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The Applications of Cloning to the Conservation of Rare and Endangered Species

BY

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A Master's Degree Project submitted to the Faculty of Environmental Design in partial fulfillment of the requirements for the degree of Master of Environmental Design (Environmental Science)

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The undersigned certify that they have read, and recommend to the Faculty of Environmental Design for acceptance, a Master's Degree Project entitled:

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ABSTRACT

THE APPLICATIONS OF CLONING FOR THE CONSERVATION OF RARE AND ENDANGERED SPECIES

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Over the last few decades, advances in plant and animal cloning technologies such as micropropagation and somatic cell nuclear transfer have resulted in potentially useful tools to address the challenges associated with conserving rare and endangered species. Given the demonstrated ability of cloning technologies to reproduce rare and endangered species, there is a need to evaluate the advantages and disadvantages of more broadly applying cloning for the purposes of species conservation. This Master's Degree Project presents a theoretical framework describing the current and potential ability of cloning applications to provide options for the conservation of rare and endangered species. An evaluation of the costs and benefits yields the conclusion that given the appropriate species and circumstance, cloning may be a useful tool to achieve specific conservation goals. Cloning may be particularly valuable in conserving critically endangered species by providing a method of reconstructing genetic variability and reproducing species that have proven difficult to reproduce by other approaches. However, cloning is not a comprehensive 'solution' as it does not address many of the challenges associated with species conservation. Cloning should only be used in the context of a comprehensive management plan capable of addressing holistic conservation issues and coordinating various conservation efforts to maximize the benefits and minimize the risks of each approach. Finally, cloning is a reactionary approach to conservation, capable only of addressing current problems rather than their prevention. Thus, cloning does not address the definitive causes of many species extinctions, including man-kind's interactions with nature.

Keywords: cloning, conservation, rare species, endangered species, somatic cell nuclear transfer, micropropagation, tissue culture, cost-benefit evaluation, management recommendations.

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List of Abbreviations

ACT Advanced Cell Technology

AI Artificial Insemination

ART Assisted Reproductive Technologies

CITES The Convention on International Trade in Endangered Species

DNA Deoxyribonucleic Acid

ET Embryo Transfer

IVP *In Vitro* Embryo Production

IVF *In Vitro* Fertilization

MDP Masters Degree Project

mtDNA Mitochondrial DNA

NT (Somatic Cell) Nuclear Transfer

PETA People for the Ethical Treatment of Animals

SARA Species At Risk Act

USFDA The United States Food and Drug Administration

WWF World Wildlife Fund

Chapter 1: Introduction

1.1 Purpose

The purpose of this Masters Degree Project (MDP) is to present a theoretical framework describing the current and potential ability of cloning applications to provide 'solutions' to the problems of conserving rare and endangered species. The results of the project are condensed in this report, which may serve as both a literature resource in an emerging field of interest, and as a decision-making tool for agencies contemplating the risks and benefits of the use of cloning applications for conservation. The project may also serve a role in providing a basis for the future re-evaluation and research regarding the role of cloning technology in conservation.

1.2 Research Need

Several problems are typically encountered in attempts to conserve rare and endangered plant and animal species. Certain categories of species are vulnerable to decline and extinction due to their biological characteristics and more immediate mechanisms such as exploitation, stochastic events, habitat changes or an alteration of biotic interactions often cause species to decline. Conservation becomes difficult as species are reduced to a few (or) small declining populations with reduced genetic diversity as a result of genetic drift and inbreeding (Primack, 1998). Individuals may be unwilling or unable to reproduce effectively enough to maintain stable populations.

Over the last few decades, advances in plant and animal cloning techniques such as micropropagation and somatic cell nuclear transfer have resulted in new options for the conservation of rare and endangered species (Mitalipov & Wolf, 2000), potentially capable of addressing some of these challenges. Although many of these applications have already been suggested, there is a need to weigh the risks, benefits and overall appropriateness of these cloning applications as conservation tools in order to provide a sound basis of decision for those contemplating their use. In essence, this MDP will aid

in providing the basis for determining whether cloning can provide viable conservation options for conserving rare and endangered species, now and in the future.

1.3 Relevance of the Research Project

This MDP theorizes about the applications of cloning in improving existing conservation practices and in providing 'solutions' to the problems encountered in attempting to conserve rare and endangered species. It explores the pertinent scientific and value-based issues that affect the ultimate employability of the theoretical solutions, and outlines the costs and benefits of using cloning for conservation.

1.4 Objectives Regarding Rare and Endangered Species

1. Identify the challenges faced by current approaches to species conservation
2. Describe the current state of cloning technology
3. Outline the present uses of cloning technology for conservation
4. Identify how cloning applications could provide specific solutions to conservation challenges
5. Evaluate the risks and benefits of using cloning technology for the conservation
6. Identify the pertinent technical, environmental and social issues that may prevent the implementation of cloning applications
7. Identify stakeholder views of how and should cloning technology be applied to conservation
8. Theorize as to the future applications of emerging and current technologies for the specific use of conserving species
9. Provide policy and management recommendations on the use of cloning for conservation

1.5 Methodology

1.51 Literature Review

Relevant refereed scientific journals and published scientific books were used as sources of technical data. In addition, magazines, websites and newsletters were used as sources for theorizing as to the possible applications, risks and benefits of using cloning for conservation.

The purposes of the literature review were to:

- Identify the challenges associated with conserving rare and endangered species
- Identify the current approaches to conservation
- Describe the current state of cloning technology and its expected and potential applications for rare and endangered species
- Identify the social and environmental risks and benefits of using cloning for conservation

1.52 Key Informant Interviews

To supplement the information gained through the literature review and to provide expert opinion and a broader perspective of the issue, non-structured and semi-structured interviews took place with key stakeholder groups, scientists and professionals working, or that have worked in the fields of biotechnology and/or conservation.

Interviewees were selected (based on their expertise and availability) from four broad categories: Industry, Regulation and Policy, Zoos and Botanical Gardens, and Conservation Groups. The specific format of the interviews varied from semi-structured interviews carried out via email to obtain information from interviewees not easily reached by telephone, to non-structured interviews that were more conversational in nature. Appendix A provides further details.

The purposes of the key informant interviews were to:

- Theorize as to the current and future applications of cloning for the conservation of rare and endangered species
- Identify the key technical, environmental and social issues that would prevent the implementation of cloning applications
- Identify stakeholder views of how and should cloning technology be applied to species conservation

1.6 Design Intervention

Analysis of the literature review and interview data results in an evaluation of the costs and benefits of applying cloning technology to the conservation of rare and endangered species. In doing so, cloning is contrasted with alternative conservation approaches to gauge its relative usefulness. The project concludes as to whether cloning should be considered a useful conservation strategy and provides recommendations as to how cloning could best be utilized to meet conservation goals.

1.7 Document Organization

Chapter 1 (Introduction): An introduction to the project, its purpose, objectives, methods, and organization.

Chapter 2 (Current Challenges and Approaches to Rare and Endangered Species Conservation): A general description of the factors that cause and contribute to species decline and extinction and the current approaches to conservation that attempt to address these conservation challenges. The chapter provides comments on the advantages and limitations of each approach.

Chapter 3 (Plant Cloning): Describes the current approaches to plant propagation including sexual reproduction and vegetative reproduction. A cost-benefit discussion of Micropropagation, a technology-intensive cloning method, is the focus of the chapter.

Chapter 4: (Animal Cloning): Provides a brief history of the development of somatic cell nuclear transfer, the current state of the technology and its most recent applications. A discussion of the costs and benefits of using this technology as a reproductive option is the focus of the chapter.

Chapter 5 (Cloning as a Conservation Approach): A theoretical and practical evaluation of the merits and drawbacks of using cloning in general as an approach to species conservation.

Chapter 6 (Recommendations and Conclusions): The final chapter provides conclusions by the investigator and the research findings and suggests whether cloning should be considered a useful conservation tool. Recommendations are made as to how the benefits of cloning can be maximized while minimizing the risks of its use.

Chapter 2: Current Challenges and Approaches to Rare and Endangered Species Conservation

2.1 Rare and Endangered Species Definitions

Endangered species are often characterized by declining numbers of populations and individuals. They are defined as having a "high risk of 'extinction in the wild' (with the species living only in captivity, cultivation or naturalized populations well outside its original range) in the near future, and may become 'critically endangered'" (with an extremely high risk of extinction in the wild in the immediate future) (Primack, 1998).

For the purposes of this document, "extinction" will be defined as when no member of the species remains alive anywhere in the world (Primack, 1998).

A species is considered to be rare if it occupies a narrow geographic range, only one or a few specialized habitats, or occurs only in small populations (Primack, 1998). In many areas/cases rare species are considered to be scarce and extirpations or local extinctions (where the species is no longer found in an area it once inhabited, but is still found elsewhere in the world (Primack, 1998)) are still of concern.

Rare and endangered species are of particular interest in the context of conservation due to the structure and abundance of their populations and the challenges that these factors generate. Although the focus of this project is the conservation of such species, the discussions of using cloning for conservation could potentially apply to any valuable species.

2.2 Conservation Challenges

2.2.1 Causes of Decline and Extinction

The factors directing species towards decline and extinction can be divided into two categories: ultimate and proximate. The vulnerability of a species to decline ultimately depends on its ecology, anatomy, behavior and reproduction characteristics. These factors are genetically determined and result from their evolutionary history. Superimposed on the ultimate factors are the proximate factors, or immediate causes of decline including exploitation, habitat change (Weddell, 2002), stochastic events (Primack, 1998), and changes in biotic interactions (Levin, 2000).

a) Ultimate Causes of Extinction and Decline

Ecologists and conservationists have observed that the following categories of species are especially vulnerable to decline and extinction (Primack, 1998) based on their reproduction and dispersal capabilities, habitat requirements, geographic range, anatomy, degree of genetic variability, behavior, and degree of exposure to evolutionary antagonists.

The ultimate factors increasing the vulnerability to decline and extinction include:

1. Reproduction:
   - Low biotic potential: Species with low intrinsic rates of natural increase may be less able to replace individuals and maintain stable population numbers (Weddell, 2002).
   - Susceptibility to external genetic influences: Reproductive mechanisms that allow external genetic influences to dilute alleles which allow adaptations to local environments (Weddell, 2002), may inhibit a species ability to replace themselves (Levin, 2000). External genetic factors may also alter the genetic composition of

the species to the point where speciation occurs (and the former species becomes extinct).

2. Dispersal:
   - Lack of strong dispersal mechanisms: Dispersal of individuals and reproductive material away from the population of origin is important in avoiding inbreeding depression. Without strong dispersal mechanisms, a species may also have difficulty finding genetically different mates (Primack, 1998), and suitable habitat in changing environmental conditions.

3. Geographic Range:
   - Large home ranges: The larger the home range, the greater likelihood that a portion of it will be altered contrary to the needs of the species (Weddell, 2002), (Primack, 1998).
   - Narrow geographic ranges: If a species only occupies a small area, it is likely that all of the habitat will be affected if a change occurs in that area (Primack, 1998).
   - Seasonal migrants: Species that rely on a number of distinct habitat patches or on the continuity of migration paths may be more vulnerable to decline if habitat patches are missing (or adversely altered) or if migration paths are blocked (Primack, 1998).

4. Habitat and Resource Requirements:
   - Higher trophic levels: Species from higher trophic levels rely on prey availability and are susceptible to bioaccumulation of toxic substances in the food chain (Weddell, 2002).
   - Specialists: Species relying on highly specific foods, habitats and resources lack versatility as compared to generalist species, and are unable to utilize as diverse a range of alternate resources in the event of environmental change (Primack, 1998).

- Narrow ranges of tolerance: An organism's physiological ability to tolerate varying environmental conditions is largely genetically controlled (Fincham & Ravetz, 1990). Species with inherently narrow ranges of tolerance to environmental characteristics will be comparatively more susceptible to extinction than those with wider ranges of tolerance as it is more likely that they will encounter environmental conditions outside of their ability to withstand the change.

5. Anatomy:
   - Large body size: Larger animals tend to have large ranges, require more food, and are more easily hunted by humans (Primack, 1998).

6. Genetic Variability:
   - Species with little genetic variability: Generally, genetic variability is required for species to adapt to changing environmental conditions (Primack, 1998).

7. Behavior:
   - Species that form permanent or temporary aggregations are more easily found, removed, exploited (Weddell, 2002), or decimated due to chance factors (Primack, 1998).
   - Allee effect: A population can become unstable as a result of the inability of the social structure to function after a decline in population size. Species that rely on group structure for defense, hunting, foraging, mate selection and reproduction may be vulnerable to decline and extinction (Primack, 1998).

8. Exposure to Evolutionary Antagonists:
   - Species that have evolved with little exposure to change may be unable to adapt to new antagonists as their evolutionary history has not required them to develop defense adaptations (Weddell, 2002). Similarly, species that are characteristically found in environments where disturbance is minimal may be unable to adapt to changes in species composition and environmental changes (Primack, 1998).

b) Proximate Causes of Extinction and Decline

The proximate or immediate causes of decline include habitat change, changes in biotic interactions, exploitation and stochastic events. These factors often play on the ultimate factors to increase the vulnerability of species to extinction.

1. Habitat Change:

Habitat change stemming from the growth of the human population and increasing levels of resource use is cited as the main cause of present extinctions (Primack, 1998). The consequences of environmental change are species-specific. In general, changes that exceed a species range of tolerance or ability to adapt to the altered environmental conditions cause their decline.

Every organism requires space and resources to live. If the habitat or resources become limited in terms of availability or quality, ensuing competition between individuals will reduce the population to a level that the limited habitat and resources can support. In addition, changes in the proximity or connectivity of habitats may result in the isolation of populations or their exposure to new genetic influences (Primack, 1998), both of which may prove detrimental.

2. Changes in Biotic Interactions:

Within their environments, species interact to form complex relationships and associations. Changes in these interactions such as the removal of species, the introduction of species, diseases, etc. can affect the overall functioning of the ecosystem and the survival of species within (Levin, 2000).

3. Exploitation:

The utilization and exorbitant harvesting of species has been the cause of decline and extinction for a number of species (Primack, 1998). Rare and endangered species may be targeted for collection due to their scarcity and associated value.

4. Stochastic Events:

Stochasticity is the "random variation in the biological and physical environment." Unfavorable stochastic events can have significant negative impacts on species, particularly for small populations (Primack, 1998), possibly resulting in drastic reductions in population sizes, or extinction.

Demographic Stochasticity describes the "random variation in the survival and reproductive success of the individuals in a finite population" (Frankel, Brown & Burdon, 1995). The chance variations in small populations in terms of the number of births, deaths, number of offspring produced, and sex ratio are of particular concern as small populations have a higher chance of going extinct simply by chance (Primack, 1998), (Weddel, 2002).

Environmental Stochasticity refers to chance fluctuations in the climate, weather, food and resource availability, and the frequency of natural disasters (Primack, 1998). It also includes fully or partially unpredictable temporal variations in the biotic environment such as the abundance of competitors, symbionts, herbivores, pathogens, parasites. For plants, environmental stochasticity is of greater importance than demographic variance (Frankel et al., 1995).

Genetic Stochasticity describes the "random changes in the genetic structure of populations due to inbreeding, random drift, founder effect, or the breakdown of species reproductive isolation." This includes alterations in the types of genes, and levels in

genetic variation. Such changes are likely to reduce the survival and reproductive capabilities of individuals (Frankel et al., 1995).

2.2.2 Rare and Endangered Species Conservation Challenges

a) Small Populations

Species with small populations (as opposed to those with large populations) are particularly vulnerable to decline and extinction by genetic and reproductive isolation and a lack of genetic variability (Primack, 1998). The smaller a population is or becomes, the more vulnerable it is to decline or extinction by stochasticity (Primack, 1998; Weddell, 2002). In addition, the fewer the number of individuals in a species, the more difficult it is to remove individuals for study, translocation or use in *ex situ* conservation efforts.

b) Declining Populations

Most endangered species (and some rare species) have populations that are declining in numbers. This trend often continues and leads to entities that are made of small isolated populations (Levin, 2000), and eventually to extinction unless the cause of decline is identified and corrected (Primack, 1998).

c) Reduced Genetic Variability

As the number of reproducing individuals in a population (ie. effective population) declines, the amount of genetic variation is expected to decrease –as the effects of genetic drift and inbreeding become greater (Levin, 2000).

2.2.3 Genetic Variability: Mechanisms and Effects of Change

a) Genetic Drift

Random genetic drift, the chance loss of alleles that occurs from one generation to the next, reduces the number of different alleles in small populations compared to those that have remained large throughout their evolutionary history. The smaller the population, the greater the likelihood that some alleles will fail to occur in the next generation. Genetic drift can lead to a substantial loss of genetic variation any time that a population is suddenly or severely reduced (Weddell, 2002). Once an allele is "lost," it is absent from the population until re-introduced by a mutation (an unlikely event), or an immigrant (Weddell, 2002; Primack, 1998).

b) Inbreeding Depression

Inbreeding is the mating among close relatives. It is common in small populations when no or few other mates are available. For animals in larger populations, individuals normally do not mate with close relatives and often disperse from their place of birth – inhibited by unique individual odors and other sensory cues. For plants, morphological and physiological mechanisms encourage cross-pollination and prevent self-pollination (in sexually reproducing species). However, when mates are unavailable, these mechanisms can fail to prevent inbreeding (Primack, 1998).

The result is a decrease in genetic variability (Primack, 1998) as fewer different alleles are combined. Inbred populations may have a higher incidence of genetic defects than progeny from large populations due to the more frequent coupling of deleterious recessive alleles (Levin, 2000). Specifically, inbred populations can experience higher mortality of offspring, fewer offspring, or offspring that are weak, sterile or have low mating success. The incidence of inbreeding depression can be decreased by the introduction of new genetic material to the population (Primack, 1998).

While the detrimental effects of inbreeding are generally recognized as something to be avoided, the specific consequences of inbreeding differ among species (Holt, Pickard & Prather, 2004).

c) Outbreeding Depression

Outbreeding is the mating between individuals of a different species, subspecies or divergent genotypes of the same species. Outbreeding (among different species) rarely occurs in the wild as there are strong ecological, behavioral, physiological and morphological isolating mechanisms that ensure mating only happens between individuals of the same species. However, outbreeding may occur when a species is rare and other mates are difficult to find, or its habitat is damaged (Primack, 1998).

Outbreeding depression may be especially significant in plants where chance is a key factor in determining what genotype of pollen arrives on the flower. A rare plant growing near a related common species may be overwhelmed by the reproductive material of the common species, resulting in genetically divergent hybrids (Primack, 1998).

Outbreeding (outcrossing) increases heterozygosity. Thus, rare homozygous alleles that provide a function in a species may be lost or overshadowed by dominant alleles from a divergent genotype. Oubreeding may result in weak or sterile offspring due to incompatible chromosomes and enzyme systems inherited from their parents. The hybrid species may also be poorly adapted to the environment of the parent species and the genetic identity of the rare or endangered species may be lost or diluted if its small gene pool is overwhelmed by and mixed with a larger pool of a more common species (Primack, 1998).

2.2.4 Significance of Genetic Variability

The degree of genetic variation required for survival varies with the species. A loss of genetic variation may be a major factor in one species decline and be inconsequential in another's (K. Prior, Personal Communication, March 3, 2004). For species with inherently low genetic variability, it may still be desirable to preserve genetic differences between populations to maintain a full range of genetic variation within the species (Schaal, Leverich & Rogstad, 1991).

In general, genetic variation provides the basis upon which natural selection can act, allowing species to adapt to current and future changes in environmental conditions, ensuring evolutionary flexibility and less of a chance of extinction (Weddell, 2002; Schaal et al., 1991).

2.3 Conservation Approaches

The following outlines some of the general approaches to rare and endangered species conservation. Conservation plans often use several of these approaches depending on the specific needs of the species and the circumstance.

2.3.1 Regulatory Approaches

Many nations have developed legislation tightening regulations for the use, removal (hunting, collection), trade, transport and alteration of habitats for species designated on 'at risk' lists. Canada's Species at Risk Act (SARA) and the Endangered Species Act in the United States are examples of such approaches. Internationally, agreements such as CITES, the Convention on International Trade in Endangered Species of Wild Fauna and Flora regulates the international trade of species that are put at risk by that trade. Such agreements can be effective in regulating exploitation, however not all nations are part of the agreements, and enforcement of the regulations is politically complex, and frequently under-funded (Weddell, 2002).

A regulatory approach to conservation is useful in providing guidelines designating how rare and endangered species can be utilized or affected by human actions, thereby mitigating exploitation as a proximate cause of decline. However many species do not benefit from such legislation until they are already in decline and designated on 'at risk' lists (Weddell, 2002). In addition, designation on such lists may be to the detriment of some species if their conservation compromises the needs or interests of private landowners (Primack, 1998).

2.3.2 Habitat Protection

Preserving habitat is the most efficient method of maintaining unique assemblages of species (Frankel et al., 1995), and biotic interactions important to the survival of the ecological community and specific species within (Weddell, 2002). It is a "catch-all" approach that protects species that have yet to be identified and that would otherwise be lost by adopting a species-specific approach to conservation (Frankel et al., 1995). Thus, the preservation of species *in situ* via habitat protection is viewed as the best method of maintaining biodiversity (Comizzoli, Mermillod & Mauget, 2000).

Habitat preservation can be used to conserve specific species as well as species assemblages. Protection of species-specific geographic ranges, migration routes etc. can prevent a range of undesirable habitat changes (including changes in biotic interactions) which may compromise the ability of a species to persist in the wild. While this approach may be effective for some species, others may require increased intervention to avert extinction (Frankel et al., 1995).

2.3.3 Habitat Modification

Habitat modification describes a general group of *in situ* conservation strategies that preserve natural communities and populations in the wild (Primack, 1998) while altering the environment in some way to promote the survival of a specific species. Approaches range from reversing detrimental habitat changes, adding needed resources, to the removal of other species. In essence, this approach attempts to manipulate the environment to reduce the major sources of mortality (Weddell, 2002).

Disadvantages of habitat modification include that the entire community may be adversely affected for the benefit of one species. This may translate into a loss of biodiversity, possibly including many unidentified species. Habitat modification may be impractical for large habitats, or when modification requires intensive work. In addition, unless all the limiting factors, including external sources of decline ex. exploitation, are identified and addressed, habitat modification will not be effective (Weddell, 2002).

2.3.4 Translocation

The relocation of individuals or groups to new populations is often used to augment declining populations (Weddell, 2002), to more widely disperse species (Primack, 1998), and to increase genetic diversity (to relieve the effects of genetic drift, inbreeding depression and counteract the effects of genetic stochasticity). An alternative to relocating individuals is to move their reproductive material. For example, the seeds and spores of many plant species can be easily collected and distributed elsewhere.

Risks associated with translocation include the transmission of diseases, pests, parasites, and the introduction of dominant genes that do not confer competitive advantages which may also dilute critical environmental adaptations (Weddell, 2002). Careful site selection is important. If species are placed in conditions outside their ranges of tolerance, for which adaptations have not been developed, their survival may be limited (Frankel et al., 1995).

Translocation relies on the normal functioning of the population to increase the abundance of rare and endangered species, often a process requiring time. If these processes do not function well or in a timely manner, another strategy is required. Although translocation is useful for some species, more critically endangered species may require a greater degree of intervention.

2.3.5 *Ex Situ* Environments

Another approach to conservation is to temporarily or permanently maintain species or populations in artificial conditions under human supervision. *Ex situ* facilities include zoos, game farms, aquaria, private breeders, botanical gardens, and arboreta (Primack, 1998). *Ex situ* conservation is useful when there is limited or no available habitat in the wild (Frankel et al., 1995), when the risk of extinction in the wild is high (Primack, 1998), for the safety of vulnerable organisms, or the rehabilitation of sick, injured or abandoned individuals (Weddell, 2002). In addition, *ex situ* conservation provides access to species for study (Frankel et al., 1995) and public education (Weddell, 2002).

The ultimate goal of many *ex situ* conservation programs is the eventual re-introduction of individuals into the wild. However, long-term confinement and reproduction within controlled environments, usually in small populations, (Primack, 1998) may have significant negative impacts on the ability of a species to maintain itself in captivity and upon release due to inbreeding depression, loss of genetic variability, and genetic adaptations to captivity (Woodworth, Montgomery, Briscoe & Frankham, 2002). In addition, behaviors learned in the wild may be absent or altered in captive populations including complex group hunting methods, migration patterns, subtle cues and rituals required to secure a mate, care for offspring, find shelter and sense danger (Primack, 1998).

Ex situ conservation often requires long-term commitments and is labor and cost intensive (Primack, 1998; Weddell, 2002). The organisms are dependent on human intervention for survival, and any disruption in their care could cause the extinction of a species if the facility contained the only remaining individuals (Primack, 1998).

This approach directly addresses the immediate sources of mortality and attempts to preserve the individuals as long as possible. However, it relies on the ability of the individuals to naturally reproduce in captive environments, which proves difficult for many species. Thus, *ex situ* conservation programs must often employ breeding programs to ensure the reproduction of captive individuals.

2.3.6 Assisted Reproductive Technology

Assisted reproductive technologies (ARTs) take on many forms –from simply assisting natural mating under controlled conditions, to the cloning of adult animals. ARTs have become common strategies for the reproduction of a number of different animal species (Long, Walker, Tang & Westhusin, 2002).

a) Selective Breeding Programs

Selective breeding does not interfere with natural reproduction. Rather, it provides a greater opportunity for reproduction by placing the prospective mates in close proximity. Careful planning and tracking of breeding pairs is often required to avoid matches between closely related individuals in attempts to circumvent the erosion of genetic variability by inbreeding (Weddell, 2002; Comizzoli et al., 2000). However, species that are not willing or able to reproduce efficiently enough to prevent decline and extinction may require further intervention.

b) Advanced Assisted Reproductive Technologies

Advanced assisted reproductive technologies such as artificial insemination, *in vitro* embryo production, embryo transfer and somatic cell nuclear transfer represent the most direct approach to increasing the reproductive output of rare and endangered animals. To differing degrees, these assisted reproductive technologies (ARTs) allow the reproduction of unwilling or unable individuals, allow more offspring to be obtained from selected genitors, and reduce the intervals between generations. ARTs can also be used as a tool to increase genetic diversity within populations (Comizzoli et al., 2000). Thus, reproductive technologies can provide a role in mitigating or addressing some conservation challenges.

Although ARTs can increase the rate of fertilization and birth, they do not address the sources of mortality post-partum. Thus, they are often used as supplementary reproductive components of a more comprehensive conservation plan.

ARTs are comparatively technology intensive and may require sterile environments, specialized equipment and trained personnel. As such, they are largely used in laboratory environments, but are increasingly being applied in the field and with wild populations.

Although all of the following techniques have been used for rare and endangered species, the application of ARTs is limited to species whose reproductive biology has been well studied (Comizzoli et al., 2000).

i) Artificial Insemination

Artificial insemination (AI) is the process by which sperm is collected from suitable males and used to impregnate females in breeding condition (Primack, 1998). This method circumvents an individual's desire to engage in reproductive behaviors and can maximize the reproductive potential of valuable males without limitations of time and distance when cryopreserved samples are used (Cseh & Solti, 2000).

The efficiency of AI depends on the precise timing of insemination which must be coordinated with the timing of ovulation. Thus, one must correctly identify when the animal is in heat (often a difficult task) (Comizzoli et al., 2000) or use drugs to synchronize ovulation (Cseh & Solti, 2000). The techniques for AI are not universal. The reproductive biology of the species must be known in order to make the required species-specific corrections for anatomy and physiology (Comizzoli et al., 2000).

ii) *In Vitro* Embryo Production

In vitro embryo production (IVP) refers to the collection and subsequent maturation of male and female gametes within test tubes, and fertilization of the ova to produce an embryo (Comizzoli et al., 2000). Once the embryo has been produced, it can be transferred to a recipient mother or cryopreserved for later use.

IVP requires less handling compared to AI (the stress of which can lead to pathologies or traumatism), and more embryos can be produced with the same amount of semen sample (Comizzoli et al., 2000). As IVP offers a convenient method to produce several

offspring from genetically valuable individuals, it may allow more rapid population growth than would be expected with natural breeding.

IVP also allows the creation of embryos from pre-pubertal, pregnant females, and individuals of both sexes after their deaths. Specific sexes can also be selected if cytometric-sorting of the X and Y chromosomes occurred before the fertilization procedure (Loskutoff, 2003).

This method can overcome some infertility problems and can be combined with other technologies such as gamete micromanipulation to use non-motile or non-viable sperm for assisted fertilization (Cseh & Solti, 2000).

iii) Embryo Transfer

Embryo transfer (ET) involves the transplantation of an embryo from one individual to another. Interspecies ET (involving the transfer of an embryo from one species to another) may be especially useful if the number of individuals is extremely limited (Cseh & Solti, 2000). Embryos from endangered species can be transferred into more common, related species to gestate, rather than subjecting the females of the endangered species to the risks of pregnancy or consuming their reproductive lives with pregnancy (Primack, 1998; Longtin & Kraemer, 2002).

A major obstacle to using interspecies ET for rare and endangered species is the availability of suitably related surrogates. Surrogate mothers must have similar body sizes, estrus cycles and gestation patterns (Comizzoli et al., 2000). Even minor differences between similar species may create immunological barriers that may prevent successful interspecies ET (Longtin & Kraemer, 2002).

iv) Somatic Cell Nuclear Transfer

Somatic cell nuclear transfer (cloning) is the next major advance in the line of animal assisted reproductive technologies. This technology is the topic of Chapter four.

2.3.7 Preservation of Genetic Information

Ex situ preservation of genetic information including pollen, seeds, explants, whole plants, gametes, cells, embryos and other genetic samples is being extensively promoted for the management and conservation of endangered species (Comizzoli et al., 2000). Seed and pollen banks, *in vitro* preservation and cryopreservation are among the methods to preserve these samples for varying periods of time ranging from a few months to several years. The samples can then be used to create new individuals through the use of reproductive technologies (or other methods) (Holt & Pickard, 1999).

The preservation of genetic information increases the efficiency of most reproductive technologies as the limitations of time and distance may be removed (Cseh & Solti, 2000). Samples taken in the field can be preserved, transported and stored until the appropriate circumstance or technology exists to take advantage of it.

In general, cryopreservation and other storage methods provide lower cost, less space and labour-intensive alternatives to maintaining large living populations through the years (Comizzoli et al., 2000; Primack, 1998). Cryopreservation also allows a much greater number of species to be included in *ex situ* conservation, whereas their inclusion in traditional *ex situ* approaches is usually limited due to financial, space and maintenance constraints (Ryder & Benirschke, 1997).

Disadvantages of preserving genetic information include that some types of storage material must occasionally be utilized and recollected to maintain the viability of the samples. For example, seeds in seed banks must be periodically germinated, grown and recollected to ensure that they maintain their ability to germinate and do not accumulate harmful mutations –an arduous task for large collections (Primack, 1998). Many species are also recalcitrant to long-term storage or have a limited storage life and are therefore not well preserved (Primack, 1998). In additions, specific preservation procedures may be unavailable for less studied species, making it difficult for many rare and endangered species to be included in banks (Holt & Pickard, 1999).

Lastly, unless genetic samples are continuously collected and utilized to generate living individuals, this conservation approach does not address the current conservation

needs of living populations. However, it is uniquely suited to address future conservation challenges through the utilization of pre-collected samples.

Chapter 3: Plant Cloning

3.1 Natural Plant Reproduction

Nearly all plants have the ability to reproduce sexually (Clark, 2004), resulting in genetically unique offspring. Many plants also have the ability to reproduce asexually, using vegetative parts to yield offspring Ex. *Populus* spp. (T. Thorpe, personal communication, March 8, 2004). Unlike sexual reproduction, vegetative reproduction yields a clone, a genetic copy of a single parent (Kyte & Kleyn, 1996).

3.2 Cloning Definition

The term 'Clone' was derived from the Greek work *clonos*, twig, and *clonizo*, 'to cut twigs.' Today the word clone denotes individuals that are (largely) genetically identical to, and derived asexually from one parent (Di Berardino, 2001). It is used to refer to a full range of types of cloning (Maienschein, 2002), including gene cloning, the cloning of cell lines, and reproductive cloning. This document will focus on the use of cloning to reproduce whole individuals, as they are more directly useful for the conservation of rare and endangered species.

3.3 Plant Cloning

The concept of artificial plant cloning has been known for centuries (Mitalipov & Wolf, 2000). It first appeared in agriculture when Herbert John Webber wrote in 1903 of "clons," groups of plants that were propagated by the use of vegetative parts such as bulbs, tubers, cuttings, grafts etc. (Maienschein, 2002) and are simply parts of the same individual (Maienshchein, 2001). Vegetative reproduction can be artificially induced by a variety of procedures including division, layering, grafting (Clark, 2004), taking cuttings and micropropagation (Kyte & Kleyn, 1996).

The simplest form of vegetative reproduction is division. Plants with many crowns or growing parts are split into several individual smaller plants. This approach is labor intensive, and is often a last option for mass production (Clark, 2004).

Cuttings take advantage of the tremendous potential of plant stems to regenerate by taking shoots or sections of stems and allowing them to root in a growing medium (Kyte & Kleyn, 1996). Cuttings can be taken from stems, leaves, roots or buds. Not all plants can be propagated by cuttings and few (if any) plants can be grown from all types (Clark, 2004). This approach has been used to propagate millions of copies of commercially desirable plants (Mitalipov & Wolf, 2000). However, cuttings only produce one clone per sample taken (Kyte & Kleyn, 1996). Thus, this approach is likely unsuitable for conserving many rare and endangered species, where samples are in short supply.

Layering encourages the development of roots on a stem while it is still attached to the parent plant. For example, tip layering is accomplished by bending a branch to ground, wounding it where touches ground and covering it with soil. Roots subsequently develop and new shoots are sent out. The connection to parent plant is then cut (Clark, 2004), yielding an independent clone.

Grafting connects two pieces of living tissue, allowing the parts to unite and grow as a single plant. A scion (a piece of stem or shoot with dormant buds that will develop branches) is joined to the stock (a root system, a sapling etc.), and the two grow together into a new individual. Grafting readily transfers disease, requires skilled personnel, and is one of the more expensive asexual reproductive options (Clark, 2004).

3.4 Micropropagation

Micropropagation is the vegetative multiplication (cloning) of plants *in vitro*, under artificial conditions (Kyte & Kleyn, 1996). It is concerned with producing whole plantlets through supporting the growth and development of isolated plant cells, tissues or organs in an artificial nutritive medium (Renfroe, 1998). The term micropropagation is often used interchangeably with *in vitro* culture or to broadly refer to 'tissue culture.' Micropropagation, and cloning in general, does not involve the deliberate changing of the genetic material of a plant, simply the multiplication of it (Kyte & Kleyn, 1996).

3.4.1 Micropropagation Procedure

The parent genetic material is obtained through the collection of an explant, a tissue sample removed from the plant which is then placed in culture (Renfroe, 1998). Samples are commonly leaves, stems, roots, buds or single cells (Kyte & Kleyn, 1996).

Before the explant is placed into the test tube and culture medium it must be sterilized to remove surface organisms. The culture medium is axenic (free of other organisms) (Renfroe, 1998), and consists of a balanced nutritive solution containing micro- and macro-nutrients, vitamins, sucrose, and plant growth regulators specific to the plant in culture (Murch, 2004). The explants are periodically transferred into fresh medium and are maintained in a controlled environment (Renfroe, 1998).

While in culture, the explant develops into plantlets (very small juvenile shoots reminiscent of seedlings) which are theoretically capable of being multiplied indefinitely (Kyte & Kleyn, 1996). Thus, a limitless number of clones may be able to be produced from a single sample. This is due to totipotency, the remarkable potential of individual cells to become whole plants (Murch, 2004).

In the final stages of micropropagation, the plantlets mature and are acclimatized to the appropriate environmental conditions and transplanted into a container or into the field. The time required for micropropagation ranges from weeks to months depending on the species (Kyte & Kleyn, 1996).

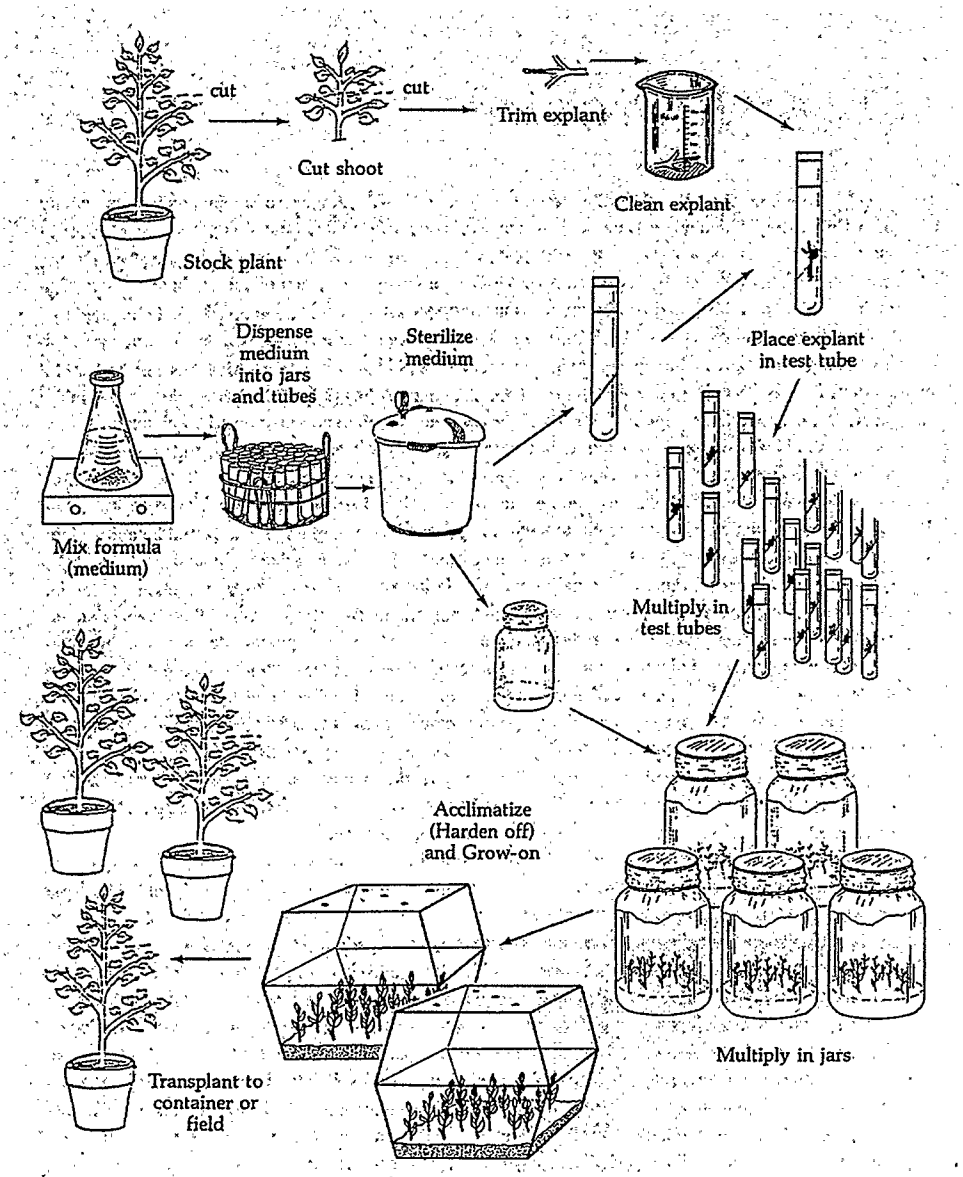

Figure 3.1 Shoot tip micropropagation procedure (Kyte & Kleyn, 1996).

3.4.2 Specific Approaches

Depending on the explant and the contents of the nutrient medium, different routes of development can be initiated and different end products can be produced (Collin & Edwards, 1998).

Organogenesis: Organogenesis involves the regeneration of plants by inducing the explant to form an organ. Specifically, organogenesis requires the formation of a shoot which is then rooted, or a root which forms a shoot that is then separated and rooted (T. Thorpe, personal communication, March 8, 2004).

Somatic Embryogenesis: Somatic (non-reproductive) cells from explants are induced to form embryos which then develop into plantlets (Kyte & Kleyn, 1996). The embryos can be encased in artificial nutrient shells to create "synthetic seeds" (Murch, 2004) which can be widely distributed in the field.

Embryo Rescue: Involves retrieving the embryo from a seed, culturing and multiplying it *in vitro* to obtain a number of embryos which can then be induced to form plantlets (Kyte & Kleyn, 1996). This may be useful for propagating rare and endangered species that do not generate (enough) mature viable seeds.

Pollen Culture: In culture, the haploid pollen can be doubled to produce a diploid plant (with the normal number of chromosomes) through chemical treatments. This cloning approach is particularly useful in restoring rare homozygous recessive traits (T. Thorpe, personal communication, March 8, 2004).

3.4.3 Applications of Micropropagation to Rare and Endangered Species

Research on plant tissue culture began more than thirty years ago (Frankel et al., 1995). It was initially developed for the rapid propagation and breeding of crop plants (Murch, 2004). Applications of this technology have expanded to include the propagation of a variety of ornamental species, forest trees (T. Thorpe, personal communication, March 8, 2004), and rare and endangered species (Mikulik, 1999; S. Murch, personal communication, April 23, 2004). However, only in the last decade has the potential use of tissue culture for long-term conservation been investigated (Frankel et al., 1995). Plant tissue culture protocols are now being applied for the mass propagation of species in imminent danger of extinction and *in vitro* conservation is being used to recover genetic diversity, and to ensure the survival of unique species (Murch, 2004).

Micropropagation has been used worldwide to conserve a number of species. One such example is *Kanaloa kahoolawensis,* a highly endangered leguminous shrub in Hawaii. The plant rarely forms complete viable seeds and is susceptible to a range of bacterial and fungal pathogens, and attempts to propagate it by cuttings have been unsuccessful. Somatic embryogenesis was used to propagate the only living specimen remaining in the wild (Murch, 2004).

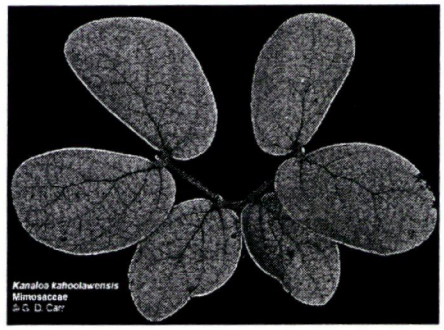

Figure 3.2 *Kanaloa kahoolawensis* (Carr, 2004).

3.4.4 Advantages of Micropropagation over Other Methods of Propagation

The use of micropropagation to conserve rare and endangered species has a number of advantages over sexual reproduction and conventional approaches to asexual reproduction including division, cuttings, layering, and grafting.

a) Efficiency

The major advantage of micropropagation is its efficiency in mass production (T. Thorpe, personal communication, March 8, 2004). If every cell in a 1 cm piece of explant were exposed to the correct culture conditions, it is possible to get more than 10000 plants. However, it is more common to recover 30-500 plants per explant, depending on the species, the choice of growth stimulant and the competence of individual cells to accept the inductive signal (Murch, 2004). Efficiency also is reduced by plants lost or removed from the culture process due to contamination by disease, fungus, virus, somaclonal variation -the differences in regenerated plants derived from the variation present in the explant cells and from culture conditions (Collin & Edwards, 1998), vitrification -the thickening and glassy appearance of the leaves due to culture conditions (Ziv, 1991), and plants that do not survive the transfer from *in vitro* to greenhouse or field conditions (Preece & Sutter, 1991).

Other methods of plant propagation are not as efficient in producing a large number of offspring. For example, seeds and cuttings produce only one plant per seed or cutting. Thus, in some cases tissue culture may be the only practical method of producing a large number of individuals (Kyte & Kleyn, 1996).

b) Obtaining Explants

With endangered species, the choice of starting material is often limited (S. Murch, personal communication, April 23, 2004). As only one or a few small samples may be required for the mass production of a desired plant, tissue culture is especially useful

when plant material is scarce or irregularly available (Kent, McKently, Adams & Langston, 2000).

The size of the explant itself is relatively small in comparison to the samples taken for other methods of vegetative propagation. Typically leaves, single seeds or small sections of stems are removed, resulting only in minimal damage to the research plant (Kent et al., 2000), no worse than the removal of the same material by herbivory (S. Murch, personal communication, April 23, 2004).

Collection of explants can occur *in situ*, allowing the parent plant to remain alive and healthy in the wild while researchers propagate it *in vitro* (Kent et al., 2000). In contrast, some other methods of plant propagation require the removal of individuals from the wild into *ex situ* environments to serve as stock plants, providing seeds and plant material for propagation. In other situations, as with the division of bulbs, the original plant is destroyed in the process of creating clones. Finally, tissue culture provides an advantage over reproduction by seeds in that explants are relatively easy to collect and handle (Kyte & Kleyn, 1996).

c) Propagation of Difficult to Reproduce Species

Reliance on natural methods of reproduction to ensure the viability of a species requires stable favorable environmental conditions –the absence of which is likely the cause of many species declines. Thus, alternative approaches are often required. The use of sexual reproduction to propagate a species may prove difficult if the species does not grow well from seed, or does not produce enough viable seeds (Clark, 2004). In addition, many species are recalcitrant to propagation by the afore mentioned artificial vegetative reproduction methods.

Micropropagation provides an alternative for species that do not reproduce well naturally or by other methods of propagation (S. Murch, personal communication, April 23, 2004). It bypasses natural reproductive mechanisms, eliminating many infertility problems and ensures the production of a new generation, regardless of the availability of seeds and *in situ* environmental conditions.

Micropropagation offers another method to resurrect extinct species if a living sample (preserved cells, tissue, seeds, pollen etc.) is available (S. Murch, personal communication, April 23, 2004). Tissue culture would likely be a valuable tool in attempting to clone an extinct species from a sample found in the wild, ex. from long-lived seeds (T. Thorpe, personal communication, March 8, 2004), as cloning can utilize a number of different starting materials, and can produce a number of individuals to increase the likelihood of obtaining plants that will survive the culture conditions and acclimatization.

d) Production of "Disease-Free" Offspring

Through the selection of the explant and surface sterilization, the tissue culture procedure can eliminate viruses, diseases, bacteria, fungi, spores and insects from the explant. As such, micropropagation can allow the use of diseased or contaminated plants as explant sources. Conversely, other procedures are limited to healthy donor plants as these factors would be readily transmitted to the next generation (Kyte & Kleyn, 1996).

Tissue culture can not eliminate all diseases. However the disease-eliminating process, combined with the axenic conditions result in tissue cultured plants that are more vigorous, disease resistant and disease free than those produced by cuttings (Kyte & Kleyn, 1996). Micropropagation is also useful in initiating virus and disease-free stock plants which can then be used for other methods of propagation (Collin & Edwards, 1998). Another major advantage in pathogen-free plants is the easier exchange of plants between provinces, states and countries. Tissue cultured plants have gained world-wide trade acceptance because the dangers of introducing disease are "virtually eliminated" (Kyte & Kleyn, 1996).

e) Faster Growth and Production

Plants grown in culture (and other *ex situ* environments) do so under ideal conditions and are not subject to limited resources or other factors that may slow their growth. As such, these plants should exhibit faster growth than those grown *in situ*. In addition,

micropropagation yields well-started plantlets with a stockpile of nutrients which exhibit more rapid growth than plants grown from seeds or cuttings (which can take months to root in greenhouses) (Kyte & Kleyn, 1996). Thus, the rapid multiplication of plants *in vitro* makes it possible to produce clones more quickly than by other means (Kyte & Kleyn, 1996; Kent et al., 2000).

Micropropagation also allows the production of plants year-round (Mikulik, 1999), without the seasonal restrictions that govern most conventional nursery-grown seeds and cuttings, although collection of the explant may be seasonally limited (Kyte & Kleyn, 1996).

f) Minimal Maintenance and Storage Requirements

In vitro propagation is less maintenance-intensive than propagation by cuttings and seeds, as cultures need only to be divided and transferred to fresh media every 2-6 weeks, requiring no watering or maintenance in the intervening period (Kyte & Kleyn, 1996).

In general, tissue culture requires less space than other propagation methods (Kent et al., 2000). It does not necessarily require the maintenance of stock plants to obtain material for cloning (Kyte & Kleyn, 1996), the explants are smaller than samples taken for other methods, and their cultures require little space (Mikulik, 1999).

g) *In Vitro* Preservation

Micropropagation using slow-growth culture media provides a method for the short and long-term storage of plants that are difficult to maintain in greenhouses, in the field, or in cryopreservation (Sudhersan, AboEl-Nil & Hussain, 2003; Chicago Botanic Garden, 2001; Kent et al., 2000). *In vitro* preservation via shoot cultures also requires less space, handling, and maintenance (with subcultures only required at intervals) than some other methods of preservation (T. Thorpe, personal communication, March 8, 2004).

3.4.5 Disadvantages of Micropropagation over Other Methods of Propagation

a) Financial and Technological Requirements

Micropropagation is more costly than the use of seeds (the cheapest method of plant reproduction), and conventional asexual reproduction (T. Thorpe, personal communication, March 8, 2004). It requires expensive technical equipment (Mikulik, 1999), specialized facilities (see Figure 3.3), and experienced personnel (Clark, 2004; T. Thorpe, personal communication, March 8, 2004). However, the cost does not preclude its usefulness as a conservation option. It is economically feasible to clone several different genotypes as well as to mass-produce individuals (T. Thorpe, personal communication, March 8, 2004).

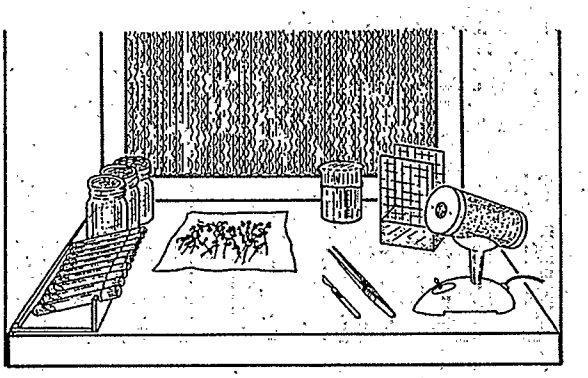

Figure 3.3 Detail of micropropagation work area -a laminar air flow transfer chamber designed to provide a sterile environment in which the technician works. Commonly used equipment includes test tubes, sterile jars, a scalpel, and sterilizing equipment (Kyte & Kleyn, 1996).

b) Research Requirements

Micropropagation is not a new technology. The scientific breakthroughs have been made, the general procedure has been established, and theoretically any species can be reproduced by this approach. However, species-specific procedures have yet to be devised for many of the lesser studied rare and endangered species. The success of developing these procedures depends on the availability of detailed biological information, which is often lacking. Thus empirical studies would have to be carried out,

and the deficiency of experimentation material may make such studies difficult (T. Thorpe, personal communication, March 8, 2004).

c) Risks of Release

There are no major controversial issues associated with using micropropagation to produce clones intended for release into wild or other environments. Micropropagation is considered to be relatively "low-tech" and viewed as a laboratory procedure that re-creates a process that occurs in nature, and as "extending what nature does." In general, it is accepted as a safe technology by the public and the scientific community (T. Thorpe, personal communication, March 8, 2004).

It is possible that clones produced in culture (and by macropropagation) can contain mutations (ex. somaclonal variations) that could adversely affect populations upon release. However micropropagation better preserves the 'genetic integrity' of species as fewer somaclonal variants are produced as compared with macropropagation (T. Thorpe, personal communication, March 8, 2004).

In either case, the subtle genetic changes are likely to alter only a few genes in an affected plant, and it is unlikely that the mutated genes could overwhelm populations (T. Thorpe, personal communication, March 8, 2004). Cultures are often screened for abnormal phenotypes (which are removed), reducing this risk (Collin & Edwards, 1998). In addition, as rare and endangered species often do not survive well in the wild, concerns about mass proliferation of mutations introduced by these individuals should be limited (S. Murch, personal communication, April 23, 2004). Furthermore, experts suggest that the translocation of species is more likely to adversely affect wild populations than the release of micropropagated plants (T. Thorpe, personal communication, March 8, 2004; S. Murch, personal communication, April 23, 2004).

Chapter 4: Animal Cloning

4.1 Animal Cloning in Nature

Natural clonal reproduction occurs in many animal species. For example, one celled organisms such as *Escherichia coli* naturally clone themselves by binary fission (DNA replication followed by fission resulting in two identical progeny). Similarly, after bisection, flatworms (*Planaria*) spontaneously regenerate yielding two identical organisms (Foote, 2002).

Cloning in mammals involves the spontaneous creation of identical embryos (Siedel, 2002). For example, nine-banded armadillos (*Dasypus novemcinctus*) regularly give birth to identical quadruplets with every pregnancy (Ryder & Benirschke, 1997), and identical twinning occurs in many other vertebrate species (Mitalipov & Wolf, 2000).

4.2 Artificial Cloning

Early efforts in mammalian cloning began by artificially inducing identical twinning by blastomere separation or embryo splitting (separation of blastomeres or embryos into groups of cells that develop into identical offspring) (Mitalipov & Wolf, 2000; Green, 2002). This approach was successful in yielding a limited number of clones, but it proved difficult to obtain more than two clones from the few cells contained in the early (blastocyst) stage of development (Tsunoda & Kato, 2002b).

4.3 Somatic Cell Nuclear Transfer

The current approach to animal cloning is Somatic Cell Nuclear Transfer. The process involves transferring the nucleus of a cell obtained from the individual to be cloned into an unfertilized ovum with its own nucleus removed (Westhusin et al., 2001). The result is a clone, with the nuclear genome of the nuclear donor. Similar to the occurrence of somaclonal variants in plants, the clones produced by NT are not perfectly identical. Variation results from different environments from conception onward, the

source of mitochondrial DNA, and mutations. Thus, although clones are genotypically and phenotypically similar, individual traits and characteristics can never be exactly duplicated (Siedel, 2002).

4.3.1 Nuclear Transfer Procedure

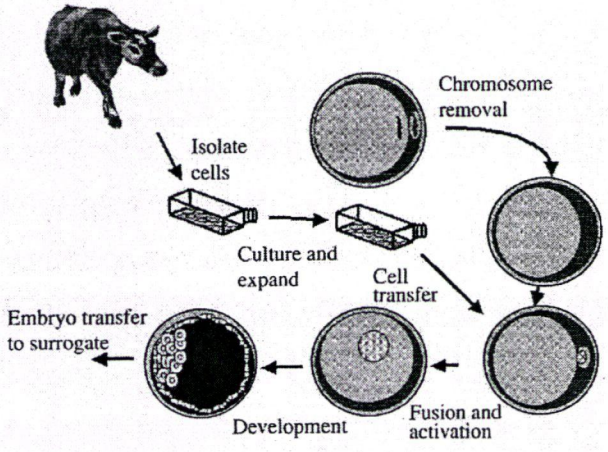

Figure 4.1 Generalized nuclear transfer procedure (Critser, Riley & Prather, 2003).

Although the specific procedure will vary with the species (Westhusin et al., 2001), the generalized protocol is outlined below:

1. Donor Nucleus Isolation: Cell samples are taken from the animal to be cloned, cryopreserved, and grown in the laboratory (Lanza, Dresser & Damiani, 2000) to produce cell lines that will be used in the cloning procedure (D. Vanderwall, personal communication, March 22, 2004). See Figure 4.1.

2. Extraction of Ova: Eggs from the surrogate mother are collected (and possibly coaxed to mature *in vitro)* (Lanza, Dresser, et al., 2000).

3. Ova Enucleation: A needle is inserted through the protective layer (zona pellucida) surrounding the egg. The chromosomes and polar bodies (remnants of the egg cell) are drawn into the needle and removed (eliminating most of the genetic material), leaving only a sac of gel called the cytoplasm (Lanza, Dresser, et al., 2000).

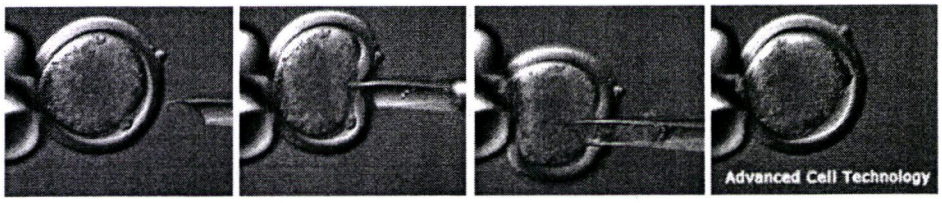

Figure 4.2 Enucleation procedure. The recipient oocyte is held by a glass pipette (left), while a glass needle (right) is used to remove the genetic material (Advanced Cell Technology, 2004).

4. Transfer: The entire donor cell is taken up into a needle, and inserted underneath the zona pellucida, where it remains separate from the egg cytoplasm (Lanza, Dresser, et al., 2000).

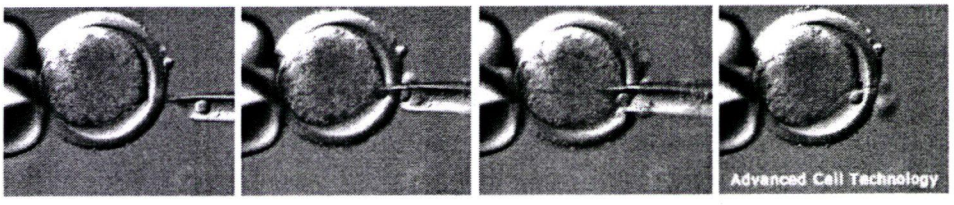

Figure 4.3 Transfer of the nucleus. A cell containing the donor genetic material is placed inside the Zona Pellucida, and an electrical pulse fuses the cells (Advanced Cell Technology, 2004).

5. Electrofusion: The injected egg is then exposed to an electric shock that fuses the donor cell with the egg cytoplasm. The donor cell's nucleus, with its genes enters the egg cytoplasm. Within a few hours the fused cell begins to divide (Lanza, Dresser, et al., 2000).

6. Embryo Culture and Transfer: The early embryo is cultured *in vitro* (Westhusin et al., 2001) for a few days until it becomes a mass of cells large enough to implant in the surrogate mother (Lanza, Dresser, et al., 2000).

4.3.2 History of Nuclear Transfer

The idea of animal cloning by nuclear transfer as a realistic scientific procedure dates back to 1938, when Hans Spemann proposed a fantastic experiment –using nuclear transplantation to produce clones from embryonic or adult cells (Maienshein, 2001). The experiment required introducing an isolated nucleus into the protoplasm of an egg devoid of its own nucleus (Downey, 1999). Early experiments with single celled amoeba and aectabularia made this proposal a technical reality with the transplantation of living cell nuclei (Gurdon & Byrne, 2003). Cloning techniques subsequently developed in small steps in various laboratories (Downey, 1999).

The 1950s and 1960s brought the cloning of cell lines (Maienschein, 2002), and in 1952, the year before Watson and Crick announced their discovery of the structure of DNA, the first animal clones were produced by nuclear transfer (Tsunoda & Kato, 2002a; Downey, 1999). Briggs and King produced feeding stage tadpoles in frogs (*Rana pipiens*) using embryonic cells. Despite numerous attempts to clone using adult amphibian nuclei, complete and normal development of such clones were never observed (Di Berardino, 2001).

The next three decades yielded considerable advances in mammalian reproductive biology, permitting the successful *in vitro* culture of embryos (prior to implantation) and the development of microsurgical techniques required for handling small fragile oocytes and embryos (Di Berardino, 2001).

In 1981, Illmensee and Hoppe claimed to have created the first cloned adult mammal by nuclear transfer. A cell nucleus from a donor blastocyst (Di Berardino, 2001) was transferred into an enucleated zygote to produce three cloned mice (Gurdon & Byrne, 2003). However, several laboratories could not repeat the results (Di Berardino, 2001).

The first uncontested mammalian clones arrived in 1986. Willadsen produced three lambs after the transfer of embryonic cells into enucleated oocytes (arrested at a particular stage in its cell cycle). During the next 10 years, numerous mammalian species were cloned from cells of preimplantation embryos including mice, rabbits, rats, pigs, goats, sheep, cattle, and the rhesus monkey (Di Berardino, 2001).

Interest grew in cloning adult cells as the phenotypes of the offspring could be predicted (such is not possible with the use of embryonic or fetal cells). In July of 1996, Ian Wilmut and colleagues at Scotland's Roslin Institute succeeded in producing the world's first animal cloned from an adult cell via somatic cell nuclear transfer. "Dolly," a Dorset sheep was produced by transferring an adult mammary cell into an enucleated ewe oocyte (Di Berardino, 2001). The process took 277 attempts to yield a single live clone (Downey, 1999). Successful cloning using adult cells was soon confirmed in cattle, mice, pigs, and goats (Di Berardino, 2001).

Dolly matured, mated and delivered a healthy lamb, "Bonnie" and two subsequent offspring (Di Berardino, 2001; Downey, 1999), proving that clones can mate and give rise to normal and healthy offspring (Holden, 2003).

In 1997 the Roslin Institute produced the first transgenic mammalian clones, defining a new path for commercial cloning. Researchers inserted human genetic sequences coding for human clotting factor IX into the DNA of lambs, and the surviving lamb "Polly" secretes the clotting factor in her milk. Subsequently, a number of cloned transgenic animals including calves, goats and sheep have been produced (Di Berardino, 2001).

4.3.3 Applications of Nuclear Transfer to Rare and Endangered Species

The twenty-first century marks the beginning of the application of nuclear transfer technology to the conservation of rare and endangered species. In January of 2001, a Massachusetts biotechnology company, Advanced Cell Technology (ACT), generated a clone of a gaur (*Bos gaurus*), an endangered wild Asian ox named "Noah." This marked the birth of the world's first endangered species cloned by nuclear transfer (Lanza, Dresser, et al., 2000).

Noah was also the first cloned animal to gestate in the uterus of another species (Mastny, 2001; Lee, 2001). The nuclear donor sample, a skin cell, was collected from a postmortem male gaur and cryopreserved by the San Diego Zoo (Nash, 2001). The sample was later thawed and transferred into an enucleated bovine egg. The cloned embryo was implanted in an Iowa cow named "Bessie" (Lanza, Cibelli, et al., 2000). The

successful birth of Noah provided proof that one animal can carry and give birth to an animal clone of another species (Lanza, Dresser, et al., 2000). The interspecies nuclear transfer also resulted in the first reported cloning of an animal with the nuclear genome of one species (gaur) and the maternally inherited mitochondrial genome of another species (bovine) (Lanza, Cibelli, et al., 2000). Noah died two days after his birth of an intestinal infection, a common cause of death among farm animals (Lee, 2001).

In April of 2003, ACT announced the birth of the world's first apparently healthy clone of an endangered species: a banteng (*Bos javanicus*), a wild bovine from Java. Researchers used a cryopreserved sample taken from a male that died in the 1980s and a cow as the egg donor and surrogate mother (Holden, 2003). The eight month old male banteng has since been released into a San Diego Zoo population consisting of three female bantengs (Yahoo News, 2004).

As of 2004, a number of rare and endangered species have been cloned, and many more projects are underway or in discussion. Appendices B and C list some of the successful and potential cloning projects involving rare, endangered, and extinct species.

4.3.4 Application of Nuclear Transfer Cloning Among Taxonomic Groups

The factors that determine the desirability, feasibility and practicality of cloning vary with the taxonomic group, depending on the species, biological system, and the chances of obtaining suitable funding (Holt et al., 2004). Mammalian species are the current focus of nuclear transfer research. They comprise just under 10% of the total number of vertebrate species, but seem to gain 90% of the attention. Fish, birds, amphibians and invertebrates rarely enter the public debate on cloning in species conservation (Holt et al., 2004). As the following illustrates, some of these taxa present distinct advantages in easing the difficulty of cloning (as compared to mammalian species), while the cloning of others may prove more difficult. In any case, a fundamental lack of research currently limits the application of cloning to most non-mammalian groups.

The following outlines the recent progress and challenges associated with cloning non-mammalian species:

a) Fish Cloning

Only a few recent publications have reported successful reproductive cloning in fish, namely in medaka (*Oryzias latipes*), and in zebrafish with success rates equal to or less than 2%. It may be straightforward to mass-produce fish as their eggs are produced in large quantities, and are relatively large in comparison to mammalian oocytes. Difficulties may arise in obtaining the eggs and in applying the technology to different fish species which are reproductively diverse. It is currently possible to culture fish cells *in vitro*, thus the collection of endangered fish cell lines could be used as a current conservation strategy (Holt et al., 2004).

b) Bird Cloning

To date, the cloning of birds has not been accomplished (Holt et al., 2004). Difficulties in bird and reptile cloning include the repatriation of the embryo into the egg, given their hard shells (D. Galbraith, personal communication, May 11, 2004).

c) Amphibian Cloning

As cloning technology was developed using amphibians, it may be possible to clone rare and endangered amphibians or restore extinct amphibians using cloning. However, the lack of studies on methods of assisted reproduction in amphibians may prevent the application of the technology to lesser-studied species (Holt et al., 2004).

d) Invertebrate Cloning

Research into insect cloning was initiated in the 1960s. However due to the fragility of the eggs, enucleation has been difficult and the offspring have developed with the characteristics of both host and donor nuclei (Di Berardino, 2001).

4.3.5 Advantages of Animal Cloning Over Other Reproductive Strategies

a) Production of Difficult to Reproduce Species

The use of cloning allows greater flexibility in terms of producing difficult to reproduce individuals. First, somatic cell nuclear transfer is able to utilize a greater range of genetic resources in producing offspring. Currently, many types of somatic cells collected from embryonic and adult sources can be utilized to create clones. With increased research, it may be possible to utilize any type of somatic cell, increasing the number of possible sample types. Even now, cloning offers the only reproductive option if viable gametes are not available.

Cloning allows the reproduction of animals that are unwilling or unable to engage in sexual behavior, or that are spatially (or temporally) separated. In addition, as cloning does not require the use of gametes, many of the infertility problems associated with other approaches can be eliminated. Specifically, cloning can permit the reproduction of pregnant, infertile, pre-pubescent, embryonic, and even dead individuals –given that an appropriate viable genetic sample is available.

Cloning can provide unique options for the conservation of critically endangered and extinct species. The cryopreservation of gametes, embryos and tissue samples have allowed AI, IVP, ET and NT to occur in the absence of living males (as the stored genetic material can be transferred into a living female yielding a new generation). NT is the only method that does not require a genetic sample from a male at all -unless the desire is to create a male clone (D. Vanderwall, personal communication, March 23, 2004).

NT may also occur in the absence of living females if a closely related surrogate species exists. Thus, interspecies nuclear transfer can theoretically resurrect extinct species *given that living genetic samples exist and a suitable surrogate is available* (Corley-Smith & Brandhorst, 1999). Cloning requires only a single cell sample, (whereas other methods rely on the genetic contribution from two parents), thus NT provides a more likely opportunity to recover a genetic sample that may result in the resurrection of an extinct species for which samples have not been preserved.

b) Ease in Obtaining Nuclear Donor Samples

Given that the most common sources of nuclear donor material are fibroblast cells, isolated from a small full-thickness biopsy of skin, the collection of nuclear donor samples is generally easier and less invasive than the collection of sperm or ova. Only small samples need to be taken for nuclear transfer and they can be obtained from live animals. Other sources of donor material are varied but range from easy to collect oral cavity gum tissue to ovarian tissue which requires surgery on a sedated animal (D. Vanderwall, personal communication, March 22, 2004). Nuclear donor samples can be collected opportunistically (Trounson, 2001), during regular handling of wild and captive populations and from recently deceased animals, for the purpose of establishing genetic resource banks (C. Hannigan, personal communication, March 29, 2004).

c) Proliferation of Genetic Samples: Establishing Cell Lines

Theoretically, animal cloning requires only a single nuclear donor sample as somatic cells can be duplicated in culture to produce a cell line, a source of infinitely available identical cells (D. Vanderwall, personal communication, March 22, 2004). Thus, regardless of the number of attempts required to produce a cloned offspring, the availability of genetic material should not be a limiting factor.

Conversely, other assisted reproductive technologies require the collection of both sperm and ova, which can not be multiplied in the same fashion. Therefore, many more samples must be taken to create the same number of offspring (D. Vanderwall, personal communication, March 22, 2004), and it is less probable that enough viable gametes can be obtained from dwindling populations and species.

d) Cryopreservation of Nuclear Donor Samples

Cryopreservation has been used successfully for the preservation of somatic cells, ova, sperm, and embryos of many species, however the species-specific procedures have not been developed for every rare and endangered species (Holt & Pickard, 1999). Nuclear

donor samples can be stored indefinitely by cryopreservation without significant loss in viability (D. Vanderwall, personal communication, March 23, 2004), and are typically easier to preserve than sperm and ova (C. Hannigan, personal communication, March 29, 2004). Difficulties have been encountered in preserving the gametes and embryos of some species. Specifically, the oocytes of some species are subject to damage by freezing (Lanza, Dresser, et al., 2000). In the absence of the ability to freeze gametes and embryos, somatic cell preservation may be the only method preserve an individual's genetic content.

In terms of storage space, somatic cells and sperm samples take up less space than oocytes, and can therefore be preserved in greater quantities. This becomes significant when a portion of the sample is damaged during freezing or thawing. Losses will not be as significant for nuclear donor or sperm cells as there are more samples to work with (D. Vanderwall, personal communication, March 23, 2004).

Many cryopreservation storage facilities or "frozen zoos," have developed worldwide (Estabrook, 2002) for the purposes of genetic research and preserving the means to produce live young through ARTs (Leibo & Songsasen, 2002). San Diego's "Frozen Zoo" began to preserve cells in 1976 and the bank currently represents over 3200 individual mammals, representing 355 species and subspecies (San Diego Zoo, 2004). The Frozen Zoo has since been used to create living clones, including the guar (Lee, 2001).

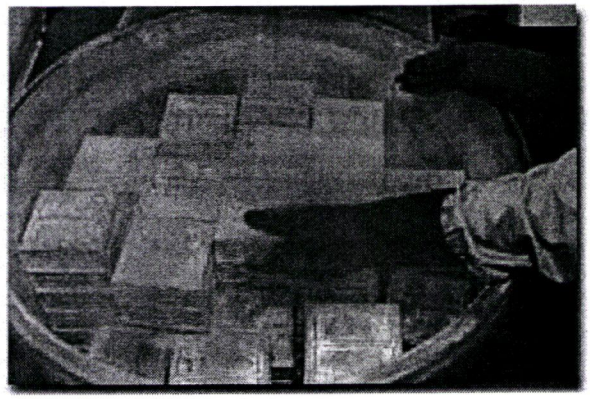

Figure 4.4 San Diego's Frozen Zoo (San Diego Zoo, 2004).

4.3.6 Disadvantages of Animal Cloning over Other Reproductive Strategies

a) Experimental Technology

Somatic cell nuclear transfer is an experimental technology (Holt et al., 2004; K. Prior, Personal Communication, March 3, 2004), still in the early stages of development. The cloning procedure has not yet been worked out for every species. Efforts have primarily focused on domestic species whose reproductive biology has been studied in depth (Corley-Smith & Brandhorst, 1999) and only a few rare or endangered species have been successfully cloned. Conversely, other reproductive technologies (ex. AI) have been established in many species, and are more widely used for animal reproduction.

b) Lack of Reproductive Biology Research

The cloning of endangered animals presents practical problems which arise out a lack of knowledge of the species basic reproductive biology. Species-specific nuclear transfer procedures must be developed to account for unique reproductive characteristics, and species-specific protocols must also be developed for many of the supporting procedures (Holt et al., 2004). Adequate knowledge of the species biology is a prerequisite for the development of these procedures, and is often lacking in less-studied rare and endangered species (Comizzoli et al., 2000). Although other ARTs also require extensive biological information, the nuclear transfer procedure is considered to be more complex and sophisticated (Leibo & Songsasen, 2002), likely requiring a greater amount of detailed research.

c) Low Efficiency

Nuclear transfer technology is currently unreliable as a method of reproduction (Tsunoda & Kato, 2002b). The efficiency varies between each attempt and depends on the genotype, type of nuclei donor cell utilized, treatment of donor cells prior to nuclear transfer, source of recipient ova, techniques employed and the people performing the work (Westhusin et al., 2001). In addition, the species, and the specific animal will lend greater and lesser degrees of success in cloning (D. Vanderwall, personal communication, March 23, 2004).

In general, only <0.1-5% of cloned embryos develop into living offspring, requiring between 20 and 1000 nuclear transfers to be performed to achieve one viable offspring (Holt et al., 2004). Higher rates of up to 12-18% efficiency have been reported in domestic cattle (Trounson, 2001). However, in general, it is currently much more efficient to conventionally breed animals (barring other complications) rather than to use nuclear transfer (Cohen, 1997b).

d) Abnormalities

The low efficiency suggests that the embryos produced by NT are developmentally compromised (Dominko et al., 1999). Since the first sheep were produced by nuclear transfer using cultured cells as sources of nuclei, many studies have revealed that cloned mammals suffer from developmental abnormalities (Holt et al., 2004). Reported abnormalities include extended gestation, large offspring syndrome (due in part to inadequate *in vitro* culture conditions that alter genes expression resulting in excessive growth *in utero* (Critser et al., 2003)), defects in most organs including the kidney, brain, cardiovascular system, and muscles (Holt et al., 2004), and higher incidences of perinatal and postnatal death (Mitalipov & Wolf, 2000).

Abnormalities:

- Large Offspring Syndrome (Estabrook, 2002).
- Longer than usual gestation periods (Estabrook, 2002).
- Gestational abnormalities (Trounson, 2001).
- Inadequate placental formation (Holt et al., 2004).
- Increased fetal wastage throughout pregnancy (Mitalipov & Wolf, 2000).
- Poor adaptation to the after-birth environment (Mitalipov & Wolf, 2000).
- Poor health of offspring (Trounson, 2001).
- Limb deformation (Illmensee, 2001).
- Histological defects in most organs (kidney, brain, cardiovascular system, muscle) (Holt et al., 2004).
- Higher incidences of perinatal and postnatal death (Mitalipov & Wolf, 2000).
- Seizures (Holden, 2003).
- Tumors (Holden, 2003).

The occurrence of abnormalities in cloned offspring differs between species. For example, fewer abnormalities have been observed in pigs and mice than in cattle and sheep. In some species, the anomalous phenotypes are not transferred to the next generation (Holt et al., 2004), thus the release of abnormal clones of these species into populations should be of little concern as the fitness of future generations should not be reduced.

Despite the inefficiency in producing clones and the high losses of clones, researchers assert that "the overwhelming majority of clones alive today are healthy and apparently normal" (Hill & Chavatte-Palmer, 2002). The United States Food and Drug Administration (USFDA) is considering the safety of cloned animals for human use for food and fiber. Currently, all indications suggest that they are moving to allow such uses of clones and their offspring, leading some researchers to believe that the risks of releasing clones and their offspring into populations are likely to be minimal (D. Vanderwall, personal communication, March 23, 2004).

e) Incomplete Nuclear Reprogramming

Recent research has pointed to the incomplete reprogramming of the donor cell nucleus as the source of many abnormalities in clones (Tsunoda & Kato, 2002b; Holt et al., 2004). During normal development, embryonic cells begin to differentiate into specialized cells (Maienschein, 2002), which only express the genes appropriate for its cell type (as the expression of information coding for other cell types is inhibited) (D. Vanderwall, personal communication, March 23, 2004). In order to produce a clone, the differentiated nuclear donor cell must be reprogrammed or "undifferentiated" to allow the expression of all cell types (mimicking the level of gene expression of cells at the time of fertilization) (D. Vanderwall, personal communication, March 23, 2004).

The transfer of the donor nucleus into the enucleated oocyte causes morphological and biochemical changes in the nuclear material (Mitalipov & Wolf, 2000), and if successful the nucleus is fully reprogrammed and able to direct the development of the embryo in a similar manner to the development of the animal from which the cell was obtained (Westhusin et al., 2001).

Currently, it is estimated that complete nuclear reprogramming takes place in only a small percentage of nuclear transfers from differentiated cells (Gurdon & Byrne, 2003). The mechanisms of nuclear reprogramming after NT are still unclear (Tsunoda & Kato, 2002b), however, researchers suggest that a second half-century of nuclear transfer research should identify the mechanisms of nuclear reprogramming (Gurdon & Byrne, 2003) and in the future it may be possible to reprogram differentiated cells *in vitro* prior to nuclear transfer (Loi et al., 2001).

f) Collection and Availability of Oocytes

The ease in obtaining eggs from live female donors varies with the species. The collection of ova from species that externally release unfertilized eggs is relatively easy. Conversely, mammalian species require procedures such as trans-vaginal follicular aspiration (a needle guided by ultrasound to remove ova from the ovaries), laparoscopy or surgery requiring general anesthesia (D. Vanderwall, personal communication, March 22, 2004).

If the efficiency of nuclear transfer is less than 1%, it is reasonable to require over 100 oocytes to obtain one cloned offspring (D. Vanderwall, personal communication, March 22, 2004). The needed oocytes may be acquired by the following strategies:

1. Collection of Mature Oocytes: Many mammals only produce and release one or a few mature oocytes per ovulatory cycle (D. Vanderwall, personal communication, March 22, 2004), thus collection from a single living donor would require several collection attempts and/or drugs to induce superovulation (Dominko et al., 1999). Alternatively, mature ova can be collected from several different (living or dead) individuals (D. Vanderwall, personal communication, March 22, 2004).

2. Collection of Immature Oocytes: Immature ova can be collected from living and dead individuals and matured *in vitro*. This approach has proven successful for some species (D. Vanderwall, personal communication, March 22, 2004), however it has yet to be established in many rare and endangered species as even the culture procedures to maintain mature ova *in vitro* have yet to be worked out (Cohen, 1997b). *In vitro* maturation of immature oocytes could greatly increase the availability of ova and increase the ease of cloning rare and endangered species. With only one or a few collection procedures, one female could potentially provide the oocytes for a number of different cloning procedures (in different animals) as there is not a problem with immune responses to foreign oocytes of the same species (D. Vanderwall, personal communication, March 22, 2004).

Collection of ova from living *in situ* rare and endangered animals poses several challenges:

- It is difficult to determine when a female is ovulating in the field or to synchronize ovulation with drugs in order to collect mature ova.
- The animal would likely have to be sedated to keep it from moving during the procedure (D. Vanderwall, personal communication, March 22, 2004).
- Some collection procedures may require sterile environments and specialized equipment, not easily used in the field
- It would be very difficult to collect anywhere near the number of mature oocytes required for a reasonable chance of successful cloning if it was only possible to collect one or a few oocytes from each animal (D. Vanderwall, personal communication, March 22, 2004).
- It may not be desirable to subject critically endangered species to potentially risky ova extraction procedures

g) Availability of Gestational Surrogates

As the number of individuals may be few in rare and endangered species, the number of recipient mothers is likely to be a limiting factor in animal cloning (Lanza, Cibelli, et al., 2000). Due to their scarcity, it may not be desirable to risk the loss of females of endangered species by using them as surrogate mothers for clones. In such cases, many researchers advocate interspecies nuclear transfer, using closely related non-endangered species as surrogate mothers and oocyte donors for the endangered species. This approach allows the females of the endangered species to available for natural breeding (Cohen, 1997b). In addition, interspecies nuclear transfer could be used to reverse extinctions using preserved genetic material.

The success of interspecies NT depends on finding a suitably related surrogate species. Subtle differences even among related species may not allow an embryo to gestate in another species (Lee, 2001), yet there are examples of its successful use (Appendix B).

h) Cytoplasmic Inheritance

Thus far, the majority of clones produced by nuclear transfer have been genetic hybrids, with the nuclear genome of the nuclear donor and the mitochondrial genome of the ooctye donor. The oocyte mitochondrial DNA (mtDNA) is transferred to the clone through the oocyte cytoplasm containing the mitochondria (Lanza, Cibelli, et al., 2000), a cellular organelle responsible for energy production (Prescott, Harley & Klein, 1996). Clones can also be heteroplasmic to varying degrees, containing both nuclear and oocyte donor mtDNA (Di Berardino, 2001). This is possible as the cytoplasm of the nuclear donor cell is combined with the oocyte cytoplasm during nuclear transfer. There is often a resulting selective loss of mtDNA from one of the two sources, commonly donor cell mtDNA (Mitalipov & Wolf, 2000).

Researchers assert that the normal development of clones is not compromised by heteroplasmy (Di Berardino, 2001), however, the long-term consequences of combining genomic and heterologous mtDNA are unknown (Loi et al., 2001). Furthermore, it is unknown how much influence the oocyte mtDNA has on cloned phenotypes (D. Vanderwall, personal communication, March 23, 2004).

The creation of genetic hybrids can be avoided entirely if the nuclear donor is also the surrogate mother, however, this is not always possible or convenient. Thus, in cloning for conservation, individuals of the same (or different) species will likely be used as surrogates. Within a species, the presence of ooctye-derived mtDNA (from another individual) is likely not significant if the goal is the reconstruction of endangered populations –as all the genetic material is within the species (Holt et al., 2004). However, the presence of mtDNA from another species (as in interspecies cloning) could more significantly affect phenotypes.

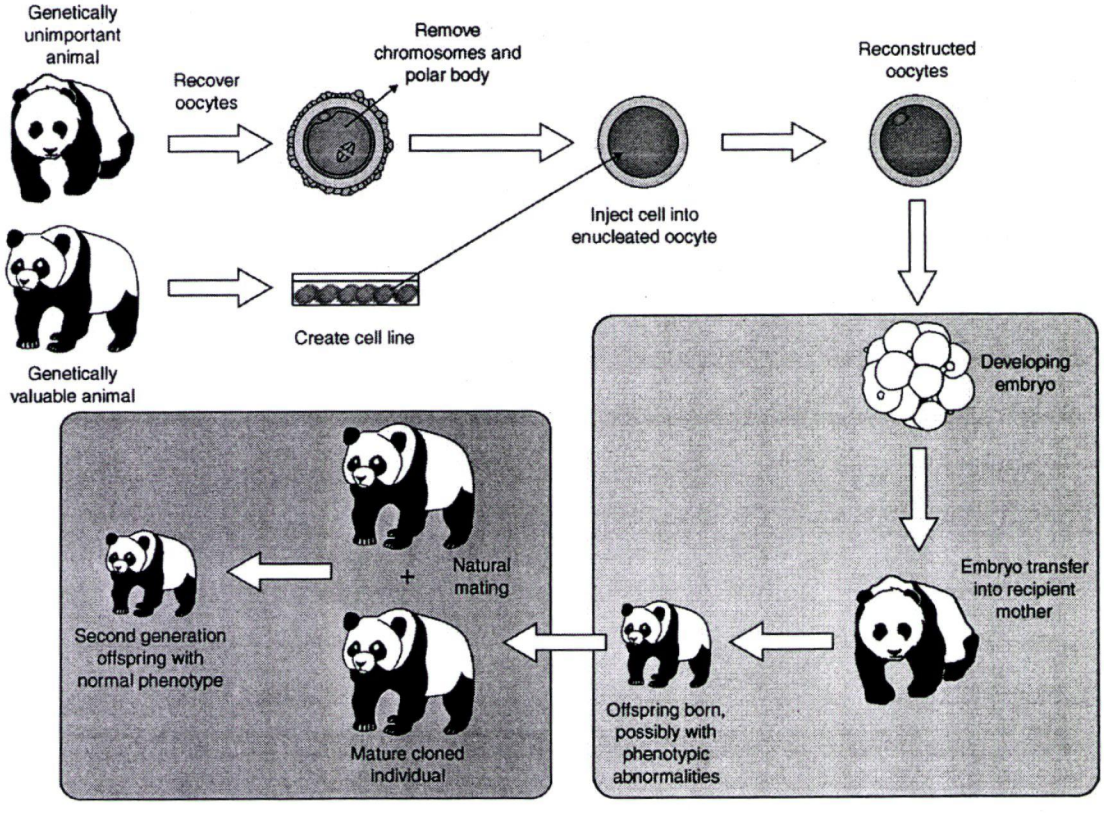

Figure 4.5 Intraspecies cytoplasmic inheritance and transmission of abnormal phenotypes. A female of the same species serves as the oocyte donor, ensuring that the clone contains the mtDNA of its species. While the offspring may exhibit phenotypic abnormalities, they are not necessarily evident in subsequent generations created by natural breeding (Holt et al., 2004).

The first generation of interspecies clones will have the mtDNA of the oocyte donor species. If the clone is a male, he can be mated with a female of his species to produce offspring with the mtDNA of his species. Thus, in theory it should be possible to use interspecies cloning to restore the nuclear genome of the male's genetic line (Holt et al., 2004).

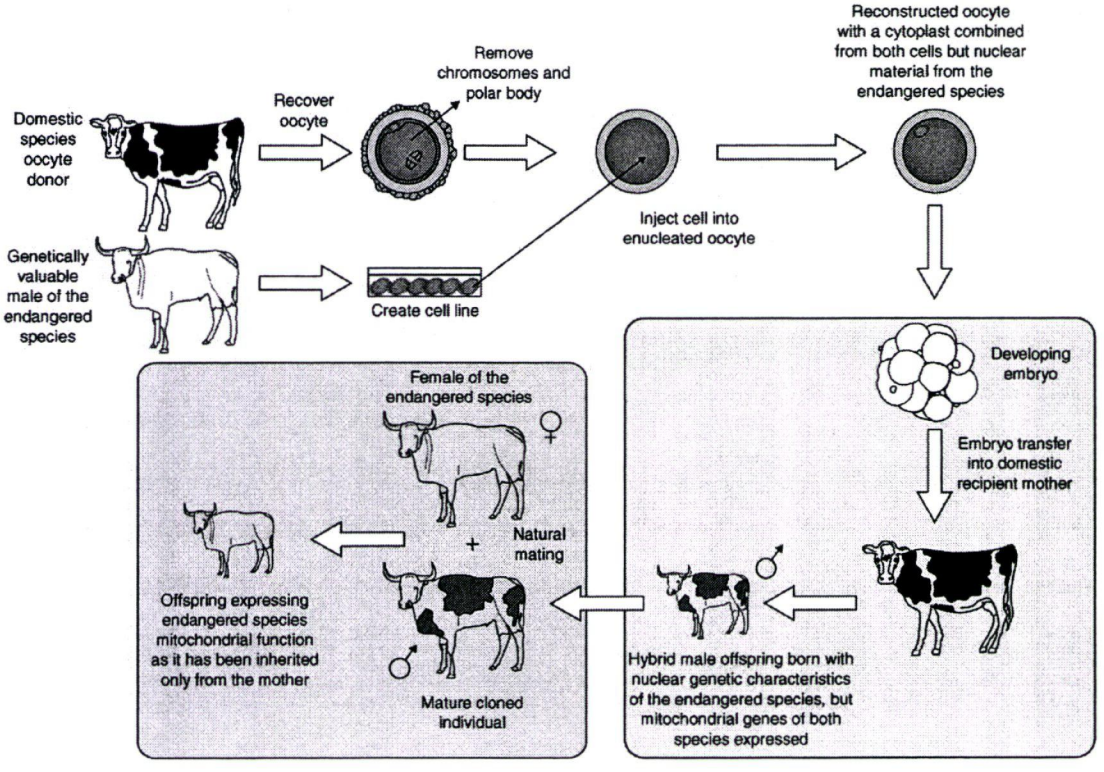

Figure 4.6 The effect of cytoplasmic inheritance on the male genetic line. A domestic species is used as the surrogate mother, transmitting its mitochondrial genome to the clone resulting in heteroplasmy. Subsequent mating of the clone with a female of the endangered species should result in offspring with the mtDNA of the endangered species (Holt et al., 2004).

Conversely, natural breeding of a first generation female clone would yield offspring possessing the mtDNA from the oocyte donor species (as the mitochondria is inherited via the maternal cytoplasm). Hence, the restoration of the female genetic line can not occur as it does for males (Holt et al., 2004).

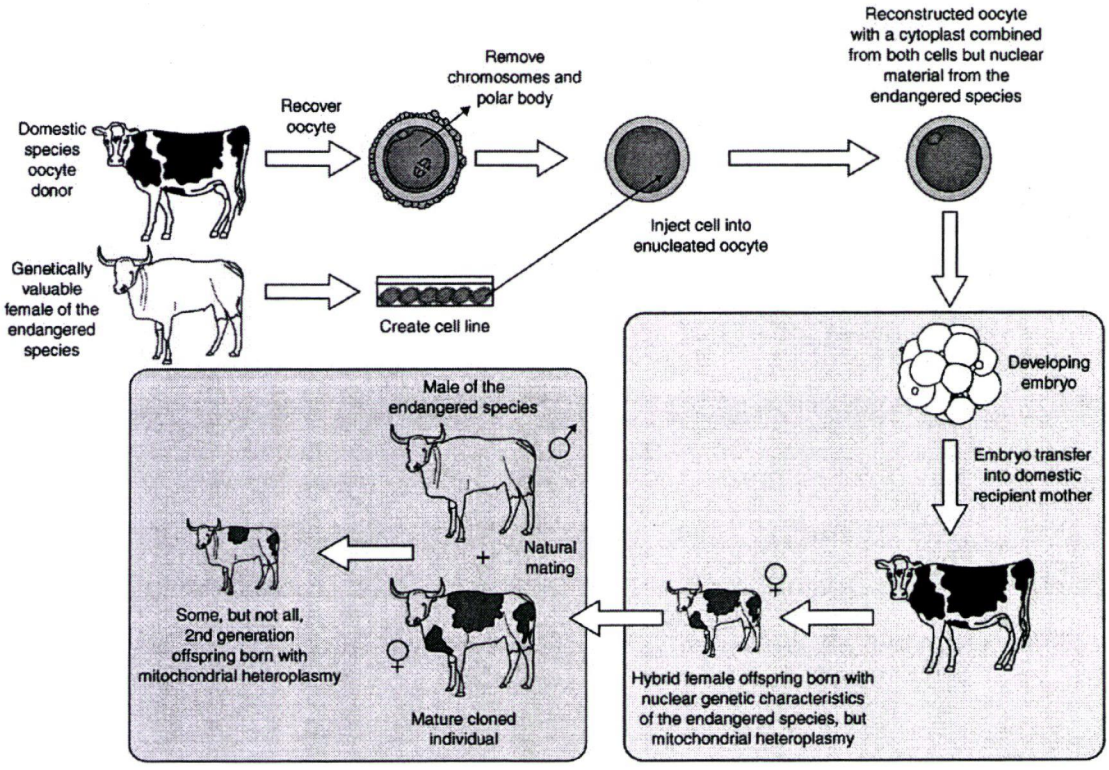

Figure 4.7 The effect of cytoplasmic inheritance on the female genetic line. Interspecies nuclear transfer results in a heteroplasmic female clone, with the mitochondrial genome of both species. Natural breeding of female clone yields heteroplasmic offspring containing oocyte-derived mtDNA due to maternal inheritance (Holt et al., 2004).

Future research may yield genetically engineered surrogate animals with the mtDNA of the species to be cloned, avoiding the creation of genetic hybrids (Lanza, Cibelli, et al., 2000; D. Galbraith, personal communication, May 11, 2004).

i) Embryonic Clone Transfer

The embryonic clones are transferred into the surrogate mother's oviduct or uterus at an appropriate time in her estrous cycle. Thus, the female must be at a point where she is naturally receptive to pregnancy or have been previously treated with hormones (Lanza, Dresser, et al., 2000). Transfer of the embryo to the oviduct requires surgery in many species, whereas transfer to the uterus may be accomplished non-surgically, although many wild species would still likely require sedation (D. Vanderwall, personal communication, March 23, 2004).

Difficulties in transferring embryonic clones to *in situ* females include:

- The lack of basic knowledge about most wild species reproductive biology - required to determine when a female is in her natural estrous cycle, to allow synchronization of the estrous cycle with hormones and transfer of the embryo (Critser et al., 2003).
- The transfer procedure may require a sterile environment and technical equipment not easily used in the field.

Chapter 5: Evaluation of Cloning as a Conservation Approach

5.1 Evaluation of the Theoretical Ability of Cloning to Address Conservation Challenges

5.1.1 Conservation Goals

For the purposes of this MDP, the ultimate aim of conservation will be broadly defined as ensuring the survival and evolutionary development of populations and species in their native habitats (without the need for human intervention) (Holt & Pickard, 1999; D. Galbraith, personal communication, May 11, 2004).

Given this broad definition, there are a number of different conservation strategies that can provide valuable contributions to the ultimate aims of conservation. Exploitation regulations, habitat preservation and modification, translocation, and ARTs (including cloning), can contribute directly or indirectly to the eventual establishment and maintenance of *in situ* populations.

5.1.2 Reconstructing Population Numbers

As the number of individuals in a species declines, the number of effective conservation options often diminishes. Cloning can directly contribute to the accomplishment of conservation goals by providing reproductive options that are particularly valuable in increasing the number of individuals in critically endangered species or rare populations.

- Clones can be added to existing *in situ* and *ex situ* populations to prevent further decline and possible extinction by avoiding the problems experienced by small populations ex. maintaining population numbers at a level where stochastic events are less significant.
- New populations can be also created from the last few remaining individuals of a species, or from genetic samples of an extinct species.

One of the major advantages of a cloning approach to increasing population numbers is that it allows the reproduction of species that have proven difficult to reproduce by other methods. Some species that do not reproduce effectively by natural breeding or other reproductive strategies due to physiological, environmental, or behavioral impediments may be efficiently reproduced by cloning. If cloning is successful but not particularly efficient, it may still be useful in producing 'founder' individuals for use in other reproductive and conservation programs. Other advantages include that the results of cloning are measurable in terms of the number of individuals created and immediate, capable of significantly increasing a population within a single generation.

5.1.3 Reconstructing Genetic Variability

If the conservation goal is the establishment of self-sustaining populations that can persist in changing environments, populations must have genetic diversity on which natural selection can act (D. Galbraith, personal communication, May 11, 2004). Its seems counter-intuitive that the production of genetically identical individuals could provide a role in increasing genetic variation, given that cloning does not increase the number of unique genetic combinations. However, cloning can preserve and increase the genetic diversity of populations through careful selection of the genotypes to be introduced (Holt et al., 2004; Lee, 2001). This targeted approach to cloning can address ultimate and proximate causes of decline and extinction by increasing genetic variation, and mitigating the adverse effects of genetic stochasticity and exposure to undesirable genetic influences.

a) Purposes

1. Restoring "Lost" Genetic Variability

For precariously small populations, the loss of individuals can mean a significant reduction in the amount of genetic diversity within a population. For example, if there are only two members of a species and one dies, half of the species genes are "lost." Similarly, half of the genetic material is lost if only one offspring is produced by sexual reproduction. Cloning ensures the retention of most all of the genetic information (Cohen, 1997a) and provides a method of re-introducing lost genes back to the population by creating a clone which serves as a conduit for the retention of genetic variation (Ryder, 2002; Lanza, Dresser, et al., 2000). The recovery of lost or under-represented genetic lines through cloning could increase the fitness of the population (Holt et al., 2004), thereby avoiding decline and extinction.

Cloning can not increase the number of different alleles, it can only re-introduce the level of diversity of the population when the sample was collected. Thus, whether a population can remain viable in the future depends on the amount of genetic variation in the last remaining founders or preserved samples (Critser et al., 2003). As such, attempts to create populations from single genetic samples will not produce self-sustaining populations (especially for species relying on sexual reproduction and for which genetic variability is important) as all of the clones will be of one sex and would have little genetic diversity.

Genetic variability could be increased despite the state of diversity available in the last founder individuals by releasing cloned populations into divergent habitats. Over time, the different environmental conditions will influence the genetic structure of the population, favoring individuals with minor genetic differences (ex. mutations) that confer advantages. This process, combined with genetic drift should result in genetically different populations and a slight increase in genetic variability.

2. Avoiding the Exacerbation of Inbreeding Depression

An important aim of any conservation oriented breeding or propagation program is to avoid inbreeding depression (Holt et al., 2004). Sexual reproduction in small populations may exacerbate the level of inbreeding depression, resulting in offspring with reduced fitness. As cloning replicates genotypes, clones will exhibit the same degree of inbreeding depression as the sample donors, thereby avoiding the exacerbation of inbreeding depression by sexual reproduction. In addition, selective use of cloning can alleviate the effects of inbreeding by introducing new genes and alleles into inbred populations (Audubon Institute, 2004).

3. Preserving Genetic Traits

Sexual reproduction may yield a generation containing some but not all of the genetic variability of its parents (Holt et al., 2004). Adaptations and rare recessive traits that may confer survival advantages may be lost (Ryder, 2002) by genetic drift or domination by external genetic influences. Cloning can allow the recovery of the entire nuclear genetic complement of the donor without the genetic dilution that would occur in biparental hybrids (Corley-Smith & Brandhorst, 1999). Thus cloning can ensure that desirable traits and the genetic content prior to exposure to adverse genetic influences are represented in the next generation (Kent et al., 2000).

b) Strategies

Depending on the species and circumstance, different strategies can be employed to increase the genetic variation and the number of individuals in a species.

1. Cloning Populations

In situations wherein only a few individuals or a small population remains, a good strategy may be to clone every individual (Holt et al., 2004). This ensures that more copies of the genes are present in the larger population, and increases the likelihood that a greater number of genes are represented in the naturally bred successive generations.

2. Genetic Translocation

For species with multiple populations, another strategy may be to sample individuals from genetically diverse populations and introduce the "fresh" genes into less diverse populations. This is essentially a higher-technology version of translocation, with the advantages of not having to remove the original organism from its population (Lee, 2001; Comizzoli et al., 2000), and requiring less disturbance/handling stress (Weddell, 2002).

This approach, combined with genetic resource banks could allow *ex situ* facilities to maintain smaller populations without a loss in genetic diversity.

3. Cloning from Preserved Samples

Preserved genetic samples can also be used to restore a population to a degree of its former diversity and abundance. The use of stored samples for nuclear transfer provides a means of introducing clones that represent lost individuals, genetic lines and genetic variability, or species.

i) Genetically Valuable Individuals: The cloning of deceased individuals from preserved samples can effectively lengthen their 'genetic life span,' allowing them to contribute to breeding programs after their deaths (Holt & Pickard, 1999).

ii) Lost Genetic Lines and Genetic Variation: Preserved samples can be used to re-introduce a previous state of genetic material prior to any adverse genetic changes that may have occurred as a result of genetic drift, hybridization and outbreeding depression etc. (Frankel et al., 1995). If the genetic samples are collected when the population's genetic diversity was high, then the original level of genetic diversity can be restored by cloning at some point in the future (Loi et al., 2001). Thus, it may be possible to recreate small genetically diverse populations if cells from wide enough genetic samples are available (W. Holt, personal communication, May 12, 2004; Corley-Smith & Brandhorst, 1999).

iii) Lost Species: As cryopreservation banks can provide a means of storing genetic material for 200 or more years (Wildt & Wemmer, 1999), samples preserved prior to species extinctions may provide a means for their resurrection.

As opposed to dynamic conservation approaches which allow a population's genetic material to evolve in response to environmental pressures, the preservation of genetic samples occurs in the absence of these factors (Frankel et al., 1995). Thus, the preserved samples may not posses the needed adaptations for the organism to survive under current environmental pressures. Thus, although genetic banking can help restore genetic variability, it can not be used as an alternative to maintaining natural populations (Primack, 1998). Care must be taken in ensuring that the preserved genetics do not dilute newly acquired survival adaptations, and that clones created from preserved samples can survive in the new environment. Additional factors to be considered include the transmission of genetic defects, abnormalities (C. Hannigan, personal communication, March 29, 2004), diseases and viruses (W. Holt, personal communication, May 12, 2004).

5.1.4 Cloning as an *Ex Situ* Conservation Approach

a) Cloning: an *Ex Situ* Approach

Currently, cloning by micropropagation and somatic cell nuclear transfer is exclusively conducted in *ex situ* environments, and many of the resulting clones (including all animal clones) remain *ex situ* for study, to maintain collections, or to ensure their survival. Although it is generally agreed that *ex situ* preservation of endangered species is secondary to their conservation within natural communities (Frankel et al., 1995; K. Prior, Personal Communication, March 3, 2004), the production and maintenance of *ex situ* clones can serve conservation goals by providing increased access to species that are economically or otherwise valued, or used for other conservation efforts, reducing the pressure on wild populations (D. Galbraith, personal communication, May 11, 2004). In addition, organisms retained *ex situ* may be of use in terms of public education and providing the basis for increased pressure to preserve habitat for species (C. Hannigan, personal communication, March 29, 2004).

b) Species-specific Approach

As a conservation approach, cloning is focused only on the reproduction of specific species. It does not consider the preservation of needed ecological relationships and interactions. Moreover, it does not address the problems that underlie the entire ecological community and cause the decline of species (K. Prior, Personal Communication, March 3, 2004).

c) Availability of Habitat

Cloning can take place in the absence of suitable habitat for that species, as clones can maintained *ex situ*. However, if the ultimate conservation goal is the establishment of self-sustaining wild populations, suitable habitat must eventually be made available. As a conservation option, cloning does not address this eventuality.

d) Addressing Sources of Mortality

Cloning reproduces individuals. As a conservation approach, it does not address the sources of mortality after the clone has been created. Cloning could create a limitless supply of offspring, but have little impact in the long-term conservation of the species as it has no method to ensure the survival of the clones.

i) Short-Term Survival of Clones

A potentially major source of short-term mortality for *ex situ* produced clones is their introduction to *in situ* environments. For plants, the process of acclimatization eases the transition from *in vitro* to *in situ* conditions. However a lack of site-specific adaptations to environmental conditions, and the presence of competitors may make transplantation to *in situ* conditions difficult.

For animals, reintroductions have proven problematic as adaptations to captive environments have resulted in the loss of survival skills and behaviors (Woodworth et al., 2002; Primack, 1998; K. Prior, Personal Communication, March 3, 2004). Due to the costs and difficulties associated with re-introductions, at best only a small number of rare species bred in institutions are successfully released into the wild (Weddell, 2002). It is likely that clones created by interspecies NT will have decreased fitness as the surrogate mothers (of a different species) fail to impart appropriate skills and behaviors to clones that must interact with others of their species raised by conspecific mothers (Ryder, 2002). Thus, interspecies NT may yield clones of endangered or extinct species that do not know how to "be" that species (Nash, 2001), raising the question of how much of a species identity is based on genetic content, instinctual or learned behavior (C. Hannigan, personal communication, March 29, 2004).

ii) Long-term Survival of Clones

The ability of clones to survive and contribute to *in situ* populations over a number of years will depend in part on the appropriateness of their genetic make-up to the prevailing

environmental conditions. Clones created with samples taken from individuals in different environments from that of the intended release may not possess the required adaptations to survive local environmental pressures. Thus, they may not be able to compete or reproduce as well as the naturally-bred members of the population.

An extreme example might be the resurrection of a long-extinct species like the Wooly Mammoth. Without the benefit of centuries of natural selection to shape the genetic content of the species, it is questionable whether the species could survive the current environmental conditions.

To help ensure that the released clones have the necessary adaptations for their long-term survival, it may be desirable to limit the sources of nuclear donors to living individuals from the area of intended release.

e) Ecological Risks of Releasing Clones into Populations

The risks of releasing clones into *in situ* and *ex situ* populations can be divided into two categories: risks to the survival of the clones (previously discussed sources of mortality) and risks to populations and ecosystems. Risks which may decrease the fitness of a population include the transmission of diseases, viruses (Critser et al., 2003), abnormalities, mutations, diluting advantageous adaptations, and adversely increasing or decreasing genetic diversity. Risks to ecosystems may include changes in species interactions and overall ecosystem functioning as a result of altering the relative abundance of species.

In general, the ecological risks associated with the release of micropropagated plants are considered to be minimal, less than to those of translocating species to new areas. The impacts of the release of animal clones into populations and their effects on species genetics are largely unknown. However, future research on the release of clones into captive environments may provide insights. In any case, there is no such thing as zero risk. All human activities occasionally have failures which can lead to accidents (Braun, 2002). Thus, the possibility of ecological disasters should not be ruled out.

5.2 Evaluation of the Practical Ability of Cloning to Address Conservation Challenges

5.2.1 Financial and Technological Requirements

Micropropagation and nuclear transfer cloning are technologically intensive, requiring specialized equipment and trained personnel (S. Murch, personal communication, April 23, 2004; Primack, 1998; K. Prior, Personal Communication, March 3, 2004). Cloning is generally more costly than sexual reproduction, traditional plant propagation (T. Thorpe, personal communication, March 8, 2004), and likely more costly than other ARTs.

5.2.2 Research Requirements and Challenges

Currently, cloning provides an opportunity to conserve only those species whose reproduction is well understood (Loi et al., 2001), including those utilized by humans, related to domestic species, or that have been kept in *ex situ* environments for long periods of time (Weddell, 2002). Some rare and endangered species have been well characterized (S. Murch, personal communication, April 23, 2004), however, the reproductive biology of the majority of such species is still poorly understood (Comizzoli et al., 2000; Holt et al., 2004). The procedures developed for well studied species are not easily transferred due to the large variance in the reproduction biology even between closely related species (Comizzoli et al., 2000; S. Murch, personal communication, April 23, 2004). Thus, the ability to clone a given species will depend on the amount of research focused on that species (Westhusin et al., 2001; D. Vanderwall, personal communication, March 23, 2004).

The realization of the lack of information about a species of interest often comes too late, when the species is in decline and is difficult to study (Holt & Pickard, 1999). For example, rare and endangered species may be difficult to remove from the wild for study due to their scarcity. There may not be enough source material for traditional scale experiments, and several smaller scale experiments must be conducted (Murch, 2004). In addition, it may be difficult to obtain funding for endangered species research for non-charismatic species and those with no immediate commercial value. Lastly, it is unlikely

that the cloning of endangered species for conservation purposes will generate money, and funding for research must come from foundations, academic institutions and governments (T. Thorpe, personal communication, March 8, 2004). Thus, it may take longer than a species can withstand to extinction to collect the detailed biological information.

5.2.3 Gaining Access to Rare and Endangered Species

As rare and endangered species are limited resources, obtaining access to these species for research or conservation may be difficult. They are often difficult to find and regulations (ex. SARA) may preclude access to species designated on 'at risk' lists. In addition, there may be disagreements on which conservation strategy should be used, and different approaches may be vying for the same individuals.

5.2.4 Cloning Patents and Intellectual Property Rights

Somatic cell nuclear transfer and micropropagation procedures have been patented (D. Vanderwall, personal communication, March 23, 2004; T. Thorpe, personal communication, March 8, 2004). However, the patents should not inhibit cloning research for rare and endangered species as it is unlikely that the research would lead to commercial sale or profit. The patent holders are usually only interested in maintaining control over the uses of the technology that generate money (D. Vanderwall, personal communication, March 23, 2004; T. Thorpe, personal communication, March 8, 2004). In addition, there are often ways around patent issues by using a slightly different method or type of genetic sample (T. Thorpe, personal communication, March 8, 2004). Patents may in fact drive cloning research for some commercially valuable species, and the benefits may extend to other un-patented species ex. endangered species.

The ownership of biological materials and clones will likely not be of concern for species in *ex situ* reproduction programs where financial gain does not exist. In such situations, the institution (ex. zoos) owns the individual organism or shares individuals in institutional consortia (shipping the organisms between institutions to increase genetic diversity of populations). In the case of multiple offspring, ownership is transferred with the organism (Critser et al., 2003). Ownership issues become controversial where patents exist and commercial interests prevail. For example, the cloning of high profile endangered and extinct species may generate an enourmous amount of publicity and financial incentives, making shared ownership of clones difficult. Such clones would likely arise out of collaborations involving zoos or botanical gardens, universities, and high-tech private industry, and involve the use of patented methodologies, giving rise to property issues. In addition, if *ex situ* held clones substantially increased the amount of money generated by gate receipts, the ownership of clones created by patented technologies may be called into question. In any case, the legal implications of the use of cloning technologies should be examined before initiating its use in conservation programs (Critser et al., 2003).

5.2.5 Stakeholder Views

The spectrum of support and criticism for cloning as a conservation approach is represented by comments from various stakeholders including conservation and environmental groups, cloning researchers, the cloning industry, zoos and botanical gardens, and regulatory agencies. These views shape public opinion and ultimately affect the utilization of cloning as a conservation approach.

As animal cloning is more controversial, most of the comments are made in this context, however the discussion is relevant to plant cloning as well.

a) Opponents

Many critics view cloning, particularly nuclear transfer, as time consuming, expensive, prone to failure (Nash, 2001) and therefore of limited value to conservation. Some assert that at best cloning could be considered an expensive, high-tech, last-ditch attempt to save a species. At worst, it is a science that will create populations of clones unfit to survive in the wild (Estabrook, 2002).

Conservationists:

It is unlikely that cloning will be readily accepted by conservationists. Many are suspicious of reproductive technologies and have been slow to recognize the benefits of ARTs and hesitant to consider cloning (Holt et al., 2004; Lanza, Dresser, et al., 2000).

Conservation Groups:

Conservation International, an organization dedicated to habitat preservation, and the World Wildlife Fund (WWF) agree that *ex situ* conservation technologies including artificial insemination, *in vitro* fertilization, and cloning may play a limited role in wildlife conservation and in producing self-sustaining captive populations (Estabrook, 2002; K. Baragona, personal communication, April 23, 2004). However, the WWF does

not see much relevance in applying such technologies to the conservation of *in situ* populations, suggesting that "cloning is interesting science. It's not conservation" (K. Baragona, personal communication, April 23, 2004).

Animal Rights Groups:

People for the Ethical Treatment of Animals (PETA) opposes animal cloning due to concerns of animal suffering. PETA cites experiments that have yielded clones with deformities and asserts that it is not right that animals should suffer for the problems that humans have created. The organization views AI, IVP and NT as forms of harassment, and suggests that going to great lengths to save a few "cute" species is not the best way to save animals in general –the best way being the protection of species *in situ* by habitat protection, and dietary and other lifestyle changes (J. Bartlett, personal communication, April 19, 2004).

b) Advocates

Supporters of the use of cloning for conservation commonly include cloning researchers, the cloning industry and some zoos and botanical gardens. Some advocates justify the use of cloning for conservation by citing the current massive species declines and suggest taking any suitable action to reverse the trend (Holt et al., 2004). Others view cloning as a tool to redress past human wrongs against nature (Mastny, 2001) and suggest that "there is a moral imperative to help prevent the decline of vulnerable species towards extinction" (Holt & Pickard, 1999).

Advocates suggest that "biotechnology might offer the best way to keep some endangered species from disappearing from the planet" and that cloning "has an important place in plans to manage species that are in danger of extinction." Many do not view cloning as an entire solution, but recognize that the technology could have important benefits such as saving some of the species that contribute to global diversity (Lanza, Dresser, et al., 2000).

c) Concerns

Circumvention of Habitat Preservation

Environmental and conservation groups including Greenpeace and the WWF view the "catch-all" approach of habitat preservation to be a more valuable conservation approach than species-specific strategies including cloning (K. Gamble, personal communication, March 31, 2004; K. Baragona, personal communication, April 23, 2004). A major concern of such groups is that cloning may be seen as a 'quick fix' to preserving species without having to protect their native habitat. Some speculate that for this reason, cloning may not move forward as quickly as the technology may permit (Fagin, n.d.).

Advocates of using cloning for conservation agree that the preservation of species within their habitats is undoubtedly the most desirable way to prevent extinctions. However, they argue that in practice this is often an unachievable goal (Leibo & Songsasen, 2002). In some cases the battle to preserve species *in situ* has already been lost or the outcome looks dire (Lanza, Dresser, et al., 2000). Habitats may be lost, or specific actions may be required to save critically endangered species (Loi et al., 2001). In these cases, cloning may be considered the only option for some species.

Distraction of Resources

Many conservationists oppose supporting cloning as it may divert resources including biological expertise, time (Mastney, 2001) and money away from more reliable conservation efforts -with higher probabilities of success, lower costs, and using less extreme techniques (Lee, 2001; K. Prior, Personal Communication, March 3, 2004). Specifically, many are concerned about cloning siphoning funds away from habitat preservation (Lee, 2001; Nash, 2001; Mastny, 2001). Conservation biologists argue that efforts to clone endangered species would be so expensive that they could derail other conservation efforts (Cohen, 1997a).

Others counter this view arguing that the funds for undertaking cloning would not have otherwise gone into *in situ* conservation (Lee, 2001; K. Baragona, personal

communication, April 23, 2004). Cloning receives support from biotechnology companies (Mastny, 2001) and from donations by those that would not normally support species conservation (Lanza, Dresser, et al., 2000).

Distraction of Attention

A major concern of both advocates and opponents of the use of cloning for conservation is that cloning could be perceived as a 'technical fix' to curb species loss, lulling the public and policy makers into a sense of complacency about protecting the remaining members of species (Mastney, 2001). Cloning may distract attention away from the causes of species declines and extinctions and from the efforts that pro-actively prevent small problems from becoming larger, requiring more drastic intervention (K. Prior, Personal Communication, March 3, 2004). Some argue that a reliance on technological solutions lets people off the hook. That *ex situ* conservation of species has already resulted in the mentality that species can always be preserved in captivity or cultivation, even if they are critically endangered or extinct in the wild (K. Baragona, personal communication, April 23, 2004). However, others state that expanding the technological capacity of *ex situ* conservation only poses a risk to the primary goals of conservation (the conservation and maintenance of functioning ecosystems) if the overall priority of conservation efforts is changed (Ryder & Benirschke, 1997).

Opponents and advocates alike do not want cloning to be viewed as a panacea, or a complete solution in itself. Moreover, they do not want cloning to be seen as a substitute for other forms of conservation (Nash, 2001; Cohen, 1997a). Advocates assert that cloning should be used in collaboration with other forms of conservation rather than to replace them (Nash, 2001). Most advocates view cloning as "another tool to help insure against extinction" (Kloor, 2000), and as a fallback or "safety net" to ensure the species are here in one form or another, in the event that habitat protection fails (Estabrook, 2002).

Chapter 6: Recommendations and Conclusions

6.1 Summary of Key Findings

Many rare and endangered species possess innate characteristics that increase their vulnerability to decline (Primack, 1998). When these ultimate causes of extinction are coupled with proximate or immediate causes of decline such as habitat change, exploitation (Weddell, 2002), changes in biotic interactions (Levin, 2000), and stochastic events (Primack, 1998), the resulting reduction of the species often presents conservation challenges. Specifically, conservation challenges stem from the few number of individuals or populations, frequently declining population trends (Levin, 2000), low levels of genetic variability (due to genetic drift and inbreeding) (Primack, 1998), (Weddell, 2002), and susceptibility to outbreeding depression (Primack, 1998).

Today, there exists a diverse array of approaches to address the unique challenges associated with conserving rare and endangered species. Such approaches include the development of conservation-oriented regulations, habitat preservation, habitat modification, translocation, maintenance and reproduction of species in *ex situ* environments, the use of seeds and artificial vegetative propagation for plant reproduction, and assisted reproductive technologies for animal reproduction. Advancements in the fields of micropropagation and somatic cell nuclear transfer have resulted in new reproductive procedures, prompting the question of where these technologies fit into the spectrum of conservation approaches.

The advantages of applying cloning technologies to the conservation of rare and endangered species are owed to its unique approach to reproduction. As cloning requires only one genetic sample, it may provide the only method to increase the populations of species with no, one, or only a few remaining individuals. Cloning also allows the reproduction of species that have proven difficult to reproduce by sexual and asexual methods (S. Murch, personal communication, April 23, 2004). A targeted approach to cloning can also preserve genetic traits and increase the level of genetic diversity of populations through careful selection of the genotypes to be introduced (Holt et al.,

2004). The use of cloning to increase the numbers in small populations can also avoid the exacerbation of inbreeding depression.

The impact of patents and intellectual property rights are likely to be case-specific – largely depending on the commercial value of the clones, but in general are not likely to significantly impede cloning research and applications for conservation. In some cases, patents may support research for valuable rare and endangered species.

Drawbacks to applying cloning as a conservation tool include that the scope of cloning is limited only to the reproduction of species. Cloning does not consider the short- or long-term survival of clones, their eventual need for *in situ* habitat and functioning ecological relationships and interactions. Moreover, it does not address the problems that underlie the entire ecological community and cause the decline of species (K. Prior, Personal Communication, March 3, 2004).

Micropropagation and nuclear transfer are expensive, technologically intensive, and require specialized equipment and trained personnel (S. Murch, personal communication, April 23, 2004; Primack, 1998; K. Prior, Personal Communication, March 3, 2004; T. Thorpe, personal communication, March 8, 2004). Cloning can only provide conservation options for species whose reproductive biology has been studied in detail, and gaining access to many rare and endangered species for research or cloning may prove difficult.

Stakeholder concerns may also prove to be an impediment in applying cloning to conservation. Many groups are apprehensive, concerned that the use of cloning for conservation may be perceived as a 'technical fix' to curb species loss, lulling the public and policy makers into a sense of complacency about protecting the remaining members of species (Mastny, 2001). Moreover, cloning may distract attention away from the causes of species declines and extinctions and from the efforts that pro-actively prevent small problems from becoming larger (K. Prior, Personal Communication, March 3, 2004).

The following provides recommendations and conclusions based upon these findings.

6.2 Comparison of Cloning Technologies

Much progress has been made in the development of nuclear transfer technology, generating widespread discussion regarding the potential applications for rare and endangered species. However, the implementation of NT has seen less dramatic gains, with cloning still playing a relatively small role in research and commercial endeavors (Stice, 2002).

Currently, nuclear transfer is an experimental technology (Holt et al., 2004; K. Prior, Personal Communication, March 3, 2004). The primary obstacle in the application of NT to endangered species remains its low efficiency (Holt et al., 2004; Dominko et al., 1999). The high incidence of abnormalities (Foote, 2002), lack of fitness for survival of cloned animals in their natural environments, and the impracticalities of applying NT to non-domestic species (Ryder, 2002) and to wild species in field environments also present challenges. Specifically, the field collection of ova and transfer of the cloned embryo to wild surrogates pose difficulties and much research will be required to apply the technology to wild animals (D. Vanderwall, personal communication, March 23, 2004).

With many hurdles remaining, NT is not at the stage where it is ready to be broadly applied to maintain population viability or to reliably conserve species for which the technology is available (Ryder, 2002). Yet it has been used to accomplish specific conservation goals and advocates predict that animal cloning will play a "modest but growing" role in preserving biodiversity as research is applied to wildlife (Fagin, n.d.), and in the future there will be instances where cloning will make critical differences for some species (Ryder, 2002).

In comparison to animal cloning, micropropagation is currently of more practical value to conservation. The technology has been worked out, with only species-specific procedures to be devised. Micropropagation is already efficient at producing clones which have been used for the conservation of populations. In addition, there are no major controversial issues. Thus, generating rare and endangered species clones by micropropagation is currently easier and more widely applied for conservation purposes than nuclear transfer.

6.3 Cloning as a Useful Conservation Strategy

Micropropagation and nuclear transfer have been successfully used to achieve specific conservation goals. Although cloning is not broadly applied to conservation, it should be considered to be a useful conservation strategy as it can provide a role in accomplishing both case-specific and broad conservation goals as defined in this document.

Addressing Conservation Challenges

Cloning provides unique 'solutions' to some of the problems of conserving rare and endangered species by addressing or mitigating some of the causes and effects of species decline and extinction.

a) Ultimate Causes of Decline and Extinction:

- Low Biotic Potential: Cloning can increase the reproductive output of individuals and even allow the propagation of an individual's genome after their death, thereby compensating for a species inherently low rate of increase.
- Susceptibility to External Genetic Influences: Cloning can reproduce lost or under-represented genetic lines after the species genome has been diluted due to outbreeding.
- Dispersal: Introducing 'new' genetic material (in the form of clones) from other populations or preserved samples to inbred populations may reduce the effects of inbreeding depression.
- Low Genetic Variability: Although cloning can not directly increase the number of new genetic combinations, it can increase the genetic variability within the current state of the species by re-introducing genotypes that have been lost.

b) Proximate Causes of Decline and Extinction:

- Stochastic Events: Cloning can mitigate the effects of demographic, environmental and genetic stochasticity by adding clones to counter the losses by these factors, specifically by adjusting the population level, sex ratio and re-introducing 'lost' genotypes.

c) Inbred Populations: Cloning can increase the genetic diversity of inbred populations by adding clones with 'fresh' genetics from other populations, or deceased individuals.

d) Declining Populations: Cloning can provide a method to reproduce species that are difficult to reproduce by other methods, supplying declining populations with new individuals.

e) Extinct Species: Cloning provides the most likely method of resurrecting extinct species.

As the preceding list demonstrates, cloning is able to address some of the challenges encountered in conserving rare and endangered species. However, it does not represent a comprehensive or holistic conservation strategy as many more conservation challenges are not addressed. Most notably, cloning approaches do not address the short and long-term survival of both individual clones and their species. In the short-term, cloning does not address various sources of mortality ranging from exploitation to the risks involved in introducing clones to *in situ* environments. In the long-term, cloning does not consider the need for species to occupy habitat, to be in the presence of other species in an ecosystem (to maintain biotic interactions), the resolution of ecosystem-level problems affecting the survival of species, and the importance of gene-environment interactions in enabling clones to survive local environmental pressures.

Despite its shortcomings, cloning should be considered as a tool among many conservation approaches, to be applied to rare and endangered species conservation when its use is warranted.

6.4 Expected Applications

6.4.1 Near Future

Currently, and in the near future, the applications of cloning to species conservation will likely be limited to select situations, used as tools to accomplish case-specific conservation goals.

Due to the required effort, amount of research and the high cost associated with cloning, it is likely that only species that are considered to be valuable will be cloned. Thus, species of high conservation value (with important ecological roles), species for which countries have a high global responsibility, endemic and emblematic species will be targeted. In addition, only well studied and species that are otherwise valued (ex. charismatic species) will likely be considered for conservation purposes. In the context of conservation, it is unlikely that cloning of extinct species will be of much interest as most conservationists are currently more concerned with extant populations and would view the resurrection of extinct species as secondary to the conservation of living species (K. Prior, Personal Communication, March 3, 2004).

The preference in conservation will always be to conserve species *in situ* (D. Galbraith, personal communication, May 11, 2004), through the lowest cost, least technological options. As such, cloning will likely only be considered when:

- *In situ* conservation approaches fail.
- Lower cost reproductive options have been exhausted ex. sexual reproduction, other forms of vegetative reproduction.
- It is the most efficient method of achieving the conservation goals.

Thus, cloning will likely be considered only as a last-resort option by most conservationists and environmental managers. As such, the role of cloning in conservation (in the near future) will likely be limited to conserving the last remaining individuals of critically endangered species. As clones (especially animals) are currently likely to be kept in *ex situ* environments –in part due to their value and for protection and research, it is likely that cloning will be used as a captive breeding/propagation tool, rather than for the conservation of wild populations.

6.4.2 Distant Future

Future research into reproductive biology and the development of species-specific cloning and supporting procedures will increase the number of species (and taxa) that can be cloned. For NT, refinements including the resolution of reprogramming problems should result in fewer abnormal clones and increased efficiency in producing offspring. In addition, the resolution of cytoplasmic inheritance issues and the development of methods to allow the use of a greater range of surrogate species should allow greater use of interspecies NT. For Micropropagation, future research may increase the general efficiency of producing clones and reduce the incidence of vitrification.

Research may also defray concerns over the risks of release, and increase the confidence of conservationists and environmental managers in using cloning in field environments. For nuclear transfer, procedures that increase the ease of ova collection and embryo transfer may allow clones to gestate in wild surrogates, avoiding the problems associated with introducing clones from captive to wild environments.

The future applications of cloning for conservation could be very different from present uses and may include:

- The use of cloning as a pre-emptive tool to avoid population decline rather than as a last-resort. As cloning becomes more efficient and less costly, particularly for nuclear transfer, it may become a more efficient reproductive option than presently used approaches.
- The use of cloning for *in situ* applications including reconstructing populations and the management of gene pools.
- Extinct species cloning. As viable samples are found or reconstructed using the DNA of related species, the resurrection of recently and long-extinct species may be accomplished.

6.5 Cloning in the Context of a Larger Management Plan

As cloning is not capable of holistically addressing conservation challenges, it should exist as a targeted approach to achieve specific conservation goals within the context of a larger management plan for a particular species, area, or ecosystem. The broader-scoped management plan should be able to attend to the conservation challenges left unaddressed by a stand-alone cloning strategy. For example, the management plan could use the spectrum of conservation approaches to ensure the protection of ecosystems and availability of habitats, and mitigate sources of mortality by reducing exploitation, modifying *in situ* environments, etc.

The integration of cloning into a larger management plan will also result in the ability to take advantage of cloning technologies while minimizing some of the ecological risks of releasing clones into *in situ* (or *ex situ*) populations and environments. Coordination of cloning with other conservation approaches can organize what species are cloned and/or released, their age, sex and genetic content and where and when they are released. This can avoid many ecological risks of release including adversely altering the relative abundance of species in ecosystems, and creating populations with disproportionate sex ratios and age distributions. As cloning results in genetically identical offspring, coordination is required to ensure no one individual is over-represented, and that the DNA samples used are genetically valuable, resulting in healthy individuals capable of sustaining populations (Critser et al., 2003).

6.6 Regulatory Recommendations

Currently there are no specific regulations governing the use of cloning for conservation. However, conservation plans for endangered species must abide by their country's species at risk regulations and international treaties governing endangered species (S. Murch, personal communication, April 23, 2004). Micropropagation experts suggest that they are not concerned by a lack of regulation, and do not advocate the development of formal laws concerning the release of clones into populations, as they consider the risks of release to be minimal (T. Thorpe, personal communication, March 8, 2004; S. Murch, personal communication, April 23, 2004). In terms of animal cloning, there does not seem to be much interest in advocating the development of formal regulations.

Due to the technical and financial requirements of cloning, in the foreseeable future it is likely that cloning for conservation will only be undertaken by governments, foundations and academic institutions that are likely to be involved in or aware of broader conservation plans for species (T. Thorpe, personal communication, March 8, 2004). The clones are likely to be considered valuable commodities due to the effort required to create them. Therefore, it is not likely that they will be indiscriminately released into populations and environments by their creators. Thus, the use of cloning in an overarching conservation plan should avoid the need to develop formal regulations regarding the release of rare and endangered species clones into populations, as many of these issues can be addressed less formally by this approach.

6.7 Feasibility Analysis

Those considering the use of cloning for conservation should conduct a feasibility analysis in order to determine/consider:

a) The probability that cloning can be used successfully to meet the case-specific conservation goals (K. Prior, Personal Communication, March 3, 2004).

b) Whether the results of cloning can justify the costs and potential difficulties (K. Prior, Personal Communication, March 3, 2004).

c) Alternative conservation options: Cloning must be a good fit for the type of conservation problem (D. Galbraith, personal communication, May 11, 2004). For every species and circumstance, the range of conservation options must be evaluated for the most effective and practical options. Often a lower technology option (such as a conventional breeding program) used earlier may be as or more effective than a technology intensive option (ex. cloning) used at a more "endangered stage." Thus, a little foresight in the management of rare and endangered species can avoid the need for expensive, "technology-intensive" options.

d) Availability of Habitat: Depending on the conservation goals, it may not be desirable to create clones without habitat. Similarly, the availability of *ex situ* space for clones should be considered prior to cloning.

e) Degree of distraction from higher priority conservation needs: Consideration should be given as to whether a cloning application will remove needed resources and attention from higher priority conservation approaches (K. Prior, Personal Communication, March 3, 2004).

f) Case-specific risks: The analysis should consider the transmission of diseases, abnormalities, unexpected results of the release of clones into populations, negative

alterations of population ecology and structure, and methods to ensure safety from ecological disasters (K. Prior, Personal Communication, March 3, 2004).

6.8 Maintaining an Experimental Approach

The applications of cloning for conservation are still being investigated. Maintaining an experimental design in producing and releasing clones will provide valuable information upon which the usefulness of cloning as a conservation option can be further evaluated (K. Prior, Personal Communication, March 3, 2004).

6.9 Future Research

It is obvious that the species requiring most urgent protection and conservation are those that are considered to be 'endangered,' yet it is for these species that we have the least amount of background physiological knowledge (Holt et al., 2004). Many agree that research into the reproductive physiology of endangered species should be a priority given that this fundamental data is missing for the majority of the earth's species (Lee, 2001). Increased reproductive biology research for rare and endangered species would benefit many approaches to conservation, and without which, cloning will not be a feasible conservation option for most species.

Future research should also investigate the impacts of inserting cloned genotypes into populations and the effects on the genetics and fitness of the species. Based on this information, strategic tools can be developed for using cloning for population conservation.

6.10 Planning for the Future

Research should occur concurrently with the preservation of habitat and genetic samples.

Preserving Genetic Samples

The collection of genetic samples in genetic resource banks can act as a hedge against the loss of genetic variation in the future (Nash, 2001). Regardless of whether cloning is currently a realistic option for conservation, the future may result in cloning becoming a more efficient, easier tool than currently practiced methodologies (Ryder & Benirschke, 1997) including sexual reproduction, assisted reproductive technologies and vegetative propagation. In any case, the collection of somatic cell samples especially for rare and endangered species should occur now, while the genetic variation is comparatively high. The future may bring better conservation tools, but it will not bring increased access to genetic resources for declining species. If the collection of genetic samples is delayed until immediately required by a technology (such as cloning), the species may become extinct or severely reduced.

DNA banking can be easily conducted in *ex situ* environments including zoos and botanical gardens, and can be integrated into the handling and collection procedures for wild populations. Regardless of whether the samples are used for cloning or any other approach, they will be useful materials for research.

Preserving Habitat

Species are unique due to their interrelatedness to their habitats, conspecifics, and other species. Complex relationships between species that arise within their natural habitats cannot be re-derived at will. Consequently, cloning can not replace conservation of species *in situ.* Thus, a fundamental priority should be the protection of native habitats (Critser et al., 2003) to simultaneously prevent extinctions and provide habitat for future re-introductions.

6.11 Final Conclusions

Cloning via micropropagation and somatic cell nuclear transfer offers exciting possibilities for the conservation of specific rare and endangered species. In the face of conservation actions that are ineffectual, these new technologies represent a hope for reconciling previously irreconcilable conservation goals (Ryder & Benirschke, 1997). However, the appropriateness of cloning as a conservation tool will vary with the species and circumstance, and it should not be considered a substitute for other forms of conservation or as a panacea.

The applications of cloning to conservation management have in part been demonstrated and it is inevitable that cloning will improve in efficiency and increase in its applications. As such, it will become increasingly useful to consider *how* the evolving technology can most appropriately be considered as a conservation tool, rather than *whether* it is applicable to conservation (Critser et al., 2003). In addition, consideration should be given to the underlying ethical issues. Many applications, including the creation of interspecies nuclear transfer clones, raise ethical concerns including altering species identities, the role of humans in the course of evolution, and whether we can comprehend the outcomes of our actions –especially in the context of complex ecosystems (Long et al., 2002).

In conclusion, cloning is a reactionary approach to conservation, limited to addressing current problems rather than their prevention. Ultimately, preventative strategies should be emphasized in avoiding the need (or desire) to use drastic approaches to species conservation. As the definitive cause of many extinctions stems from man-kind's interactions with nature, it is reasonable to conclude that this is where the emphasis in conserving rare and endangered species should lie.

Personal Communications

Baragona, K. April 23, 2004. Deputy Director of Species Conservation, U.S. World Wildlife Fund. Personal Communication on the use of cloning for conservation.

Bartlett, J. April 19, 2004. Street Team Administrator with People for the Ethical Treatment of Animals. Personal communication on the use of animal cloning for conservation.

Galbraith, D. May 11, 2004. Head of Scientific Development at the Royal Botanical Gardens, member of COSEWIC Reptile and Amphibian Taxon Specialist Group. Personal communication of the use of plant and animal cloning for conservation.

Gamble, K. March 31, 2004. Greenpeace. Personal Communication on the use of *ex situ* approaches for species conservation.

Hannigan, C.J. March 29, 2004. Adjunct Assistant Professor in the Faculty of Environmental Design, University of Calgary, former Calgary Zoo Veterinarian. Personal Communication on the use of cloning for conservation.

Holt, W.V. May 12, 2004. Institute of Zoology, Zoological Society of London. Personal Communication on the use of nuclear transfer technology for animal conservation.

Murch, S.J. April 23 2004. Institute for Ethnobotany of the National Tropical Botanical Garden in Hawaii. Personal Communication on *in vitro* conservation.

Prior, K. March 3 2004. Canadian Wildlife Service. Personal Communication on the use of cloning for conservation.

Thorpe, T. March 8 2004. Faculty Professor of Plant Biology, University of Calgary. Personal communication on the use of micropropagation for conservation.

Vanderwall, D.K. March 22 2004. Northwest Equine Reproduction Laboratory, Department of Animal and Veterinary Science, University of Idaho. Personal communication on the use of somatic cell nuclear transfer for conservation.

Vanderwall, D.K. March 23 2004. Northwest Equine Reproduction Laboratory, Department of Animal and Veterinary Science, University of Idaho. Personal communication on the use of somatic cell nuclear transfer for conservation.

Bibliography

Advanced Cell Technology (2004). Retrieved August 14, 2004, from http://www.advancedcell.com/scnt.htm

Audubon Nature Institute Homepage (2004). Retrieved February 2, 2004, from http://www.audubooninstitute.org/rcenter/res_cloning.htm

Bird, M., Barnes, E., Cray, D., Skari, T., & Walker, J. (2001, January 8). Noah's New Ark. *Time,* 157 (1), 60-61.

Braun, R. (2002, September). People's Concerns about Biotechnology: Some Problems and Some Solutions. *Journal of Biotechnology,* 98 (1), 3-8. Retrieved July 5, 2004, from Science Direct database.

Carr, G.D. (2004). *Hawaiian Native Plant Genera.* Retrieved August 14, 2004, from http://www.botany.hawaii.edu/faculty/carr/kanaloa.htm

Chicago Botanic Garden Homepage (2001, September 21). Retrieved February 2, 2004, from http://www.chicago-botanic.org/research/breeding/tissue_culture.html

Clark, T. (2004). *Plant Propagation.* Mount Holyoke College Botanic Garden. Retrieved April 21, 2004, from http://www.njha.org/pdfs/studman/16.pdf

Cohen, J. (1997a, May 30). Can Cloning Help Save Endangered Species? *Science*, 276 (5317), 1329-1330. Retrieved July 5, 2004, from Academic Search Premier database.

Cohen, J. (1997b, May 30). Would-be Cloners Face Daunting Hurdles. *Science*, 276 (5317), 1329-1330. Retrieved July 5, 2004, from Academic Search Premier database.

Collin, H.A., & Edwards, S. (1998). *Plant Cell Culture.* Oxford: Bios Scientific Publishers Limited.

Comizzoli P., Mermillod, P., & Mauget, R. (2000). Reproduction Biotechnologies for Endangered Mammalian Species. *Reproduction, Nutrition and Development*, 40 (5), 493-504.

Corley-Smith, G.E., & Brandhorst, B.P. (1999). Preservation of Endangered Species and Populations: A Role for Genome Banking, Somatic Cell Cloning, and Androgenesis? *Molecular Reproduction and Development*, 53, 363-367.

Critser, J.K., Riley, L.K., & Prather, R.S. (2003). Application of Nuclear Transfer Technology to Wildlife Species. In W.V. Holt, A.R. Pickard, J.C. Rodger, D.E. Wildt (Eds.), *Reproductive Science and Integrated Conservation* (pp. 196-208). Cambridge: Cambridge University Press.

Cseh, S., & Solti, L. (2000). Importance of Assisted Reproductive Technologies in the Conservation of Wild Rare or Indigenous Ungulates: Review Article. *Acta Veterinaria Hungarica,* 48 (3), 313-323.

Di Berardino, M.A. (2001). Animal Cloning –the Route to New Genomics in Agriculture and Medicine. *Differentiation,* 68, 67-83.

Dominko, T., Ramalho-Santos, J., Chan, A., Moreno, R.D., Luetjens, C.M., Simerly, E., Hewitson, L., Takahashi D., Martinovich, C., White, J.M., & Schatten, G. (1999). Optimization Strategies for Production of Mammalian Embryos by Nuclear Transfer. *Cloning,* 1 (3), 143-152.

Downey, R. (1999). The Social Construction of Dolly in the Canadian Media. Master's Degree Thesis, University of Calgary, Calgary, Alberta, Canada.

Estabrook, B. (2002). *Staying Alive.* Retrieved September 30, 2003, from http://www.audubоninstitute.org/rcenter/stayingalive.htm

Fagin, D. (n.d.). *Gene Bank Acts as a High-Tech Noah's Ark.* Retrieved September 15, 2003, from http://future.newsday.com/4/fmon0412.htm

Fincham, J.R.S., & Ravetz, J.R. (1990). *Genetically Engineered Organisms: Benefits and Risks.* Toronto: University of Toronto Press.

Foote, R.H. (2002). Historical Perspective. In J. Cibelli, R.P. Lanza, K.H. Campbell, M.D. West (Eds.). *Principles of Cloning* (pp. 3-14). London: Academic Press.

Frankel, O.H., Brown, H.D. & Burdon, J.J. (1995). *The Conservation of Plant Biodiversity.* Cambridge: Cambridge University Press.

Green, R.M. (2002). Ethical Implications of Cloning. In J. Cibelli, R.P. Lanza, K.H. Campbell, M.D. West (Eds.). *Principles of Cloning* (pp. 477-491). London: Academic Press.

Gurdon, J.B., & Byrne, J.A. (2003, July). The First Half-Century of Nuclear Transplantation. *Proceedings of the National Academy of Sciences of the USA,* 100 (14), 8048-8052. Retrieved July 5, 2004, from PubMed database.

Hayhurst, C. (1999, November-December). Life after Death. *E Magazine: The Environmental Magazine,* 10 (6), 23-25.

Hill, J.H., & Chavette-Palmer, P. (2002). Pregnancy and Neonatal Care of Cloned Animals. In J. Cibelli, R.P. Lanza, K.H. Campbell, M.D. West (Eds.). *Principles of Cloning* (pp. 247-262). London: Academic Press.

Holden, C. (2003). Banteng Cloned. *Science Now,* August 4, 1-2. Retrieved July 5, 2004, from Academic Search Premier database.

Holt, W.V., & Pickard, A.R. (1999). Role of Reproductive Technologies and Genetic Resource Banks in Animal Conservation. *Reviews of Reproduction,* 4, 143-150.

Holt, W.V., Pickard, A.R., & Prather, R.S. (2004). Wildlife Conservation and Reproductive Cloning. *Reproduction,* 127, 317-324.

Illmensee, K. (2002). Biotechnology in Reproductive Medicine. *Differentiation,* 69, 167-173.

Illmensee, K. (2001). Cloning and Risk Factors. *Journal of Assisted Reproduction and Genetics,* 18 (8), 477.

Kent, D.M., McKently, A.H., Adams, J.B., & Langston, M.A. (2000). Tissue Culture and Outplanting of Rare Florida Scrub Plant Species. *Ecological Restoration,* 18 (4), 249-253.

Kloor, K. (2000). *Field Notes*. Retrieved March 4, 2004, from http://magazine.audubon.org/fieldnotes/fieldnotes0003.html

Kyte, L., & Kleyn, J. (1996). *Plants From Test Tubes: An Introduction to Micropropagation* (3rd ed.). Portland: Timber Press.

Lanza, R.P., Cibelli, J.B., Diaz, F., Moraes, C.T., Farin, P.W., Farin, C.E., Hammer, C.J., West, M.D., & Damiani, P. (2000). Cloning of an Endangered Species (*Bos guarus*) Using Interspecies Nuclear Transfer. *Cloning,* 2 (2), 79-90.

Lanza, R.P., Dresser, B.L., & Damiani, P. (2000, November). Cloning Noah's Ark. *Scientific American,* 283 (5). Retrieved September 15, 2003, from Academic Search Premier database.

Lee, K. (2001, April 3). Can Cloning Save Endangered Species? *Current Biology,* 11 (7), R245-R246. Retrieved July 5, 2004, from Science Direct database.

Leibo, S.P., & Songsasen, N. (2002). Cryopreservation of Gametes and Embryos of Non-Domestic Species. *Theriogenology*, 57, 303-326.

Levin, D. A. (2000). *The Origin, Expansion, and Demise of Plant Species*. New York: Oxford University Press.

Loi, P., Ptak, G., Barboni, B., Fulka, J., Cappai, P., & Clinton, M. (2001). Genetic Rescue of an Endangered Mammal by Cross-species Nuclear Transfer Using Post-Mortem Somatic Cells. *Nature Biotechnology,* 19 (10), 962-964.

Long, C.R., Walker, S.C., Tang, R.T., & Westhusin, M.W. (2003). New Commercial Opportunities for Advanced Reproductive Technologies in Horses, Wildlife, and Companion Animals. *Theriogenology*, 59 (1), 139-149.

Longtin, D., & Kraemer, D.C. (2002). Cloning Red Herrings: Why Concerns about Human-Animal Experiments are Overblown. *Policy Review*, Feb/March, 59-69.

Loskutoff, N.M. (2003). Role of Embryo Technologies in Genetic Management and Conservation of Wildlife. In W.V. Holt, A.R. Pickard, J.C. Rodger, D.E. Wildt (Eds.), *Reproductive Science and Integrated Conservation.* Cambridge: Cambridge University Press.

Maienschein, J. (2001). On Cloning: Advocating History of Biology in the Public Interest. *Journal of the History of Biology,* 34, 423-432.

Maienschein, J. (2002). Part II –What's in a Name: Embryos, Clones, and Stem Cells. *The American Journal of Bioethics,* 2 (1), 14-19.

Mastny, L. (2001). Scientists Cloned Endangered Species. *World Watch,* 14 (1), 8. Retrieved September 15, 2004, from Academic Search Premier database.

Mikulik, J. (1999). Propagation of Endangered Plant Species by Tissue Cultures. *Biologica*, 37, 27-33.

Mitalipov, S.M., & Wolf, D.P. (2000). Mammalian Cloning: Possibilities and Threats. *Ann Med,* 32 (7), 462-468.

Murch, S. J. (2004). *In Vitro* Conservation of Endangered Plants in Hawaii. *Biodiversity,* 5 (1), 10-12.

Nash, S. (2001, September). New Tools, Moon Tigers, and the Extinction Crisis. *Bioscience*, 51 (9), 702-707.

Preece, J.E. & Sutter, E.G. (1991). Acclimatization of Micropropagated Plants to the Greenhouse and Field. In P.C. Deburgh, R.H. Zimmerman (Eds.), *Micropropagation Technology and Application* (pp. 71-93). Dordrecht: Kluwer Academic Publishers.

Prescott, L.M., Harley, J.P., & Klein, D.A. (1996). *Microbiology* (3rd ed.). Dubuque: Wm C. Brown Publishers.

Primack, R.B. (1998). *Essentials of Conservation Biology* (2nd ed.). Sunderland: Sinauer Associates Inc.

Renfroe, M. (1998). *Getting Started in Plant Tissue Culture.* Retrieved February 2, 2004, from http://www.jmu.edu/biology/pctc/tcstart.htm

Ryder, O.A. (2002, June). Cloning Advances and Challenges for Conservation. *Trends in Biotechnology,* 20 (6), 231-232.

Ryder, O.A., & Benirschke, K. (1997). The Potential Use of "Cloning" in the Conservation Effort. *Zoo Biology,* 16, 295-300.

San Diego International Conservation Zoo Project: Frozen Zoo. Retrieved June 4, 2004, from http://www.sandiegozoo.com/conservation/frozen.html

Schaal, B.A., Leverich, W.J., & Rogstad, S.H. (1991). A Comparison of Methods for Assessing Genetic Variation in Plant Conservation Biology. In D.A. Falk, K.E. Holsinger (Eds.), *Genetics and Conservation of Rare Plants* (pp.123-134). New York: Oxford University Press.

Siedel, G.E. (2002). Genetic and Phenotypic Similarity among Members of Mammalian Clonal Sets. In J. Cibelli, R.P. Lanza, K.H. Campbell, M.D. West (Eds.), *Principles of Cloning* (pp. 215-223). London: Academic Press.

Stice, S.L. (2002). Current Research and Commercial Applications of Cloning Technology. In J. Cibelli, R.P. Lanza, K.H. Campbell, M.D. West (Eds.), *Principles of Cloning* (pp. 443-455). London: Academic Press.

Sudhersan, C., AboEl-Nil, M., & Hussain, J. (2003). Tissue Culture Technology for the Conservation and Propagation of Certain Native Plants. *Journal of Arid Environments,* 54, 133-147.

Trounson, A. (2001). Nuclear Transfer in Human Medicine and Animal Breeding. *Reproduction, Fertility and Development,* 13 (1), 31-39.

Tsundoa, Y., & Kato, Y. (2002a). Donor Cell Type and Cloning Efficiency in Mammals. In J. Cibelli, R.P. Lanza, K.H. Campbell, M.D. West (Eds.), *Principles of Cloning* (pp. 267-275). London: Academic Press.

Tsunoda, Y., & Kato, Y. (2002b). Recent Progress and Problems in Animal Cloning. *Differentiation,* 69, 158-161.

Weddell, B. J. (2002). *Conserving Living Natural Resources in the Context of a Changing World.* Cambridge: Cambridge University Press.

Weidensaul, S. (2002). *Raising the Dead.* Retrieved March 16, 2004, from http://magazine.audubon.org/features0205/thylacine.html

Wells, D.N., Misica, P.M., Tervit, H. R., & Vivanco, W.H. (1998). Adult Somatic Cell Nuclear Transfer is used to Preserve the Last Surviving Cow of the Enderby Island Cattle Breed. *Reproduction, Fertility and Development*, 10 (4), 369-378.

Westhusin, M.E., Long, C.R., Shin, T., Hill, J.R., Looney, C.R., Pryor, J.H., & Piedrahita, J.A. (2001). Cloning to Reproduce Desired Genotypes. *Theriogenology,* 55, 35-49.

Wildt, D.E., & Wemmer, C. (1999). Sex and Wildlife: The Role of Reproductive Science in Conservation. *Biodiversity and Conservation*, 8, 965-976.

Woodworth, L.M., Montgomery, M.E., Briscoe, D.A., & Frankham, R. (2002). Rapid Genetic Deterioration in Captive Populations: Causes and Conservation Implications. *Conservation Genetics,* 3, 277-288.

Yahoo News. *San Diego Zoo to be the First to Take a Cloned Endangered Animal* (2004). Retrieved January 22, 2004, from http://story.news.yahoo.com/news?tmpl=story&cid=1850&ncid=737&e=12&u=/cpress/20040122/ca_pr_on_sc/sci_zoo_cloned_animal

Ziv, M. (1991). Vitrification: Morphological and Physiological Disorder of *In Vitro* Plants. In P.C. Deburgh, R.H. Zimmerman (Eds.), *Micropropagation Technology and Application* (pp. 45-69). Dordrecht: Kluwer Academic Publishers.

Appendix A

Key Informant Interview Methodology

The main purposes of the interviews were to identify the key issues associated with using cloning for conservation and to gain a broader perspective on the topic. As such, four major categories of stakeholders were identified representing the Cloning Industry, Regulation and Policy Groups, Zoos and Botanical Gardens and Conservation Groups.

Within these categories, interviews took place with scientists and professionals working, or that have worked in the fields of biotechnology and/or conservation. Specifically, individuals were recruited based on their area of expertise, experience and availability. The ten interviews were not designed to be statistically representative of the stakeholder groups, rather they were intended to simply outline various points of view on the topic.

The specific format of the interviews varied from semi-structured interviews carried out via email to obtain information from interviewees not easily reached by telephone, to non-structured interviews that were more conversational in nature. Although the specific questions varied with the interview, the following interview guide provides a general outline of the line of questioning.

Interview Guide:

- Do you think that cloning technology could play a role in conserving rare and endangered species –now or in the future?

- How do you view cloning as compared to other conservation strategies (including habitat preservation, other reproductive technologies) in terms of providing options to address conservation challenges?

- What do you see as the major advantages and disadvantages of using cloning for species conservation?

- What (if any) policy or management guidelines do you suggest to address the potential risks and disadvantages of using cloning for conservation?

- What do you view as the major obstacles to using cloning for conservation –now and in the future?

- Should cloning be considered a conservation option? If so, how should it fit into the spectrum of conservation approaches?

Appendix B

Examples of Recent Progress in Cloning Endangered Species by Nuclear Transfer

Enderby Island Endangered Breed of Cattle:

The first practical application of adult cloning for animal conservation was demonstrated in the production of a calf from the last surviving cow of the Enderby Island cattle breed. The endangered breed developed adaptations to the harsh Sub-Antarctic conditions of Enderby Island. Despite attempts using AI, ET, and IVF, the species dwindled to a single female 'Lady' (Wells, Misica, Tervit & Vivanco, 1998). Researchers successfully used a somatic cell sample from Lady and a domestic cow as a surrogate to create a cloned calf by nuclear transfer (Comizzoli et al., 2000). Future plans to re-establish the herd center around the use of the clone's ova (as Lady was 13 and had poor quality ova) and cryopreserved sperm samples to yield male offspring and re-introduce lost genetic lines (Wells et al., 1998).

Guar (*Bos guarus*):

In January of 2001, the first clone of an endangered species by nuclear transfer was born. "Noah," the clone of an endangered ox-like animal, was created using frozen skin cells from a guar that died at the San Diego Zoo in 1992. Noah was also the first cloned animal to gestate in the uterus of another species (domestic cow) (Mastny, 2001; Lee, 2001). 692 attempts were required for one live birth (Estabrook, 2002). Noah died two days after his birth of an intestinal infection, a common cause of death among farm animals (Lee, 2001).

Mouflon (*Ovis orientalis musimon)*:

In 2001, this endangered wild species of sheep was cloned from cells collected from a dead (18-24 hour post-mortem) mouflon found in a pasture. Domestic ewes were used as surrogate mothers (Loi et al., 2001).

Banteng (*Bos javanicus)*:

In April of 2003, ACT announced the birth of the world's first apparently healthy clone of an endangered species: a banteng, "Jahava," a wild male bovine from Java. Researchers used a cryopreserved sample taken from a male that died in the 1980s (without having reproduced) and a cow as the egg donor and surrogate mother (Holden, 2003). "Jahava" has since been released into the San Diego Zoo's banteng population of three females (Yahoo News, 2004).

African Wildcat:

"Ditteaux," the first cloned endangered African wildcat and wild carnivore, was born on August 6, 2003 to its surrogate mother, a domestic cat. "Ditteaux" is the clone of "Jazz" who is the result of the first successful interspecies IVF frozen/thawed embryo transfer in 1999 (Audubon Institute, 2004; Kloor, 2000).

Appendix C

Examples of Current and Prospective Animal Cloning Projects:

Wooly Mammoth:

The mammoth has been extinct for millennia. Recent projects are attempting to resurrect this species through the use of nuclear transfer technology and samples collected from frozen carcasses. Thus far, only incomplete genetic samples have been obtained, the result of eons of freezing and cooling of the samples. Researchers have not yet been able to fill in the genetic gaps (Lanza, Dresser, et al., 2000).

Giant Panda:

Based on the advancement in NT technology, China has announced the initiation of a program to clone their giant pandas to increase their populations - possibly using bears as surrogate mothers (Corley-Smith & Brandhorst, 1999).

Thylacine:

The Tasmanian tiger was declared extinct in 1936. The Australian Museum in Sydney is undertaking experiment to create a live animal from a pickled baby specimen and have reported that they have extracted unusually good-quality DNA. However, it is unlikely that a suitable nuclear donor sample will be obtained from this specimen as it was preserved in alcohol which removes water from the cells causing irreversible cellular structure collapse (Weidensaul, 2002).

Researchers plan to tackle the problem of having an incomplete genetic code by filling in the gaps with DNA from closely related marsupial species including the Tasmanian devil and the numbat (Weidensaul, 2002).

Huia:

Researchers in New Zealand plan to clone an indigenous bird, the Huia, declared extinct in the 1920s as the result of a craze for its feathers. The goal is to create a genetically diverse wild population from a number of well-preserved skins in museums (Hayhurst, 1999).

Bucardo (*Capra pyrenaica pyrenaica):*

In 2001, ACT and Spanish officials began a program to resurrect the extinct mountain goat, the bucardo. The last of the species was killed when a tree fell on it (Lanza, Dresser, et al., 2000). The cells from this animal represent the only preserved sample for the species, thus every cloned bucardo would be genetically identical (Estabrook, 2002). Researchers plan to use another species of goat as the surrogate (Bird, Barnes, Cray, Skari & Walker, 2001).