamoto et al. 1994; Takedomi et al. 1995).

A regimen involving two or three injections of prostaglandin F$_2\alpha$ or its analogs is frequently used in estrous synchronization protocols for domestic and nondomestic ungulates. Although progestogens may be more effective for synchronization, application of progestogen pessaries can be more stressful to nondomestic ungulates than the use of prostaglandins (Haigh 2001). Orally administered progestogens could minimize handling stress since they can be added to the regular diet. For example, altrenogest has been used to control estrous cycles in pigs and cattle (Estienne et al. 2001; Ferguson et al. 2002) and to support pregnancy in cases of luteal insufficiency in horses (Daels et al. 1998) and okapi (Schwarzenberger et al. 1999). However, its effectiveness in controlling the estrous cycle of antelopes has not been evaluated.

Behavioral training, combined with improved handling systems, has been used to perform procedures without immobilizing nondomestic ungulate species. Phillips and co-workers (Phillips et al. 1998) have indicated that conditioning can often replace chemical immobilization with subsequent reductions in potential risks to the animal and the handlers. This group successfully
conditioned lowland nyala and bongo in 97 and 118 days, respectively (Grandin 2000). Animals cooperated with procedures such as blood sampling, injections, milking and treatment of wounds without chemical or manual restraint. The method of conditioning involved acclimation of animals to the presence of a shipping crate, habituation to walking through the crate, providing treats in the crate and gradual training (conditioning) to veterinary procedures over a period of time.

Other species have been trained to comply with various tasks by applying the principles of positive reinforcement or operant conditioning (Stevens 1978; Bloomsmith et al. 2003). Ungulates, including giraffe, eland, wildebeest and Thompson’s gazelle have been trained to associate audio cues with a food reward at the Disney’s Animal Kingdom (Joe Kalla and Marty Sevenich, personal communication). However, there are no reports on the use of behavioral training to facilitate ART procedures in nondomestic ungulates.

Advanced handling chutes have been used to do clinical procedures in many large mammals, including the eland (Read et al. 1993). A prototype device is the hydraulic chute, The TamerTM, originally developed for handling deer and later modified for larger hoofstock (Citino 1995; Atkinson et al. 1999). The latter investigators have used the device to perform many veterinary procedures that would have otherwise required immobilization in eland, bongo and kudu antelopes. In addition, another type of device, the drop-floor chute, has been used to restrain addax for ART procedures. After handling females in the chute, ultrasound-guided oocyte retrieval was done under acepromazine-induced tranquilization and epidural analgesia (Junge 1998). Mechanical or manual restraint has also been used to perform artificial insemination, with subsequent production of offspring, in the giraffe, gaur and addax [(Densmore et al. 1987; Godfrey et al. 1990) see (Pope and Loskutoff 1999)]. However, there is no report on the use of such restraint devices for ART procedures in tragelaphine antelopes.

In the present study, we evaluated the development of in vitro-derived bovine embryos in protein-free media and identified several factors affecting embryonic development in vitro (Chapter
3). Moreover, behavioral training and handling of female elands in a hydraulic chute were used to facilitate ultrasound-guided oocyte retrieval. It is demonstrated that ART procedures can be carried out in elands without inducing general anesthesia. Some parameters associated with handling of female elands, and factors affecting ovarian response after gonadotropin treatment and the \textit{in vitro} development of eland oocytes after IVP are also presented (Chapters 4 and 5).
Chapter 3. Development of In Vitro-Derived Bovine Embryos in Defined (Protein-free) Media

3.1. Introduction

In vitro-produced cattle embryos display morphological, biochemical and functional deviations when compared with those derived in vivo. These anomalies are more severe in embryos produced in media containing complex components, such as serum. Thus, many laboratories are attempting to develop and use simple and defined or semi-defined embryo culture media (Chapter 2).

The use of chemically defined culture media has multiple benefits (Bavister 1995). It reduces variations among different lots of media and minimizes risks of contamination with pathogens. Complex additives such as serum or serum protein preparations often contain ill-defined inhibitory or stimulatory modifiers of in vitro embryo development. Testing the effects of such modifiers, or eliminating them, can be achieved only by using chemically defined culture systems. Information generated using chemically defined media (without serum, BSA or co-culture) is thus helpful for improving our understanding of the requirements of embryos and subsequently for optimizing culture systems (Bavister 1995; Keskintepe et al. 1995; Liu and Foote 1995b; Holm et al. 1999; Hernandez-Fonseca et al. 2002).

Results of recent studies of cattle IVP using chemically defined culture systems are encouraging. In vitro embryo developmental frequencies similar to those achieved in complex culture media have been reported (Abe et al. 1999; Holm et al. 1999). Moreover, pregnancies have been established (Keskintepe et al. 1995; Hernandez-Fonseca et al. 2002) and healthy calves born (Holm et al. 1999; Hernandez-Fonseca et al. 2002) after transferring embryos cultured in chemically-defined media to recipient cows. Even so further refinement of chemically defined media is needed to increase the efficiency of embryo production and improve our understanding of early embryo development.
Amino acids, energy substrates and osmotic pressure and/or NaCl concentration are among the main factors influencing *in vitro* development of mammalian pre-implantation embryos (Bavister 1995; Liu and Foote 1995a; Lee and Fukui 1996; Steeves and Gardner 1999) (also see Chapter 2). Thus, the hypothesis of the present study is: the *in vitro* development of bovine embryos in protein-free media is affected by groups of amino acids, base media, supplement type and/or osmotic pressure. Specific objective were to:

1. compare the effects of supplementing two base media (modified KSOM, BM-3) with two groups of amino acids (20aa, 11aa) on the *in vitro* development of *in vitro*-derived bovine embryos; and

2. determine the effects of osmotic pressure of BM-3-20aa and four selected supplements on the *in vitro* development of *in vitro*-derived bovine embryos.

### 3.2. Material and Methods

#### 3.2.1. General

All chemicals were purchased from Sigma Chemicals Co. (St. Louis, MO, USA) unless indicated otherwise. All media were prepared in ultrapure (Milli-Q) water fortified with 1% penicillin-streptomycin-amphotericin solution and passed through a syringe filter with 0.22 µm pore-size before use. During IVF and IVC, a group of 10 to 15 oocytes/embryos were incubated in 50-µL droplets in 60 x 15 mm Falcon® polystyrene petri dishes (Becton Dickinson Labware, Franklin Lakes, NJ, USA). The droplets in the dishes were covered with embryo-tested mineral oil and equilibrated for ≥2 hr in the incubator before placing gametes or embryos in them. Incubation temperature was maintained at 39°C for IVM, IVF and IVC.
3.2.2. *In Vitro* Maturation

Cattle oocytes were collected by aspirating follicles (<10 mm in diameter) from ovaries obtained at a slaughterhouse. The commercial supplier (Cyagra, Inc., Gibbons, NE, USA) collected and shipped the oocytes to our laboratory. Oocytes underwent IVM for 23 to 25 hr while en-route to our laboratory. For IVM, 50 to 100 oocytes were placed in a 2-mL glass tube containing IVM medium and gassed with 5% CO₂ and shipped in a battery-operated portable incubator (Minitüb, Tiefenbach, Germany). The temperature of the incubator was maintained at 39°C. The IVM medium was bicarbonate-buffered TCM-199 with 10% fetal calf serum, bovine LH (0.01 U/mL) and estradiol (1 µg/mL).

3.2.3. *In Vitro* Fertilization

At 23 to 25 hr of IVM, oocytes with homogeneous ooplasm and three or more expanded cumulus layers were selected and washed at least three times in modified Tyrode’s solution supplemented with lactate, HEPES and PVA (TL-HEPES-PVA, Bavister 1989) supplemented with 1.25 mM pyruvic acid and 1% Minimal Essential Medium (MEM) non-essential amino acids before undergoing IVF. The latter was done in TALP [modified Tyrode’s solution + bovine serum albumin (6 mg/mL) + DL-lactic acid (10 mM) + pyruvic acid (0.25 mM)] (Bavister and Yanagimachi 1977) containing 20-µg/mL heparin (Parrish *et al.* 1988), PHE [penicillamine (20 µM) + hypotaurine (10 µM) + epinephrine (1 µM)] (Ball *et al.* 1983; Susko-Parrish *et al.* 1990) and 1% MEM amino acids.

Cryopreserved bull spermatozoa, which had been previously validated for IVF, were used. A 0.5 mL straw of semen was thawed in a water bath (37°C, 30 sec) and its contents were placed in a 35-mm dish. The semen was layered gently over a 1 mL of PureSperm® (GenX, Guilford, CT, USA) gradient in a 15-mL centrifuge tube consisting of 45% (top) and 90% (bottom) solutions in TL-HEPES-PVA. The tube was then centrifuged (500 x g, 15 min). Then the pellet was recovered and diluted in TL-HEPES-PVA to give a final insemination concentration of ~1.5 x 10⁶ motile
spermatozoa/mL and volume of 1 µL. An atmosphere of 5% CO₂ in air with high humidity was used for IVF.

3.2.4. In Vitro Culture and Experimental Design

At 16 to 20 hr of sperm-oocyte co-incubation, presumptive zygotes were vortexed (1800 rpm, 2 min) to remove cumulus cell layers and attached spermatozoa, washed (≥3x), and randomly allocated to in vitro culture treatments. Oil covered microdrops of IVC media were prepared as described above (Section 3.2.1). The same dish contained all treatments. To prevent cross-contamination of microdrops during the allocation of ova to IVC treatments, at least three extra microdrops of the simplest treatment (i.e., HECM-6 in Experiment 3.1 and BM-3-20aa in Experiment 3.2) were used for additional washings. Immediately before placing the ova in the IVC drop, a final washing was done in one of the extra microdrops of the IVC treatment.

In vitro culture was conducted in an atmosphere of 5% CO₂, 10% O₂ and 85% N₂ with high humidity in culture medium as specified in the experimental designs below. Embryos were moved to fresh culture medium on day 4 post-insemination.

3.2.4.1. Experiment 3.1 (Effects of Amino Acids and Base Media)

This experiment was a 2 x 2 factorial arrangement (Table 3.1) designed to compare the effects of two groups of amino acids and two base media on the development of in vitro-derived embryos. Treatment 1 (HECM-6) was used as a control. The two base media were BM-3 (McKiernan et al. 1995), and KSOM (Erbach et al. 1994) as modified previously (Yang et al. 1995). The first group of 20 amino acids (20aa) consisted of glutamine and commercial preparations of Basal Medium Eagle (BME) essential and MEM non-essential amino acids. [The classification of amino acids as essential and non-essential is based on nutrient requirements in rats and “is not relevant to embryos” (Liu and Foote 1997)]. However, it is helpful to use this nomenclature as a simple way to identify these groups of amino acids.
The BME and MEM amino acids, purchased as 50x and 100x solutions, respectively, were supplemented at 1% in the IVC media. The second group was the 11 amino acids developed specifically for hamster IVC [HECM-6, McKiernan et al. 1995]. Modified 11 amino acids had the same components as HECM-6 but concentrations were increased to equal those in BME or MEM. Glutamine, taurine and cysteine, which are not components of BME/MEM, were used at 1.0, 0.5 and 0.05 mM, respectively (Table 3.2).

Table 3.1. Design of Experiment 3.1

<table>
<thead>
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<th>Treatment</th>
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<th>Amino acid (aa) supplements</th>
<th>Designated as</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>BM-3</td>
<td>11 (HECM-6) aa</td>
<td>HECM-6</td>
</tr>
<tr>
<td>2</td>
<td>BM-3</td>
<td>modified 11 aa</td>
<td>BM-3-m11aa</td>
</tr>
<tr>
<td>3</td>
<td>BM-3</td>
<td>20 aa (BME + MEM + glutamine)</td>
<td>BM-3-20aa</td>
</tr>
<tr>
<td>4</td>
<td>modified KSOM</td>
<td>modified 11 aa</td>
<td>mKSOM-m11aa</td>
</tr>
<tr>
<td>5</td>
<td>modified KSOM</td>
<td>20 aa (BME + MEM + glutamine)</td>
<td>mKSOM-20aa</td>
</tr>
</tbody>
</table>

3.2.4.2. Experiment 3.2 (Effects of Glucose, Pyruvate, Lactate and Phosphate in BM-3 Having Two Osmolarities)

In Experiment 3.1, interaction between base medium and group of amino acids was apparent, with 20aa supplemented in modified KSOM supporting the highest frequency blastocyst development. Therefore, Experiment 3.2 was designed to evaluate the differential effects of four selected components of KSOM and the 20aa in BM-3 having two osmolality levels. The experiment was a 7 x 2 factorial design with the following supplement treatments and two osmotic pressures:

1. KSOM-PVA + 20 amino acids (= KSOM-20aa)
2. BM-3 + 20 amino acids (= BM-3-20aa)
3. BM-3 + 20 amino acids + 0.2 mM glucose (= BM-3-20aa-gluc)
4. BM-3 + 20 amino acids + 0.2 mM pyruvic acid (= BM-3-20aa-pyr)
Table 3.2. Components and concentrations of modified KSOM, BM-3 and three groups of amino acids

<table>
<thead>
<tr>
<th>Component</th>
<th>mKSOM</th>
<th>BM-3</th>
<th>Amino acids, final concentration (mM)</th>
<th>Component</th>
<th>Twenty&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Eleven&lt;sup&gt;c&lt;/sup&gt;</th>
<th>mEleven</th>
</tr>
</thead>
<tbody>
<tr>
<td>PVA (mg/mL)</td>
<td>0.1</td>
<td>0.1</td>
<td>MEM</td>
<td>L-Alanine</td>
<td>0.10</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>NaCl</td>
<td>95.0</td>
<td>113.6</td>
<td>L-Asparagine</td>
<td>0.11</td>
<td>0.01</td>
<td>0.11</td>
<td></td>
</tr>
<tr>
<td>KCl</td>
<td>2.5</td>
<td>3.0</td>
<td>L-Aspartic acid</td>
<td>0.10</td>
<td>0.01</td>
<td>0.10</td>
<td></td>
</tr>
<tr>
<td>KH&lt;sub&gt;2&lt;/sub&gt;PO&lt;sub&gt;4&lt;/sub&gt;</td>
<td>0.35</td>
<td>--</td>
<td>L-Aspartic acid</td>
<td>0.10</td>
<td>0.01</td>
<td>0.10</td>
<td></td>
</tr>
<tr>
<td>MgSO&lt;sub&gt;4&lt;/sub&gt;</td>
<td>0.2</td>
<td>--</td>
<td>L-Glutamic acid</td>
<td>0.09</td>
<td>0.01</td>
<td>0.09</td>
<td></td>
</tr>
<tr>
<td>MgCl&lt;sub&gt;2&lt;/sub&gt;</td>
<td>--</td>
<td>0.5</td>
<td>Glycine</td>
<td>0.10</td>
<td>0.01</td>
<td>0.10</td>
<td></td>
</tr>
<tr>
<td>CaCl&lt;sub&gt;2&lt;/sub&gt;·2H&lt;sub&gt;2&lt;/sub&gt;O</td>
<td>1.7</td>
<td>1.9</td>
<td>L-Proline</td>
<td>0.10</td>
<td>0.01</td>
<td>0.10</td>
<td></td>
</tr>
<tr>
<td>HEPES</td>
<td>2.5</td>
<td>--</td>
<td>L-Serine</td>
<td>0.10</td>
<td>0.01</td>
<td>0.10</td>
<td></td>
</tr>
<tr>
<td>EDTA (Na salt)</td>
<td>0.01</td>
<td>--</td>
<td>BME</td>
<td>L-Arginine HCl</td>
<td>0.06</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Pyruvic acid (Na salt)</td>
<td>0.2</td>
<td>--</td>
<td>L-Cystine</td>
<td>0.03</td>
<td>--</td>
<td>--</td>
<td></td>
</tr>
<tr>
<td>Glutamine</td>
<td>1.0</td>
<td>0.2</td>
<td>L-Histidine</td>
<td>0.03</td>
<td>0.01</td>
<td>0.03</td>
<td></td>
</tr>
<tr>
<td>Glucose</td>
<td>0.2</td>
<td>--</td>
<td>L-Isoleucine</td>
<td>0.10</td>
<td>--</td>
<td>--</td>
<td></td>
</tr>
<tr>
<td>DL-Lactic acid (Na salt)</td>
<td>10.0</td>
<td>4.5</td>
<td>L-Leucine</td>
<td>0.10</td>
<td>--</td>
<td>--</td>
<td></td>
</tr>
<tr>
<td>NaHCO&lt;sub&gt;3&lt;/sub&gt;</td>
<td>25.0</td>
<td>25.0</td>
<td>L-Leucine</td>
<td>0.10</td>
<td>--</td>
<td>--</td>
<td></td>
</tr>
<tr>
<td>HCl (1 mol/L)</td>
<td>--</td>
<td>(1.4 µL/mL)</td>
<td>L-Lysine HCl</td>
<td>0.10</td>
<td>0.01</td>
<td>0.10</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>L-Methionine</td>
<td>0.03</td>
<td>--</td>
<td>--</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>L-Phenylalanine</td>
<td>0.05</td>
<td>--</td>
<td>--</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>L-Threonine</td>
<td>0.10</td>
<td>--</td>
<td>--</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>L-Tryptophan</td>
<td>0.01</td>
<td>--</td>
<td>--</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>L-Tyrosine HCl</td>
<td>0.04</td>
<td>--</td>
<td>--</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>L-Valine</td>
<td>0.10</td>
<td>--</td>
<td>--</td>
<td></td>
</tr>
<tr>
<td>Other</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Taurine</td>
<td>--</td>
<td>0.5</td>
<td>0.5</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>L-Glutamine</td>
<td>1.0</td>
<td>0.2</td>
<td>1.0</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>L-Cysteine.HCl.H&lt;sub&gt;2&lt;/sub&gt;O</td>
<td>--</td>
<td>0.01</td>
<td>0.05</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Osmolality 255 ± 5 275 ± 5

<sup>a</sup>Base media were prepared with glutamine and stored at 4°C for ≤1 week. <sup>b</sup>Premixed MEM/BME amino acids were from Sigma Chemicals Co. <sup>c</sup>Eleven amino acids were prepared (100x) and stored at -80°C before use.
5. BM-3 + 20 amino acids + 5.5 mM lactic acid (= BM-3-20aa-lact)
6. BM-3 + 20aa + 0.35 mM potassium bisphosphate (= BM-3-20aa-phos)
7. BM-3 + 20aa + 0.2 mM gluc + 0.2 mM pyr + 5.5 mM lact + 0.35 mM phos (= BM-3 + All).

The two osmotic pressure groups of BM-3 were ~255 (designated “low”) and ~275 (designated “high”). The “high” osmotic pressure BM-3 was the regular BM-3 formulation (McKiernan et al. 1995). However, treatments containing additional lactate required adjustments for osmotic pressure. This was achieved by replacing the medium with ultrapure water (~7.3%, vol/vol). In “low” BM-3, 95 mM NaCl (as in KSOM) was used and the osmolality in Treatments 2, 3, 4 and 6 was adjusted using sodium sulfate (Na$_2$SO$_4$). The latter has no known toxic or beneficial effects on embryo development in vitro (Monis and Bavister 1990). Lactate-supplemented treatments in low BM-3 did not require further adjustments. HEPES was excluded from modified KSOM (Control) in Experiment 3.2.

3.2.5. Evaluation of Embryonic Development and Cell Counts

Frequencies of cleavage and development to 8-cell were evaluated on day 2 and day 3 post-insemination. Advanced development was evaluated on day 7, day 8 and day 9. Blastocysts were subjectively classified as “Expanded” when increment in diameter was noticeable during microscopic examination. Blastocysts were fixed on day 9 and subjected to nuclear staining using a combination of propidium iodide and Hoechst stains (Thouas et al. 2001) to achieve differential cell counts on trophectoderm and inner cell mass. However, reliable differentiation could be achieved on only a small fraction of embryos, so the values were combined and used as total cell counts. In the latter part of the experiment, Hoechst staining alone was used for nuclear staining. Cells were counted under a fluorescent microscope equipped with Nomarski differential interference optics.
3.2.6. Data Analyses

Percentage values for embryonic development were arcsine transformed before analyses. Data were analyzed using the SAS® software (SAS 2001). In Experiment 3.1, the general linear model was used. In Experiment 3.2, the mixed model was used with supplement and osmotic pressure groups included in the model as fixed effects and replicate and its interaction as random variables. Tukey’s test was used for pair-wise comparisons of significantly different means. Data on cell counts in Experiment 3.1 were not normally distributed and were analyzed using the Kruskal-Wallis test. For the analyses, blastocysts observed escaping from their zonae pellucidae and those that had completed this process were considered “hatched”. Differences in treatment means were considered significant at $P \leq 0.05$.

3.3. Results

3.3.1. Experiment 3.1 (Effects of Amino Acids and Base Media)

A total of 1,109 IVM oocytes were subjected to IVF in five replicates across days. Cleavage frequency ranged from 61% (mKSOM-m11aa) to 75% (BM-3 + 20aa) ($P>0.05$; Table 3.3). The frequency of blastocyst development continued to increase from days 7 to 9 in all treatments (Table 3.3). However, the percentage of total and expanded blastocysts was significantly higher in mKSOM-20aa than in the other four treatments during these three days ($P<0.05$; Tables 3.3; 3.4).

The frequency of hatched blastocysts on day 7 was virtually zero in all treatments. On days 8 and 9, hatching frequency was significantly higher in mKSOM-20aa than in the other treatments (Table 3.4). Blastocysts from this treatment also had the numerically highest cell numbers; however, this was not significantly different from the three other treatments (Tables 3.3).
Table 3.3. Development frequencies and cell count of *in vitro*-derived bovine embryos cultured in protein-free BM-3 or mKSOM supplemented with two groups of amino acids

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>n</th>
<th>≥2-cell*</th>
<th>Blastocysts*</th>
<th>Cell count</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>day 7</td>
<td>day 8</td>
<td>day 9</td>
</tr>
<tr>
<td>HECM-6</td>
<td>223</td>
<td>70.8 ± 7.4</td>
<td>5.9 ± 2.0(^a)</td>
<td>13.6 ± 1.8(^a)</td>
</tr>
<tr>
<td>BM-3-m11aa</td>
<td>218</td>
<td>64.9 ± 8.8</td>
<td>1.7 ± 0.8(^a)</td>
<td>7.6 ± 0.8(^a)</td>
</tr>
<tr>
<td>BM-3-20aa</td>
<td>230</td>
<td>75.0 ± 8.7</td>
<td>1.6 ± 1.1(^a)</td>
<td>10.2 ± 1.6(^a)</td>
</tr>
<tr>
<td>mKSOM-m11aa</td>
<td>203</td>
<td>60.9 ± 8.6</td>
<td>2.8 ± 1.8(^a)</td>
<td>7.9 ± 1.6(^a)</td>
</tr>
<tr>
<td>mKSOM-20aa</td>
<td>235</td>
<td>73.9 ± 7.8</td>
<td>15.9 ± 6.5(^b)</td>
<td>31.1 ± 7.1(^b)</td>
</tr>
</tbody>
</table>

\(^{ab}\)Values in the same column with different superscripts are significantly different (P<0.05). *Figures indicate % of oocytes ± SE.

Table 3.4. Blastocyst expansion and hatching frequencies of *in vitro*-derived bovine embryos cultured in protein-free BM-3 or mKSOM supplemented with two groups of amino acids

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Expanded blastocysts*</th>
<th>Hatched blastocysts*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>day 7</td>
<td>day 8</td>
</tr>
<tr>
<td>HECM-6</td>
<td>1.1 ± 1.1(^a)</td>
<td>7.0 ± 1.1(^a)</td>
</tr>
<tr>
<td>BM-3-m11aa</td>
<td>0.4 ± 0.4(^a)</td>
<td>3.4 ± 0.9(^a)</td>
</tr>
<tr>
<td>BM-3-20aa</td>
<td>0(^a)</td>
<td>4.2 ± 1.7(^a)</td>
</tr>
<tr>
<td>mKSOM-m11aa</td>
<td>0.9 ± 0.6(^a)</td>
<td>4.4 ± 1.3(^a)</td>
</tr>
<tr>
<td>mKSOM-20aa</td>
<td>8.3 ± 4.4(^b)</td>
<td>19.7 ± 6.8(^b)</td>
</tr>
</tbody>
</table>

\(^{ab}\)Values in the same column with different superscripts are significantly different (P<0.05). *Figures indicate % of oocytes ± SE.
3.3.2. Experiment 3.2 (Effects of Glucose, Pyruvate, Lactate and Phosphate in BM-3 Having Two Osmolalities)

A total of 1,921 oocytes were used in six replicates. Neither supplement type nor osmotic pressure affected the frequencies of initial cleavage and development to the 8-cell stage (Table 3.5). Supplement but not osmotic pressure affected the frequency development to at least the morula stage (day 7). The latter was lowest (36 to 38%) in pyruvate-supplemented media and highest (68%) in glucose-supplemented media (Tables 3.5; 3.6). Similarly, supplement treatment affected the frequency of total (day 9), expanded (day 8) and hatched (day 9) blastocysts. Effects of supplement on the frequencies of total blastocysts on days 7 and 8, and expanded blastocysts on day 9 also approached statistical significance (Table 3.5).

Regardless of treatment type, blastocyst development frequency peaked on day 8 (Figures 3.1; 3.2). The frequency of hatching continued to increase through day 9 (Tables 3.5; 3.6). Blastocyst cell count was affected by both supplement type and osmotic pressure; however, supplement exerted a more pronounced effect (Tables 3.5; 3.6). Osmotic pressure affected the frequency of blastocyst expansion on day 7. Its effect on the frequencies of day 8 blastocyst expansion and day 9 hatching also approached statistical significance. Generally, low osmotic pressure BM-3 treatments tended to increase frequency of blastocyst expansion and hatching as well as cell counts. However, a significant difference was detected only between the frequencies of day-7 expanded blastocysts in BM-3-20aa treatments and between blastocyst cell counts in BM-3-20aa-phos treatments (Tables 3.6; 3.7).

Among the BM-3-based treatments, glucose-supplemented media supported (at least numerically) superior embryonic development (Tables 3.6; 3.7; Figures 3.1; 3.2). In high BM-3, the frequency of day-9 hatched blastocysts in glucose-supplemented media was significantly greater than in all non-glucose supplemented treatments. Similarly, among low BM-3 treatments, the frequency of
day 9 hatching was significantly higher in glucose-supplemented medium than in all other BM-3 treatments (Table 3.7). Blastocysts produced in glucose-supplemented treatments also had greater cell numbers than those in other treatments (Table 3.6). The effect of interaction between osmotic pressure and supplement groups was not significant for any of the parameters evaluated (Table 3.5).

Table 3.5. Effects of osmotic pressure of modified BM-3 and supplements on the development of in vitro-derived bovine embryos at different stages

<table>
<thead>
<tr>
<th>Variables</th>
<th>Osmotic pressure</th>
<th>Supplement</th>
<th>Interaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>≥2-cell (day 2)</td>
<td>0.8761</td>
<td>0.3870</td>
<td>0.8111</td>
</tr>
<tr>
<td>≥8-cell (day 3)</td>
<td>0.2518</td>
<td>0.3362</td>
<td>0.8638</td>
</tr>
<tr>
<td>≥Morula (day 7)</td>
<td>0.2237</td>
<td>0.0317</td>
<td>0.4047</td>
</tr>
<tr>
<td>Blastocysts</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>- Total (day 7)</td>
<td>0.3507</td>
<td>0.0978</td>
<td>0.5743</td>
</tr>
<tr>
<td>- Total (day 8)</td>
<td>0.1536</td>
<td>0.0592</td>
<td>0.6531</td>
</tr>
<tr>
<td>- Total (day 9)</td>
<td>0.4766</td>
<td>0.0414</td>
<td>0.8967</td>
</tr>
<tr>
<td>- Expanded (day 7)</td>
<td>0.0137</td>
<td>0.4699</td>
<td>0.5066</td>
</tr>
<tr>
<td>- Expanded (day 8)</td>
<td>0.0953</td>
<td>0.0360</td>
<td>0.1677</td>
</tr>
<tr>
<td>- Expanded (day 9)</td>
<td>0.3879</td>
<td>0.0904</td>
<td>0.7504</td>
</tr>
<tr>
<td>- Hatched (day 7)</td>
<td>0.1790</td>
<td>0.5017</td>
<td>0.6030</td>
</tr>
<tr>
<td>- Hatched (day 8)</td>
<td>0.3879</td>
<td>0.0904</td>
<td>0.7504</td>
</tr>
<tr>
<td>- Hatched (day 9)</td>
<td>0.0904</td>
<td>&lt;0.0001</td>
<td>0.8615</td>
</tr>
<tr>
<td>- Cell count (day 9)</td>
<td>0.0279</td>
<td>&lt;0.0001</td>
<td>0.6769</td>
</tr>
</tbody>
</table>
Table 3.6. Effects of glucose, pyruvate, lactate and phosphate in high or low osmotic pressure modified BM-3 on the frequency of cleavage (day 2) and development to morula (day 7) and blastocyst (day 9) stages of in vitro-derived bovine embryos cultured in protein-free media

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>High</th>
<th>Low</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n (≥2-cell)</td>
<td>≥Morula</td>
</tr>
<tr>
<td>KSOM-20aa</td>
<td>154 (84.7 ± 2.1)</td>
<td>63.2 ± 8.5&lt;sup&gt;abc&lt;/sup&gt;</td>
</tr>
<tr>
<td>BM-3-20aa</td>
<td>140 (80.9 ± 2.4)</td>
<td>43.0 ± 7.9&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td>BM-3-20aa-gluc</td>
<td>154 (85.9 ± 4.1)</td>
<td>68.3 ± 9.2&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>BM-3-20aa-pyr</td>
<td>153 (76.0 ± 3.2)</td>
<td>37.5 ± 6.1&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>BM-3-20aa-lact</td>
<td>149 (80.1 ± 3.1)</td>
<td>45.3 ± 12.1&lt;sup&gt;abc&lt;/sup&gt;</td>
</tr>
<tr>
<td>BM-3-20aa-phos</td>
<td>138 (80.6 ± 7.1)</td>
<td>34.8 ± 10.5&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>BM-3aa + All</td>
<td>155 (82.0 ± 3.0)</td>
<td>51.3 ± 9.3&lt;sup&gt;abc&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Values under 2-cell, Morula and Blastocyst headings represent mean (%) of oocytes inseminated ± SE. <sup>abcd</sup>Values in the same column with different superscripts are significantly different (P<0.05). There was no statistical difference within a supplement treatment across osmotic pressure group except for cell count values of in BM-3-20aa-phos.
Table 3.7. Effects of glucose, pyruvate, lactate and phosphate in high or low osmotic pressure modified BM-3 on the frequency (%) of blastocyst expansion (days 7 and 8) and hatching (day 9) in vitro-derived bovine embryos

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>High</th>
<th>Low</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>day 7 expanded</td>
<td>day 8 expanded</td>
</tr>
<tr>
<td>KSOM-20aa</td>
<td>8.4</td>
<td>18.9&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>BM-3-20aa</td>
<td>2.2</td>
<td>5.8&lt;sup&gt;bc&lt;/sup&gt;</td>
</tr>
<tr>
<td>BM-3-20aa-gluc</td>
<td>5.3</td>
<td>19.1&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>BM-3-20aa-pyr</td>
<td>3.6</td>
<td>6.6&lt;sup&gt;bc&lt;/sup&gt;</td>
</tr>
<tr>
<td>BM-3-20aa-lact</td>
<td>2.6</td>
<td>7.2&lt;sup&gt;bc&lt;/sup&gt;</td>
</tr>
<tr>
<td>BM-3-20aa-phos</td>
<td>2.9</td>
<td>8.7&lt;sup&gt;bc&lt;/sup&gt;</td>
</tr>
<tr>
<td>BM-3 + All</td>
<td>3.8</td>
<td>11.5&lt;sup&gt;ac&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a,b,c</sup> Values in the same column with different superscripts are significantly different (P<0.05). There was no statistical difference within a supplement treatment across osmotic pressure group except for frequencies of day 7 expanded blastocysts in BM-3-20aa.
Figure 3.1. Blastocyst development of *in vitro*-derived bovine embryos cultured in high osmotic pressure BM-3-20aa with different supplements (see Table 3.6 for day 9 development data).
Figure 3.2. Blastocyst development of *in vitro*-derived bovine embryos cultured in low osmotic pressure BM-3-20aa with different supplements (see Table 3.6 for day 9 development data).
3.4. Discussion

In the present study, effects on *in vitro* embryonic development of selected supplements and osmotic pressure of culture media were evaluated. Protein-free HECM-6 (i.e., BM-3 + 11 amino acids) supports complete preimplantation development of hamster embryos that are capable of developing into normal offspring after embryo transfer; however, HECM-6 components have not been systematically evaluated in bovine IVC. In Experiment 3.1, the much higher blastocyst development on day 9 in modified KSOM-20aa than in the other treatments (Table 3.3) was puzzling; hence, Experiment 3.2 was designed. We demonstrated that 20 amino acids and glucose (0.2 mM) are crucial for culturing bovine embryos in BM-3.

In Experiment 3.1, mKSOM-20aa supported blastocyst development (42%) at similar frequencies to other commonly used culture media [e.g., M199 or SOF plus serum or BSA, G1/G2 or coculture: see (Holm *et al.* 1999; Krisher *et al.* 1999; Steeves and Gardner 1999; Lane *et al.* 2003)]. The frequency of day 7 blastocysts was relatively low (16%) indicating slow embryonic development in mKSOM-20aa; but the frequency increased on subsequent days and reached 42% on day 9 (Table 3.3). The slow development indicates reduced developmental competence as reported for bovine and other species (Massip *et al.* 1995; Bavister 2002b).

KSOM has been used to culture bovine embryos under defined (Liu and Foote 1995a; Liu and Foote 1997) or complex (Al Katanani *et al.* 2002) conditions. *In vitro* development in protein-free KSOM in previous studies (Liu and Foote 1995a; Liu and Foote 1997) was lower than in the present study, possibly due to variations in the concentration of amino acids used or the stage at which they were supplemented. For example, at concentrations employed for tissue culture (2%), BME amino acids inhibit early-stage development of bovine embryos (Keskintepe *et al.* 1995; Liu and Foote 1995b; Steeves and Gardner 1999).

Increasing the concentration of HECM-6 amino acids to that of BME/MEM did not improve embryonic development beyond that seen in HECM-6 (Table 3.3). The greater frequency of embryo
development in the presence of 20 amino acids as compared with 11 amino acids suggests that bovine and hamster embryos differ in their amino acid requirements. Other investigators (Liu and Foote 1997) also did not observe further improvements in the in vitro development of bovine embryos when the concentration of non-essential amino acids was increased up to five times. This is paradoxical, considering that the concentration of free amino acids in the reproductive tract of cows is >30 mM (Elhassan et al. 2001). The total concentration of amino acids used in the present study was only 2.5 mM (20 amino acids), 0.8 mM (11 amino acids) and 2.3 mM (modified 11 amino acids). Methionine, an amino acid that is essential to initiate protein synthesis is not one of the 11 (HECM-6) amino acids. Blastocyst expansion and, even at times, hatching occurred in media supplemented with HECM-6 amino acids, suggesting that protein synthesis did take place without the addition of external methionine. Thus, like mouse oocytes (Wassarman and Albertini 1994), bovine oocytes probably store methionine.

In Experiment 3.1, blastocyst hatching was rare (<1%) in treatments other than mKSOM-20aa (15% on day 9; Table 3.4). Others have also observed reduced hatching frequencies in bovine embryos cultured in protein-free media (Krisher et al. 1999). Hatching is also reduced in bovine blastocysts resulting from cumulus denuded oocytes matured in defined media (Holm et al. 1999; Landim-Alvarenga et al. 2002). Nevertheless, modifications of defined media can improve hatching frequencies [(Holm et al. 1999); the present study]. For example, using low osmotic pressure BM-3-20aa with glucose increased the hatching frequency more than threefold (Table 3.7).

In Experiment 3.2, BM-3-20aa supplemented with glucose alone stood out in supporting a high frequency of development comparable to KSOM-20aaain (Table 3.6; Figures 3.1; 3.2). This is in contrast to the widely held dogma that glucose inhibits early embryonic development, especially at concentrations around physiologically normal blood levels and/or in the presence of phosphate (Pinyopummintr and Bavister 1991; Takahashi and First 1992; Kim et al. 1993).

Most investigators agree on the need for glucose during culture of bovine morula and blastocyst stage embryos. In the present study, 0.2 mM glucose during the entire IVC period
(including the pre-cleavage stage) was also beneficial. Although data on temporal metabolism of glucose during the entire pre-elongation development of bovine embryos are limited, gene transcripts and proteins involved in glucose transport and metabolism are present during all stages. Hence, it has been suggested that substrate availability and not proteins are determinants of glucose metabolism in early stage mammalian embryos (Gardner et al. 2000). The incubation environment or condition could also modify the effect of glucose (Yamashita et al. 1999).

In the present study, the higher frequency of in vitro embryonic development in pyruvate-free but glucose-supplemented medium, as compared with pyruvate-containing medium, suggests a more important role of glucose in pathways other than glycolysis and/or the Krebs cycle. Moreover, endogenous pyruvate could be adequate for energy metabolism. In fact, cleavage stage mammalian embryos have a high ATP/ADP ratio and additional ATP is probably not required until after embryonic genome activation (Gardner et al. 2000). Bovine embryos with reduced glycolytic activity are developmentally more competent than those with high glycolysis (Rieger et al. 1995) and embryos cultured in defined medium have low glycolytic activity that is similar to in vivo-derived embryos (Krisher et al. 1999). Similarly, the gene expression pattern of bovine embryos cultured in chemically defined medium is more similar to that of in vivo derived cattle embryos when compared with embryos cultured in serum-supplemented medium (Wrenzycki et al. 1999). Furthermore, morphologically, bovine embryos produced in defined or semi-defined media are more similar to those derived in vivo than those embryos produced in complex media (Abe et al. 2002).

Alternative routes of glucose utilization include the pentose-phosphate pathway, which generates intermediates for nucleic acid and lipid biosynthesis, and the hexosamine pathways which are required for post-translational glycosylation of proteins [(Gardner et al. 2000; Ludwig et al. 2001); Figure 3.3]. During fertilization, oxidation of glucose is more important than that of pyruvate (Pantaleon et al. 2001). The beneficial effects of glucose during mouse embryo culture also led to a recommendation for increasing its concentration in KSOM from 0.2 to 3.4 mM (Biggers and McGinnis 2001).
Pyruvate, phosphate and glucose are not components of HECM-6 (i.e., BM-3 + 11aa), which has been shown to support bovine embryo development during the first 2 to 4 or 7 days of culture (Pinyopummintr and Bavister 1996; Krisher et al. 1999). In the latter study (Krisher et al. 1999), 21% of inseminated oocytes developed to blastocysts with an average cell number of 73 after IVF, and subsequent IVC in HECM-6 supplemented with pyruvate (0.25 mM) and glucose (2 mM). The blastocyst frequency and cell count of embryos cultured in BM-3-20aa containing 0.2 mM glucose in the present study were higher than in the previous report by Krisher et al. (1999), when HECM-6 was supplemented with higher concentrations of glucose and pyruvate. Furthermore, in the present study, pyruvate appeared to be inhibiting blastocyst development in high BM-3-20aa compared with the same medium with glucose (Table 3.6).

Others (Yoshioka et al. 1993) also reported improved development of in vitro-derived bovine embryos in a semi-defined medium when the concentrations of lactate and pyruvate were reduced (from 20.1 to 3.0 mM and from 0.5 to 0.3 mM, respectively). Pyruvate was also reported to be either unnecessary or toxic to bovine embryos in a semi-defined medium (Rosenkrans, Jr. et al. 1993) and it is not a crucial supplement in sheep IVC medium (Thompson et al. 1992). Taken together, these data underscore the uncertainty surrounding the beneficial role of external pyruvate, although it is believed to be the preferred energy substrate for bovine embryos (Massip et al. 1995; Khurana and Niemann 2000). Anti-oxidant properties of pyruvate have also been reported (Gardner et al. 2000); however, such a role of pyruvate in defined IVC medium awaits confirmation.

Glycolysis and Krebs cycle are closely related such that imbalances in key substrates could affect either pathway. The “Crabtree effect” is a prototype example in which upregulation of glycolysis inhibits oxidative phosphorylation (Seshagiri and Bavister 1991). Data are scant on the kinetics of the Krebs cycle enzymes during pre-elongation embryonic development; however, the regulatory enzyme of glycolysis (phosphofructokinase) has low activity prior to mouse embryonic genome activation (Barbehenn et al. 1974). High glucose (5.6 mM) in IVC media could prematurely up-regulate glycolysis and/or activate the polyol pathway (Moley et al. 1996). The latter depletes
Figure 3.3. Pathways of glucose metabolism and related factors influencing embryonic development: The regulatory enzyme of glycolysis, phosphofructokinase (PFK), has low activity in early cleavage-stage mammalian embryos. High glucose could lead to premature activation of PFK and/or up-regulation of aldose reductase and subsequent accumulation of sorbitol in the cytoplasm. Osmotic stress may also stimulate the conversion of glucose to sorbitol, the formation of which depletes glutathione (GSH, an antioxidant) and up-regulates apoptotic pathways. Removal of sorbitol from cells requires its conversion to fructose, a step that produces NADH that can subsequently inhibit oxidative phosphorylation in the mitochondria. The Pentose Phosphate Pathway produces several important intermediates, including fructose-2,6-bisphosphate- the key allosteric activator of PFK [(Roskoski, Jr. 1996) and see also text for relevant references].
NADPH and could stimulate apoptotic pathways [(Galvez et al. 2003); Figure 3.3]

Compared with observations in Experiment 3.1, embryonic development in KSOM was reduced in Experiment 3.2. The reason for this is not clear. The exclusion of HEPES was an unlikely reason because adding it back in a sideline trial did not improve embryonic development during Experiment 3.2 (personal observation). A possible reason is seasonal variation in oocyte quality, which is poor during the hot (Summer) season (Zeron et al. 2001; Al Katanani et al. 2002). (Experiment 3.1 was done in Fall and Experiment 3.2 was performed in Spring/Summer seasons). Nevertheless, embryonic development in the control KSOM-20aa treatment was similar during replicates run with high or low osmotic pressure BM-3 treatments.

Previous studies (Lim et al. 1994; Liu and Foote 1996) recommended reducing the osmotic pressure of bovine IVC media to <270 but the most widely used media (SOF, TCM199, CR1) have higher osmotic pressures than this. Reduced osmotic pressure with a concomitant reduction in [NaCl] is among the factors responsible for improved development of mouse embryos in KSOM (Summers et al. 1995). The use of low osmotic pressure BM-3 appeared to minimize or eliminate treatment differences (Tables 3.6; 3.7) observed in the frequency of embryonic development in high BM-3. Moreover, although there were large variations in embryonic development, low osmotic pressure BM-3-20aa supported blastocyst development at a similar frequency as the same medium containing glucose (Table 3.6; Figure 3.2). This may indicate the beneficial role of low osmotic pressure for culturing bovine embryos as demonstrated previously (Lim et al. 1994; Liu and Foote 1996).

In most instances, increased osmotic pressure of culture media is synonymous with high [NaCl]; however, extracellular Na+ affects intracellular pH via its effect on the Na+/H+ anti-porter (Lane and Bavister 1999). Reducing extracellular [NaCl] also alters gene expression (Ho et al. 1994) and protein synthesis (Liu and Foote 1997). Osmotic stress could activate the polyol pathway and apoptosis [(Galvez et al. 2003); Figure 3.3]. The higher frequency of blastocyst expansion and hatching, and mean cell numbers per blastocyst, in low BM-3 treatments suggests a similar mechanism is operating.
In the present study, 20aa and glucose (0.2 mM) used during the entire IVC period were beneficial. Glucose supported superior development in medium with low lactate (4.5 mM) concentration and without pyruvate or phosphate. Although the mechanism by which glucose exerts its effects during embryo development is not fully elucidated, its effects are evident as early as the fertilization stage in mice and bovine (Pantaleon et al. 2001; Comizzoli et al. 2003). Moreover, glucose metabolism via the pentose phosphate pathway provides intermediates for the synthesis of key macromolecules and is important for redox regulation (Figure 3.3).

In another study (Wirtu et al. 2004), further supplementation of protein-free mKSOM-20aa with insulin improved cleavage and blastocyst formation frequencies of in vitro-derived bovine embryos. In addition, it increased cell numbers per blastocyst. With additional serum supplementation during IVC of bovine embryos, BM-3-20aa containing glucose supported up to 41% blastocyst formation (Wirtu et al. 2003). Similarly, up to 27% blastocysts developed in this medium after culturing interspecies SCNT couplets reconstructed by using giant eland cells and bovine oocytes (Damiani et al., 2003).

The respectable levels of in vitro development of bovine embryos in medium with reduced substrates (i.e., no pyruvate and phosphate, low lactate and glucose concentrations; Tables 3.6; 3.7) are also consistent with the hypothesis that reducing embryonic metabolism by restricting medium supplements improves viability (Leese 2002). In conclusion, the beneficial effects of amino acids on the in vitro development of bovine embryos are influenced by the base medium. Low osmotic pressure facilitates blastocyst expansion, while medium supplements are more important in determining the frequency of total blastocysts and cell number per blastocyst. Further evaluation of the viability of such embryos by using additional end points, such as development following embryo transfer, is needed.
Chapter 4. Handling Elands for Reproductive Technology Procedures without Inducing General Anesthesia: Roles of Behavioral Training and Hydraulic Chute

4.1. Introduction

Handling of most nondomestic ungulates for clinical or other procedures has traditionally been carried out using complete chemical immobilization. Most assisted reproductive technology studies in tragelaphine (spiral-horned) antelopes also used general anesthesia to perform semen collection (Merilan et al. 1977; Merilan et al. 1978; Schiewe et al. 1991a; Schiewe et al. 1991b), embryo recovery (Dresser et al. 1985; Gelwicks et al. 1989; Schiewe et al. 1991b), embryo transfer (Dresser et al. 1985; Gelwicks et al. 1989) and artificial insemination (Bartels et al. 2001). However, several risk factors limit the routine use of general anesthesia for the development and application of reproductive technologies.

General anesthesia predisposes animals to the risk of cardiac and respiratory arrest, and long-term cell/organ injuries. It can also interfere with normal endocrine responses and gamete and/or embryo transport (Loskutoff and Betteridge 1992). Moreover, narcotic agents used for immobilization pose a potential health hazard to the personnel participating in procedures. Animals have also been known to renarcotize and die subsequent to narcotic general anesthesia (Domínguez and Aguilar 2001). Elands (Taurotragus oryx) are among the bovid species considered difficult to immobilize (Citino 2003). In addition, their athletic ability and flighty behavior complicates the induction phase and could affect the outcome of anesthesia event.

Behavioral training and habituation (“conditioning and desensitization”) of animals has been used to avoid the need for inducing general anesthesia while conducting selected procedures in many mammalian species including ungulates, carnivores, primates and marine mammals (Dumonceaux et al. 1998; Grandin et al. 1995; Phillips et al. 1998). Conditioning of common eland to sound cues has
been incorporated into ungulate management at Disney’s Animal Kingdom (Joe Kalla and Marty Sevenich, personal communication).

While the use of advanced mechanical handling devices has enabled clinicians and scientists to perform certain procedures without inducing general anesthesia in several ungulate species (Read et al. 1993; Citino 1995; Atkinson et al. 1999), the use of these devices combined with behavioral training has not been evaluated for performing reproductive technology procedures in tragelaphine antelopes. Data on the safety or stressful effect of such handling are not available. Moreover, although it has been reported that individual elands have unique identifying physical and behavioral attributes (Kiley-Worthington 1978), it is unknown if physical characteristics influence the taming potential or the manner in which specific eland respond to training or handling. Thus, the hypotheses of the current study were:

1. female elands in a small group can be conditioned to associate specific sound cues with food treats; and
2. sedated female elands will tolerate handling in a hydraulic chute during assisted reproductive technology procedures.

Specific objectives were to:

1. evaluate the feasibility of conditioning individual female elands in a group of 10 to specific sound cues;
2. assess the use of conditioned stimuli to move female eland into a hydraulic chute;
3. evaluate the physiological responses of elands during sedation and handling in a hydraulic chute; and
4. determine, retrospectively, if the degree of cooperation with training and handling (taming potential) is associated with two selected physical attributes of the elands.
4.2. Materials and Methods

4.2.1. Study Animals and Management

Animals were maintained at the Freeport-McMoran Species Survival Center, a facility of the Audubon Nature Institute, located on the outskirts of New Orleans, Louisiana. The area has a semi-tropical climate. The study was carried out over a period of three years (June 2000 to June 2003) using a group of 10 adult elands. Animals were housed on an open yard (45 m x 56 m, plot A; Figure 4.1) surrounded by a 2.4 m high woven wire fence.

The yard has a feeding area (10 m x 12 m, see plot B) with a concrete floor. Feed was placed in a trough on both sides of a shade structure (7.6 m x 2.5 m) built in the center of the feeding area. The herd was fed daily as a single group with a bale of grass hay and 41 kg of pelleted concentrate (Mazuri® CU ADF-16, PMI Nutrition International, Brentwood, MO, USA). The concentrate contained a minimum of 17 % crude protein and 3 % crude fat, and a maximum of 15 % crude and 16 % acid detergent fiber. Water and mineral lick block were provided ad libitum.

Routine veterinary care included anthelminthic treatments twice a year by alternating among fenbendazole (Panacur®, DPT Laboratories, San Antonio, TX, USA), pyrantel pamoate (Strongid®-T, Pfizer Animal Health, Exton, PA, USA) and ivermectin (Ivomec®, Merial Ltd, Iselin, NJ, USA). Vitamin E and selenium (BO-SE®, Schering Plough Animal Health Corp, Union, NJ, USA) were also administered twice yearly. Animals received annual vaccinations against rabies (Rabvac™-3, Fort Dodge Animal Health, Fort Dodge, IA, USA), Clostridia (Fortress®-7, Pfizer Animal Health, Exton, Pennsylvania, USA) and Leptospira (Leptoferm-5®, Pfizer Animal Health). Fecal samples were examined quarterly for parasite ova, and insecticides/repellents were applied as needed. Animal procedures were carried out with the approval of the Institutional Animal Care and Use Committee of the Audubon Center for Research of Endangered Species, New Orleans, Louisiana 70131, USA.
Figure 4.1. Sketch of the eland holding area and associated structures:

Plots include the main holding area for females (A), feeding area with a shade (B), temporary holding area with a shade (C), holding and feeding areas for a male eland (D, E), keepers area (F) and barns (G, H) leading into a hydraulic chute (I). Gates are numbered from No. 1 to No. 27 and doors inside the structure are remotely operable. Gates No. 25 and No. 26 lead to loading and unloading areas. Barn H is equipped with a remotely operable push-wall that can be moved between the areas of Gate No. 14 and Gate No. 16. Sketch not drawn to scale.


4.2.2. Conditioning to Sound Cues

During animal training, classical or Pavlovian conditioning is often used to “bridge” primary reinforcers (e.g., feed) with secondary stimuli such as clickers (www.wagntrain.com/OC/#Operant, accessed 23 November 2003). Thus, feed rewards (treats) other than the regular diet were used to condition animals to specific sound cues. Elands readily ate treats such as tree branches, spinach, carrots, alfalfa hay and bananas; however, alfalfa and banana were preferred the most. We chose bananas as a treat item because they can be hand-fed or by throwing it into the holding area; and medications can be embedded in them for feeding them to a specific animal. Bananas are also stable for several weeks when stored at 4°C, which makes them desirable.

The two criteria used to select sound producing tools were: 1) no two tools should produce similar sounds to prevent confusion among animals of sound cues and 2) at least one hand should be free for providing treats and/or recording observations. Examples of sound cue tools matched to each female (tool: eland ID) were: clicker (KP-600R, Sunshine Books, Inc, Waltham, MA, USA): 125; tapping pant leg with a hand: 140; coach’s whistle: 124; mouth whistling: 139; etc. We did not attempt to condition one female (eland 128) because it was not possible to train her to accept handheld treats; however, in an attempt to get treats, she would always approach the training area and chase away subordinate females. She was also the most aggressive female and often attempted to charge people standing outside of the fenced pen.

Conditioning to sound cues was conducted daily between June and August 2000 in sessions of up to 4 hr each, in the vicinity of Gate No. 1 (Figure 4.1), while monitoring the response of animals through the fence. To condition each animal to sound cues, treats were thrown into the holding area near an animal or hand-fed to those females (elands 125, 123, 129, 139 and 140) already accepting hand-fed treats. The specific sound cue was then produced continuously while the animal was eating the treat. To avoid competition for treats and confusion over the conditioned sound cues, a
second person kept other females as far away as possible from the animal being conditioned.

Most training was conducted in the morning (before animals were fed). We also determined, in a pilot study, the interval from producing conditioned audio cues to acceptance of handheld treats by females. This response was evaluated in the morning (before feeding) and afternoon (3 to 4 hr after feeding) by inviting each female, using the conditioned cue, on at least three different days. The interval was recorded in whole number minutes.

4.2.3. Chute Training

After achieving positive association between sound cues and treats, the cues were then used to move females, initially around the holding area (plots A and B, Figure 4.1) and then to entice each female to the chute area, and subsequently, into the chute (plot I, Figure 4.1). The chute (The Tamer™, Fauna Research, Inc., Red Hook, NY, USA) was padded internally and could be operated using a hydraulic pump to restrain (squeeze) and lift an animal. Atkinson and colleagues have provided a more detailed description of this device (Atkinson et al. 1999).

Animals were “called” or “invited” into the vicinity of Gates No. 15 to No. 18 via Gates No. 8 and No. 20. Invitation of females and observation of their responses was done through one of the two sliding doors on either side of the chute. After repeated and consistent voluntary entry into the chute, females were allowed to remain in the chute during its activation. Chute use was gradually increased from simply turning on the hydraulic pump to partially squeezing animals. The response of animals to chute training was graded from 0 to 6 scores as described next:

0 = does not approach the chute/does not enter the barn via Gate No. 18 (Figure 4.1);
1 = enters the barn but does not come close to the chute gate (Plot I, Figure 4.1);
2 = approaches the chute gate, takes hand-held treat at the gate before retreating back;
3 = approaches the chute gate and stays taking handheld treat;
4 = partially enters the chute, gets treat and stays for a prolonged period;
5 = enters the chute voluntarily; and,
6 = fulfills criterion No. 5 and allows being touched and/or tolerates closure of chute gate,

noise of hydraulic pump and partial squeezing of sides of the chute.

4.2.4. Use of Sedatives and Physiological Effects of Handling in a Hydraulic Chute

Tranquilization protocols and the safety of carrying out procedures in the chute were initially established from trials done on the three most cooperative females (elands 123, 125, 140). Body weights, taken by placing a flatbed Altralite™ scale (Rice Lake Weighing Systems, PA, USA) on the floor in the hydraulic chute, were used for calculating dosage of sedative agents. Two separate neuroleptic agents (haloperidol, acepromazine), an α-2 agonist (xylazine) alone, and a combination of xylazine with the opioid analgesic (butorphanol) were evaluated. The regular feed of the group was reduced by one half on the day before sedation trials.

Each female was treated with 1 mg/kg of haloperidol lactate (Geneva Pharmaceuticals, Inc. Broomfield, CO, USA) given orally embedded in bananas. Animals were monitored both inside and outside the chute for approximately 6 hr after treatment and for the following three days. Attempts were made to partially squeeze the animals in the chute. Due to inadequate sedation with the initial regimen, the dosage was increased to a single treatment of 1.5 mg/kg or three consecutive daily treatments of 1.25 mg/kg.

One animal was hand-injected in the chute with 10 mg of acepromazine maleate (Boehringer Ingelheim, St. Joseph, MO, USA), i.m. and monitored during the first hour after injection. Sufficient sedation to allow animal handling for reproductive procedures was not achieved with either haloperidol or acepromazine. Thus, xylazine HCl (Xyla-Ject®, Phoenix Pharmaceutical, Inc., St. Joseph, MO, USA) was chosen as the next sedative agent for evaluation.

Literature information on the use of xylazine alone for sedating eland was not available;
however, up to 150 mg of xylazine (~0.4 mg/kg) is often used to supplement other drugs when inducing general anesthesia in the eland (Citino 1995; Nielsen 1996). We began with a dosage of 0.2 mg/kg i.m., which was increased based on individual response to the highest dose, 0.7 mg/kg. Xylazine alone was used during the first five procedures. In subsequent procedures (n = 36), xylazine was combined with a total dose of 25 mg of butorphanol tartrate (Torbugesic®, Fort Dodge Animal Health, Fort Dodge, IA, USA) to achieve complementary sedative and analgesic effects.

Medications were hand-injected after positioning and slight squeezing of animals in the chute. Time from injection to onset of sedation (unresponsiveness to hand movements in frontal area, loss of prehensile ability when treats were provided, salivation, loss of ocular stare) and events surrounding the sedation were recorded. Once sedated, animals were positioned by squeezing and lifting with the chute. A bale of hay was placed across the brisket to restrict back and forth movement of the animal. An epidural block was induced for doing transvaginal ultrasound-guided follicular aspiration to recover oocytes (Chapter 6).

Heart rate, blood oxygen saturation and body temperature were monitored using a pulse oximeter (Model 340V, Palco Labs, Inc, Santa Cruz, CA, USA) probe introduced into the nasal cavity. Respiratory rate was determined by hearing and feeling each breath at the muzzle. After completion of each oocyte retrieval procedure, blood samples were collected from the tail vein, prophylactic treatment of 3.6 million U penicillin-G (G.C. Hanford Mfg. Co, Syracuse, NY, USA) was administered and xylazine sedation was reversed using 600 mg of tolazoline HCl (Tolazine™, Lloyd Laboratories, Shenandoah, IA, USA).

Blood gas analyses were performed on samples (n = 4) collected from coccygeal vessels using a 20 g needle and 5-mL heparinized syringe. Analyses were completed within 10 min of collection using an IRMA blood analysis system (Diametrics Medical, Inc. St. Paul, MN, USA). Blood glucose (n = 18), hematocrit (n = 14) and creatine phosphokinase, CPK (n = 18) levels were analyzed by a commercial laboratory (Antec Diagnostics, Memphis, TN, USA).
4.2.5. Taming Potential

Certain physical attributes influence the potential to tame/domesticate wild animals (Hemmer 1976; Hemmer 1990; Price 2002). Determination of such features in the eland or for related antelopes has direct relevance to captive management or future domestication endeavors. Thus, an attempt was made to determine if specific physical attributes of female eland are associated with taming potential.

Animals that were well conditioned to sound cues and consistently moved at least to the entrance of the chute gate (Figure 4.1: Gate No. 16) when invited were categorized as ‘Cooperating’ or having “Good” taming potential. The remaining animals were classified as having “Poor” taming potential (“Not cooperating”).

Preliminary observations revealed two physical attributes that varied among the female eland. These characteristics were the pattern of horn growth (generally, either splayed or straight) and the number of vertical stripes on the sides of the body (ranged from 2 - 13). Thus, the association between the taming potential and the pattern of horn growth and/or number of body stripes was evaluated.

4.2.6. Data Analyses

Intervals from production of conditioned stimuli to acquisition of handheld treats in the morning and afternoon sessions were compared using two-way ANOVA and Duncan’s test. Data on the interval from injection of either xylazine or xylazine-butorphanol combination to onset of sedation were compared using an unpaired t-test. Count data were analyzed using the Chi-square test and/or Fisher’s Exact test. Averages were presented as mean ± SD. Variations in heart rate, respiratory rate, body temperature and oxygen saturation during sedation were tested using repeated measures ANOVA after excluding data points with missing values. Hematological values of animals that underwent oocyte retrieval were also compared using t-test with values obtained in three females
opportunistically handled similarly but without doing oocyte retrieval.

All analyses were done using the GraphPad Instat® version 3.00 for Windows 95 (GraphPad Software, San Diego, CA, USA) except for two-way ANOVA using SAS (SAS Software Release 8.2, Cary, NC, USA). Descriptive statistics were also used to present data. A P-value of 0.05 was considered statistically significant.

4.3. Results

4.3.1. Conditioning to Sound Cues

All nine females recognized their specific sound cues and accepted handheld treats. Visible indications of response were increased alertness, attempting to locate food treats and moving toward the sound cue. However, the extent of acceptance of a handheld treat varied among the females. Elands 126, 124 and 131 were more cautious and did not take handheld treats consistently. Some degree of non-specific response to sound cues, which was more marked during training before feeding was also observed. However, two females often stopped eating their regular diet and came to the trainer upon hearing their specific sound cue. Dominant animals usually interfered with conditioning by displacing subordinate females.

Conditioning to sound cues was done over a period of seven weeks. Animals seemed to associate the sound cues with treats on the first session of training. Intervals from producing conditioned audio cue to acquiring handheld treats varied from immediate (<0.5 min) to over an hour (65 min). Mean intervals from activating audio cue to animals accepting handheld treat, during morning or afternoon sessions, are presented in Table 4.1. The model effect was highly significant (P <0.0001), but animals had a more marked (P <0.0001) effect than time of training (P = 0.058).

The interaction between animal and time of day was not significant (P = 0.867). The mean (± SD) interval in the morning and afternoon was 13.6 ± 17.1 and 17.7 ± 19.5 min (overall mean = 15.7), respectively, and ranged from less than 2 min (eland 125) to almost 1 hr (eland 124; Table
Table 4.1. Mean interval (min) from conditioned sound cue to acceptance of handheld treats during morning and afternoon training sessions in elands

<table>
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<tr>
<th>Eland ID</th>
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<th>Overall</th>
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<td>11.7</td>
<td>27.0</td>
<td>19.3&lt;sup&gt;bc&lt;/sup&gt;</td>
</tr>
<tr>
<td>140</td>
<td>14.5</td>
<td>18.8</td>
<td>16.6&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>abcde</sup> Values in the same column with different superscripts are significantly different (P<0.05).

4.1). Although the effect of time of day was not significant, mean intervals were consistently shorter in the morning sessions, suggesting the fasting overnight was beneficial for training.

4.3.2. Chute Training

All females, including eland 124, which was not conditioned to associate specific sound cue with treat, came into the barn and within close proximity of the chute during the chute-training period. Five females voluntarily entered the chute when invited between the 10<sup>th</sup> and 16<sup>th</sup> training sessions over a period of 2 months. The eventual scores of response to chute training varied from 1 - 6 (scale 0 to 6). Four females (eland 123, 125, 129, 140) attained the highest score of 6; one female (eland 139) was at score 5 and another was at 4 (eland 132). The remaining four elands were more difficult to train to the chute and were assigned scores of 2 (elands 124, 28, 131) and 1 (eland 126).
4.3.3. Use of Sedatives and Physiological Effects of Handling in a Hydraulic Chute

At the doses used, neither haloperidol (1.0 - 1.5 mg/kg) nor acepromazine (0.036 mg/kg) produced adequate sedation for handling the eland in the chute. Xylazine induced sedation at all dose ranges evaluated. Intervals from i.m. injection of xylazine or xylazine/butorphanol to the observation of sedation ranged from 5 to 20 min, and did not differ between the two treatments (P = 0.612; Table 4.2). Excitement immediately before sedative treatment tended to prolong the interval to onset of sedation.

The frequency of recumbency after administration of sedatives and before positioning them in the chute was not different between the two treatments (P = 0.362; Table 4.2). A single sedative dose was adequate to handle females during most of the procedures (34/41, 83%). The frequency of procedures requiring a second dose to maintain sedation did not differ between the two treatments (P = 1.00; Table 4.2). After prompting, all recumbent animals returned to standing and were able to be repositioned to continue with the procedure.

Individual behavior influenced the dose of xylazine required for sedation. Eland 128 required as much as 250 mg (0.7 mg/kg) of xylazine, while 100 to 150 mg was adequate for the other females.

Table 4.2. Effect of sedative treatments on onset of sedation, frequency of recumbency or a need for second dosing of sedative agents in elands during handling and oocyte retrieval in a hydraulic chute

<table>
<thead>
<tr>
<th>Drug(s)</th>
<th>Number treated</th>
<th>Dose, mg/kg</th>
<th>Onset of sedation, mean ± SD, min</th>
<th>Recumbent females, n (%)</th>
<th>Redosing needed, n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Xylazine</td>
<td>5</td>
<td>0.2-0.7</td>
<td>11.0 ± 2.2</td>
<td>3 (60)</td>
<td>1 (25)</td>
</tr>
<tr>
<td>Xylazine/Butorphanol*</td>
<td>36</td>
<td>0.2-0.7/0.07</td>
<td>11.9 ± 3.9</td>
<td>13 (36)</td>
<td>6 (17)</td>
</tr>
</tbody>
</table>

*This protocol plus atropine was used for sedating eland in most oocyte retrieval procedures; initial trials (n = 10) using haloperidol or acepromazine did not produce adequate sedation.

Some vital physiological parameters observed during sedation and handling for oocyte retrieval are shown in Table 4.3. Animals subjected to sedation and oocyte retrieval had a
significantly greater blood glucose concentration (14.4 ± 3.1 vs. 9.3 ± 2.7 mmol/L; P = 0.0067) than females opportunistically sedated and handled without oocyte collection. Other parameters, including CPK activity (1084 ± 972 vs. 354 ± 133 U/L; P = 0.156), hematocrit (36 ± 4 vs. 42 ± 13 %; P = 0.121) and plasma osmotic pressure (290 ± 7 vs. 284 ± 5 mmol/kg; P = 0.298) were not different between the two groups.

Mean blood glucose levels (mmol/L) per eland during sedation and handling for oocyte retrieval varied between 10.9 (eland 125) and 19.1 (eland 128). Each female showed glucose response that was typical for herself (Figure 4.2). For example, glucose levels in eland 125 were close to 11 mmol/L (range: 10.3 to 11.4) while eland 124 and 128 consistently had values over 16.5 mmol/L (range: 16.7 to 20.6). Moreover, when animals were classified into two cooperative groups (good or poor), there was a significant difference in blood glucose levels with the higher levels in elands in which handling was more difficult (Table 4.4). The CPK profile also reflected the pattern of glucose profile (Figures 4.2; 4.3) in all females except one animal (eland 139).

During the sedation and handling for oocyte retrieval, the mean intranasal temperature, heart and respiratory rates and oxygen saturation of blood did not differ significantly among the time points evaluated. However, slight increases in heart rate toward the end of the procedure and reduction in oxygen saturation during the procedure were observed (Figures 4.4 – 4.7). Recovery after reversal of sedation was uneventful in all handling procedures and occurred without complication.

4.3.4. Taming Potential

It was possible to divide the animals into an equal number of cooperating (elands 123, 125, 129, 132, 140) and non-cooperating (elands 124, 126, 128, 131, 139) females. [Note that this classification does not necessarily relate to the possibility of performing reproductive procedures on a female or her chute training score; see Discussion].
Figure 4.2. Blood glucose (mean ± SD) and hematocrit (mean or single sample values) in sedated elands handled in a hydraulic chute for oocyte collection (n = 18).
Figure 4.3. Mean blood CPK level in sedated elands handled for oocyte retrieval (n = 18).
Table 4.3. Some physiological parameters of female elands during sedation and handling in a hydraulic chute for oocyte retrieval.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Mean ± SD</th>
<th>Range</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood glucose (mmol/L)</td>
<td>14.4 ± 3.1</td>
<td>9.8 - 20.6</td>
<td>18</td>
</tr>
<tr>
<td>Hematocrit</td>
<td>35.8 ± 4.3</td>
<td>28.3 - 42.7</td>
<td>14</td>
</tr>
<tr>
<td>Blood CPK (U/L)</td>
<td>1084.3 ± 972</td>
<td>232 - 3766</td>
<td>18</td>
</tr>
<tr>
<td>Blood pH</td>
<td>7.44 ± 0.05</td>
<td>7.37 - 7.49</td>
<td>4</td>
</tr>
<tr>
<td>Blood osmolality (mmol/kg)</td>
<td>290.0 ± 7.3</td>
<td>278 - 306</td>
<td>16</td>
</tr>
<tr>
<td>Heart rate/min</td>
<td>58.8 ± 16</td>
<td>32 - 99</td>
<td>20</td>
</tr>
<tr>
<td>Breathing rate/min</td>
<td>30.6 ± 10</td>
<td>12 - 55</td>
<td>18</td>
</tr>
<tr>
<td>Rectal temperature</td>
<td>37.6 ± 0.3</td>
<td>37.4 - 37.8</td>
<td>2</td>
</tr>
</tbody>
</table>

*Temperature was measured 15 min after administering sedative agent; other data were collected at the end of oocyte retrieval.

Table 4.4. Some variations between female elands with good (n = 5) or poor (n = 5) taming potential.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Taming potential</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Good (cooperating)</td>
<td>Poor (non-cooperating)</td>
</tr>
<tr>
<td>Stripes, mean ± SD (range)</td>
<td>6.4 ± 2.3 (4 - 10)</td>
<td>11.2 ± 1.6 (9 - 13)</td>
</tr>
<tr>
<td>Straight-horned (n/group total)</td>
<td>4/5</td>
<td>1/5</td>
</tr>
<tr>
<td>Blood glucose (mmol/L)*</td>
<td>12.0 ± 2.8</td>
<td>16.0 ± 3.6</td>
</tr>
</tbody>
</table>

*Collected during handling.
Figure 4.4. Heart rate (mean ± SD) profile during xylazine-butorphanol-induced sedation in elands handled for oocyte retrieval (n = 25).
Figure 4.5. Blood oxygen saturation (mean ± SD) profile during xylazine-butorphanol-induced sedation in elands handled for oocyte retrieval (n = 23).
Figure 4.6. Respiratory rate (mean ± SD) profile during xylazine-butorphanol-induced sedation in elands handled for oocyte retrieval (n = 23).
Figure 4.7. Peripheral body temperature profile during xylazine-butorphanol induced sedation in elands handled for oocyte retrieval (n = 13).
The number of lateral stripes varied from 2 (left side of eland 129) to 13 (right side of eland 124). The mean of the higher number of stripes on either side of the animal was 8.8 ± 3.2 (range: 4 - 13). Non-cooperating females had almost twice as many stripes as the cooperative ones. The cooperative group females had horn growth pattern that was straighter, but the association between this factor and cooperation by the eland was not significant (Table 4.4). Nevertheless, there was only one female with splayed-horn appearance that was cooperative and only one female with straight horns that was not cooperative (Table 4.4).

Blood glucose levels of all elands during handling was significantly lower in cooperating than in non-cooperating females (Table 4.4). However, there was no correlation between the number of stripes and mean blood glucose level of all females (r = 0.068; P = 0.851) or for females subjected to oocyte retrieval (r = 0.3541; P = 0.436). When comparing all 10 females, blood glucose level (mmol/L) was higher in elands with splayed horns than in females with straight horns (15.9 ± 3.2 vs. 11.5 ± 2.6; P = 0.002). Similarly, among females subjected to oocyte retrieval, glucose levels were higher in elands with splayed horns than females with straight horns (16.3 ± 3.2 vs. 12.6 ± 1.7; P = 0.008) and in non-cooperating elands than in the cooperating females (16.5 ± 3.6 vs. 13.1 ± 1.9; P = 0.018).

Over time, handling of the eland for oocyte retrieval reduced their readiness to voluntarily enter the chute. During the early phases of the study, five females readily entered the chute. After repeated procedures (sedation, handling in the chute and oocyte retrievals), all five animals were hesitant to enter the chute and required the use of mild enforcements such as water sprayed from a garden hose and push walls to encourage entry into the chute. However, there was no indication of reduced response to conditioned audio cues by the females when they were away from the chute area.

4.4. Discussion

The present study demonstrated that individual elands in a group can be conditioned to
identify specific sound cues. Subsequently, the conditioned cue was used to move animals from one location to another, including into the handling chute, thus confirming the potential benefits that classical and operant conditioning can offer to improve the management and handling of nondomestic mammals in captivity (Stevens 1978; Fridman 2001; Bloomsmith et al. 2003). We have continued to effectively use conditioned audio cues to separate individual females from the group for various purposes.

There were marked variations in the time taken from audio cue to accepting handheld treats [ranging from < 2 min to > 60 min] and in the response to the chute training. One female (eland 125) often ran to the trainer in response to cues. This female and another (eland 129) also stopped eating their regular diet and come to the trainer when prompted. It was also possible to get the attention of other females by producing specific sounds during feeding. Thus, elands can discriminate between two or more audio cues. Bongo and nyala antelopes have also been reported to recognize and become agitated by the voice of the veterinarian who administers medications by darting (Grandin 2000). The individual variation in the responses of eland to conditioning could be due to previous experiences with people and/or may have a genetic basis.

Animals maintained excellent memory of conditioned stimuli even after reinforcement was interrupted for several months. Maintenance of memory of conditioned stimuli after interrupting reinforcement for a long time has been reported in other mammalian species. For example, an elephant performed conditioned tasks after 8 yr without reinforcement (Stevens 1978). Bongo and nyala antelopes also responded in a similar manner as in preceding sessions during monthly reinforcements with conditioned stimuli (Grandin 2000), indicating animals maintained good memory of the conditioning. Such memory in ungulates has implications for handling since animals can remember, for a prolonged period, the manner in which they were handled.

Interference by dominant animals during training of subordinates has been observed in other species, including bongo, but social rank did not interfere with training the nyala. In the nyala,
handlers could safely enter the room in which animals were kept to facilitate conditioning; however, it was not safe to enter the bongo pen (Grandin et al. 1995; Phillips et al. 1998). Eland in the present study are similar to the bongo in this respect because two females (elands 128 and 139) made active attempts to charge people. However, during training, two persons could easily keep animals in different corners of the holding area so that a single female could be trained in a separate area, without interference by dominant females.

Training of the five eland to enter the chute required 10 to 16 training sessions (days) over a period of 2 months. Bongo and nyala have been trained to enter a handling crate in 21 days (Phillips et al. 1998). The aggregation of multiple females at the area of the gate of the chute, when training a single female to the chute, was a problem because subordinates had to retreat when dominant females approached the area. Also, the elevated entrance into the chute delayed voluntary entry. Thus, considering the active pursuit of treats (e.g., more so than members of our bongo antelope) by eland, a shorter interval of training them to enter the chute can be expected. However, they are more prone to flight and more active than bongos and may not readily accept desensitization for handlings.

Initially, excessive physical movement by the three most cooperative females during restraint in the Tamer chute precluded performing clinical exams and other procedures on non-sedated animals. Oral treatment with 1 mg/kg of haloperidol has been used to sedate female elands for manual handling (Boyd et al. 2000). Intramuscular or i.v. administration of 20 to 40 mg of haloperidol tranquilized eland for 8 to 18 hr (Citino 1995). The reason for the failure of haloperidol treatment to successfully sedate eland in the present study is unclear. In the holding area, only one of the females treated with haloperidol displayed mild sedation (associated with signs of dizziness), which was not apparent after she was moved into the chute. Individual animal variation, or possibly the noise of the electric motor of the hydraulic pump may have affected the sedative effects of haloperidol. Unpredictable effects of haloperidol have also been reported in the eland (Ebedes and Raath 1995).
The eland is more tolerant to xylazine (up to 0.7 mg/kg in the present study) as compared with domestic ruminants (≤0.3 mg/kg, [www.merckvetmanual.com/mvm/index.jsp](http://www.merckvetmanual.com/mvm/index.jsp), accessed December 31, 2003). Although we used butorphanol tartrate with xylazine to improve analgesia/sedation, the onset of satisfactory sedation was not different, nor were there other noticeable differences in response when using xylazine alone or in combination with butorphanol tartrate. Thus, the actual benefit of butorphanol at the dosage used remains to be determined. The duration of sedation (~1 hr) allowed sufficient time to perform ultrasound-guided oocyte retrieval and other procedures.

Xylazine suppresses heart and respiratory rates in domestic ruminants (Mogoa et al. 2000; Ndeereh et al. 2001). In the present study, heart rates and respiratory rates recorded at three intervals during sedation were not significantly different (Figures 4.4; 4.6). Whether these differences are attributable to species differences or the method of data collection remains to be determined. Blood oxygen saturation declined early during sedation (e.g., up to 63% in one procedure) but oxygen supplementation allowed a return to normal. Thus, cardiopulmonary parameters during sedation and handling of the eland were within manageable ranges.

Body temperature, heart and respiratory rates, hematocrit, blood glucose, CPK and cortisol levels are often used to assess acute stress during animal transport or handling (Knowles and Warris 2000). Establishment of baseline values for most of these variables in the eland is difficult because data/sample collection requires either immobilization or vigorous physical restraint, both of which alter normal parameters. Remote assessment of heart rate and respiratory rates on alert (non-restrained) animals may be possible; however, we could count jugular pulse only on two of the elands in the present study (mean rates were 35 and 51 for each female) and we could not remotely count breathing movements. The good body condition of the animals may have reduced the remote visibility of jugular pulse and breathing movements.
Thus, it is difficult to compare the present cardiopulmonary parameters and body temperature with baseline values. Nevertheless, body temperatures ranging from 39.4 - 42.9 (mean = 41.0) have been reported in immobilized females (Denney 1970). In the present study, mean intranasal temperature varied by less than 2°C. During two procedures, simultaneous recordings of rectal temperature (37.4 to 37.8°C) in the eland were as much as 2.7°C higher than the intranasal temperature.

The mean blood glucose level (14.4 mmol/L, 260 mg/dl) of elands during oocyte retrieval was higher than in females handled similarly by us but without oocyte retrieval (9.3 mmol/L). It was also higher than the mean value of multi-institute data (10.4 mmol/L) gathered by ISIS/MedARKS for adult female elands in North America. Similarly, eland immobilized by darting from a helicopter or those manually restrained after chasing into nets in South Africa (Ganhao et al. 1988) had lower blood glucose levels (7.9 and 8.7 mmol/L, respectively) than values found in the present study. Bongo antelope subjected to traditional immobilization methods had a blood glucose level of 9.3 mmol/L, which was 2.7 times higher than bongos conditioned to handling (Phillips et al. 1998).

In domestic cattle, the normal blood glucose level is <4.5 mmol/L and levels similar to those recorded in the present study are observed only in animals subjected to i.v. glucose tolerance tests or animals receiving glucocorticoid therapy (Abdel-Fattah et al. 1999; Panicke et al. 2002). The numerically higher CPK activity in elands handled for oocyte retrieval as compared with those handled without oocyte retrieval is also reflective of greater muscle exertion in the former group.

These results indicate that elands have a large pool of glucose and/or glucose precursors and possess the ability to mobilize them during physical exertion. However, since high blood glucose level is an indicator of stress during handling, and we noted a tendency for a higher glucose level in eland that were more difficult to handle, alternative methods of minimizing the elevated glucose response should be investigated. The higher-than-physiological blood glucose level observed during oocyte collection may also lead to an increased follicular glucose level, due to increased
vascularization and permeability of preovulatory follicles (Larsen et al. 1996; Barboni et al. 2000). Moreover, bleeding caused by aspiration of follicles occurs during ultrasound-guided oocyte retrieval and may expose oocytes to supra-physiological glucose levels that could possibly affect the developmental potential of the oocytes.

Initially, five females readily entered the hydraulic chute, but sedation followed by oocyte retrieval negatively affected the readiness of voluntarily entry into the chute during subsequent procedures. Whether a more intense habituation and desensitization or strict adherence to principles of operant conditioning during training could prevent or minimize this negative effect remains to be determined. Nevertheless, the response of elands to sound cues away from the chute area did not show any sign of subsidence. Thus, the conditioned sound cue could be used throughout the study to relocate animals outside the barn area and to direct them toward the chute.

More recently, after separating individual animals from the group, a combination of sprayed water from a garden hose and push walls have been used to move animals into the chute. Guidance of animals toward the chute was facilitated by additional modifications made to the holding area, including the addition of two sliding doors in Plot B and the construction of an alley between Gates No. 8 and No. 13. The first modification enabled us to separate an animal on one side of the feeding area for subsequent movement through the alley into the barn. A push wall was then used to encourage entry of non-compliant females into the chute. Thus, it was possible to consistently guide animals into the chute and handle them, and oocyte retrievals could be done not only on the animals trained to enter the chute but also on two additional untrained females.

The fact that eland 128 could be used for ultrasound-guided oocyte retrieval without conditioning to audio cue and without training to voluntarily enter the chute underscores the importance of knowing the “personality” profile of each eland. Because of her voracious appetite, she was among the first to come to the feeding yard and hence it was easy to separate her for providing estrous synchronization treatments. Eland 124 could also be treated similarly but isolating
her often required more time. Because of the tendency of elands 128 and 139 to charge people, increased caution was required when around them. Elands 126 and 131 were excluded from oocyte retrievals because of their excessive flighty behavior and since it was not possible to consistently provide hormone treatments. Eland 132 was not used for oocyte retrieval because of her geriatric state and poor body condition. Nevertheless, the previously described modifications to the holding area enabled handling of all 10 animals in the chute at least once.

“Genetic and experiential” factors determine the degree of tameness of an animal (Price 2002). Behavioral modifications leading to tameness and domestication are associated with certain anatomical and biochemical changes. For example, domestic counterparts of some wild species (e.g., domestic cats vs. wildcats or dogs vs. wolves) have evolved to have a smaller (up to 30%) brain. Agouti rats and deer mice are tamer (easier to handle) than those with non-agouti phenotypes (Hemmer 1990; Price 2002). Selection and subsequent breeding of animals with certain phenotypes, is therefore, a viable approach to establish a tame or even domesticated line of wild animals (Cottle and Price 1987; Hemmer 1990; Prasolova and Jing 1999; Trut et al. 2000).

Observations in the present study suggest that females with fewer vertical stripes, and possibly, straighter horns may be good candidates for taming. Similarly, among another group of eight female elands at our facility that were not included in the present study, an approach by strangers induced shorter flight distance in females with fewer stripes and straighter horn growth pattern (personal observation). The characteristics described in the present study may serve as a starting point for characterizing additional phenotypic and genetic factors influencing eland tameness. We have also demonstrated that social rank did not affect taming potential (Wirtu et al., In Press).

Stripes are the main criteria for differentiating Northern (East African) and Southern (Zambezi and Cape) strains of eland, with the Southern strains having fewer stripes. Thus, the variation in stripe number among adult female eland in the present study may have a genetic basis.
Horns are understood as instruments for defense against predators, for establishing dominance hierarchy and for breaking tree branches by tragelaphine antelopes (Kingdon 1982). They may also have thermoregulatory functions (Picard et al. 1999). The behavioral variation among individuals with different patterns of skin color may be explained by the close association of some genes and pathways influencing both skin color and certain behavior of animals (Hemmer 1990; Price 2002). It is unknown if horn growth pattern is also affected similarly.

Data are not readily available on whether the Northern and Southern strains of eland vary in their horn growth pattern or whether they differ in their tameability. Interestingly, a successful eland domestication endeavor at the Askanya-Nova Zoo in Ukraine was achieved with the southern (Cape and Zambezi) strains (Treus and Lobanov 1971). On the other hand, a well-funded project designed by international team members to domesticate eland in East Africa (Kenya) led to the conclusion that eland were not a good candidate for domestication (Spinage 1986). An obvious question is whether the strain of eland used in the Kenya study influenced the conclusions reached by the team.

In conclusion, a combination of behavioral conditioning/training and restraint of sedated eland in a hydraulic chute was a reliable and repeatable method for performing minimally invasive assisted reproductive techniques. Moreover, targeted audio conditioning of individual elands among a group of females can be used to facilitate the administration of medications to specific animals and can be helpful in the management of captive eland, such as fast translocation or separation of specific animals from a herd without using expensive alley systems. During sedation and handling for oocyte retrieval, several physiological parameters were either normal or within clinically manageable range. However, blood glucose levels were elevated and may be used as a marker in reducing and/or evaluating handling-induced physical exertion or stress in elands.
Chapter 5. Transvaginal Ultrasound-guided Oocyte Collection, *In Vitro* Oocyte Maturation and Embryo Production in the Eland Antelope

5.1. Introduction

There is a growing interest in using reproductive technologies, such as artificial insemination, embryo transfer, and the more recently developed methods of *in vitro* embryo production in the captive breeding of rare and endangered species of mammals. Moreover, many investigators believe that these technologies can facilitate genetic transfer between captive and free-ranging populations of nondomestic species (Lasley *et al.* 1994; Loskutoff *et al.* 1995; Wildt *et al.* 1997; Holt and Pickard 1999; Lanza *et al.* 2000b; Pope 2000; Berg *et al.* 2002; Ptak *et al.* 2002). Compared with natural mating or the first generation of ART (i.e., AI and ET), *in vitro* embryo production, combined with cryopreservation and ET, offers more flexibility in sire-dam selection and, theoretically, would maximize the number of offspring that can be produced per male or female gamete donor.

Techniques for *in vitro* production of domestic ungulate (cattle, sheep, goats, pigs) embryos have advanced greatly during the last three decades, such that viable embryos can now be produced consistently and repeatably. The ease with which gametes or embryos can be collected *in vivo* and the availability of reproductive organs from abattoirs have contributed immensely to this progress.

Direct extrapolation of techniques developed in domestic species (e.g., cryopreservation of spermatozoa or embryos, IVF, embryo culture) to nondomestic ungulates has mostly resulted in low success as evaluated by production of offspring (Hammer *et al.* 2001; Berg and Asher 2003), suggesting the need for development of species-specific methods. Application of ART to large nondomestic ungulates using captive animals can be facilitated by developing minimally stressful handling methods for collecting biological samples (blood, feces, urine, gametes, embryos etc), administering treatments and monitoring responses.

Applying traditional approaches of handling (e.g., frequent darting and complete chemical immobilization) to ART procedures is stressful to large nondomestic ungulates. For example,
subjecting four species of antelopes (oryx, bongo, eland and kudu) to ART procedures by
confinement, daily treatment with hormones and multiple administration of anesthetics led to weight
losses of up to 0.4 kg/day (Schiewe et al. 1988) and a probable reduction in superovulatory response
and fertilization rate (Schiewe et al. 1988; Schiewe et al. 1991b). The multiple hormone treatments
and associated handling for estrous synchronization and ovarian stimulation are a major cause of
stress in nondomestic ungulates [e.g., the bison (Dorn 1995)] and among the factors that need
improvement for increasing the efficiency of commercial cattle embryo transfer (Hasler 2003).

The limitations of traditional handling methods for ART in wild ungulates has been
emphasized by Solti et al. as follows: “...there have been more losses of nondomesticated animals in
attempts to collect embryos than there have been offspring produced by the transfer of in vivo-
derived embryos…” (Solti et al. 2000). Thus, besides the possibility of using behavioral training and
modern handling devices to reduce the need for general anesthesia (Chapter 4), there is also a need to
develop approaches to reduce the number of hormone injections required for ovarian stimulation.

Ultrasound-guided oocyte collection offers a safe, relatively non-invasive, repeatable (twice
per week in domestic cattle) approach for recovering oocytes from large ungulates. In nondomestic
ungulates, the technique, in conjunction with in vitro embryo production and subsequent transfer of
the embryos to recipient females, has resulted in guar (Armstrong et al. 1995; Hammer et al. 2001)
and wapiti (Berg and Asher 2003) offspring. In vitro embryo development after IVF of oocytes
recovered by ultrasound-guided methods were also reported in several large nondomestic species
including the bongo (Pope et al. 1998b), addax (Asa et al. 1998), Burchell’s and Hartmann’s zebra
(Meintjes et al. 1997), red hartebeest, tsessebe, sable and African buffalo (Loskutoff et al. 1995).

The eland antelope is a useful model species for the study and application of ART in rare
tragelaphine antelopes. It is a large bovid and hence amenable to non-surgical, transrectal and/or
transcervical accessibility and manipulation of the female reproductive tract (Chapter 2). In vitro
embryo production has been reported in at least 13 species of bovidae (Hall-Woods 2001) and in
vitro-derived embryos have resulted in offspring after transfer to recipient females in eight species of bovidae (Table 2.1). Recently, we reported the initial demonstration of ultrasound-guided oocyte retrieval and some embryonic development in vitro in the eland (Wirtu et al. 2002a; Damiani et al. 2003). Thus, the hypotheses of the present study were:

1. transvaginal ultrasound-guided follicular aspiration can be used to collect oocytes in sedated eland antelope; and
2. eland oocytes recovered by an ultrasound-guided approach are capable of in vitro maturation and subsequent embryonic development after in vitro fertilization, intracytoplasmic sperm injection or somatic cell nuclear transfer.

Specific objective were to:

1. determine ovarian response and oocyte recovery rates following treatment with pituitary-derived FSH dissolved in a polyvinyl pyrrolidone carrier given four and/or two days before oocyte retrieval,
2. compare effects of targeting preovulatory or postovulatory follicular waves on ovarian response and oocyte recovery after incorporating an oral progestogen (altrenogest) in the estrous synchronization and ovarian stimulation protocol; and
3. evaluate the in vitro developmental competence of eland oocytes after in vitro maturation, and subsequent in vitro fertilization, intracytoplasmic sperm injection or somatic cell nuclear transfer.

5.2. Materials and Methods

5.2.1. Study Animals

Seven adult eland females among a group of 10 animals were used for oocyte collection. Animals lived in a 45 m x 56 m open yard fenced with woven wire. The holding area was equipped with a hydraulic handling chute (Tamei™, Fauna Research, Inc., Red Hook, NY; USA) and gates
with remotely operable doors (see Chapters 4 for detailed descriptions of the holding area, herd management and history). The seven females were selected based on the ability to consistently administer estrous synchronization and ovarian stimulation treatments and move them to the handling chute using conditioned audio cues and/or the gate and push-wall systems (Chapter 4).

5.2.2. Estrous Synchronization and Ovarian Stimulation

5.2.2.1. Experiment 5.1 [Single (day –2) vs. Double (day –4 and day –2) FSH Injections]

Before conducting oocyte retrieval procedures, the elands were treated to synchronize their estrous cycle and to induce ovarian stimulation. Figure 5.1 shows the treatment schedule.

Starting at unknown stages of the estrous cycle, three consecutive doses of prostaglandin F₂α, (PGF₂α, 25 mg of dinoprost tromethamine, Lutalyse®, Pharmacia and Upjohn Company, Kalamazoo, MI, USA), were administered i.m. The first (day 0), second (day 11) and third (day 22) injections were given 11 days apart, with the third injection given two days (~48 hr) before the expected day of oocyte retrieval. Thus, assuming ovulation occurs between 2 and 3 days after treatment with prostaglandin, females were at days 8 to 9 of the estrous cycle during the last two prostaglandin treatments. All parenteral treatments were hand-injected while females were in the hydraulic chute.

For ovarian stimulation, a porcine pituitary folltropin extract (Folltropin®-V, 400 mg NIH-FSH-P1 per vial, Bioniche Animal Health Canada, Inc., Belleville, Ontario, Canada) was used. A 40% solution of polyvinyl pyrrolidone (PVP, mol. wt = 40,000) was prepared in ultrapure (NANOpure) water. Aliquots of the PVP solution were sterilized by autoclaving in glass vials, and were stored at 4°C. On the day of use, the powdered contents of a vial of Folltropin®-V was dissolved in 1.25 mL of saline and aspirated into a 3-mL syringe. The Folltropin®-V vial was then rinsed with 3.75 mL of 40% PVP solution, aspirated into a 5-mL syringe and then mixed with the 1.25 mL solution of Folltropin®-V.
Figure 5.1. Estrous synchronization and ovarian stimulation treatment schedule before ultrasound-guided oocyte collection in elands in experiment 5.1.
After thorough mixing of the solution, animals received either a single (5.0 mL) injection or two injections of two-thirds (3.3 mL) and one-third (1.7 mL) of the total volume, depending on the experimental design (see below). The remaining quantity of re-constituted FSH was stored at −20°C. Thus, the final concentration of PVP used as a gonadotropin carrier was 30%.

In Treatment 1 (single injection), animals were treated i.m. with 400 mg of FSH (Folltropin®-V) on day 22 of treatment (day 0 = first prostaglandin injection), 2 days before oocyte retrieval (Figure 5.1). In Treatment 2 (double injections), 267 mg (two-thirds) and 133 mg (one-third) of FSH were administered on days 20 and 22 of treatment 4 and 2 days before oocyte retrieval, respectively (Figure 5.1). In both treatments (n = 13), prostaglandin administration was similar.

5.2.2.2. Experiment 5.2a (Targeting Preovulatory or Postovulatory Follicular Wave)

In this experiment, the effects of an oral progestogen (altrenogest) combined with a single injection of prostaglandin F₂α on estrous synchronization and subsequent ovarian response to two Folltropin®-V treatments were evaluated. The two follicular waves (preovulatory and postovulatory) that are universally found in ruminants studied to date (Adams 1999) were targeted. In Treatment 1, the follicular wave that leads to ovulation (preovulatory) immediately following ovarian stimulation and prostaglandin treatments was the target. In Treatment 2, the expected ovulation following estrous synchronization using altrenogest and prostaglandin treatments was bypassed and ovarian stimulation treatment was initiated one day after the expected day of ovulation. Thus, the immediate postovulatory follicular wave was the target in Treatment 2.

Figure 5.2 shows the treatment schedules targeting each follicular wave. In Treatment 1 (preovulatory), females were fed 6 mL of 2.2% altrenogest (Regu-mate®, DPT Laboratories, San Antonio, TX, USA) embedded in banana for 7 days (day 0 = day of first treatment). Animals received 267 mg and 133 mg of FSH in 30% PVP on days 4 and 6, respectively. Prostaglandin was also given on day 6 and ultrasound-guided oocyte retrieval was performed on day 8.
Figure 5.2. Treatment schedules to target preovulatory or postovulatory follicular waves in elands before conducting ultrasound-guided oocyte retrieval in Experiment 5.2a.
In Treatment 2 (postovulatory), altrenogest and prostaglandin were given as in Treatment 1. Day 9 was the expected day of ovulation and emergence of a new postovulatory follicular wave. Thus, 267 mg and 133 mg of FSH were given on days 10 and 12, respectively, and oocytes were recovered on day 14 (Figure 5.2). Eight procedures were conducted and compared in Experiment 5.2a. Subsequently, an additional 16 procedures were conducted using Treatment 1 (preovulatory) for estrous synchronization and ovarian stimulation.

5.2.2.3. Experiment 5.2b (Individual and Seasonal Variation in Response to Ovarian Stimulation)

Experiments 5.1 and 5.2a produced results that were suggestive of individual variation among the elands to ovarian stimulation treatments. To further explore this possibility, data from Experiments 5.1 and 5.2a were combined with data from 16 additional ovarian stimulation/oocyte retrieval procedures done as in Treatment 1 (preovulatory) of Experiment 5.2a. Data were combined because there were no treatment effects on the number of follicles and oocytes recovered. These data were used to test the effect of animals or months (season) on ovarian response to gonadotropin treatment (follicle development) and oocyte production.

5.2.3. Ultrasound-guided Follicular Aspiration for Oocyte Retrieval and Evaluation of Oocytes

5.2.3.1. Custom-made Adaptor for Ultrasound Transducer

The first two attempts to do oocyte retrievals were not successful because the commercially made adaptor of a 5-MHz, curvilinear ultrasound transducer (model UST-9111-5, Aloka Co. Ltd, Tokyo, Japan) could not be passed through the vestibule of the two eland. Also, the adaptor for a human transducer was not long enough to be able to scan the ovaries of either of the two females. Thus, an in-house custom-molded plastic adaptor (see Figure 5.3) was constructed for mounting the transducer. The design was based on the original adaptor but was made with smaller
Figure 5.3. Ultrasound transducer and a needle guide enclosed in the custom-made adaptor (A); B: Close-up views of the transducer tip with the custom-made (left) and original (right) adaptors. Note the ~1 cm reduction in the vertical dimension of the tip of the device in the custom-made adaptor as compared with the original adaptor.
dimensions. After encasing the transducer and the needle guide in the modified adaptor, the device was wrapped in self-fusing elastic tape (Tommy Tape, Manco, Inc Avon, OH, USA). Thus, an Aloka SSD-500V real time ultrasound scanner (Corometrics Medical Systems, Inc., Wallingford, CT, USA) and the 5 MHz transducer in a custom-molded plastic adaptor were used for ultrasound scanning of ovaries and follicular aspiration to collect oocytes.

5.2.3.2. Oocyte Collection and Evaluation

For oocyte retrieval, females entered a hydraulic chute (Chapter 4) and were positioned by adjusting the sides to prevent animals from turning around. Sedation was induced using 100 to 250 mg of xylazine with or without butorphanol tartrate. After sedation, females were further positioned and lifted using the hydraulic chute (Chapter 4). The sacrococcygeal area was prepared for administering epidural analgesia by clipping the hair and applying disinfectants. For the epidural block, 4 mL of 2% lidocaine HCl (Biomedica Inc, Riverside, MO, USA) was injected into the epidural space at the sacrococcygeal space or between the first and second coccygeal vertebrae. Paresis of the tail was used as an indication of a successful epidural block.

After manually cleaning the rectum and the perineal region of the animal, the vagina was rinsed with 30 mL of saline solution containing 1% lidocaine. Ultrasound transmission gel (Universal Medical Systems, Inc., Bedford Hills, New York, USA) was applied to the tip of the transducer. The latter, attached to the ultrasound unit, was lubricated (Lubogel-V™, Agtech, Inc, Manhattan, KS, USA) and covered with a condom before introducing into the vagina.

During rectal palpation, the dimensions of ovaries were estimated by using pre-calibrated distances on the index and thumb fingers of the technician performing the palpation. The number and size of ovarian follicles were recorded during ultrasound scanning.

An 18-g, 64-cm needle connected to a 50-mL centrifuge tube with a 65-cm long Teflon tubing was used for follicular aspiration, The needle and tubing were rinsed with aspiration medium (see later) and introduced through the needle guide attached to the transducer. All ovarian follicles
(3 mm) visible on the monitor of the ultrasound unit were punctured and aspirated using a negative vacuum pressure of 70 mm Hg, generated using a regulated pump (Model V-MAR-5000B, Cook Veterinary Products, Eight Mile Plains, Australia).

Follicular contents from each ovary were aspirated into separate tubes and held at ~39°C. The aspiration medium was TL-HEPES solution (04-616F, BioWhittaker Co, Walkersville, MD, USA) supplemented with 50 µg/mL of gentamicin and 10 U/mL of heparin. After each oocyte retrieval procedure, blood samples were collected, prophylactic penicillin-G was administered and sedation was reversed using tolazoline (Chapter 4). Initially, the transducer and needle guide were dismantled from the adaptor for cleaning; however, removing several centimeters of tape from the scanning portion of the transducer was found to provide adequate exposure of the device for cleaning.

After transporting aspirated fluid to the laboratory, oocytes were located using a stereomicroscope and graded as previously described (de Loos et al. 1989), but with slight modifications. The four quality grades were; A: multilayered, uniformly translucent cumulus investment and homogenous ooplasm, B: multilayered, less translucent cumulus investment and mostly homogenous ooplasm with some dark cortical areas, C: darker cumulus investment and irregular ooplasm, and D: dark and irregular cumulus investment and nonhomogenous ooplasm. Also, the degree of cumulus expansion was graded on a scale of 0 to 3: 0 (compact), 1 (slightly expanded), 2 (moderately expanded) and 3 (fully expanded) (Hinrichs and Williams 1997); however, expansion grades of 0 and 1 were categorized as “Compact” and 2 and 3 as “Expanded” for data analysis. Diameter of oocytes was measured on an Olympus inverted microscope equipped with a pre-calibrated eyepiece micrometer.
5.2.4. Oocyte Maturation and Embryonic Development in Vitro

5.2.4.1. Experiment 5.3 (In Vitro Maturation of Oocytes)

Initially (first portion of the study), IVM was done in medium 199 (TCM199, Gibco, Grand Island, NY, USA) supplemented with 10% heat inactivated bovine calf serum (BCS, Hyclone Laboratories, Logan, UT, USA), FSH, LH, estradiol, pyruvate and EGF (Krisher et al. 1999). Later (second portion of the study), the IVM medium was modified because of unsatisfactory maturation rates when using the previous protocol.

The modifications included use of TCM 199 (cat. # 9102, Irvine Scientific, Santa Ana, CA, USA), 1 mM L-glutamine, 1.2 mM L of cysteine and 5 µg/mL of insulin with LH, estradiol, pyruvate and EGF, as described above, but without FSH. In addition, either heat inactivated female eland serum collected during the oocyte retrieval procedures, or BCS were used. The modified IVM medium was used for oocytes obtained after incorporating altrenogest into the estrous synchronization protocol.

For IVM, ~10 oocytes were put into each 50 µL drop of medium under mineral oil in a 60 x 15 mm Falcon® polystyrene petri dish. Incubation was conducted at 38.5 ºC in an atmosphere of 5% CO₂ in humidified air.

In most experiments (n = 34 procedures), oocytes underwent IVM for ~24 hr before IVF, ICSI or SCNT (intraspecies, or interspecies using giant eland somatic cells). However, during four procedures, all or a sub-set of oocytes (n = 22) with fully expanded cumulus cell layers were selected and used for in vitro embryo production on the day of oocyte collection (~ 6 hr post collection) after visually determining their nuclear maturation status.

5.2.4.2. Experiment 5.4 (In Vitro Embryo Production and Embryo Culture)

In vitro fertilization was done in Tyrode’s-albumin-pyruvate (TALP) medium supplemented with heparin and PHE (Chapter 3) using frozen-thawed eland semen. The semen samples were
collected from two eland bulls using either electroejaculation or rectal massage. The sample collected by electroejaculation was cooled to 4°C in TEST yolk buffer. Four to 6% glycerol in TEST yolk buffer (Irvine Scientific) was added and the sample was loaded in 0.25 ml straws. Samples were then frozen on dry ice and then plunged into liquid nitrogen for storage.

Samples collected by rectal massage were extended in Tris-citrate based extender (Bilady®, Minitüb, Tiefenbach, Germany) containing 20% egg yolk. After cooling to 4°C (3 to 12 hr), the same extender containing 14% glycerol (final concentration =7%) was sequentially (10%, 20%, 30% and 40%) added at 10 min intervals and samples were loaded in 0.5 ml straws. Freezing was done by placing straws 8-cm above the surface of liquid nitrogen for 20 min and then plunging the straws in liquid nitrogen for storage.

The frozen-thawed eland spermatozoa were generally characterized by low motility (<40%) and reduced longevity of progressive motility during incubation at 38.5°C. Accordingly, the IVF procedures were modified by 1) supplementing the IVF microdrop with spermatozoa that were maintained at room temperature for 3 to 4 hr after initial IVF; 2) centrifugation (400 x g/15 min) for concentrating/cleaning semen samples, with a less frequent use of gradient centrifugation columns for sperm separation.

Moreover, during the latter part of the study, heparin was excluded due to preliminary observations suggesting a negative effect on the longevity of progressive motility of eland spermatozoa during IVF. Attempts were also made to increase the percentage of motile spermatozoa in IVF microdrops by depositing concentrated spermatozoa in two to three peripheral microdrops connected to the oocyte-containing drop via channels of medium under oil.

Nuclear transfer was carried out using cumulus cells of the oocyte donor or frozen-thawed epithelial cells of common eland bull (collected during rectal massage) or frozen-thawed skin fibroblast cells of a giant eland bull. Enucleation of the oocyte chromatin and injection of somatic
cells was carried out as described previously (Lanza et al. 2000a). Micromanipulation was done using a Nikon Eclipse (TE2000-S, Nikon Corp., Japan) inverted microscope with a Nikon UV light source (C-SHG1, Nikon Corp., Japan). Couplets were fused electrically (Lee et al. 2003) and chemically activated using 5 or 10 μM ionomycin and 2 mM 6-dimethylaminopurine (Lanza et al. 2000a; Lee et al. 2003).

Micromanipulation for intracytoplasmic sperm injection (ICSI) was carried out using the Nikon Eclipse inverted microscope described above for nuclear transfer. Frozen-thawed common eland spermatozoa were subjected to swim-up in a 15-mL centrifuge tube and a 5 to 10 μL aliquot from the top layer was placed in a 100-μL microdrop of 10% PVP. A single spermatozoon was immobilized using the injection pipette and injected directly into the ooplasm. After sperm injection, oocytes were activated using ionomycin and 6-DMAP as described above for nuclear transfer.

Embryo culture after IVF, ICSI or SCNT was carried out at 38.5°C in a humidified atmosphere of 5% CO₂, 5% O₂ and 90% N₂. Initially, HECM-6 was used for the first 2 to 3 days with further IVC in TCM-199 supplemented with pyruvate and 10% BCS (Krisher and Bavister 1999). However, due to poor embryonic development in the eland, different media were used during the course of the study with the goal of improving in vitro development. Additional media used for IVC included KSOM-20aa or BM-3-20aa-glucose supplemented with either bovine or eland serum (Chapter 3), modified Tyrode’s solution (Gomez et al. 2000), CR1aa, deer SOF (Berg and Asher 2003) and alpha minimum essential medium (Gibco, Grand Island, NY, USA) supplemented with 5% BCS.

5.2.4.3. Evaluation of Oocyte Maturation and Embryonic Development

Determination of in vitro maturation was made using Hoechst staining (before nuclear transfer) or by microscopic evaluation of the first polar body extrusion (during ICSI). Meiotic status of oocytes subjected IVF was retrospectively determined based on cleavage and/or by staining 48 hr
post insemination. Oocytes were stained using Hoechst 33342 (Lee et al. 2003) or Giemsa after a two-step fixation (Pope et al. 1993).

The nuclear maturation status of oocytes subjected to nuclear transfer was determined during the routine Hoechst staining done to identify the recipient oocyte chromatin. Determination of embryonic development after nuclear transfer was based on morphological evaluation for cleavage and on Hoechst staining. Staining involved fixing couplets or embryos in a paraformaldehyde-Triton X-100 solution, washing in PBS-BSA and mounting them in Hoechst-glycerol solution, as described previously (Lee et al. 2003).

Chromatin structure was examined and photographed using bright (Giemsa stain) or UV (Hoechst stain) light on an Olympus BX60F-3 (Olympus Optical Co. Ltd, Japan) connected to a Sony color video printer (Model UP-1800MD, Sony Corporation, Japan). An Olympus UV light source (Model BH2-RFL-T3) was used during fluorescent microscopy. Images were also taken using an Olympus digital camera (model DP11-N) attached to an inverted Olympus microscope (model IX70-S8F2).

The chromatin structure of oocytes was classified as germinal vesicle (GV), metaphase I or metaphase II. When the chromatin structure was difficult to determine, it was classified as “Not determined”. Embryonic development was evaluated both morphologically and by counting the number of nuclei after staining by one of the two methods described above.

5.2.5. Data Analyses

Effects of Folltropin®-V treatment (single vs. double injection) on ovary size were tested using the Mann-Whitney test while effects on the total number of follicles or oocytes were compared using the t-test. Treatment effects on the distribution of follicles by size, the morphological appearance of oocytes at recovery and the meiotic status of oocytes after IVM were tested using Chi-square analysis. The effects of animal and month on ovarian response and number of oocytes
recovered were compared using one-way ANOVA and the Kruskal-Wallis test (nonparametric ANOVA), respectively. Simple regression analysis was used to test the correlation between number of follicles observed and oocytes recovered. Analyses were done using the GraphPad Instat® version 3.00 for Windows 95 (GraphPad Software, San Diego, CA, USA). A P-value of 0.05 was considered statistically significant. Descriptive statistics were also used to present data.

5.3. Results

5.3.1. Experiment 5.1 [Single (day –2) vs. Double (day –4 and day –2) FSH Injections]

Ovaries were ovoid to spherical in shape. The size (= longest diameter) of the left and right ovaries were not different after ovarian stimulation when compared within single (1.7 ± 1.2 cm vs. 1.8 ± 0.8 cm; P = 0.8892) or double (2.7 ± 1.0 cm vs. 3.4 ± 1.0 cm; P = 0.2576) Folltropin®-V treatments, respectively. Thus, data on the size of both ovaries were combined to evaluate effects of treatment.

The type of Folltropin®-V treatment affected the size of ovaries, with the ovaries in the double treatment being nearly twice the size of ovaries from the single treatment (3.2 ± 1.0 cm vs. 1.7 ± 0.9 cm; P = 0.0013). Similarly, treatment affected the size distribution of follicles; however, there was no effect on the mean number of follicles or oocytes recovered or the oocyte recovery rate (oocytes recovered/follicles aspirated) (Table 5.1). After gonadotropin treatment, the number of follicles observed on either ovary ranged from 4 to 17.

Most of the recovered oocytes (89%, 98/110) in both treatments had intact zona pellucidae, homogenous ooplasm and multiple layers of cumulus cells (Table 5.2). The number of oocytes recovered ranged from 3 to 17 with an average of 8.0 and 8.9 oocytes recovered per procedure in single and double injection treatments, respectively (Table 5.1). Oocyte recovery rates ranged from 44 to 100% (mean = 73 to 78%). FSH treatment also affected (P = 0.0497) the morphological distribution of oocytes, with more degenerate/damaged oocytes in the single than in the double
injection treatment group (Table 5.2). Analysis of data after excluding degenerate/damaged oocytes showed that treatment type did not influence the degree of cumulus expansion (P = 0.5259).

5.3.2. Experiment 5.2a (Targeting the Preovulatory or Postovulatory Follicular Wave)

Whether gonadotropin treatment targeted the preovulatory (Treatment 1) or postovulatory (Treatment 2) follicular wave did not affect ovarian size, number of follicles or number and percentage of oocytes recovered. However, there was a tendency for more small-sized follicles and lower oocyte recovery rates when targeting postovulatory follicular waves (Table 5.3). From 1 to 14 and 3 to 9 oocytes were recovered per procedure in Treatments 1 and 2, respectively. The average number of oocytes recovered was numerically higher when targeting the preovulatory follicular wave, but the difference was not statistically significant.

Moreover, when the postovulatory wave was targeted, ovarian bleeding tended to increase during follicular aspiration, presumably due to the presence of corpora hemorrhagica, causing blockage of the aspiration tubing. Treatment affected the percentage of follicles >10 mm in diameter, but did not affect other parameters (Table 5.3).

In the 16 oocyte retrieval procedures subsequently done on seven elands, the preovulatory follicular wave was targeted for stimulation by gonadotropin treatment. The overall mean oocyte recovery rate in the 16 procedures was 80.7% (total oocytes = 192). The mean numbers of follicles observed and oocytes recovered were 14.9 ± 6.9 (range = 6 to 28) and 12.0 ± 8.0 (range: 3 to 28), respectively. The mean ovary size was 3.34 ± 0.95 cm (range = 1.5 to 5.0). When evaluated by the degree of expansion of cumulus cell layers, cumulus oocytes complexes recovered after estrous synchronization with altrenogest were more morphologically uniform in appearance than those recovered after estrous synchronization with 3x prostaglandin treatment.

In Experiments 5.1 and 5.2a, since treatment did not affect ovarian response (i.e., number of follicles) or the number of oocytes recovered, data from both experiments were combined to evaluate
effects of animal and month (season) in Experiment 5.2b. These factors were evaluated because of an apparent pattern of individual variation in ovarian response to gonadotropin treatment.

Table 5.1. Ovarian response and oocyte recovery rates in elands (n = 4) treated with Folltropin®-V given 2 (single) or 4 and 2 (double) days before ultrasound-guided oocyte retrieval

<table>
<thead>
<tr>
<th>FSH treatment</th>
<th>Procedures, n</th>
<th>Follicles, n (%) by size (mm)</th>
<th>Total follicles, n (mean ± SD)</th>
<th>Oocytes recovered, Total, n (Mean ± SD)</th>
<th>%*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Single</td>
<td>6</td>
<td>63 (10.5 ± 3.7)</td>
<td>48 (8.0 ± 3.2)</td>
<td>78</td>
<td></td>
</tr>
<tr>
<td>Double</td>
<td>7</td>
<td>84 (12.0 ± 4.6)</td>
<td>62 (8.9 ± 4.1)</td>
<td>73</td>
<td></td>
</tr>
<tr>
<td>P-value</td>
<td>-</td>
<td>0.0498</td>
<td>0.8483</td>
<td>0.0076</td>
<td>0.3338</td>
</tr>
</tbody>
</table>

*Values indicate percentage of oocytes recovered among the follicles detected.

a,b Values in the same column with different superscripts are significantly different (P<0.05).

Table 5.2. Morphological distribution of cumulus oocyte complexes recovered from eland donors (n = 4) treated with Folltropin®-V for 2 (single) or 4 and 2 (double) days before oocyte retrieval

<table>
<thead>
<tr>
<th>FSH treatment</th>
<th>Procedures, n</th>
<th>Cumulus oocyte complex morphology, n (%)*</th>
<th>Degenerate/damaged</th>
<th>Compact cumulus</th>
<th>Expanded cumulus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Single</td>
<td>6</td>
<td>22 (46)</td>
<td>9 (19)a</td>
<td>17 (35)</td>
<td></td>
</tr>
<tr>
<td>Double</td>
<td>7</td>
<td>38 (61)</td>
<td>3 (5)b</td>
<td>21 (34)</td>
<td></td>
</tr>
<tr>
<td>P-value</td>
<td>-</td>
<td>0.0295</td>
<td>0.2562</td>
<td>1.00</td>
<td></td>
</tr>
</tbody>
</table>

*Oocytes classified as having compact and expanded cumulus had uniformly homogenous ooplasm.

a,b Values in the same column with different superscripts are significantly different (P<0.05).
Table 5.3. Effect of targeting the preovulatory or postovulatory follicular wave on ovarian response to FSH treatment and oocyte recovery rates in elands (n = 6)

<table>
<thead>
<tr>
<th>Procedures, Ovarian size, cm</th>
<th>Follicles, n (%)&lt;sup&gt;a&lt;/sup&gt; by size, mm</th>
<th>Oocytes recovered Total, n (mean±SD) %&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment n</td>
<td>&lt;5</td>
<td>5 to 10</td>
</tr>
<tr>
<td>Preovulatory</td>
<td>5</td>
<td>3.2</td>
</tr>
<tr>
<td>Postovulatory</td>
<td>3</td>
<td>3.4</td>
</tr>
<tr>
<td>P-value</td>
<td>-</td>
<td>0.875</td>
</tr>
</tbody>
</table>

<sup>a</sup>Values in parentheses indicate percentage of total follicles per treatment. <sup>b</sup>Of total follicles/treatment. <sup>c,d</sup>Values in the same column with different superscripts are significantly different (P<0.05).

5.3.3. Experiment 5.2b (Individual and Monthly Variations in Response to Ovarian Stimulation)

There was a significant animal effect (P = 0.0105) on the number of follicles observed. This was mainly due to one female (eland 123) having more follicles than three other females (eland 128, 129 and 139; Figure 5.4). Similarly, there was a slight animal effect on the mean number of oocytes recovered (P = 0.0532).

Three to 10 oocyte retrieval procedures were done on each of the seven females. Overall, an average of 12.8 follicles was observed and a mean of 9.8 oocytes was recovered (363/475 or 76.4%). There was a strong correlation between the number of follicles observed and the number of oocytes recovered (r = 0.9079; Figure 5.6; P< 0.0001). Also, there was a significant correlation between percentage of oocyte recovery and the number of follicles observed (r = 0.4025; P = 0.0135); however, the latter correlation may be an artifact because of the difficulty of accurately estimating the number of follicles in highly responsive females.

Oocyte retrieval procedures were done in all months of the year, except December. There was no effect of month on the number of follicles observed (P = 0.4417) or number of oocytes recovered (P = 0.7692). The monthly distribution of procedures, follicles observed and oocytes recovered are shown in Figure 5.5.
Figure 5.4. Effect of animal on follicles observed and oocytes recovered from eland donors. 
\(^{a,b}\)Means with different letters are significantly different (P<0.05).
Figure 5.5. Mean number of follicles observed and oocytes recovered from eland donors during each month of the year.
Figure 5.6. Correlation between the number of follicles observed and oocytes recovered from eland donors.
5.3.4. Morphology of Eland Oocytes

Most eland oocytes (91.7% or 333/363) were morphologically normal in appearance as judged by criteria used for domestic ungulate oocytes (uniform or non-degenerate ooplasm, intact zona pellucida and multiple layers of cumulus cells). A small proportion had degenerate ooplasm (3.9%), lacked a cumulus cell layer (3.3%) or had damaged zonae pellucidae (1.1%; Figure 5.7).

The mean diameter of eland oocytes (n = 14) was 134.6 ± 10.3 µM. The mean thickness of the zona pellucida was 16.9 ± 5.3 µM; and the mean total diameter, including the zona pellucida, was 172.5 ± 22.4 µM. The only effect of estrous synchronization and ovarian stimulation treatments on cumulus oocyte complex morphology was on the degree of cumulus cell layer expansion.

5.3.5. Experiment 5.3 (In Vitro Maturation of Eland Oocytes)

The frequency of oocytes maturing to the metaphase-II stage varied between 56% and 73%. None of the three variables evaluated (course of the study, serum type or interval post-oocyte recovery) affected the meiotic status of oocytes (P > 0.05; Table 5.4).

Analysis of the data after excluding oocytes in which chromatin structures could not be determined indicated that the post-oocyte recovery interval affected the distribution of oocytes by meiotic status (P = 0.0446) with a tendency for a higher percentage of metaphase II oocytes at 24 hr post-recovery. The other two variables, course of study and serum type, did not affect the distribution of oocytes by meiotic status.

5.3.5. Experiment 5.4 (In Vitro Embryonic Development of Eland Oocytes)

When three methods (IVF, ICSI and somatic cell nuclear transfer, SCNT) of in vitro embryo production were evaluated, from 18 % to 55 % of the metaphase II oocytes developed to 2-cell stage, most of which stopped development at ≤8-cell stage (Table 5.5; Figure 5.8). The only
blastocysts were derived after SCNT of common eland cells into enucleated eland oocytes. Three of the four blastocysts were produced using male epithelial cells and the other blastocyst developed after SCNT using cumulus cells from the oocyte donor.

Initially, the consistently poor in vitro development after IVP of eland oocytes was considered to be due to an inappropriate culture medium. Therefore, in an effort to improve cleavage frequency and embryo development in vitro, several different media were examined during the course of the study (See Material and Methods). Even so, no further improvement in in vitro development was observed in any single medium or combination of media.

Accordingly, it is inappropriate to discuss the effects of media, other than to say that, under our culture conditions, none of the media used in the present study were able to adequately and reliably support high rates of cleavage and subsequent development in vitro of eland embryos. Nonetheless, development of one SCNT embryo to the blastocyst stage occurred during culture in CR1aa medium supplemented with 5% eland serum (Figure 5.9). The other three blastocysts developed during culture in alpha-MEM supplemented with pyruvate (0.25 mM) and 5% bovine calf serum. Some of the developmental stages of eland oocytes after IVP are shown in Figures 5.8 and 5.9.

Figure 5.7. Morphological appearance of eland cumulus-oocyte complexes recovered by ultrasound-guided transvaginal follicular aspiration:
A: fully expanded cumulus cell mass
B: expanded cumulus cell mass with most cells dissociated
C: few cumulus cells, a damaged zona pellucida and ruptured oolemma
Bar = 50 µm
Table 5.4. Effect of three factors on the in vitro maturation (meiotic status) of eland oocytes recovered after Folltropin®-V-induced ovarian stimulation.

<table>
<thead>
<tr>
<th>Factors *</th>
<th>Procedures, n</th>
<th>Oocytes evaluated, n</th>
<th>GV, n (%)</th>
<th>Metaphase I, n (%)</th>
<th>Metaphase II, n (%)</th>
<th>Not determined, n(%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Portion of the study</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>First</td>
<td>13</td>
<td>81</td>
<td>4 (4.9)</td>
<td>11 (13.5)</td>
<td>45 (55.5)</td>
<td>21 (25.9)</td>
</tr>
<tr>
<td>Second&lt;sup&gt;a&lt;/sup&gt;</td>
<td>20</td>
<td>228</td>
<td>13 (5.7)</td>
<td>25 (11.0)</td>
<td>157 (68.9)</td>
<td>33 (14.5)</td>
</tr>
<tr>
<td>Serum type</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Eland</td>
<td>14</td>
<td>161</td>
<td>10 (6.2)</td>
<td>14 (8.7)</td>
<td>113 (70.2)</td>
<td>24 (14.9)</td>
</tr>
<tr>
<td>Bovine</td>
<td>6</td>
<td>67</td>
<td>3 (4.5)</td>
<td>11 (16.4)</td>
<td>44 (65.7)</td>
<td>9 (13.4)</td>
</tr>
<tr>
<td>Duration post recovery&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>~6 hr</td>
<td>4</td>
<td>22</td>
<td>3 (13.6)</td>
<td>4 (18.2)</td>
<td>12 (54.5)</td>
<td>3 (13.6)</td>
</tr>
<tr>
<td>~24 hr</td>
<td>16</td>
<td>139</td>
<td>7 (5.0)</td>
<td>10 (7.2)</td>
<td>101 (72.7)</td>
<td>21 (15.1)</td>
</tr>
</tbody>
</table>

*Factors did not affect the distribution of oocytes by each chromatin structure; however, the duration post recovery affected the distribution when data were analyzed after excluding “not determined” oocytes.

<sup>a</sup>The IVM medium was modified from that used in the first portion of the study (see text).

<sup>b</sup>Some oocytes with fully expanded cumulus cell layer were selected for evaluation on the day of recovery (~6 hr).

<sup>c</sup>Excludes degenerate or damaged oocytes or those lost during handling.

<sup>d</sup>Includes oocytes for which the meiotic status could not be determined after staining.
Figure 5.8. (A-J) Status of eland oocytes after applying IVM, IVF (A-H) or SCNT with giant eland somatic cells (I, J):

A-H: note the presence of one (A, E, F, G) or two (B, H) polar bodies; 0 (A, E, H) or ≥ 1 (B, C, D, F, G) spermatozoa (arrows) attached; and a 3-cell (C) and a 5-cell (D) IVF/IVC-derived eland embryos.

I, J: 3- and ~8- Hoechst-stained nuclei, respectively, present after SCNT with giant eland cells and IVC.

K. Day-9 blastocyst that developed after SCNT of a giant eland cell into enucleated bovine oocyte.

Bar = 50 µm (all except H).

H = 400x.
Table 5.5. Development of eland oocytes subjected to three methods of *in vitro* production (IVP) of embryos.

<table>
<thead>
<tr>
<th>IVP type</th>
<th>Procedures, n</th>
<th>Metaphase II oocytes, n</th>
<th>≥2-cell</th>
<th>3- to 8-cell</th>
<th>&gt;8-cell</th>
<th>Morula</th>
<th>Blastocyst</th>
</tr>
</thead>
<tbody>
<tr>
<td>IVF</td>
<td>15</td>
<td>55</td>
<td>10 (18.2)</td>
<td>6 (10.9)</td>
<td>1 (1.8)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>ICSI</td>
<td>9</td>
<td>44</td>
<td>19 (43.1)</td>
<td>9 (20.5)</td>
<td>1 (2.3)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>SCNT</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Common eland</td>
<td>9</td>
<td>48</td>
<td>26 (54.1)</td>
<td>22 (45.8)</td>
<td>8 (16.7)</td>
<td>7 (14.5)</td>
<td>4 (8.3)</td>
</tr>
<tr>
<td>Giant eland</td>
<td>2</td>
<td>11</td>
<td>6 (54.5)</td>
<td>5 (45.5)</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

*The remaining MII oocytes were used for activation experiments (data not shown), which indicated that eland oocytes were activated at a higher rate in 10 µM of ionomycin than in 5 µM of ionomycin (Dr. Philip Damiani, personal communication).

Figure 5.9. Day 7 blastocyst (400x) that developed after SCNT into an eland oocyte using a cumulus cell from the same oocyte donor.
5.4. Discussion

In Chapter 4, the feasibility of handling female eland in a hydraulic chute for the purpose of conducting assisted reproductive procedures was demonstrated. However, initially, it was not possible to access the eland vaginal vault for ultrasound-guided oocyte retrieval with the commercially available adaptor widely used in domestic cattle. A similar instrument has been successfully used for transvaginal oocyte retrieval in several ungulate species that are smaller in size than common eland, including mountain bongo, red deer and wapiti (Pope et al. 1998b; Berg and Asher 2003).

It was not possible to determine if the difficulty in accessing the vaginal vault was related to parity status because that information was not known for the eland used in the present study. Since considerable body size variation does exist among female eland, it is certainly possible that the commercially available adaptor may be usable on some females; however, we were not able to use it with 5 females. The custom-molded modified adaptor we designed/built proved to be a reliable instrument and was used throughout the course of the study. Unfortunately, several weeks were required to design, build and validate the modified adaptor, which delayed the start of the ovarian stimulation/oocyte retrieval experiments. Therefore, based on our experience, for similar experiments in nondomestic ungulates, it is recommended that the size and patency of the vestibular/vulvar passage be determined before initiating treatments/procedures.

In Experiment 5.1, it was demonstrated that one (single) or two (double) injections of porcine FSH induce ovarian stimulation and that both treatments produce a similar yield of oocytes after ultrasound-guided follicular aspiration. The significant increase in the size of ovaries in the double FSH injection treatment group was clearly indicative of the ovarian response to treatment. Although ultrasound-guided oocyte retrieval in domestic cows is commonly done without inducing ovarian stimulation using exogenous gonadotropins, an attempt to collect oocytes in an eland female without
gonadotropin treatment was unsuccessful because there were no follicles of sufficient size for aspiration. Moreover, a maximum of 4 follicles were detected during 4 weeks of twice-weekly ultrasound scanning of the ovaries of an eland that did not receive gonadotropin treatment (personal observation).

In domestic cattle, oocyte production without ovarian stimulation is maximized by aspirating follicles at 2 to 3 day intervals because removal of the dominant follicle leads to the growth of a new cohort of small follicles. While it was demonstrated that eland can be conditioned for handling in a chute, they are still nondomesticated animals; therefore, it may not be possible to perform multiple oocyte retrievals at frequent intervals as practiced in domestic cattle. Thus, it appears that inducing follicular development with exogenous gonadotropins is an essential component of the treatment protocol for successful ultrasound-guided retrieval of oocytes in the eland.

Previous studies on FSH-induced ovarian stimulation in the common eland indicate that the average ovulation rate (number of corpora lutea) or number of ova/embryos recovered have ranged from 8.4 to 12.3 after twice-daily injections of FSH over a period of 5 days (Schiewe et al. 1991b; Pope and Loskutoff 1999). In the present study, after single or double injections of FSH, an average of 12.8 follicles was detected and an average of 9.8 oocytes was recovered, suggesting that ovarian response after single or double injections of FSH may be similar to that obtained after twice daily FSH injections for 5 days. However, the proportion of eland follicles that would eventually ovulate after ovarian stimulation with a reduced number of FSH injections, as described in the present study, remains to be determined. In domestic cows, ovulation frequencies were reported to be similar when FSH dissolved in saline was administered in multiple injections or it was dissolved in 30% PVP and given as single injection (Yamamoto et al. 1994).

In ungulates, cumulus cell layer expansion and progression of meiosis from the germinal vesicle to the metaphase II stage are mediated by LH. In domestic cows, cumulus cell expansion after FSH (Folltropin®-V) treatment is minimal when pulsatile release of endogenous LH is suppressed by
concomitant administration of a GnRH agonist (Lindsey et al. 2002). In Experiment 5.1 of the present study, regardless of the treatment type, ~35% of eland oocytes had expanded cumulus cell layers at the time of recovery (Table 5.2). Oocytes with similar morphology were also frequently observed during Experiment 5.2. The cumulus cell expansion observed in eland oocytes after gonadotropin treatment may be a result of increased frequency of the endogenous pulsatile secretion of LH, a surge of endogenous LH or a direct consequence of the LH present in the exogenous FSH used for injection.

In Experiment 5.1, we anticipated that the longer exposure of follicles to FSH (96 hr vs. 48 hr) in the double FSH treatment group would produce oocytes with more cumulus cell expansion. However, cumulus cell expansion was similar in both the single and double FSH injection treatment groups. A possible explanation for the lack of difference between treatments may be that the extent of cumulus expansion is determined mainly by the length of time between the preovulatory administration of prostaglandin and oocyte collection. This interval was ~48 hr in both treatment groups.

In Experiment 5.2, the stimulation of follicular development obtained by targeting the follicular wave that emerges immediately after ovulation provided indirect evidence for the emergence of a follicular wave following ovulation. This evidence of a postovulatory follicular wave, along with the known preovulatory follicular wave that leads to ovulation, is indicative that the common eland has at least two follicular waves, as seen in other ruminants (Adams 1999). Confirmation of this will require frequent monitoring of follicular dynamics throughout the estrous cycle. Nevertheless, there was no specific advantage of targeting the first (postovulatory) follicular wave in terms of oocyte yield. Moreover, this treatment required a longer treatment period (15 days) as compared with targeting the preovulatory wave (9 days).

Oral administration of altrenogest, combined with a single injection of prostaglandin, was effective for estrous synchronization in the eland. Altrenogest treatment required fewer handling
events than needed for estrous cycle synchronization using multiple (3x) prostaglandin injections. Furthermore, its use appeared to produce a more uniform group of oocytes, although its effects on oocyte development are not known. Another indication that the estrous synchronization protocols were effective was the basal levels of serum progesterone found in samples (n = 30, data not shown) taken at the time of oocyte recovery.

The oocyte recovery rate obtained in the present study (76%) was relatively higher than most rates reported in domestic cattle. In most of the eland procedures with the lowest oocyte recovery rates, technical problems occurred, such as blockage of the aspiration tubing. Since it appears that almost all aspirated follicles can produce oocytes, the high recovery rate suggests that the cumulus oocyte complex is loosely attached to the follicular wall. Cell-to-cell contact may be loosened by ovarian stimulation treatment, as previously suggested to occur in gonadotropin-treated domestic cattle (Gordon 1994).

In domestic cattle, the mean number of oocytes recovered by ultrasound-guided follicular aspiration in heifers was 9.1 after twice daily treatment with FSH (20 mg divided into six equal doses) for three consecutive days or 7.1 oocytes when FSH was administered in decreasing doses (Sirard et al. 1999). In another study using crossbred domestic cows (Goodhand et al. 2000), it was reported that the average number of oocytes recovered after single (5.3 oocytes) or multiple (5.9 oocytes) FSH injections were similar and did not differ from that of nontreated females (4.1 oocytes); however, more follicles were observed in FSH treated cows.

One of the highest numbers of oocytes recovered using ultrasonography in an ungulate species was achieved after a single FSH injection in gaur cows primed with Synchromate-B® for 5 to 7 days (Armstrong et al. 1995). The females received a single injection of 200 mg of Folltropin®-V dissolved in 1% carboxymethylcellulose and 2% Tween-80. An average of 34.3 (n = 4) oocytes/cow were recovered on the third day after gonadotropin treatment and 15 to 17 % of the oocytes developed to the blastocyst stage after IVF/IVC. A pregnancy was produced after transferring one of
the blastocysts to a domestic cow. Perez (2003) reported an average of 17.6 follicles per treatment after administering a single injection of FSH dissolved in 30% PVP to postpartum beef cows. After IVF/IVC, ~30% of the oocytes developed to blastocysts. These studies indicate that gaur and bovine oocytes recovered after a single injection of FSH are developmentally competent.

Several factors influence ovarian response and oocyte recovery rate, including season, individual animal variation and ovarian status at the initiation of gonadotropin treatment (Adams and Pierson 1995). Another factor affecting oocyte recovery rate is technician efficiency; however, this effect was not evaluated in the present study because one person (the author) did all of the oocyte recovery procedures. In the present study, month of the year (or season) did not have a detectable influence on ovarian response to FSH treatment; however, there was a significant animal effect on the number of follicles observed after gonadotropin treatment (Figure 5.4). Moreover, the effect of animal on the number of oocytes recovered approached statistical significance (P = 0.0532).

Although the cause for such variation is unknown, similar individual differences in embryo yield among eland donors after gonadotropin-induced ovarian stimulation have been documented (Dresser et al. 1984). In the latter report, transcervical flushing of the uterus produced an average of 8 to 10 ova/embryos, including one female from which, 31 embryos were recovered. Thus, individual differences in superovulatory response, rather than disparate fertilization rates, may have been the primary cause of the variation in embryo yield.

The mean diameter of eland oocytes (135 µm) was larger than that of domestic cattle oocytes [(114 µm, (Otoi et al. 1997)] collected from antral follicles, but smaller than that of Asian buffalo oocytes [146 µm, (Raghu et al. 2002)]. Most domestic bovine oocytes (82%) recovered from slaughterhouse ovaries had diameters ranging between 110 and 125 µm, and only 5% were larger in size than 130 µm (Otoi et al. 1997).
Some embryonic development occurred after IVF, ICSI and SCNT of eland oocytes. Even though *in vitro* maturation rates were satisfactory, embryonic development was very poor after homologous IVF of eland oocytes. The almost complete lack of development after IVF was, to some extent, due to the low motility of the eland spermatozoa. Alternatively, standard domestic bovid IVF methods may not produce satisfactory results in the eland.

Cleavage frequency was similar after nuclear transfer into enucleated common eland oocytes of either common eland or giant eland somatic cell nuclei. The development of blastocysts in the former group may be because more replicates were attempted. This poor development is in contrast to the more acceptable *in vitro* development (up to 77% cleavage, and 27% blastocyst formation) found when giant eland nuclei were transferred into enucleated bovine oocytes and cultured under similar conditions (Damiani *et al.* 2003). These results demonstrate that the ooplasm environment is a critical factor influencing the development of eland nuclear transfer couplets.

Clearly, methods for activation of couplets and subsequent *in vitro* culture of eland embryos are suboptimal. Nevertheless, the present study presents the initial demonstration that *in vitro* derived eland embryos can be produced and provides the framework for further research. Additional studies focusing on ultrastructural, molecular and biochemical events will provide further insights into the biology of eland gametes/embryos and such information may be used to improve methods of *in vitro* embryo production in this species.

To conclude, the present study has demonstrated that an average of 9.8 oocytes can be collected in sedated common elands after a single or double injection(s) of FSH. The number of follicles available for oocyte collection after one or two injections of FSH was similar to observations in other eland studies in which FSH was given as multiple (~10) injections over several days. Eland oocytes are capable of undergoing *in vitro* maturation and some embryonic development *in vitro*. 
Future work to define and understand factors that influence *in vitro* developmental competence of oocytes is warranted, including studies on basic aspects of eland gamete physiology, cryobiology of eland spermatozoa and molecular/cytological analysis of embryos to verify fertilization and embryonic genome activity. Moreover, endocrine profiles associated with ovarian stimulation and their effect on the yield and developmental competence (*in vitro*) of eland oocytes require characterization.
Chapter 6. Summary and Conclusions

The first part of the study (Chapter 3) evaluated several key components and attributes of embryo culture media to determine the requirements of bovine embryos in protein-free media. In Experiment 1, the effects of a group of either 20 (glutamine + essential + non-essential) or 11 (HECM-6) amino acids were evaluated in modified KSOM or Basic Medium (BM)-3. Results showed that modified KSOM containing 20 amino acids (mKSOM-20aa) supported the highest frequency of total, expanded (days 7, 8, and 9) and hatched blastocysts. This experiment demonstrated that the beneficial effects of amino acids on the *in vitro* development of bovine embryos are influenced by the base medium. The huge difference in embryonic development between two base media (BM-3 and KSOM) was puzzling. Thus, a second experiment was designed to identify the factors responsible for the developmental disparity.

In Experiment 2, the effects of glucose, pyruvate, lactate, phosphate or all four supplements were evaluated in low (255) or high (275) osmotic pressure BM-3 containing 20 amino acids (BM-3-20aa). Supplement type affected the frequency of development to at least the morula stage (day 7), expanded (day 8), hatched (day 9) or total blastocysts and cell number per blastocyst. Osmotic pressure affected the frequency of expanded blastocysts (day 7) and blastocyst cell number, with a tendency for higher frequencies and more cell numbers in the low osmotic pressure treatments.

Regardless of the osmotic pressure, BM-3-20aa containing glucose (0.2 mM) supported the highest frequency of blastocyst development. The interaction between supplement type and osmotic pressure was not significant; however, treatment mean differences were more discernible in high than in low osmotic pressure medium. Glucose was identified as the most crucial supplement of BM-3-20aa and its addition led to embryonic development equivalent to that observed in protein-free KSOM. The higher frequency of embryonic development in glucose containing media as compared
with pyruvate- and/or phosphate-supplemented media suggests that glucose plays more important roles in non-energy generating pathways.

Difficulties and risks associated with restraining large nondomestic ungulates are limiting factors to the development and application of assisted reproductive technologies, such as artificial insemination and embryo transfer. Thus, the next studies evaluated the use of behavioral training and handling of common eland females in a hydraulic chute on the possibility of doing transvaginal ultrasound-guided oocyte retrieval without inducing general anesthesia. Some physiological responses of elands to the handling and factors influencing them were assessed. The *in vitro* developmental potential of eland oocytes was also evaluated.

In Chapter 4, 9 of 10 females were conditioned to associate specific sound cues with food treats. The interval from the audio cues until acceptance of handheld treats varied among females. Animals also differed in their response to training for voluntary entry into the chute.

Handling eland for doing oocyte retrievals in the hydraulic chute required sedation, which was achieved using xylazine, either alone or in combination with butorphanol. During sedation and handling, elands undergoing oocyte retrieval procedures had higher blood glucose levels than females handled similarly but without doing oocyte retrieval. Females that were more difficult to train to the chute had higher blood glucose levels than the more cooperative animals.

Other procedures including embryo transfer, ultrasound examination of the reproductive tract, hoof trimming, body weight measurement and surgical correction of a persistent vaginal hymen were also done without inducing general anesthesia and, in some cases, without sedation. There were no complications, injuries or capture myopathy in more than 40 handling procedures. However, the high blood glucose (and slightly elevated CPK) levels occurring during sedation and handling for oocyte retrieval suggest the need to minimize the level of physical exertion and the associated stress. Preliminary data suggested that certain physical attributes (number of lateral stripes) might be
associated with taming potential of elands; however, the confirmation of actual associations requires the study of larger sample sizes.

In Chapter 5, data on ovarian response to gonadotropin treatments, the number and rate of oocytes recovered and the *in vitro* developmental potential of eland oocytes were presented. Ultrasound-guided oocyte retrieval in the eland required designing an adaptor for the transducer that was smaller than the commercial adaptor. A single or double injection of FSH dissolved in 30% polyvinyl pyrrolidone induced acceptable ovarian stimulation for oocyte retrieval. Modifications of the estrous synchronization or ovarian stimulation protocol did not affect ovarian response or number of oocyte recovered; however, daily oral administration of altrenogest combined with a single injection of prostaglandin was effective for estrous synchronization and enabled oocyte collection within 9 days of the start of synchronization/ovarian stimulation treatments.

In 37 oocyte retrieval procedures, an average of 9.8 oocytes were recovered (76% recovery rate) by transvaginal ultrasound-guided follicular aspiration. Most of the oocytes appeared morphologically normal and up to 73% matured to the metaphase II stage after 6 or 24 hr of *in vitro* maturation. On applying three methods of *in vitro* embryo production (IVF, ICSI, SCNT) to the eland oocytes, cleavage frequency varied between 18% after IVF to 55% after nuclear transfer. Most of the cleaved embryos stopped development at ≤ 8-cell stage. The only blastocyst development (n = 4; 8.3%) was observed after SCNT using common eland cells.

In addition to determining the role of key attributes and/or components of defined (protein-free) embryo culture media, the experiments on bovine embryo culture served as a background quality control for the eland IVP studies. Many of the eland embryo production experiments were simultaneously carried out either on the same day or during the same week as the domestic bovine studies, using similar embryo culture components.
The poor *in vitro* embryonic development in elands using protocols that produced acceptable bovine embryo development in the same laboratory by the same technician suggests that either 1) factors other than embryo culture media are more important in supporting the development of eland embryos *in vitro*, or 2) eland oocytes/embryos differ in their *in vitro* culture requirements. Thus, additional studies are required to improve the development of eland oocytes *in vitro*. 
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Appendix A: Attributes of Species of Tragelaphine Antelopes

<table>
<thead>
<tr>
<th>Species/common name</th>
<th>Gestation length, d&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Chromosomes, 2n male : female</th>
<th>Adult female weight, kg</th>
<th>Age at puberty, mo&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Estrous cycle length, d&lt;sup&gt;d&lt;/sup&gt;</th>
<th>Estrus duration, d&lt;sup&gt;d&lt;/sup&gt;</th>
<th>Population status (IUCN)&lt;sup&gt;e&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>T. derbianus</em>, Giant eland</td>
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<td></td>
<td></td>
<td></td>
<td>356&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>LR/NT</td>
</tr>
</tbody>
</table>

<sup>a</sup>Figures in brackets, [], indicate the reference number; <sup>b</sup>Our observation; <sup>c</sup>months; <sup>d</sup>days; <sup>e</sup>Lower risk (LR), Near threatened (NT), Conservation dependent (CD), Endangered (EN).
References for Appendix A


Appendix B: Abbreviations

aa = amino acids
AI = Artificial insemination
ART = Assisted reproductive technology
BCS = Bovine calf serum
BM = Basic medium
BME = Basal medium Eagle
BSA = Bovine serum albumin
CPK = Creatine phosphokinase
d = day(s)
EGF = Epidermal growth factor
ET = Embryo transfer
FCS = Fetal calf serum
FSH = Follicle stimulating hormone
GIFT = Gamete intrafallopian transfer
GV = Germinal vesicle
HECM = Hamster embryo culture medium
hr = Hour(s)
i.m. = Intramuscular (injection)
i.v. = Intravenous (injection)
ICSI = Intracytoplasmic sperm injection
iET = interspecies embryo transfer
iSCNT = interspecies somatic cell nuclear transfer
ISIS = International Species Information System
IUCN = International Union for Conservation of Nature and Natural Resources
IVC = In vitro culture of embryos
IVF = In vitro fertilization
IVM = In vitro maturation of oocytes
IVP = \textit{In vitro} production (of embryos)

KSOM = Potassium simplex optimized medium

LH = Luteinizing hormone

m (e.g., 56 m) = Meter

m (e.g., mKSOM) = modified

MI or MII = metaphase I or II stage oocyte

Medarks = Medical Animal Records Keeping Systems

MEM = Minimal essential medium

min = Minute(s)

mo = Month(s)

mol wt = Molecular weight

PBS = Phosphate buffered saline

PCR = Polymerase chain reaction

PHE = Penicillamine-hypotaurine-epinephrine

PVA = Polyvinyl alcohol

PVP = Polyvinyl pyrrolidone

SART = Society for Assisted Reproductive Technology

SCNT = Somatic cell nuclear transfer

sec = Second

TALP = Tyrode’s solution containing bovine serum albumin, lactic acid and pyruvic acid

TCM = Tissue culture medium

TL-HEPES-PVA = Tyrode’s solution supplemented with lactic acid, HEPES and PVA

yr = Year
Vita

Gemechu Wirtu was born in Ethiopia in 1968. He is the son of Wirtu Gerba Amba and Degetie Waqjira Nono. He grew up in rural Ethiopia. In 1992, he graduated in veterinary medicine from Addis Ababa University. Subsequently, he was employed by the same university where he taught courses to veterinary students. He also held different administrative positions and was involved in several research projects. In 1997, he won the Fulbright Scholarship to complete two years of graduate studies in the USA and received a Master of Science degree from Virginia Polytechnic Institute and State University in 1999. His thesis research work focused on xenogenous transfer of equine gametes and was conducted at the Virginia-Maryland Regional College of Veterinary Medicine.

In Fall 1999, Gemechu was accepted as a doctoral student through a joint program between Louisiana State University School of Veterinary Medicine and Audubon Institute Center for Research of Endangered Species. His dissertation focused on the development of methods of gamete collection in the eland antelope and in vitro production of embryos in cattle and eland. Drs. Barry Bavister, Robert Godke, Earle Pope and Charles Short supervised his doctoral research. Gemechu will receive his doctoral degree in May 2004.

He is married to Enkutatash Tadesse. Together, they are parents of Fidu, Milki and Kulani.