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Faculdade de Medicina Veterinária

MEXICAN GRAY WOLF COURTSHIP AND MATING – BEHAVIOR & BASIC ENDOCRINOLOGY DURING BREEDING SEASON

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Dissertação de Mestrado Integrado em Medicina Veterinária

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MEXICAN GRAY WOLF COURTSHIP AND MATING - BEHAVIOR & BASIC ENDOCRINOLOGY DURING BREEDING SEASON

Abstract

The Mexican gray wolf is the rarest subspecies of gray wolf in North America. It is officially "endangered" and its survival relies on good captive management and breeding programs. The present study's main purpose is behavior evaluation and hormonal profile assessment during proestrus and estrus, in this species. Behavioral data and feces were obtained during the breeding season at the Endangered Wolf Center, and analyzed at the Saint Louis Zoo.

Several behaviors presented moderate correlations. Differences were found between the frequencies of some behaviors in the pre and post conception periods. The average number of days between first detected Mount and first Copulatory Tie was three. Most frequent behaviors were described as well.

A progesterone peak, associated with the onset of estrus, often coincided with the occurrence of Mounts and Copulatory Ties. Our predictions for conception dates were mostly in agreement with the existing hormonal data.

These observations can be a basis for future reproductive situations – they allow for a better estimate of the ideal timing for Artificial Insemination and they add knowledge on reproductive patterns that characterize the breeding season of this species.

Key Words

Mexican wolves; breeding season; behavior; reproductive hormones; conservation

REPRODUÇÃO EM LOBOS MEXICANOS - COMPORTAMENTO & ENDOCRINOLOGIA BÁSICA DURANTE A ÉPOCA REPRODUTIVA

Resumo

O lobo cinzento Mexicano é a subespécie mais rara de lobo cinzento na América do Norte. É oficialmente considerado "em perigo" e a sua sobrevivência depende de bons programas de gestão e reprodução em cativeiro. O principal objetivo deste estudo foi a avaliação do comportamento e perfis hormonais, durante o proestro e estro, nesta espécie. Os dados sobre comportamento e hormonas foram obtidos durante a época de reprodução, no Endangered Wolf Center, e analisadas no Jardim Zoológico de Saint Louis.

Vários comportamentos apresentaram correlações moderadas. Também foram encontradas diferenças entre as frequências de alguns comportamentos nos períodos pré e pós-conceção. O número médio de dias entre a primeira "monta" e cópula detetadas foi três. Os comportamentos mais frequentes foram descritos.

Um pico de progesterona, associado ao início do estro, coincidiu frequentemente com a ocorrência de montas e copulação. As previsões efetuadas das datas de conceção estiveram, geralmente, de acordo com os dados hormonais existentes.

Estas observações podem vir a permitir uma melhor estimativa do momento ideal para Inseminação Artificial e acrescentam conhecimentos sobre os padrões reprodutivos que caracterizam a época reprodutiva desta espécie.

Palavras-chave

Lobos mexicanos; época de acasalamento; comportamento; hormonas reprodutivas; conservação

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ºC: Degrees Centigrades
AI: Artificial Insemination
AZA: American Zoo Association
CEO: Chief Executive Office
cc: Cubic Centimeter
CITES: Convention on the International Trade of Endangered Species of Flora and Fauna
Copulatory Tie: Copulatory Tie
E2: Estrogen
EEP: European Endangered Species Program
EIA: Enzyme-immunoassay
ESA: Endangered Species Act
EWC: Endangered Wolf Center
FGE: Founder Genome Equivalents
FSH: Follicle-stimulating Hormone
FTD: Female Tail Deflect
FU: Female Urinate
g: Grams
GnRH: Gonadotropin-releasing Hormone
h: hours
IA: Inseminação Artificial
ID: Identification
Kg: Quilograms
Km: Quilometres
LH: Luteinizing Hormone
LHRH: Luteinizing hormone-releasing Hormone
ml: milliliter
MM: Male Mount
MMA: Male Mount Attempt
MN: Minnesota
MSLG: Male Sniff/Lick Genitals
MSU: Male Sniff Urine
MUM: Male Urine Marking
MUMO: Male Urine Mark Over
ng: Nanogram
P4: Progesterone
pg: Picogram
PGF: Prostaglandin-F
PMC: Population Management Center
PVC: Polyvinyl Chloride
RIA: Radio-immuno-assay
RPM: Rotations per minute
SLZ: Saint Louis Zoo
SSP: Species Survival Plan
TAG: Taxon Advisory Groups
US: United States
USA: United States of America
USFWS: United States Fish and Wildlife Services
WAZA: World Association of Zoos and Aquariums

WSC: Wolf Science Center

Introduction

Throughout the ages and still nowadays, the wolf has never had a neutral relationship with mankind. Most human cultures consider him a charismatic animal that gave rise to multiple myths, legends, folklore and fairy tales. It has either been revered, respected and protected, or hated, despised and persecuted. Wolves keep making headlines year after year and polarize public opinion. Scientific research plays an important role in wolf-human dynamics, because it provides the basis for a rational common ground. Research efforts within the wolf's range have been diverse, with the majority of data concerning North America (Mech and Boitani 2003).

Wolves are a key part of many ecosystems, and can live almost anywhere in the Northern Hemisphere. The Mexican gray wolf (*Canis lupus baileyi*) is, according to the US Fish and Wildlife Service, "the smallest, southern-most occurring, rarest and most genetically distinct subspecies of gray wolf in North America". It is officially considered "endangered" and its survival relies on good captive management and breeding programs (USFWS – Red Wolf Recovery Program 2015; USFWS – Mexican Wolf Recovery Program 2015).

Reproduction is essential for the continuation and evolution of life. Therefore, this subject naturally becomes a high research priority in the general field of species conservation biology. Even though the theory behind ensuring sustainable populations may appear straightforward, the study and propagating of endangered species is extremely hard to put into practice. Challenges include lack of specimens, dangerous behaviors, stress susceptibility, need for genetic management and, most significantly, an enormous lack of scientific knowledge (Comizzoli et al 2009). To provide the best care and produce optimal reproductive rates, systematic data must be collected, analyzed and shared. Reproductive management is particularly critical to the conservation of small populations such as the Mexican and Red wolves, in order to monitor reproductive success and evaluate why certain genetically valuable wolf pairs fail to reproduce. Hormone monitoring can be used to help identify reproductive problems in these individuals (Asa 2010).

There are two main venues where conservation efforts take place – *in situ* (in natural habitats) and *ex situ* (in zoos and other captive facilities). In both settings, the goal is to maintain sustainable populations, with zoos playing a stewardship role in creating reservoirs of wildlife that are genetically as close as possible to the free-living counterparts (Comizzoli et al 2009).

The present dissertation emerges within this context. To the best of our knowledge, no study has yet systematically described the hormonal and behavioral profiles of reproductive estrus cycles of Mexican gray wolves. The hormonal ones can be used as a valuable reference tool for captive breeding facilities mainly for reproductively unsuccessful females, and for determination of the correct time for Artificial Insemination (AI). The behavioral part of the study will allow us to assess the compatibility of the pairs designated to breed by the Mexican Wolf SSP; to record the dates of mating, so the time of parturition can be predicted; to identify behavioral changes that might be used as markers of estrus for timing of artificial insemination; and to add to the knowledge on courtship and mating behavior, used to establish species "norms" (Asa 2015).

This dissertation has, therefore, the main purpose of describing the behaviors that occur during proestrus and estrus, and characterizing the hormonal profiles during breeding season in Mexican gray wolves. In general, the behaviors that occurred most frequently in different parts of the reproductive cycle were assessed, and their description made, as well as that of the hormonal changes that characterize the breeding season of Mexican wolves.

All the information (behavioral data and fecal samples for hormone determination) was obtained at the Endangered Wolf Center and processed at the Saint Louis Zoo Research Department, under the supervision of Dr. Cheryl Asa. The study and collection of data for the breeding season 2015, in which I took part, started on January 26th and ended on March 21st, but the information used in this dissertation goes back to 1998.

Hopefully this study can be a small contribution for the knowledge on wolves, in particular of this endangered subspecies, since only through research and investigation will we be able to understand and help these unique animals.

Brief Description of the Activities performed throughout the Internship

Wolf Science Center – WSC (Ernstbrunn, Austria)

The first three months of my curricular internship were spent at the Wolf Science Center, in Ernstbrunn (Austria). This research facility focuses mostly on studying Intelligence, Cognition and Cooperation of wolves and dogs, comparing both species and trying to understand the effects of domestication on the development of behavioral traits observed on these animals today. I worked here a total of 472 hours, having Dr. Sarah Marshall (postdoc researcher) as my project supervisor. The project I was assigned to was "Conflict Reconciliation in Wolves and Dogs". Conflict management is a crucial component of social systems, therefore behavioral mechanisms that mitigate conflicts should be strongly selected in animals living in stable social organizations (such as wolves) (Aureli and de Waal 2000). The first aim of this study was to compare wolves and dogs raised in the same way on their tendency to reconcile after conflict. The second one was to test the validity of the hypothesis currently proposed to explain the occurrence of reconciliation.

My job within this project was to collect behavioral data on two wolf packs (one consisting of three adult males and the other of three adult males and two females) and two dog packs (six elements each, including adults and six month old pups). All the information was recorded on the Pocket Observer 3.2, using the software Observer XT 10.5 installed on a handheld device (Samsung Galaxy Note 2). The Ethogram included 60 behaviors, the majority of which related with social interactions, and every two minutes I had to register the proximity between individuals, in order to later calculate affiliative scores. Every time a conflict would occur, I had to record the victim for 10 minutes and on the next day, at the same time as the original conflict, record another 10 minutes for the 'Match Control' observation. Later on, all the videos were coded using the software Solomon Coder. This project allowed me not only to gain vast experience on live behavioral observations, but also on working with the above mentioned softwares. It really gave me the opportunity to learn and understand a lot about wolf and dog behavior and social interactions.

Besides working on my project, I also performed other general tasks, common to all interns, namely: helping prepare the food and feeding the animals, changing the water, providing enrichment, filming experiments, shifting the wolves between enclosures and test rooms, cleaning the working areas, working at the shop, assisting the trainers on the guided wolf walks, writing the blog for the website, preparing various experiments and mostly assisting trainers and other interns or researchers on their projects and tests. It was also required of each intern to do a presentation on their project for the whole team, including the three founders of the WSC. Once a week, we were given the chance to join the trainers on a ‘pack visit’, where we could go inside the enclosure and interact with the wolves (pat them, let them lick us, etc…) without any fences in between.

The time I spent at the WSC definitely helped me understand and get familiar with the reality of working in behavior-related research and learn a lot about how to take care of captive wolves.

St. Louis Zoo (Missouri, USA)

I spent three month and a total of 478 working hours at the St. Louis Zoo Research Department. This was where I collected part of the data and worked on the project for the present dissertation. I worked both in Behavior and Endocrinology, but the Behavioral field work was conducted at the Endangered Wolf Center – EWC.

The St. Louis Zoo (SLZ) opened to the public in 1910, but its origins date back to 1904, when a bird cage, presented in a World Fair that took place in the city, had such a huge success that creating a Zoo here seemed to be a good idea. In the 1920’s, the SLZ became a world reference for being one of the first zoos to build enclosures that tried to resemble natural settings, instead of having just cages. Nowadays, it occupies an area of 36,4 hectares and lodges over 18 000 animals of 700 different species. It is one of the most visited zoos in the USA, receiving over three million people per year. Its prime mission is to help Conservation through quality animal care, research (*in* and *ex situ*), recreation and educational programs that raise awareness and enrich public knowledge on this topic.

The Association of Zoos and Aquariums (AZA) approved Research Department, currently headed by Dr. Cheryl Asa, and where I worked throughout this period, has been active since 1992. Its primary focus is Reproduction. This includes studies of behavior, physiology and endocrinology, and gamete biology. In the same building there are also the Veterinary Hospital, the Veterinary Technicians and pathologist laboratories, and the quarantine area.

It is relevant to mention the extensive work and resources the zoo puts on Research, Education and Conservation. While at SLZ I attended lectures on the state of the Zoo, Polar bears, and outreach programs in several Alaskan villages and schools. I also joined the "Zoo Orientation day", mandatory for all new employees, volunteers and interns, where we learned a bit about the SLZ history, its policies (regarding dealing with costumers and co-workers, dressing code, etc.) and where we had the chance to visit some "backstage" and restricted areas, such as the Nutrition Building (where food is stored and prepared), some general maintenance facilities and even some exhibits still closed to the public.

With respect to the actual purpose of my internship here, I had the opportunity to work at the Endocrinology laboratory for some days, where I learnt to perform progesterone and estrogen determinations on fecal samples collected from Red and Mexican gray wolves (from the EWC). This allowed me to develop practical laboratory experience and to be directly involved in obtaining all data that was used on the work conducting to this dissertation.

Data informatization, organization and processing was also done during my time at SLZ, as well as a lot of the theoretical research and writing.

Endangered Wolf Center[1] – EWC (Eureka, MO)

I did the Behavioral Observations[2] on both Mexican and Red Wolves at the EWC, having spent 70 working hours at this facility. This center forged partnerships with zoos, non-governmental organizations and government entities in Mexico and the United States, to standardize husbandry on both sides of the border and therefore enhance recovery for the Mexican gray wolf. It also conducts nutritional research that benefits endangered canids (in partnership with Purina Mills, Inc.; developed Mazuri Exotic Canine Chow, used widely in the industry, and Mazuri Maned Wolf Diet). Besides, it sponsors noninvasive behavioral research on wolves onsite and in their natural habitat, which has become an invaluable resource for high school, undergraduate and graduate students, as well as professionals in this field. The EWC also does important work to recover Red Wolves, having several animals and breeding pairs.

Usually I worked at this center three days a week, approximately from 8.30 a.m. to 17.00/17.30 p.m., doing both morning and afternoon observation shifts, and also using the time in between observations for entering the data (recorded manually on paper sheets by all the observer team) onto the computer. I observed all the four pairs approved for breeding this season.

[1] For more details on the EWC see "2.2.1. Characteristics of the EWC"
[2] For more details on the Protocol for the Behavioral Observations see "2.2.2. Procedure used to collect the Behavioral Data"

Occasionally I volunteered to work in events organized by the EWC, such as the "Trivia Night" and the "Campfire Howl", which have the objective of earning money for the center and also raising people's awareness for wolf conservation.

Working here also gave me the opportunity to talk to the staff and volunteers and learn firsthand a great deal about how to run such a facility, and the day-to-day work that is conducted on the EWC. I was given the opportunity to accompany the keepers for one morning, to see how the daily routine of taking care of the animals is, and I helped with the feeding and changing of the water. Besides I was allowed to watch the training of the Maned Wolves and Painted Dogs. This experience allowed me to see a different way of handling captivity animals than the one I had learnt in Austria, and these different methods really reflect the very distinct goals of each facility.

Wildlife Science Center (Columbus, MN, USA)

I was given the opportunity of joining a Zoo team on a seven-day trip to Minnesota, to the Wildlife Science Center, for collection of Gray wolves' sperm. These animals serve as a model for research that can later be applied to their more endangered relatives, the Mexican and Red wolves.

The Wildlife Science Center was founded in 1976 as federal research facility dedicated to documenting the behavior and physiology of captive Gray wolves. When the funding ended, in 1991, Peggy Callaghan, its current owner and manager, took over the place. The center has over seven acres, 48 animals (including not only Gray wolves but also Pumas, foxes, bears, lynxes, wolf/coyote hybrids and dogs) and receives an average of 25.000 visitors per year. They focus on education and research, and some of the projects include training people on anesthesia and blood collection of Pumas, studying wolf/coyote hybrids and making semen collections on Gray wolves. For this last one, the center has a partnership with the St. Louis Zoo, hence our work trip there.

The wolves are kept in packs of two to seven animals each. They are usually fed with donated carcasses (of deer for instance) and are given deworming medicine regularly, but are not subjected to any other medical procedures unless in case of emergency or during the sperm collection, time when they are also vaccinated (Wildlife Science Center 2015).

During our stay, we made sperm collections on ten Gray wolves (twice on each), two Red wolves and six wolf/coyote hybrids, including four F1 and two F2. For the procedure, all animals were under full anesthesia and were gaged and blind-folded, for

protection and to decrease outside stimuli. Several displayed convulsions due to the Ketamine used in the anesthetic protocol. Before the collection, all the animals were weighted and their rectal temperature measured. Some developed high body temperatures while waiting for their turn on the collection, so they were cooled down with ice bags under the legs and/or were taken outside, where it was extremely cold. After being put on the table, the urethra was catheterized (catheter number 6 or 8 according to the individual's size) and the bladder emptied, washed with saline solution until the extracted liquid had a translucent appearance. In cases of minor urethral bleeding, the animals were administered prophylactic Enrofloxacin. At the same time, another person was measuring the testicles. Finally, the penis was exposed and held by one person, while another held the cup for the collection, and a third one did the electrical rectal stimulation. A polyvinyl chloride (PVC) cloth with only a small hole for the penis was positioned on the wolf's belly, in order to reduce contaminations of the semen. Pudendal nerve stimulation was done with a Model 12 (G & S Instrument Co., Duncan, TX, USA) electroejaculator, using a rectal probe (PT Electronics, Boring, OR, USA) with three linear electrodes located ventrally. The intensity of the stimulus was slowly increased until extension of the hind limbs. Then was put back to 0 and the sequence was repeated. The semen was collected in transparent plastic glasses that were frequently changed, in order to separate fractions rich in spermatozoids from the ones contaminated with blood or urine. The obtained samples were immediately examined under the microscope (x200) and the percentage of sperm motility estimated. Slides with a sperm smear for assessing morphology were also prepared and stained with eosin-nigrosine (concentration 1:1). To calculate sperm concentration, a Markel camera and dilutions of 1:1 to 1:5 of semen and 2% glutaraldehyde were used. Semen was then centrifuged, the extender added and finally frozen with liquid nitrogen (Asa *et al* 2007).
After all the procedure, the wolves were kept in metal crates until fully recovered from the anesthesia.

This experience allowed me to learn wolf sperm collection in a very practical way, both by observing and by helping the experienced staff who were conducting the procedures. I'm thankful to have been invited to join this trip.

I. Literature Review

1.1. Aspects of Conservation and Importance of Zoos, Parks and Recovery Programs

Zoos have suffered a considerable evolution. They went from being places where the public could see strange and unusual animals, to organizations that directly contribute to the conservation of species. Nowadays, captive programs support conservation in many ways – education, captive breeding, reintroduction, scientific research and funding. There has been much effort from the Zoo community to better integrate captive programs with the needs of conservation in the wild, but there is still a lot of work to be done, especially when it comes to canids. It is crucial that the captive breeding community and field biologists work closely to establish priorities regarding their activities and research. Continued support of field research initiatives is essential, and not only for species held by zoos in their collections. And besides research in the areas of husbandry, behavior, contraception and population control, immobilizations, vaccines, animal health, nutrition, and genome banks, it is also important to create a process in which captive canid programs can be objectively evaluated. The evaluation should cover the genetic and demographic goals of the captive population, research efforts and contribution and link to field conservation efforts. This will assist zoos in remaining focused on the relationship between captive populations and conservation needs of wild canids (Bauman *et al* 2004).

Zoos also play an essential role in educating the public about conservation issues and on implementing captive breeding programs. These are utilized as an *ex situ* conservation tool, in a proactive manner, and should preferably always be combined with *in situ* conservation actions. The importance of captive breeding has evolved as zoos themselves have. Besides allowing captive populations to be self-sustaining, zoos contribute to species recovery and reintroduction by improving reproductive rates and developing monitoring techniques that provide critical data to the understanding of reproductive processes. Several threatened canid species, such as the Mexican and Red wolves, have been saved through captive-breeding efforts in partnership with reintroduction programs. Both were believed to be extinct in the wild, yet small numbers of individuals still existing in captivity were able to serve as founders in the recovery of the species. Focused captive breeding programs provided individuals to the United States Fish and Wildlife Service (USFWS) reintroduction programs. Also in both cases, despite generations in captivity, the reintroduced wolves were able to hunt appropriate game, reproduce and form social groups. The motivations and skills required to survive in the wild had not been lost or compromised during their decades of captive breeding. However, some researchers are still concerned that captive breeding compromises the "wildness" of a species. The absence of natural selection raises the question of whether animals born and raised in captivity can cope successfully if reintroduced to their original habitat. Debate continues regarding the suitability of including captive breeding in endangered-species recovery programs. Yet, with some species, such as the ones here mentioned, there has been no alternative and the results have been very positive (Bauman *et al* 2004).

There are several possible roles that reproductive science can play in captive breeding. One is reproductive monitoring, which may include detecting the onset of puberty, diagnosing pregnancy, assessing fertility and behavioral observations for compatibility, time of mating, and quality of parental care. Genetic preservation through creation of frozen banks for semen, embryos and ova is another service that can be provided to recovery programs. A less well-known component of reproductive management is limiting or reducing reproductive rates, either for the genetic health of the animal or the population or to prevent the birth of animals beyond carrying capacity. Contraception is widely used in US zoos, and is becoming more common in European and Australasian zoos as well. It is especially important for animal welfare on monogamous species such as wolves, because it allows them to not have to be separated during several months during the breeding season. The most commonly used product is Suprelorin (deslorelin), a GnRH (gonadotropin-releasing hormone) agonist that first briefly stimulates then down-regulates reproductive hormones. Although it can be effective for both genders, it is more often used on females, because only one ovulatory event needs to be prevented rather than continual suppression of spermatogenesis (Asa 2010).

Characterizing biological parameters has been useful to advance certain assisted reproductive technologies, including AI, *in vitro* fertilization and embryo transfer. Assisted breeding, especially AI, has a significant role in ensuring genetic heterozygosity, especially when linked to the ability to move cryopreserved spermatozoa, thereby offering an approach to transfer genes between geographically distant specimens or populations (Pukazhenthi and Wildt 2004 *in* Comizzoli *et al* 2009). Other methods, such as non-invasive monitoring of fecal hormonal metabolites (feces or urine) have provided valuable information on reproductive status and function. Wild carnivores are undeniably benefiting from modern reproductive science in various ways

(Comizzoli *et al* 2009). Incorporating features of the species' social systems and mating rituals that allow them to express natural behaviors may improve captive reproduction in a larger percentage of animals. Although this may appear obvious, it is surprising how little is actually known about the social and behavioral needs of many species in captivity (Asa 2010).

Zoos began having a more active role in species conservation in the early 1970's, with the U.S. Endangered Species Act (ESA) and establishment of the Convention on the International Trade of Endangered Species of Flora and Fauna (CITES). The Red wolf, declared endangered in 1967, was actually the first species to provide zoos with the opportunity of directly participating on a recovery program. It was also one of the first to have a Species Survival Plan (SSP). In other regions of the world, similar conservation programs started to be developed, for example, Europe created the European Endangered Species Program (EEP) (Bauman *et al* 2004).

The SSP focuses captive breeding and conservation strategies at a species level. Its main function is coordinating captive efforts. For instance, the AZA Mexican wolf SSP meets every year with the respective Mexican zoo team to discuss progress and problems. Captive wolves in the two countries are managed as one population, with international transfers becoming more common, as genetic pairings are recommended between wolves residing in both places. Funding for species-specific research and *ex situ* projects is also facilitated through the SSP (Bauman *et al* 2004). There are currently more than 450 SSP Programs, each managed by their corresponding Taxon Advisory Groups (TAGs), within AZA. Many of these SSP Programs represent species that urgently need to be conserved and protected in the wild. Each SSP is also responsible for developing a comprehensive population Studbook and a Breeding and Transfer Plan which identifies population management goals and recommendations to ensure the sustainability of a healthy, genetically diverse, and demographically varied AZA population (AZA 2015).

Studbooks became prevalent in the late 1960's and early 1970's. They are the foundation for all captive programs. Accurate pedigree data are vital to genetic management of captive populations, and breeding decisions are based on the genetic and demographic information contained in the Studbook (Bauman *et al* 2004). Studbooks dynamically document the pedigree and entire demographic history of each individual in a population of species. Each one is maintained by a Studbook Keeper, appointed by its corresponding TAG or SSP. All Studbook functional and management processes are specified in the Studbook Keeper Handbook. The primary functions include: creating and maintaining a current Studbook, developed in coordination with the Population Management Center (PMC); presenting general biology and species ecology data; presenting status and distribution of *in situ* populations; developing of a bibliography of relevant publications; monitoring and documenting all *ex situ* births, deaths, and transfer information; maintaining an accurate database that allows detailed genetic and demographic analyses; recommending breeding decisions to enhance genetic diversity; and assessing the population status (e.g. stable, increasing, or decreasing) (AZA 2015).

Even though wild animals have been maintained in captive conditions for centuries, there is still a lot to learn. In order to provide the best care and produce optimal reproductive rates, systematic data must continue to be collected, analyzed and shared (Asa 2010).

1.1.1. The Particular Case of Wolves

Today, *Canis lupus* is considered "vulnerable" globally, according to the new criteria of the World Conservation Union's threat categories, and several small, isolated populations are considered endangered locally. The main factor responsible for the decline of the wolf population is human persecution, usually done with the objective of reducing predation on domestic animals. The greatest long-term threat to the wolf and the second biggest cause of decline in almost all countries is habitat destruction. Suitable habitat means, firstly, habitat that can provide food. Its destruction leads to destruction of the wolf's prey base or the prey's habitat. However, depletion of prey base has not seriously threatened any wolf population to date. Another important characteristic of a proper wolf habitat is that it is a place where humans do not kill them faster than they can reproduce. Wolf distribution has sometimes also been defined by habitat features such as road density, human population density, forest cover or a combination of these factors. For some wolf populations, such as the Red wolf, another threat to the subsistence of the species is the hybridization with coyotes (Mech and Boitani 2003).

Besides facing the above mentioned threats and having to survive in extreme climatic conditions and throughout natural disasters, wolves must also survive infections by parasitic, viral and bacterial organisms that can either kill them outright or impair their prey-catching ability. Even though the role of diseases in limiting wolf populations remains unknown, they are certainly a concern, as is any mortality factor when populations are small and threatened. Rabies, canine distemper, sarcoptic mange and canine parvovirus have been shown to be potential mortality factors that can have substantial effects on wolf populations. Other diseases and parasites with importance in wolf conservation are: protozoa, helminthes, ectoparasites, infectious canine hepatitis, papillomatosis, canine coronavirus, brucellosis, Lyme disease, leptospirosis, and fungal diseases such as blastomycosis and dermatomycosis (Kreeger 2003).

Nowadays, in North America, there is essentially one single continent-wide wolf population, which extends over most of Alaska and Canada and southward into Minnesota, Wisconsin, Michigan, Montana, Wyoming and Idaho. Wolves have also been dispersing to the Dakotas. The only separate populations are those of the Mexican wolves in Arizona and New Mexico and the Red wolves in North Carolina. However, this was not always the case. By 1930 the wolf had disappeared from almost all the 48 contiguous states. Except in Minnesota and Alaska, wolf extermination continued until the last wolf was killed. The campaign to finish off wolves started when the Pilgrims arrived from England, with all the prejudices, beliefs, laws and devices that had just eradicated the wolf back in their home countries. Before this, wolf's relationship with humans (American Indians) was actually a very positive one for the wolf. Although they were hunted, they were also very appreciated and respected. But populations could not survive the "war" against the wolf, which officially began in 1609. Besides, the later westward expansion of the livestock industry, around 1870, coincided with the disappearance of the huge buffalo herds. The scarcity of natural prey resulted in the increase of predation on domestic cattle by wolves, which led to wolf hunting being permitted, even in protected areas. Finally, in Minnesota, Wisconsin and Michigan, 1974, and throughout the 48 contiguous states in 1978, wolves were declared officially protected by the federal ESA of 1973 (Mech and Boitani 2003).

After the publication of the ESA, American federal agencies were charged with recovering threatened or endangered species to the point at which they could be removed from the Endangered Species List. Recovery teams were appointed by the USFWS for four regions and for certain wolf subspecies, namely the Eastern Timber wolf, the Northern Rocky Mountain wolf, the Mexican and the Red wolf. Even though originally these plans were meant to work by subspecies, in 1978 they started applying to geographic areas instead, therefore affecting whatever wolves inhabited the zone with which each plan dealt. The four plans together are an excellent example of a coordinated, extended effort with a strong base of technical, organizational and political tools (Mech and Boitani 2003).

Even today, human-wolf conflict is still one of the biggest problems in wolf management. Prejudice, ignorance and superficial knowledge of the wolf are still widespread, both among wolf "adversaries" and supporters. Another management problem derives from the wolf's complex biology and ecology, which is sometimes hard to understand. Moreover, conservation is a multidisciplinary process, therefore it benefits from a team with experts on different fields, such as biologists, sociologists, land use planners, representatives of "stakeholder" groups, economists, among others. Unfortunately, management is usually only in the hands of specialists of one sector or another. Ultimately, wolf's survival will depend on the public's attitude towards it, and informed decisions, based on actual facts, must be made (Mech and Boitani 2003).

1.2. Social Organization and General Aspects of Wolf Behavior and Ecology

The wolf is the most widely distributed land mammal of all and one of the most adaptable species. It can live in all kind of Northern hemisphere vegetation types – from forest, prairies and tundra, to mountains, deserts and swamps (some even visit large cities) – and tolerate a wide range of environmental conditions, such as temperatures ranging from -56 to +50° C. In the wild wolves can live around 13 years, sometimes more, whereas in captivity their longevity can be up to 17 years of age. They are able to travel more than 72 Km/day, run at 56-64 Km/hour and swim as far as 13 Km. Males are generally larger and around 20% heavier than females (Mech and Boitani 2003).

Wolves lead a "feast-or-famine" existence. They can eat as much as 10 Kg at a time or spend very long periods fasting. They prey on large mammals, as well as on small animals, always tending to kill the less fit individuals, and also scavenge and eat fruits and berries (Mech and Boitani 2003). It is reasonable to assume that, at least to some extent, hunting in groups increases efficiency, even if no cooperative strategy is used. The down side is that multiple hunters must also share the profits, which means that the larger the pack, the less food obtained per individual (Schmidt and Mech 1997). Even when there is sufficient food for the whole pack, the breeding pair intimidates their older offspring and limits their access to it until they have eaten enough to feed their pups. The individuals least likely to be intimidated are the ones most likely to gain access to the food. When sustenance is scarce, breeders usually maintain their nutritional condition and the other family members, especially non-pups, are the ones who go hungry (Packard 2003).

Actually, most wolf hunts are unsuccessful. Most preys have many effective anti-predator traits and strategies that are greatly responsible for this. But there are many other factors that influence hunting success, such as season; time of day; weather; terrain; predator experience; prey species, numbers, age, sex, associates and vulnerability; and past and immediate prey history, just to name a few. One other factor that might influence wolf hunting success rate is motivation, based on time since last kill. However, wolves can sometimes show interest in attacking prey within minutes of leaving a kill, or even stop feeding on fresh kills to take advantage of new opportunities to catch prey. Captive-raised wolves with no experience can also hunt and kill wild prey and survive for years when released into the wild, since this behavior is instinctive to them. Reintroduced captive-reared Mexican wolves are an example of this (Packard 2003). Their hunting technique is different from a dog's (in which they keep their snout close to the ground). Wolves keep their ears up and the nose in the air, paying attention to every scent and noise that the wind might bring. They can detect the smell of a moose about 300 meters away. For hunting larger prey, wolves attack the back of the body, focusing on the ventral region. When hunting smaller animals, they bite and rip the neck, trachea and glottis. Wolves also fish in shallow waters (Mech 1987).

Socially, wolves are organized in packs, which can have up to around 40 animals, although most have much fewer members, but they also can survive temporarily as lone individuals. The average pack size is between three and eleven elements. It tends to be larger where wolves prey on larger ungulates. However, the relationship between pack and prey size is not definitive. When prey availability is reduced, large packs can be reduced in size through lower reproduction and/or survival and through dispersal, and when packs enlarge they sometimes split or proliferate. Pack size does not seem to be a serious constraint on whether wolf population increases or decreases (Rausch 1967 *in* Mech and Boitani 2003).

Food competition could be the feedback mechanism that regulates pack size through dispersal. Prey size and abundance would set up the upper limit to the number of individuals that could share without excessive competition. If food is scarce, the young should disperse earlier (as young as five months), if plentiful they can remain longer (up to three years old). Ideally, they will stay until they are sexually mature, moment when sexual competition and aggression might be the triggering factor for dispersal. Longer term prey fluctuations translate into adjustments in the territorial mosaic (Mech and Boitani 2003).

Packs are logically largest after the birth of the pups and this is the major annual increment to wolf populations. During the summer, some pups and a few adults die, reducing pack size, and the adult mortality usually peaks during fall and winter, which are also the major times of dispersals. This will lead to an even smaller number of pack members. But since some wolves also join packs, single individuals can pair with others, and youngsters often make pre-dispersal trips, pack size fluctuates throughout the year (Mech and Boitani 2003).

The basic social unit of a wolf pack is the mated pair, which has a strong tendency to long-term allegiance (often for life) and the natural extension of the mated pair is their progeny (Asa 1997). The pack functions as a tight unit year round. Pups reach adult size by winter, which gives the pack the appearance of a group of adults. The offspring usually stays with the parents until 10-54 months of age and after that, except under special circumstances, they all disperse. The explanation to why they stay with the natal pack for so long (when compared to other mammals) can be that it is a way for them to mature while still being subsidized by their parents. This also gives them the opportunity to learn the more subtle components of hunting and foraging behavior that are not innate. As for the parents, the benefit can be that this might be the best way to ensure their original investment (Mech and Boitani 2003). It can happen, though not frequently, that strange wolves – that we will here refer to as adoptees – join packs already containing a breeding pair. Most adoptees are males and most adoptions take place from February through May. Some packs can also have a post-reproductive female (Fritts and Mech 1981; Peterson *et al* 1984; Ballard *et al* 1987; Mech 1991; Boyd *et al* 1995; Meier *et al* 1995 *in* Mech & Boitani 2003).

Most packs (especially in the wild) are monogamous, which means only one pair breeds per season. This exclusive breeding by the dominant pair, even in a large nuclear family (i.e. having more than one mature member of each sex) seems to result from a delicate balance of asymmetric mate choice and same-sex rivalry. In these large nuclear families, monogamy is likely to be maintained at least as long as the offspring is not sexually mature. The breeders are more attracted to each other than to their descendants and courtship between siblings is interrupted. If these conditions are not met, multiple breeding may occur (Packard 1989; Solomon and French 1997 *in* Mech and Boitani 2003). Incest is not likely to occur in wild wolves, where they can choose mates other than close relatives and genetic studies tend to confirm this hypothesis (Smith 1997 *in* Mech and Boitani 2003). Another important fact to mention is that there is no evidence that non-breeding adults are physiologically suppressed, even under extreme conditions in which there is no option for dispersal (like in captive packs). They actually show the same typical hormonal cycles as the individuals that reproduced (Packard *et al* 1985 *in* Mech and Boitani 2003).

Packs are usually territorial but, where and if necessary, they can migrate hundreds of kilometers between where they raise the pups and where they take them for the winter, to follow their prey (Mech and Boitani 2003). Pack's territories are usually very large areas (sometimes tens to thousands of square kilometers) with high numbers of prey. Therefore, encounters between neighbors are rare, even along the edges of the territory, although most studies indicate a certain amount of overlap among pack's territories. Wolf movements are primarily dispersive, and marking frequency is low in the absence of foreign marking but, with foreign marking, movement is toward an organizing center and scent marking increases. The concept of territory implies the need for defense, as by definition a territory is a defended area. Wolves have developed very successful physical and behavioral strategies – a combination of three types of "defenses": howling, scent marking and direct attacks. Since wolves hunt and mark as they travel, and since marks are effective for long periods, this behavior allows efficient defense. The main disadvantage of scent marking is that it has little effect over long distances, so howling at various locations along their routes, including homesites, efficiently complements this defense (Peters and Mech 1975 *in* Mech and Boitani 2003). Although howling has several other functions, informing neighboring packs that a territory belongs to a certain clan seems to definitely be one of them (Joslin 1967; Harrington and Mech 1979 *in* Mech and Boitani 2003). These two techniques minimize the encounters between packs. However, when they do happen, the result is often wolves being killed. Since the consequences of territorial encounters are so severe and because systems are in place to avoid them, it is believed that wolves from different packs meet as a result of either desperation (most likely because of hunger) or deliberate aggressiveness (wolves seeking out others to kill or displace). Most wolf deaths resulting from attacks take place near territory boundaries or within buffer zones, and killing by another wolf is one of the most common causes of natural wolf mortality (Mech *et al* 1998).

In order to breed successfully, wolves must find not only a mate but also a territory with sufficient food resources. One of the main ways for the creation of a breeding pair is for dispersing wolves from opposite sexes to find each other in nature. However, there are several other strategies of pair formation such as: multiple breeding, budding and splitting, carving out new territories, usurping a breeder, and distant dispersal. The first one, where some maturing wolves breed in addition to the pack's established breeders while remaining in the natal pack, is definitely an exception to the usual rule of one breeding pair (the dominant pair) per pack. Despite the uncertainties regarding many aspects of extra litters per pack, this strategy proves to be viable and allows some wolves to successfully breed (Mech and Boitani 2003). The budding and splitting consists of a dispersed wolf and its mate to try to set up a new territory along the edges of the one belonging to the natal pack. The splitting implies a group of wolves, whereas the budding refers to a single individual that leaves and reestablishes with a mate. It is probably when two related breeding pairs are present that the pack splits. The split of territory and resources might be a solution for the mortal competitions among kin, and could only be necessary when food is scarce. This would explain why large packs do not always split (Mech 1970). The third strategy happens whenever dispersers establish new territories out of the existing pack's territorial mosaic. The wolves that utilize this method ("floaters") wander around the population and attend areas along the interstices of territories until they find a member of the opposite sex, mate, and attempt to set up a new territory (Rothman and Mech 1979; Fritts and Mech 1981; Meier *et al* 1995 *in* Mech and Boitani 2003). Usurping an established breeding position is undoubtedly the most dangerous strategy. It happens for instance when yearling sons challenge their fathers and breed with their mothers. The fights can become mortal in captivity, but might never get to that in the wild, since the beaten contender can escape (Smith 1997 *in* Mech and Boitani 2003). Finally we have the distant directional dispersal, which is to move long distances in more a less a single direction. Wolves of both genders can disperse to areas up to 886 Km away (Fritts 1983, Ballard *et al* 1987, Boyd *et al* 1995 *in* Mech and Boitani 2003). Dispersers can leave temporarily and return one to six times before leaving the pack for good. When they settle, they may attempt to squeeze into the territorial mosaic of a distant population, join an existing pack, or pair with a member of the opposite sex in an area uninhabited by breeding wolves. Generally, dispersing wolves of both sexes have a high rate of success in settling and pairing in new areas

(Rothman and Mech 1979; Fritts and Mech 1981; Meier *et al* 1995 *in* Mech and Boitani 2003).

The preparations for pup care start before the litter is born. Dens may even be dug already in the autumn. They are usually located away from peripheral zones, where hostile encounters with neighboring packs are most likely to occur (Ciucci and Mech 1992 *in* Mech and Boitani 2003). Adults and yearling from both genders participate in den digging and provisioning the pregnant female (Packard 2003). Litters have an average size of five to six pups, weighing 300-500 g at birth. Compared with smaller canids, wolf litters have fewer individuals and of bigger size. One explanation for this could be that larger cubs may be more resistant to wet and cold weather (Mech 1993 *in* Mech and Boitani 2003). The pups live around the den during the first eight weeks, although the mother might move them from one den to another during this period (Mech *et al* 1997 *in* Mech and Boitani 2003). From around eight to twenty weeks of age, they inhabit an area above ground that includes a "nest", where they huddle together, a network of trails, and various play areas. These areas, along with the dens, are considered "homesites" (Harrington and Mech 1978 *in* Mech and Boitani 2003). At the homesites, pack members provide indirect care to the pups through general defense (during denning, aggressiveness towards intruders increases in both reproductive and non-reproductive males), hunting and provisioning for lactating females. Although all elements participate, they do not do it equally. Mothers provide direct care during the first month after birth, in the form of milk, warmth, and by choosing and maintaining a dry, clean environment (Packard 2003). When the father wolf obtains food, he presents it to his mate either by carrying it in his mouth or by regurgitating it to her from his stomach. When the pups are out of the den, both the breeding male and other adults regurgitate food to them as well (Mech 2000 *in* Mech and Boitani 2003).

Since wolves are highly social animals and spend most of their lives in the company of the packmates, communication is an essential part of their ecology. Wolf pups possess an initial repertoire that guarantees their critical needs will be met. Obviously this repertoire must show a significant developmental plasticity and the use of signals is increasingly more sophisticated as they get older. They learn their packmates' identities and personalities, as well as to predict their subsequent actions. They will end up developing a great deal of sensitivity to different signals. Wolves communicate in many ways – there is auditory, olfactory, visual, tactile and gustatory communication. Olfactory clues can be produced by the wolf's entire body, both inside and out (skin glands, feet, back and tail, ears, anal sacs, preputial glands, vagina, saliva, feces and urine) and contain information about species or individual identity, gender, breeding condition, social and emotional status, age, condition and even diet. The sense of smell is probably the most acute of the wolf's senses (Asa and Harrington 2003).

Wolves can be affected by both social and physical factors in their environment. They share genetic propensities for certain kinds of social behavior, so the basic social reasons for pack dynamics are likely to be similar across wolf populations. Popular educational materials have perpetuated the concept of a linear dominance hierarchy. According to this, the most dominant wolf is the one that wins fights over all others, and is called "alpha". The "beta" is the one that loses a fight with the alpha but wins over all others, and so on. The last ranking wolf is the "omega" (Packard 2003). However, in most wolf packs family dynamics are far more complex. Several researchers (Lockwood 1976, 1979; Packard 1980; Zimen 1981; Mech 1999) who have observed large wolf packs over several years in a wide range of contexts have rejected the hypothesis that all packs fit a linear dominance hierarchy. Zimen (1982) suggests an "age-graded dominance hierarchy" model of conflict within packs. According to this, in interactions with adults, juveniles are more humble. Pups are disciplined by older family members. As juveniles mature, conflict is more likely to occur among members of the same gender (Packard 2003).

As for leadership, Mech defines it as "the behavior of one wolf that obviously controls, governs or directs the behavior of several others". This author has emphasized the concept of one-way, autocratic control by both parents in each family, which is consistent with a deterministic perspective. On a stochastic perspective, parents would influence offspring but the contraire would also be true (Packard 2003). "The autocratic leading wolf does not exist" (Zimen 1981 *in* Mech and Boitani 2003). Packs can actually be considered a "qualified democracy", since pack subordinates can protest their leader's actions. No member decides alone when activity starts or ends, or which way or speed to move, or exercises sole power of command in any other activities important to the pack. In spite of this, we can generally consider that the alpha male is the leader and main decision-maker of the pack. Other wolves, even older ones, respond to him submissively and affectionately. Allegiance to the leader helps keep the pack together (Packard 2003). On a sum-up we can say that wolves are interdependent, exhibit a lot of cooperation in the daily routines, and show tolerant dominance relationships (Mech and Boitani 2003).

1.3. Specific Characteristics of the Species in Study (Mexican gray wolf)

The Mexican gray wolf (*Canis lupus baileyi*), also known as “El Lobo” is, just like all other wolf species, a top predator that plays an essential role on the preservation of ecosystems. It is the smallest subspecies of the gray wolf (weighting between 60 to 90 pounds – 27 to 40 Kg) and unfortunately the most endangered wolf in the world. Their preferred habitat is the ponderosa pine-covered mountains, oak woodlands and adjacent grasslands above 4,000 feet (approximately 1200 meters) in elevation. They can only be found in North America, and their historical range was the Southwest, including Arizona, New Mexico, Texas and northern Mexico (Endangered Wolf Center 2015).

Packs have generally less elements than other gray wolf ones, which can be related to the Mexican wolves’ preference for smaller prey, such as elk, mule deer, whitetail deer, pronghorn, javelina, rabbits and other small animals. Mexican wolves can live up to 16-19 years of age and the oldest wolf currently in the population is 17 years old. Both males and females are able to breed up to 13 years of age. Males and females reach sexual maturity at around one year of age though first reproduction often does not occur until they are two years old (Siminski *et al* 2014). Pups are usually born in late April to early May and the litters’ average size is five to seven pups. Just as with other wolf subspecies, the breeding pair rears the cubs with the assistance of the entire pack (Endangered Wolf Center 2015).

During the late 1800s, Mexican wolf populations suffered from the arrival of the livestock industry in the Southwest. Farmers and land owners started using rifles, traps and poisons to get rid of the wolves, and this virtually eliminated the species. They were therefore included on the Endangered Species List in 1976 and the recovery of the species became a federal concern, which led to the hiring of Roy T. McBride by the U.S. Fish and Wildlife Service, to capture the remaining Mexican wolves. Between 1977 and 1980, in Durango and Chihuahua (Mexico), he caught four males and one female – Nina – which was pregnant and gave birth already in captivity, though none of the cubs survived. There were multiple unsuccessful attempts to breed Nina, and she finally ended up being transferred to the EWC where finally, in 1981 she bore the first Mexican wolf pups conceived in captivity. These animals made up the “McBride” lineage, which formed the early nucleus of a captive breeding program designed to increase the Mexican wolf’s numbers. Nowadays, their descendants still live there and raise litters of their own (Endangered Wolf Center 2015).

In 1995 it was determined that two more lineages were also genetically true Mexican gray wolves – the “Ghost Ranch” and “Aragon” lineages (Endangered Wolf Center 2015). This last lineage includes three wolves that were not wild-caught, but were “found in captivity.” Based on genetic evaluation, this captive lineage is not known to share common ancestry with the other two captive Mexican wolf lineages (Siminski 2011). The three lineages have currently a representation of 80.1%, 5.4% and 14.6% for the McBride, Aragon and Ghost Ranch, respectively (Siminski *et al* 2014).

All of the Mexican gray wolves that exist today are descended from captive animals in the studbook population. In late March 1998, 11 Mexican gray wolves from three independent packs were released into the Apache National Forest in eastern Arizona. In the spring of 2014, the first pups in over 30 years were born in the wild in Mexico, as a result of this new reestablishment effort. A census conducted at the end of 2015 estimated a total of at least 104 wolves surviving in the wild. As for captive individuals, there are 243 animals, distributed among 54 institutions (Siminski *et al* 2015).

From the 1st of January 1991 to 16th July 2014, the Mexican wolf population has shown growth rates capable of achieving up to 11%-14% annually. The potential for growth in this population can actually be much greater than this, given that many of the wolves have not been in breeding situations and breeding has been reduced in the past due to space limitations. The population is currently below its carrying capacity of 300 Mexican wolves, as determined in the Canid TAG Regional Collection Plan, and growth rates can be increased (Siminski *et al* 2014).[3]

All the successful wolf restorations prove that reintroduction can indeed be a viable option for reestablishing wolves in suitable parts of their former range (Bangs *et al* 1998). However, it is important to notice that this is always a controversial matter, and that a correct preparation to deal with the people in the areas where the wolves are released is vital (Mech and Boitani 2003).

Based on many genetic studies, it is known that the Mexican wolves are the most highly differentiated gray wolf taxon in North America, and the genetic diversity of naturally recolonized populations is likely to remain high. One of the implications suggested by these genetic results is that the breeding of pure Mexican wolves in captivity for reintroduction into the wild is well justified (Mech and Boitani 2003). When gene diversity falls below 90% of that in the founding population, reproduction may be

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[3] For more information concerning demography please see Annex II.

increasingly compromised by, among other factors, lower birth weights, smaller litter sizes, and greater neonatal mortality. The current gene diversity of this population is 83.4%. Since this population is descended from only seven founders, maintained as three separate lineages, much of the existing genetic variability has been lost within lineages. Combination of the lineages within the SSP has helped to preserve a higher proportion of gene diversity. Increasing the founder representation from the under-represented lineages and increasing the carrying capacity of the captive population could extend gene diversity (Siminski *et al* 2014). The Mexican wolves have their own Species Survival Plan – the AZA Mexican Wolf SSP – initiated on the 3rd of December 1993 and currently coordinated by Peter Siminski. It is a bi-national program, with cooperative planning between the U.S. and Mexico. The wolves in the U.S. are jointly owned by USFWS and the Mexican wildlife authority.

The breeding recommendations (as decided by the SSP) are described on the "Population Analysis & Breeding and Transfer Plan – Mexican Wolf (*Canis lupus baileyi*) AZA Species Survival Plan® Yellow Program" book, which contains also other important information such as demography, genetics and management strategies. Here, one can also find the Inbreeding Coefficient for each wolf – the lower the value, the more genetically valuable the animal is.

The AZA Wildlife Contraception Center is also working closely with the Mexican Wolf SSP to develop safe and effective contraception recommendations. The current contraceptive recommendation for female Mexican wolves that receive a 'Do Not Breed' request from the SSP, and cannot be separated from a male, is to receive two six-month formulation implants (4.7mg) of Suprelorin® (deslorelin) prior to the breeding season. Moreover, the females should be implanted mid-October to late October, early November at the latest, to ensure they go through the stimulation phase while the male is still infertile. To prevent ovulation and pseudo-pregnancy, it is strongly recommended these females also receive oral megestrol acetate – Ovaban (Siminski *et al* 2014).

In 1990 the Saint Louis Zoo Research Department was assigned by the U.S. Fish & Wildlife Service Mexican Wolf Recovery Program to evaluate fertility of individual males and to establish and maintain a frozen semen bank. All samples are held at the Saint Louis Zoo and at the Chapultepec Zoo in Mexico City. The males are designated for semen banking based on genetic value (equalization of founder representation), representation in the bank, and location (this last one due to the fact that the short breeding season – only around one month of good semen – limits the amount of places that the staff responsible for the semen collection is able to attend to) (Asa and Bauman 2014).

Wolves that are potential candidates for release to the wild are evaluated based on a number of behavioral and physiological criteria including genetic makeup, age, health, reproductive performance and status, proven parental skills and appropriate social behavior, and aversion to humans. Generally, wolves of high mean kinship and genetically well represented in the SSP population are designated for release. Additional analyses are performed to assure that released populations are receiving wolves of appropriate and balanced genetic history (USFWS 2015).

Before release, the wolves go through a process of acclimatization on "pre-release" sites, which include the Sevilleta Wolf Management Facility, Wolf Haven International, the Ladder Ranch in the United States, Rancho La Mesa and La Michilia Biosphere Reserve in Mexico (Asa and Bauman 2014; Siminski *et al* 2014).

1.4. Reproductive Physiology and Behavior

"*The canid reproductive system includes many features that are unusual or even unique among mammals. (...) on gray wolves, for example, these include monogamy, monoestrum with exceptionally long proestrus and diestrus phases, a copulatory lock or tie, incorporation of adult young into the social group, behavioral suppression of mating in these subordinate young, obligate pseudo-pregnancy in subordinate females, and alloparental care.*"

(Asa & Valdespino 1998)

Much of the knowledge about wolves comes from captive animals. Although some argue that captivity can somehow alter the wolf's physiology, years of studies comparing captive and wild individuals failed to show any evidence of a difference (Kreeger 2003). However, reproductive characteristics do vary within and between wolf populations, as well as during an individual's lifetime. Wild females usually do not ovulate until their second, third or even forth winter, and deliver the first litter at two to five years of age. Most wolves do not come into estrus or breed until 22 month of age or older. The maximum breeding age in unknown, but it is documented that litter size declines from age nine on. In the wild, older breeding females are sometimes replaced by their daughters (Kreeger 2003).

Nutritional condition affects physiological reproductive maturation – if extremely good, it can accelerate it by a year, both in captivity and in the wild, whereas nutritional deficiencies or other forms of stress can delay it one or two years (Packard 2003). Physiological suppression of reproduction can occur under high stress. Since the Luteinizing Hormone (LH) in this species is responsive to the opioid antagonist naloxone, it is hypothesized that stress suppression of wolf reproduction could be explained by endogenous opioids suppressing Luteinizing hormone-releasing Hormone (LHRH) in the hypothalamus, resulting in depressed LH secretion and consequently in the failure to ovulate. This mechanism may have implications for captive breeding of wolves. Stress can also cause progesterone (P4) release, probably from the adrenal gland, both in the wolf and in other animals. Its function is still unknown, but it could serve to maintain pregnancy under adverse conditions (Kreeger 2003).

One particularity of some canid species, such as wolves, is that they exhibit monogamy. The primary social unit is the mated pair, with a strong tendency toward long-term fidelity, often for life. Monogamy may also be related with paternal investment, because a male is more likely to invest in the care of youngsters if he can be certain he is their sire (Asa and Valdespino 1998). The tenure of breeders in a wolf pack is typically three to four years, but it can vary from one to eight. A pack may contain several reproductively mature females but in most cases only one reproduces. In unsaturated populations it is unlikely that two or more sisters will remain reproductively active in the same pack for more than one/two years. However, in a small percentage of packs, more than one female may reproduce in a given year. Studies have shown that social behavior is implicated in this inhibition of breeding in subordinate animals, but it is proven that physiological suppression of reproduction does not occur. As for outsider wolves, these individuals are more likely to be accepted into a family in cases of a widowed breeder seeking a new mate (Asa 1999; Kreeger 2003; Packard 2003).

Unlike the female dog, that may come into estrus twice a year and breed year-round, the female wolf is strictly monoestrus and highly photoperiodic. Monoestrus may imply the risk of limited chance for conception. However, this risk appears to be reduced by the relatively long proestrus and estrus periods, as well as by monogamy (Asa and Valdespino 1998). Another aspect of canids reproductive systems that can help explain why monoestrus can be an advantage is the role of postpubertal offspring in the social group – the existence of helpers (alloparents) is associated with an increase of the survival rates of youngsters. The presence of additional members is also profitable for group hunting strategies. Monoestrum can also contribute to social cohesion. Being monoestrus and having synchronized estrus periods, intrapack aggression to suppress subordinate sexual behavior becomes limited to a short period of time (Asa 1999).

In autumn, testosterone in males and estrogen in females begin to rise, priming the reproductive organs for a sequence of behavioral and physiological phases. In North America wolves generally come into estrus between late January and early April and, the further north, the later the cycle starts (Kreeger 2003).

There are several distinct stages on a reproductive cycle: proestrus, estrus, metestrus and anestrus. These phases reflect, respectively, follicular phase rise in estrogen, the initial luteal phase rise in progesterone and decline in estrogen, the remainder of the luteal phase, and the interval between loss of luteal function and onset of next cycle (Concannon 2011).

The duration of each phase is determined by the rate at which ovarian follicles develop and mature within each female, each season. Observations of captive wolves help detail how the courtship behavior of a pair becomes synchronized when male and female are sexually naïve (Packard 2003).

Some authors also mention a pre-proestrus phase, occurring in late autumn or early winter. During this period it is not unusual for either a male or female wolf to express unreciprocated interest in a potential mate. One theory is that flirtatious female behavior is affected by the hormonal changes associated with rising gonadotropin levels and waves of incomplete follicular development. At this time, plasma estradiol rises above the 10 pg/ml typical of anestrus. More frequent scent-marking throughout this phase may be correlated with elevated testosterone levels prior to proestrus in females. During this time, fights between males are more likely to occur.

For the two month prior to estrus, paired wolves sleep within one meter of each other, significantly closer than after mating. Usually the breeding female in each pack is followed more closely by her mate than by other pack members. Each courting pair engages in reciprocal nuzzling, prancing, genital investigation and scent-marking. Pairs scent-mark more frequently than lone wolves and newly formed pairs scent-mark more often than established pairs (Packard 2003).

Proestrus

Proestrus in captive wolves lasts an average of 15.7 +/- 1.6 days, so about double the length in dogs, while in wild wolves it can last up to 45 days. Hormonally, proestrus in wolves and dogs is similar. That is, there is an initial rise in plasma estradiol-17β, varying between 10 to 20 pg/ml, and a peak of 30-50 pg/ml later in this phase. Progesterone remains low, usually below 1 ng/ml, but occasionally increasing to 3 ng/ml. There might be a minor luteinizing hormone (LH) surge 9-24 days prior to the major preovulatory LH surge that occurs during estrus (Packard 2003). Vaginal smear epithelial cell profiles change from being dominated by parabasal cells (accompanied by varying numbers of neutrophils), to being dominated successively by small intermediate squamous cells, large intermediates, and then large cornified cells until finally becoming entirely (98–100%) composed by cornified cells, with virtually no neutrophils on the thickened epithelieum. Cornification reaches 100% at one–six days before the LH surge. The serosanguinous discharge involves serous fluid containing intact and lysed erythrocytes and their hemoglobin, originated by diapedesis in the uterus. By vaginoscopic exam, the mucosa appears edematous, changing progressively from pinkish to white, with serosanguinous fluid on the surface and in deepening vaginal folds that become more prominent in both axes, creating a smooth cobble-stone appearance. Although it is common to see vaginal bleeding, in some animals a closer inspection or vaginal swab may be necessary to confirm it (Concannon 2011; Kreeger 2003; Packard 2003).

Behaviorally, proestrus is characterized by an increased attractiveness to males and proceptive behavior. This is also called “active soliciting”, which is courtship behavior such as the female prancing, body-rubbing, pawing, nuzzling, placing her chin on her mate’s back or presenting her rear near his nose, but refusal to allow mounting. This is attributed to increased blood concentrations of estrogen. However, the frequency of active solicitation varies greatly among individuals, as does female attractiveness to males. The most solicitous females are not necessarily the most attractive (Kreeger 2003; Packard 2003).

During proestrus adult males usually become very attentive to odors in the urine and vulva of their mates. Since experienced males copulate, even if they cannot smell their mates, it is more likely that this olfactory communication functions primarily in behavioral synchronization of sexually naïve, newly formed pairs (Packard 2003). Proestrus finishes with the onset of receptive behavior usually half to three days after the peak in estradiol and within a day of the preovulatory LH surge (Concannon 2011).

<u>Estrus</u>

Estrus is the phase characterized by positive sexual behavior towards the male, including standing in place, presenting the vulva in a lordosis-like manner, reflex deviation of the tail to one side, and permitting mounting and pelvic thrusting. An unreceptive female may snap, growl, pull away, lie down, roll over or shove the male away. Experienced females may spread the rear legs slightly, enhancing their stability as the male mounts and the penis is inserted into the vulva. If a male is inattentive, an estrus female may paw at him, rub against him, or even mount him. These behaviors are attributed to a rise in progesterone after priming by estrogen during proestrus. Males respond to the females' visual and olfactory stimuli by licking her genitals, then mounting them. Inexperienced males may direct mounting behavior to the head or side of the female before learning to mount at the rear. Preovulatory LH surges in wolves reach 5-15 ng/ml in blood, which is lower than in dogs, and lasts one to three days in both canids. The LH surge enlarges and luteinizes mature ovarian follicles, which results in ovulation. It usually occurs within a day of the transition from behavioral proestrus to estrus. During the LH surge, the estrogen-secreting follicles are transformed into progesterone-secreting *corpora lutea*. After the LH peak, estradiol-17β concentrations fall precipitously to about 10-20 pg/ml, and progesterone begins rising rapidly above baseline (Kreeger 2003; Packard 2003).

Ovulation occurs in response to an abrupt end-of-proestrus gonadotropin surge, resulting in a one to three days elevation in LH and a one to four days elevation in FSH. Ovulation has been timed to occur about 48–60 h after the LH surge. Determining the time of ovulation is often critical in breeding management, timing AI, monitoring ovulation-induction, and reproductive experimentation. Since access to rapid LH assays is not available, ovulation is best timed as occurring two days after the first abrupt rise in progesterone of > 0.5 ng/ml, an event that occurs simultaneously with the LH surge in over 95% of cycles in bitches. When early and frequent measurements are not available, the first day with concentrations ≥5 ng/ml is often considered indicative of ovulation in breeding management. Intense crenulation of the vaginal mucosa due to declining estradiol is informative, as it becomes maximal two/three days after ovulation, and recedes thereafter. Ovarian ultrasound can also determine the time of ovulation with considerable accuracy, based on the temporary one/two days marked increase in echogenicity of previously anechoic follicles at ovulation, followed by a return of anechoic structures. Whether echogenicity at ovulation is due to bleeding, follicle collapse, or change in follicular fluid composition is not known. The LH-surge to ovulation interval is characterized by a rapid increase in follicle mural cell luteinization, in growth of theca and blood vessels, abrupt increases in serum progesterone and 17-hydroxyprogesterone, and typically further declines in estradiol. Increased follicular progesterone is likely to be critically involved in ovulation, which also occurs in other species. The vaginal cytology shows uniform cornification of epithelial cells with pyknotic nuclei and disappearance of erythrocytes and leukocytes. The vulva is soft or swollen in this phase (Concannon 2011).

Estrus can last between nine and fifteen days in wolves, whereas in dogs it averages one week. There is not much information about the length of estrus in wild wolves, but it is known that the same female in different years can breed over a span of a month (Kreeger 2003; Packard 2003).

Metestrus

Metestrus, or diestrus, is the period that generally encompasses the luteal phase of the pregnant and non-pregnant wolves. It lasts until parturition or the decline of progesterone to basal concentrations. Plasma Estradiol-17β fluctuates from 10 to 30 pg/ml but, unlike in dogs, there is no prepartum rise of this hormone. Progesterone peaks 11-14 days after the LH peak at 22-40 ng/ml (lower than dogs). This hormone remains elevated for 56-68 days, which is about the same length of gestation. There is a slow increase in prolactin throughout Metestrus, both in pregnant and non-pregnant wolves, and the mean progesterone levels are also similar in pregnant and non-pregnant females. Elevated plasma progesterone levels are invariably maintained through gestation and decline to non-detectable levels at parturition (Kreeger 2003; Packard 2003). Metestrus is considered to last until evidence of the ongoing luteal phase becomes minimal. The end of metestrus and anestrus onset are generally defined as when uterine endometrium has undergone histological "repair", when mammary enlargement in response to luteal phase progesterone recedes, and serum progesterone declines to levels persistently below 1 or 2 ng/ml (Concannon 2011).

On the vaginal cytology there is an abundance of leukocytes, plus round noncornified epithelial cells and neutrophils reappear. Metestrus females that are not pregnant are said to be "pseudopregnant", since some individuals show physical (slight growth of mammary glands that can be accompanied by lactogenesis and lactopoesis) and behavioral (den construction, pup care) changes usually associated with pregnancy (Kreeger 2003; Packard 2003).

In wolves, pregnancy lasts approximately 60 to 65 days. One or two days before parturition, progesterone falls below 3 ng/ml and prolactin increases. Pups are usually born early in spring, so that their nutritional needs coincide with a birth pulse of herbivores, providing relatively easy prey for the adult wolves to hunt (Kreeger 2003).

Anestrus

Anestrus in wolves usually occurs from June to December (except in India) and it is generally a period of endocrine quiescence. On the vaginal cytology one can observe noncornified epithelial cells having light blue cytoplasm with distinct, uniformly sized nuclei; and leukocytes in relatively high numbers (Kreeger 2003). There are also sparse numbers of parabasal cells and degenerate "squames" and variable but modest numbers of neutrophils. The vaginal mucosa appears thin and red with visible capillaries; the surface is easily traumatized and vaginal cytology difficult to monitor without inducing bleeding with spurious erythrocytes in smears. The apoptotic index and percent of degenerated epithelial cells in the endometrium are high during the mid-luteal phase, low in early anestrus and absent by day 120. In bitches, serum estradiol is reported to be variable but generally low and serum progesterone remains below 1 ng/ml, with a nadir near 400 pg/ml at 30–40 days before proestrus (Concannon 2011).

In dogs, after the three to ten month obligatory anestrus period, a pool of LH-sensitive follicles is selected from a group of dominant small antral follicles that would otherwise undergo atresia. Especially on the last 50 days of anestrus, these follicles increase in number and size. During this period the already high concentrations of FSH become further elevated, which is likely important in maintaining, if not stimulating, overlapping waves of dominant follicles. At the end of anestrus, the increase in the frequency of high-amplitude LH pulses causes the final selection and terminal development of the follicles (Concannon 2009).

Males

On the subject of male reproduction, wolves are physiologically capable of breeding at ten months of age, although that rarely happens. At this age there is a high percentage of immature spermatozoa and testicles are small. Even at 22 months old they may still appear undeveloped. Age of reproductive senescence is not known. Just as for the female, male wolves demonstrate a photoperiodic reproductive cycle relative to LH and testosterone secretion and testicular morphology. In North America, testosterone fluctuates from 10 to 560 ng/dl during the year, with zeniths from December to March and nadirs from June to September. LH, stimulated by luteinizing hormone-releasing hormone (LHRH), shows a similar cycle. Unlike the male dog, which is reproductively viable all year long, spermatogenesis in the male wolf is seasonal. The cyclic production of testosterone explains why sperm production is also cyclic, reaching a maximum during breeding season (Kreeger 2003).

In a successful copulatory sequence, rapid pelvic thrusts follow the insertion of the penis into the vulva, while the male forelegs clasp the female behind the ribcage. When ejaculation occurs, the final thrust is prolonged a bit and the male may raise his chin and/or rear legs slightly (Packard 2003). The ejaculate has three distinct fractions: the first and third are originated in the prostate, whereas the second is the one that contains mainly sperm. It is thought that this sperm-rich portion is deposited into the cranial vagina and then flushed to, or possibly through, the cervix by the large volume of the prostatic fluid. Studies with frozen semen actually showed greater pregnancy rates when prostatic fluid was used to increase the volume of the inseminate (England *et al* 2006).

During pelvic thrusting the bulbous gland at the base of the penis engorges with blood and locks the pair in a copulatory tie. Usually, the male dismounts and the two stand or lie rear-to-rear until the swelling declines, which can happen in five to 36 minutes. The tie is shorter if the female struggles and tries to pull away, or if other wolves interact with the tied pair. The ejaculation followed by the expansion of the penile bulb are spinally mediated reflexes facilitated by androgens. Females respond to stimulation by the penile bulb with rhythmic contraction of the smooth muscle of the uterus, such that the sperm are squeezed toward the ovaries. This happens presumably due to a short-term pulse of oxytocin. Vaginal contractions are present during normal coitus, and can be stimulated in the estrus female by digital palpation/dilation of the vagina. The function of the copulatory tie may be to help avoid post-copulatory sperm competition and/or reinforce the pair bond. The total number of copulations per estrus varies among individuals (Packard 2003).

1.4.1. Importance of Olfactory Communication and Urine Marking in Reproduction

Olfactory communication plays an important role on reproduction, in wolves as in other species.
Secretions from the anal sacs, preputial glands and vagina, as well as urine, can contain important information throughout the breeding season. The observation that female wolves rarely investigate another wolf's anal area except during the breeding season suggests the involvement of circumanal glands or anal sac secretions in reproductive communication (Asa unpublished data). As for the preputial glands secretions, its production in male dogs is stimulated by androgens and inhibited by estrogens, which implicates these secretions in reproductive communication (Sansone-Bassano and Reisner 1974; Van Heerden 1981 *in* Asa; Harrington 2003). In females similar glands exist on the clitoris, and they contribute to the attractiveness to males of the perineal area on estrus females. However, although females sometimes sniff and lick the preputial area during breeding season, what is more common is dominant males standing over subordinates, presenting this area for investigation (Mech 2001; Asa, unpublished data).
The vagina and uterus also secrete substances that play a role in reproductive communication. During proestrus and estrus, due to the influence of estrogens, the blood flows from the uterus through the vagina and incorporates vaginal secretions. This sanguineous discharge lasts about six weeks, and communicates the reproductive status of the female. It is attractive to males even from considerable distances. Male interest in urine may also be influenced by vaginal secretions (Asa and Harrington 2003).
Although spacing is the primary function of urine marking in most species, wolf's urine also carries a message about gender and reproductive condition. Wolves of both sexes urine-mark considerably more often during the breeding season, and their marks often overlay each other. First one individual marks, then the other sniffs the mark and marks close to it, and sometimes each marks two or three times (Peters and Mech 1975 *in* Asa; Harrington 2003). Double or tandem marking seems to be related to the formation and maintenance of the pair bond. The frequency of double marking is the highest in newly formed pairs (Rothman and Mech 1979 *in* Asa; Harrington 2003). Furthermore, reduced rates of double marking in captive wolf colonies were associated with failure to bond and reproduce (Mertl-Millhollen 1986 *in* Asa; Harrington 2003). So it appears that urine marking in both genders is related to reproduction, advertising proestrus and estrus and establishing a pair bond. A pair's double marks may even serve a triple purpose: to a

partner they convey a courting message, to single individuals it indicates a mated pair and to other pairs it warns against territorial intrusions (Peters and Mech 1975; Rothman and Mech 1979 *in* Asa; Harrington 2003).

Compared with dogs, the relatively long proestrus period of the female wolf (average of six weeks versus one on dogs) may facilitate pair bonding. Proestrus is preceded by a transient increase in testosterone and accompanied by elevated estrogens. During this phase, vaginal secretions incorporated in the urine, together with an increase in urination frequency, make the female attractive and appear to stimulate double marking. This is probably an important time for the female to evaluate the suitability of the male as a mate and for fortifying the pair's commitment. It is important to note though, that despite proestrus and estrus being focal and particularly important times for exchange of olfactory information between male and female, wild wolves can and do pair-bond and double mark at any time of the year, and year-round (Mech unpublished data *in* Asa; Harrington 2003). After the sexual experience, the importance of olfaction is reduced and visual and social cues may be enough, which demonstrates the role of learning (Asa; Harrington 2003).

Urine marking and raised leg urinations are also intimately related with position within the pack. The onset of urine marking at puberty is displayed only by dominant males. The subordinates continue to use a juvenile standing posture throughout adulthood except in the eventuality of an individual challenging the alpha male. Testosterone is required, but not sufficient, to permit raised-leg urination – it is needed for the organization but not the activation of the behavior. For that, the interaction between social status and testosterone are required. Urine marking behavior in females also seems to be influenced by testosterone, not estrogen (Asa *et al* 1990). Female wolves are much more likely to urine-mark than bitches. Since social status is critical, only dominant females urine-mark. Males display this behavior significantly more often than females though (Asa, Mech and Seal 1985; Asa *et al* 1990). Both dogs and wolves can discriminate between the urine of individual conspecifics (Brown and Johnston 1983 *in* Asa; Harrington 2003).

1.5. Brief Reference to some Aspects related with Reproductive Success

1.5.1. Inbreeding and main associated Problems

Even though the primary threats for the survival of several species (including the wolf) are anthropogenic, inbreeding within the resulting small and isolated populations can increase their susceptibility to possible threats (Lacy 1997 *in* Holt *et al* 2002). Inbreeding depression has been confirmed or strongly inferred for inbred carnivore populations such as wild and captive gray wolves and captive brown bears (Laikre & Ryman 1991; Peterson *et al* 1998 *in* Holt *et al* 2002).

Therefore, and given the conservation history of Mexican wolves' populations, it seemed relevant to make a quick mention to Inbreeding. Mexican wolves provide an excellent example of the successful use of scientific tools in captive population management, as well as the practical difficulties of conserving such critically endangered species (Asa *et al* 2007).

Inbreeding, and consequent increased homozygoty, is pretty much inevitable in small populations. Even if mating with close relatives is generally avoided by the individuals, after just some generations the number of relatives will exceed the number of founders contributing to the population's gene pool, and consequently the genomes of all possible mating pairs will share many alleles identical by descendant, which will result in the offspring being highly homozygous (Asa *et al* 2007, Taylor 2002 *in* Holt *et al* 2002). However, on a good note, population genetics theory indicates that, even in cases where each member of the pair is highly inbred, but not with each other, their offspring will not be inbred and, most likely, will be healthier than their inbred parents (Asa *et al* 2007).

When a population descends from only a few founders (such as in the case of Mexican wolves) it will have large inbreeding coefficients. If the population remains small and isolated, its members will also have a high kinship coefficient. Decreased reproductive rates, commonly observed in such kind of populations, have generally been attributed to inbreeding depression (Ryan and Lacy; Margulis 2002 *in* Holt *et al* 2002).

Sperm quality is an important indicator of fertility and reproductive success in Mexican wolves. A study conducted by Asa *et al* (2007) analyzed levels of inbreeding relative to two primary indicators of sperm quality – motility and morphology – and compared those parameters with reproductive success (that is, the production of young, as a measure of fertility) and also sperm quality of Mexican and generic gray wolves. They proved that inbreeding has a significant effect on sperm quality and they also related both inbreeding and sperm quality to reproductive success. High levels of inbreeding were inversely correlated with two of the major indicators of semen quality, percentages of motile sperm and of sperm with normal morphology, with the effect on morphology being stronger. The level of inbreeding was associated not only with poor semen quality, but both variables were significantly correlated with reproductive success, indicating that the level of defective sperm observed could depress fertility. It is almost certain that males with less than 10% of normal sperm are functionally infertile (Asa *et al* 2007).

Males from both McBride and Ghost Ranch had significantly lower percentages of normal sperm than did either the Mexican wolf lineage crosses or the generic gray wolves, but the very low number of samples from Aragon males may have prevented detection of a difference for that lineage. The good news is that sperm quality has improved on male offspring from lineage crosses (Asa *et al* 2007).

The environment plays an important role on inbreeding depression, which means that sometimes this can only be detected if populations are translocated or subjected to notorious environmental changes. Captive animals that appear to be healthy may manifest inbreeding problems upon release into the wild. An accurate assessment of the absolute effect of inbreeding on individual fitness can only be achieved by examining lifetime reproductive success, which obviously is very hard on most wild populations, since it requires long-term studies of individually marked animals, plus precise methods of determining reproductive success and inbred status (Taylor 2002 *in* Holt *et al* 2002).

The consequences of inbreeding on juvenile survival are well documented, being the most common cause of increased mortality (Ralls *et al* 1979; Ballou and Ralls 1982 *in* Holt *et al* 2002). However, there is a need for further investigation on the impact of inbreeding and kinship on adult individuals, and the mechanisms of these effects. The results of such studies would be very helpful on the management of captive and wild populations of threatened species. What is known is that individuals that survive to adulthood suffer from reduced adult survival, poor performance in mating competition, reduced fecundity and less capable parental care (Holt *et al* 2002).

1.5.2. Methods used to improve Reproductive Success in Wolves

Assisted reproduction has received relatively small attention in the context of conservation of wild canids. In many cases, this is due to the fact that most of them reproduce well both in the wild and in captivity, but also because progress in reproductive biotechnology has encountered major problems particularly concerning *in vitro* models for female gametes and embryos. The particular characteristics of canid gamete physiology have complicated the adaptation of biotechnological knowledge gained on other species (Farstad 2000). Working with endangered wolves, such as

Mexicans, also imposes other restrictions. For instance, handling Mexican wolves for research requires using model species, either domestic dogs or generic gray wolves (Asa and Bauman 2014).

In most mammals, oocyte maturation *in vivo* occurs when the oocyte has reached the metaphase of the second meiotic division. This is the second stage of a temporary developmental arrest, and the second meiotic maturation can only proceed after penetration of the oocyte by a spermatozoid. The oocytes can be fertilized and develop *in vitro*, but at a reduced rate and to a limited stage of embryo development. Oocyte maturation *in vitro* has shown limited success in canids, probably because of the oocyte quality (there is a high prevalence of degenerate oocytes), factors related with the hormonal environment and supplementation, and characteristics of the cumulus granulosa cells (Farstad 2000).

Oocyte vitrification is an important technique used in the context of endangered canids' conservation. Preservation of oocytes and ovarian slices by vitrification, as well as semen freezing, are part of the official recovery team's program for the species, as a gene or gamete bank (Asa and Bauman 2014).

Gamete collection and genome bank is justified on Mexican gray wolves, since there are very few of these animals in the world (only about 240 in captivity and 100 in two reintroduced populations in Mexico and the U.S.) and they all descend from only seven individuals. Because of the small size of the population and the very few founding animals, genetic diversity is being lost, generation by generation. The primary purpose of the Mexican wolf semen bank is as genetic insurance for the future. Each year, the Mexican Wolf SSP program, in a bi national planning effort, makes recommendations for the captive population. These recommendations include the collection of semen from some males and of eggs from some females. Males are selected based on their representation in the bank, the value of their semen based on mean kinship within the bank, their capability for providing quality sperm, and the logistics for collecting semen during the narrow seasonal collecting period each year and the availability of skilled collectors. Female wolves are selected based primarily on advancing age, because the collection process involves removing the ovaries of the female, resulting in the permanent sterilization of that wolf. Since a female wolf can be considered old at ten years of age, and therefore no longer likely to conceive naturally, any female wolf this age or older may receive this recommendation. The procedure is normally no more risky than a typical sterilization process for female dogs. If any male or female wolf is being considered for euthanasia or if reproductive organs are being removed for medical reasons, the collection of germ tissue should be considered too (Siminski. *et al* 2014).

To date, samples have been frozen from 137 males (30 in Mexico and 111 in the USA). However, since the number of samples needed to achieve a pregnancy by artificial insemination varies, it is hard to establish a target number of samples. Therefore, to increase the likelihood of achieving pregnancies in the future, multiple semen collections will probably be necessary. Semen collection involves electroejaculation under general anesthesia during the breeding season – typically throughout February – when males are fully spermatogenic (Siminski *et al* 2014).

As for females, there have been 42 (10 in Mexico and 32 in the USA) with collections of sufficient quality and quantity to have frozen samples for future use. Ovarian tissue slices are also vitrified, to provide an additional source of germ cells for potential future use in assisted reproduction procedures. Ova must be collected early in the breeding season (usually mid to late January), after follicle growth is underway but before ovulation occurs (Siminski *et al* 2014).

Each preserved egg or sperm allows the potential continued genetic contribution from that wolf after its natural death. This may be accomplished through assisted reproductive technologies such as Artificial Insemination (AI) or *in vitro* maturation and fertilization at some time in the future. These techniques can greatly slow the loss of genetic diversity over a very long period of time, a period much greater than through natural reproduction and natural life spans. The gamete bank is vitally important for the long term perpetuation of the Mexican wolf, not only in captivity but primarily in the wild (Siminski *et al* 2014).

AI's main advantages are: the fact that it allows to accomplish genetic management without disrupting pair bonds or moving animals among facilities (which is particularly important when it comes to international shipments between US and Mexico, such as in the case of Mexican wolves); and also that it allows to add a male's genes to the population even after he dies (Asa and Bauman 2014).

Because separation of pairs can result in considerable stress, and achieving desired heterozygosity through transfer of individuals to accomplish recommended genetic pairings presents various difficulties, detection and induction of ovulation can also be extremely helpful. Detecting ovulation in wolves is not easy – monitoring fecal hormones helps in this species, but fecal steroid assays take longer to complete than those for serum, and wolves are often housed in groups in large outdoor enclosures, which makes collection of daily fecal samples from selected individuals very difficult to achieve, if not impossible. Hence the importance of ovulation detection for AI and induction of estrus or ovulation, followed by timed insemination (Asa *et al* 2006; Asa and Bauman 2014).

One aspect that is also worth mentioning is the effect of mate choice. In their natural environment, animals usually have the opportunity to choose their own mates, depending upon the social and breeding system of the species. Reproductive failure of breeding pairs is often a result of pair incompatibility and since the possibility of choice is a component of most mating systems, it is reasonable to assume that providing a choice of mates could improve the sustainability of captive populations through increased fecundity and offspring survival while enhancing animal welfare. Yet, it is important to note that allowing mate choice can also compose a problem, since it might undermine genetic goals if the choices are inconsistent with genetic management objectives (Asa *et al* 2011). Allowing mate choice could be particularly valuable to zoos when the relationships of individuals are uncertain or unknown, and when it is not possible to identify genetically optimal pairings from pedigree analysis. The survival of offspring born as a result of a non-preferred mating may be compromised by inadequate parental care – especially in monogamous species, in which males share parental duties (as it is in the case of wolves), pair compatibility could be particularly critical not only to successful courtship and mating but also to the care provided to offspring. In spite of being undeniable that allowing animals to choose partners increases pregnancy rates, litter sizes and offspring survival, the mechanisms and factors affecting mate choice are not yet well understood (Keane 1990; McClain 1998; Drickamer *et al* 2000; Ryan and Altmann 2001; Anderson *et al* 2007 *in* Asa *et al* 2011).

The standard strategy for pairs that do not reproduce is to assign another breeding partner and transfer one or both animals to another location. A better understanding of mate choice will definitely be a great help to population managers in achieving their goals for viable, genetically healthy populations, and also help minimizing selective changes to captivity. Potentially, it will provide insight into developing a more effective breeding management strategy for captive-animal populations. Further research is still needed to determine whether incorporation of mate choice in breeding programs can increase reproductive success without compromising genetic health and to evaluate its potential to contribute to the conservation of wild populations (Asa *et al* 2011).

II. Mating and Courtship Study on Mexican wolves

2. Materials and Methods

2.1. Endocrine Determinations

2.1.2. Sample Collection and Advantages of using Non-Invasive Methods

The Endocrine data used and collected for the present dissertation was obtained through fecal samples analyses. There are several reasons for choosing this method. The evaluation of steroid metabolite contents in feces or urine represents a snapshot of hormone activity, enabling the execution of longitudinal and long term studies (such as the present one) of reproductive patterns (including seasonality, ovarian cyclicality, pregnancy and even fecundity) in individuals and also in populations, without disturbing the animals. Collecting blood for measuring circulating hormones on free-living wildlife or non-socialized individuals, such as the wolves at the EWC, would require physical restraint or anesthesia, which is stressful, costly and sometimes dangerous. Another advantage of using fecal (or in some cases urine) samples is that it reduces episodic secretory patterns that normally occur in blood circulation. Additionally, this method also allows daily sampling, which will potentially provide more significant statistical results (Holt *et al* 2002).

Steroid monitoring in feces was first described in humans by Adlercreutz & Martin (1976) and then applied to the domestic mare (Bamberg *et al* 1984) and cow (Mostl *et al* 1984) before being used in macaques (Risler *et al* 1987). Nowadays this technique is widely used, and some of the most innovative studies have integrated behavior, genetics and hormone patterns to get a better understanding on several issues such as dominance, social stress and reproductive suppression, and also to give some insights on the evolution of mating systems. More and more studies confirm that non-invasive endocrine monitoring is extremely useful in increasing knowledge on free-living wildlife by providing information on reproductive status, health and even on the impact of human disturbance in animal welfare, which has enormous repercussions on the conservation and management of wildlife species. Conservation endocrinology is a valuable emerging discipline that can provide wildlife managers and decision-makers with critical information to ensure the survival of viable wildlife populations (Holt *et al* 2002).

On the EWC, the procedure for collection of the fecal samples varies amongst animals. Some individuals actually come close enough to be fed by the trainers with beaded meat, which "marks" the feces and makes it easier to know which set of feces belongs to each animal. Later on, the trainers go inside the enclosure and collect the stool, which will be frozen (-20ºC) and sent to the Endocrinology lab at SLZ. For the animals that cannot be fed "marked" food, the samples are collected based on the information provided by the observers. Each observer gets a map of the enclosure, on which the spot where the animal defecated is marked. Afterwards, the trainers will collect the indicated samples. Ideally, the stool samples should be collected as quickly as possible after defecation, followed immediately by treatment to minimize continued bacterial degradation (Holt *et al* 2002).

During previous years, steroid determinations were done using commercially available RIA kit (Coat-A-Count© Progesterone 125I Kit). The lower detection limit was 2.5 ng/ml, and the upper detection limit was 625 ng/ml. Assays were run according to kit directions, with the exception that the progesterone kit standards, which are supplied in human serum, were replaced by standards obtained from Sigma Chemical (Saint Louis, MO) and diluted in 10% steroid-free calf serum (Murti *et al* 2013). However, for the present year (2015), EIA was the utilized method. The protocol used is featured on Annex III.

2.2. Collection of the Behavioral Data

2.2.1. Characteristics of the EWC

2.2.1.1. Brief History and Partnerships

The Endangered Wolf Center (EWC) has existed for more than 40 years and its main goal is introducing releasable wolves into their native habitats to help restore harmony to the ecosystems. It has been helping to preserve and protect Mexican and Red wolves, as well as other wild canid species (such as Swift Foxes and African Painted Dogs) through carefully managed breeding, reintroduction and educational programs. It is the only Association of Zoos and Aquariums (AZA) certified wolf facility in the world, and it was the first institution to participate in the U.S. Fish and Wildlife Service's Red wolf and Mexican gray wolf managed breeding programs. It is a committee member of the Red wolf, Mexican gray wolf, and Maned wolf Species Survival Plan© of the AZA, and leads several three-year Action Plan projects for the U.S.F.W.S. and SSP©: semen collection, cryopreservation and assisted reproduction, and husbandry training.

More Mexican gray wolves selected for release in the southwestern United States have been born at this Center than in any other facility in the USA or Mexico. It helped recover the Mexican gray wolf population from seven wolves to 243 in managed care and about 104 in the wild. Today, every pack of Mexican gray wolves roaming free in the Southwest can trace its lineage back to the EWC.

2.2.1.2. General aspects of Animal Keeping

At the moment (May 2015) there are 14 enclosures at the EWC (some of them can be divided into two by closing the connecting gates). Three of them are, respectively, for the Painted Wild Dogs (four animals), Swift Foxes (five animals) and Maned Wolves (two sisters). They have 15 Mexican gray wolves and four Red wolves. The size of the enclosures varies between one and half an acre, and they are cleaned on average twice a month. These are "natural" enclosures, which help the animals to be less stressed in captivity, and to maintain wild behaviors.

The animals are fed once a day (at variable times) with "Mazuri Exotic Canine Diet", a commercial diet with high levels of protein, especially suitable for this kind of carnivores. Each wolf gets around two pounds of food per day. Occasionally they also get dear carcasses or bones (obtained from donations) and these can also serve as enrichment. For the Mexican and Red wolves, all the enrichment has to be done with natural resources, such as the above mentioned items, or sometimes the keepers can also use scented sprays (mint, herbs…). The other three species present at the Center can get different toys, pipes, even Easter eggs (made of cardboard or cellophane), among other things, for enrichment. The water is changed daily and the drinkers are heated, in order to keep the water from freezing.

The animals that are not here for the purpose of future reintroduction (Maned Wolves, Foxes and Painted Dogs) are taught basic commands by the keepers (such as sit, lay down, paw…), to facilitate the daily health checkup. Since the ultimate goal of having Mexican and Red wolves in this facility is to allow future reintroduction, the human contact with these individuals is kept to a minimum, so there is no interaction with the keepers. The more their instinctive natural shyness around humans is preserved, the safer they will be if they are able to be released into the wild.

As far as veterinary care goes, the wolves are captured once a year, during the fall, for blood collecting in order to do a general health check. At the same time, the animals are vaccinated for Rabies, Distemper, Parvovirus and Heartworm. Four times a year, fecal samples for parasitology control are also taken. Whenever there is an unexpected surgery or if the wolves have to be captured for semen collection, they are administered

Frontline® (for ectoparasite's control). Ivermectine is monthly added to the food for internal deworming.
Every year there is an exchange of individuals between facilities, according to the SSP instructions, so the EWC receives and ships wolves regularly. It is one of the few institutions that have a direct partnership with Mexico, and that interchanges animals with that country. The purpose of this is to maintain a varied genetic pool, hence avoiding inbreeding, and at the same time to create one "global" population, instead of various separate ones.
Usually the EWC does not do quarantine of new animals because the sending institution has to do blood and fecal analyses before any transfer is allowed. The animals get acquainted to their new pack members by being put in adjacent enclosures, separated by a fence that allows them to see and smell each other. Depending on behavior and other aspects, they can be put together as fast as 24 hours after the first encounter, or the process can take much longer.

2.2.1.3. Some Reproduction Aspects on the EWC

The Mexican and Red wolf breeding pairs on each year are chosen by the respective SSP, based on genetic factors (which explains the above mentioned frequent exchange of animals between facilities). So there is not always the guarantee that the pair will get along well enough to actually want to naturally breed. Off breeding season, the wolves are usually kept in the same packs as the ones recommended by the SSP. However, in some occasions, the EWC staff regroups the wolves according to the personalities they think will work better together.
No tests are done on the wolves before the breeding season. However, there is usually a sperm collection around February on the non-breeding males, but not on the breeding ones because those are supposed to be left alone as much as possible. Throughout the pregnancy no tests are performed on the mothers-to-be, unless in case of health problems such as extensive vaginal bleeding. The staff does not interfere with parturitions either, unless the female is in real life danger or if it is an animal of extreme genetic value. The same applies for pups while growing up – the staff only helps if it is a very valuable individual/litter.
The number of pups born here per year is very variable, depending on how many pairs are recommended for breeding on that particular occasion. A regular litter (both for Red and Mexican Wolves) has around five to six puppies. The pups usually stay with the

parents until they are two years old (to mimic what would happened in the wild) and after that they can either be sent to another approved facility, or be changed to another enclosure within the EWC. Sporadically, it can happen that they stay with the parents for a longer period of time. The ideal scenario is when it is possible to reintroduce the youngsters to the wild.

The EWC is actively part of reproductive research programs such as semen collection, artificial insemination and egg vitrification.

2.2.2. Procedure used to collect the Behavioral Data

For each breeding season, the Director of Animal Care and Conservation (at the present Regina Mossotti, PhD) makes a schedule with the shifts and observers who will be working at each day and time. Before the start of the observations, the observers have an introductory class, explaining the basic rules and procedures.

The shifts are always two hours, 9.00-11.00 a.m. and 2.30-4.30 p.m., every day of the week. The length of the observations cannot be shortened by any reason. After signing in, and before each shift, the observers pick up a clipboard for their respective enclosure (it has the data sheets[4], the ethogram[5], a key to the enclosure, a map of the enclosure, and pictures of the wolves in the enclosure). Before and after each observation, the observers (who are also provided with radios and binoculars) have to radio the keepers, to let them know the beginning and ending moment of the observation. If the female wolf defecates, this should also be radioed to the keepers after the shift ends, so they can collect a fecal sample as fresh as possible.

The Ethogram provided contains eleven behaviors, all related to mating and courtship. Every behavior is recorded on paper, using predefined codes, and that data will be entered on the computer later on.

During observations, observers are seated inside a very small blind/tent, that partially protects from the wind, and that is always left in the exact same spot, in order to get the wolves comfortable and familiar with it. Observers are not allowed to leave the blind (even if that leads to losing some behaviors, which happens when the animals are at a spot of the enclosure that is not visible from their position) because that could disturb the wolves and the goal is to record the "natural" behaviors, as much as possible. It is also strictly forbidden to interact with the animals in any way, as well as eating,

[4] Please see Annex I

[5] Please see Annex I

drinking, taking pictures and using cell phones or any other devices, since this could unsettle them.

Each breeding season, the only pairs that are observed are the ones chosen for reproduction on that particular year.

For the Breeding season 2015, observations started on January 26th and officially ended on March 21st. However, the final date for each pair varies, since observations stop one week after the last observed tie. This year there were two Mexican wolf pairs observed: Rogue (female 1300) & Male 1297, and Sibi (female 1266) & Lazarus (male 1177); and two Red wolf pairs: Rozene (female 1795) & Itabi (male 1916), and Sprint (female 1586) & Don Mack (male 1402).

3. Processing of the Data

3.1. Creation of General Database with all the Hormonal Information

The Research Department of the St. Louis Zoo, and more specifically its Endocrinology Laboratory, receives samples from all over the world and from all kinds of animal species. Since the SLZ has such a close partnership with wolf related organizations, multiple wolf fecal samples arrive from various zoos and parks, and from more than one wolf species. Considering this, the first part of the work conducting to this dissertation was to organize all the information available, from 1998 to the present time.

From all the existing information, all the data about Mexican wolves were separated from the other wolf species, and posteriorly selected only the ones that were on the EWC at the time of samples collection. This allows to compare the same individuals, living in the same conditions, and also crossing references between hormonal and behavioral information (which was only available for Mexican wolves from the EWC).

All the data was introduced on a single Excel Sheet containing the following columns: Sample Number (whenever existent), Species (always Mexican Gray Wolf), Source (always EWC), Animal Name, Animal ID Number (according to the species Studbook), Date (of the sample collection), Progesterone level in ng/g and Estrogen level (whenever measured) also in ng/g.

3.2. Statistical Tests used

Since the present data do not follow a regular distribution, non-parametric tests are more appropriate. The main statistical tests used were Correlation Matrixes, GLM analyses, repeated measures ANOVA and two sample t-test. The statistical program utilized was NCSS. For doing the Correlation Matrix, data were first divided into two groups: the pairs where only "Mountings" were observed and then the ones where there were "Copulatory Ties". This was done in order to try to normalize the data, that is, to line up the datasets so the phases of the estrus cycle would more closely match. Then, correlation matrixes were ran for each female, in each group. The downside of this approach is that there were some days in which behavioral or hormonal data were lacking. As so, to try to increase the number of females with complete hormone or behavioral data, and that therefore could be used for analysis, possibilities for choosing a better estimate of ovulation and estrus were discussed. This led to selecting females with pups and estimating their conception dates (assuming 62 days gestations). From a total of 20 sets with pups, analyses were ran using the 17 sets with any behavioral data. In these sets, data were considered from a 13 day period representing the six days before estimated conception, day of conception, and the six days after it. These figures were generated using the rates of behavior, not the total counts themselves; by using the rates, it was feasible to include data from days that only included one data sheet (assuming four hours for both AM and PM or just two hours for only one session).

Regarding the GLM, we focused our analysis of the NCSS output on the Spearmen's correlations. For this test, we have chosen to organize the data by grouping together the seven days before estimated conception and the seven days after it, so it was possible to focus on the pre and post conception periods rather than on particular days. The seven sets selected were years with pups and behavior data for the full fourteen days. The behavior counts were converted into rates per hour. The seven sets used were: Wolf 204, 2003; Wolf 204, 2006; Wolf 685, 2003; Wolf 685, 2004; Wolf 685, 2005; Wolf 685, 2008; and Wolf 882, 2011.

Repeated measures ANOVA was used to determine whether the frequency of behaviors differed before and after the first day of copulation (N=8 pairs), the day that we considered to define the transition from proestrus to estrus.

For the Two Sample t-test, the pairs were divided in two groups: the first included the females that produced pups and in which copulatory ties were seen (namely Frijole in 2001, 2002, 2003 and 2005; Tanamara in 2001, 2003, 2004 and 2005; Anna in 2003, 2005 and 2008; Abby in 2011; and Madre in 2013), totaling thirteen sets from five females. The second included the females that did not produce pups and in which copulatory ties were not seen (namely Desert Song in 1998 and 1999; Saguaro in 2002 and 2003; Frijole in 1998, 1999, 2000 and 2007; Sport in 2001; Nakomis in 2003; Abby in 2006 and 2008; and Corazon in 2006) totaling thirteen sets from seven females.

Excel was used to make the graphics. For the ones regarding “Behaviors around first copulatory tie”, all the pairs with litters and in which copulations were seen were considered (N=14). Only the six days before and after the first Copulatory Tie, plus the day of first Copulatory Tie itself, were taken into account. For the graphics featuring reproductive hormones, given the limitations regarding fecal sample collection, hormonal data from most of the paired females was not enough to allow proper analysis. However, several graphics were made with the most complete datasets, plus graphics for all the females not housed with males – N=21 (from which more frequent sampling was possible), were also done.

4. Results

The frequency of courtship and mating behaviors that occurred during the morning versus afternoon observation periods were compared and no significant differences were found. Eight different pairs that had pups were considered (some pairs were observed in more than one year, which allowed the calculation of 19 different rates). The results are displayed on Table 2 of Annex IV.

Observation's Success Rate (based on pairs that produced pups (N=19) and pairs observed copulating (N=14)) was 74%.

Correlations amongst behaviors were calculated and the significant ones are presented on table 1, below. A possible correlation between behaviors and progesterone was also investigated but no relationship was found, possibly due to the lack of consistent hormonal data.

Table 1. CORRELATIONS AMONGST BEHAVIORS

Behavior		***r*-value**
Female Urinating	Male Urine Mark Over	0.59
Female Urinating	Male Sniff Urine	0.55
Female Tail Deflect	Male Sniff/Lick Genitals	0.82
Male Sniff/Lick Genitals	Male Mount Attempt	0.58
Male Sniff/Lick Genitals	Male Mount	0.54
Male Sniff Urine	Male Urine Mark Over	0.42
Female Tail Deflect	Male Mount Attempt	0.46
Female Tail Deflect	Male Mount	0.47
Male Mount	Male Mount Attempt	0.48

In regard to the behavior “Female Tail Deflect”[6], several counts were made and are presented on the following table (2).
The average number of days between first displayed FTD and first seen Copulatory Tie was 14.6 days. FTD always happened at least three days before the first Copulatory Tie.

Table 2. Female Tail Deflect	
Total number of FTDs for the females that had litters (N=19)	2200
Total number of FTDs for the females that did not have litters (N=19)	392
Average number of FTDs for the females that had litters	115.79 ≈ 116
Average number of FTDs for the females that did not have litters	20.63 ≈21
Median number of FTDs for the females that had litters	124
Median number of FTDs for the females that did not have litters	9

Regarding Male Urine Mark Over, which is crucial for pair-bonding, and given that previous studies have shown there is a higher frequency of this behavior in pairs that were together for the first time (Mech and Boitani 2003), the MUMO rates were calculated for each pair/year [7]. Then, the ones with frequency rates above three were selected (N=6) and their history checked on the 2014 Studbook and Endangered Wolf Center records. Five out of these six pairs were indeed together for the first time.

In the analysis of frequency of behaviors before and after the first day of copulation, that is, between proestrus and estrus (N=8 pairs), only Male Urine Mark (p=0.03) differed significantly, although there was a trend for Female Urinate (p=0.07) and Male Sniff Urine (p=0.08). In all three cases, the direction of change was lower during estrus. However, using first Mount date as the day of transition between proestrus and estrus (N=10 pairs) resulted in FU (p=0.05), MSU (p=0.03), and Male Mount (p=0.04) being significantly different between proestrus and estrus. MUM remained significantly different (p=0.004) and Male Mount Attempt approached significance (p=0.06). FU, MSU and MUM were lower during estrus, whereas MM and MMA were higher il.oon this period.
Estimating date of conception was another approach taken in order to incorporate more pairs in the analysis comparing proestrus to estrus behavior. Because 62 days is

[6] Absolute values displayed on Table 3 of Annex IV.
[7] Table 5, 6, 7; Annex IV.

commonly used by the Mexican Wolf SSP as approximate gestation length, 62 was subtracted from date of pup birth for pairs that produced litters, including those that had been used in the other analyses (the ones based on first day of mount or copulation) adding up to a total of 17 pairs. FU (p=0.06), MSU (p=0.02), MUM (p=0.03) and MM (p=0.03) remained significantly different between proestrus and estrus.

The comparison made between "Females that produced pups and where copulatory ties were seen" (N=13) and "Females that did not produce pups and where copulatory ties were not seen" (N=13) showed significant differences among the groups. FU (p=0.01), MUM (p=0.03), MUMO (p=0.02), FTD (p=0.00), MSLG (p=0.00), MSU (p=0.002) and MMA (p=0.01) frequencies are different for reproductively successful pairs, versus non successful ones (assuming "success" as having litters). Mean values were higher for all the above mentioned behaviors in the pairs that produced pups. For these calculations, the time period taken into account included proestrus and estrus – all the existing data from the whole "reproductive season" for each female/year were considered.

Mean duration of estrus (assuming, for the purpose of this analysis, estrus as being the period between first and last day copulation was observed), was 2.9 days (range 1-7 days). For estrus periods longer than one day, mating was not always observed daily. Parturition occurred an average of 63.4 days (range 57-73) after the last or only copulatory tie.

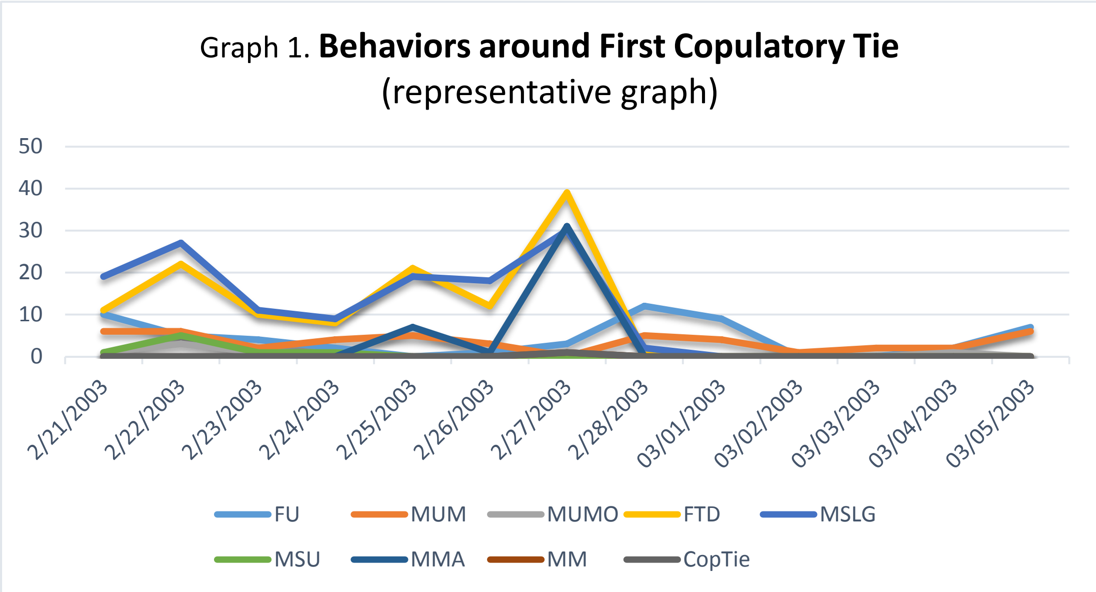
Considering all the females in which both mounting and copulation were observed, regardless of them having had pups or not (N=18), the Mean of the number of days between first Mount and first Copulatory Tie was 2.89 days[8].

Frequencies of FU (female urinate, p=0.06), MUM (male urine mark, p=0.03), MSU (male sniff urine, p=0.02) and MM (male mount, p=0.03) differed significantly between the pre and post conception periods. According to the obtained p-values, there seems to be less FU, MUM and MSU, and more MM, in the post-conception period.

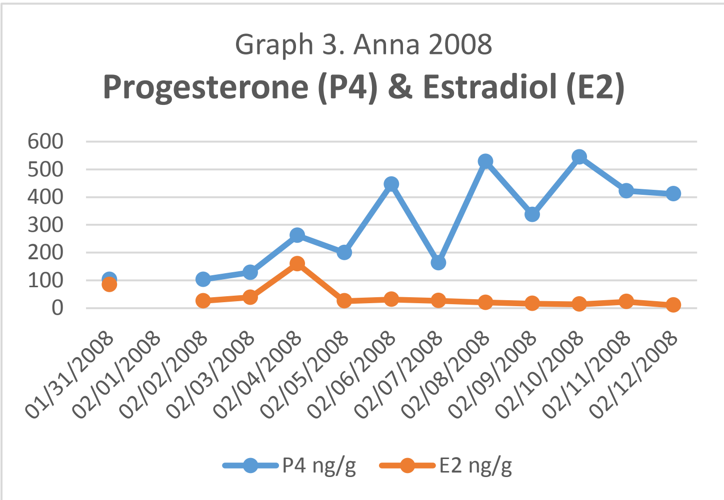
The following graphics were chosen for being representative of the obtained results.[9]

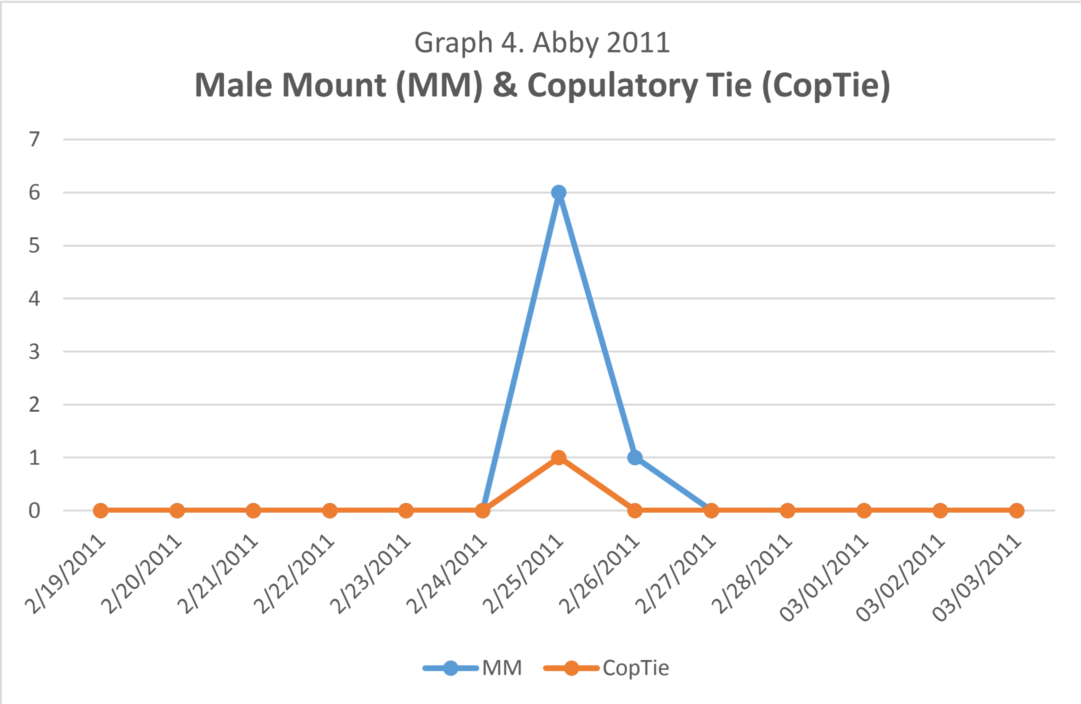
[8] Full table on Annex IV, table 4.

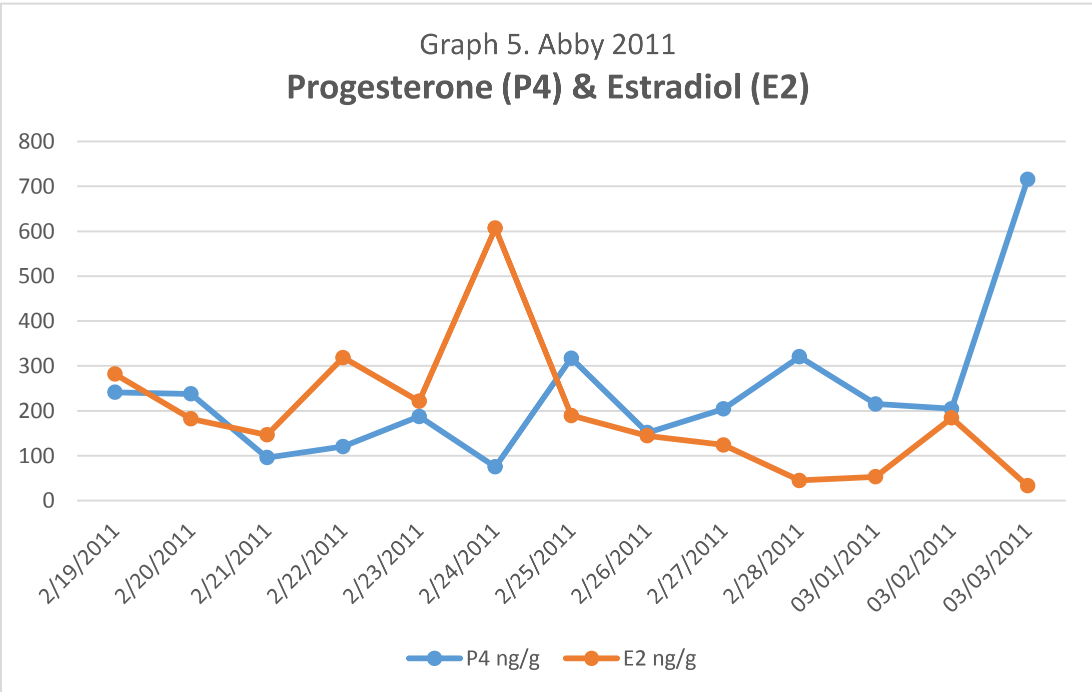
[9] Some of the most representative graphs and respective tables of values can be consulted on Annex V.

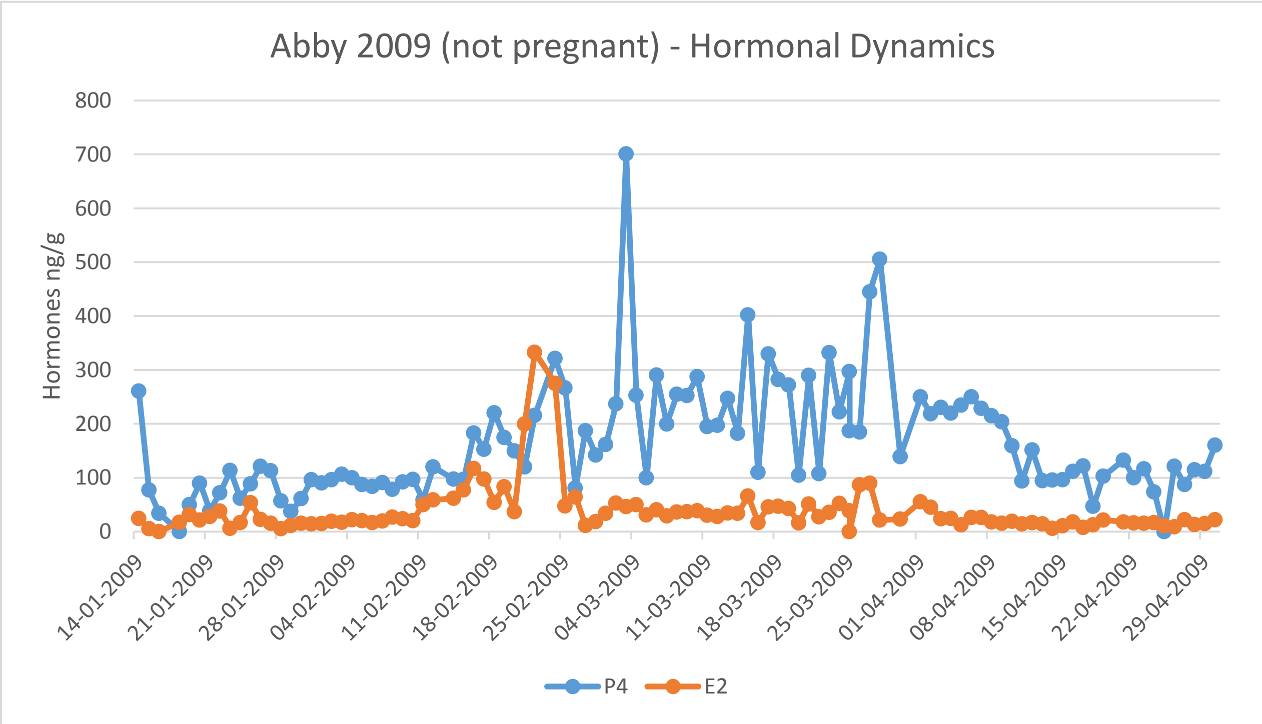


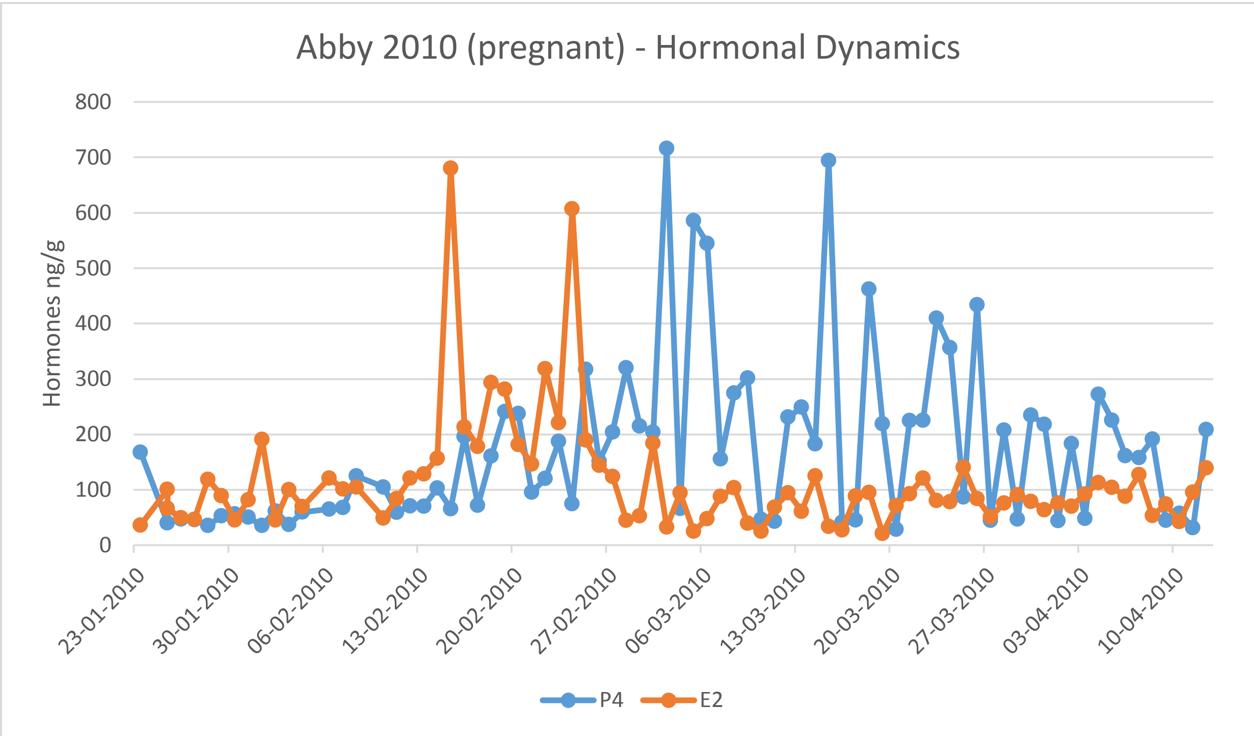
First Copulatory Tie: 2/27




Graph 4. Abby 2011
Male Mount (MM) & Copulatory Tie (CopTie)
7
6
5
4
3
2
1
0
2/19/2011
2/20/2011
2/21/2011
2/22/2011
2/23/2011
2/24/2011
2/25/2011
2/26/2011
2/27/2011
2/28/2011
03/01/2011
03/02/2011
03/03/2011
MM
CopTie


Graph 5. Abby 2011
Progesterone (P4) & Estradiol (E2)
800
700
600
500
400
300
200
100
0
2/19/2011
2/20/2011
2/21/2011
2/22/2011
2/23/2011
2/24/2011
2/25/2011
2/26/2011
2/27/2011
2/28/2011
03/01/2011
03/02/2011
03/03/2011
P4 ng/g
E2 ng/g


Abby 2009 (not pregnant) - Hormonal Dynamics
Hormones ng/g
800
700
600
500
400
300
200
100
0
14-01-2009
21-01-2009
28-01-2009
04-02-2009
11-02-2009
18-02-2009
25-02-2009
04-03-2009
11-03-2009
18-03-2009
25-03-2009
01-04-2009
08-04-2009
15-04-2009
22-04-2009
29-04-2009
P4
E2


Abby 2010 (pregnant) - Hormonal Dynamics
Hormones ng/g
800
700
600
500
400
300
200
100
0
23-01-2010
30-01-2010
06-02-2010
13-02-2010
20-02-2010
27-02-2010
06-03-2010
13-03-2010
20-03-2010
27-03-2010
03-04-2010
10-04-2010
P4
E2

5. Discussion

This study focused primarily on collection of observational data on courtship and mating in Mexican gray wolves. Fecal samples were collected when possible from the females, but most of those datasets were incomplete. Mexican wolves are part of a reintroduction program which restricts human interaction or habituation. The standard method for identifying fecal samples from individuals in social situations is to feed them inert, colored markers in a food treat, a practice that is not allowed with this species. Thus, fecal sample collection had to rely on direct observation of defecation, which was not sufficiently frequent for most individuals. Hormone data from females in the behavioral study were thus confronted with complete hormonal profiles generated from individually housed females.

No significant differences were found between morning and afternoon observations, regarding rates of the courtship and mating behaviors, so data from the two periods were combined for analysis. The schedule for the observations was determined according to husbandry and management considerations, as well as by time of day. To try to evaluate the reliability of the data collected, in terms of capturing key behaviors, a "success rate" was calculated. This rate allowed us to measure how well the four hours of observation were able to detect at least one occurrence of copulation. Since the animals were observed every day during a period of four hours per day, the obtained result of 74% success rate can be considered satisfactory. Moreover, even the pairs that were not seen copulating were observed mounting.

The existence of relationships amongst courtship and mating behaviors was investigated. No relationship between either "Day" or "P4" (progesterone) variables and any of the behaviors was found. The most probable explanation for the absence of a correlation between progesterone and the behaviors in the ethogram is the frequent lack of hormone data (for the reasons previously explained). Nevertheless, some significant relationships were found. The substantial correlation between FU, MSU and MUMO appears to be logical from a biological perspective. It would be impossible for the male to sniff or mark over female urine if she had not urinated first. The male sniffing the urine shows that he is interested in it, which is typical during proestrus and estrus, due to the vaginal secretions incorporated in the female's urine. The male marking over it has been associated, in previous studies, with making a sort of claim to that female, and as being an indication to other animals that that pair is mated. The weak correlation between MSU and MUMO is not unexpected and indicates that the male is marking over urine that he has sniffed. The strong correlation between MSLG and both MM and MMA indicates that the males' response to the females' visual and olfactory cues (by sniffing and licking her genitals) may have prompted mounting. The moderate correlation between MM and MMA shows that many attempts to mount are not successful. Attempts to mount may fail because of the animal's inexperience, because the male is just testing the female before mounting fully, or because the female moved away or refused mounting. The correlation between FTD and MSLG suggests that tail deflection may be an effective proceptive behavior – the combination of vulval secretions and proceptive behavior seems to attract the male's attention, so he "inspects" the female's genitals. There was also a moderate correlation between FTD, MMA and MM, which makes sense from a biological standpoint.

"Female tail deflect" is displayed during proestrus and estrus – that is, both when the female is exhibiting proceptive and receptive behaviors. The obtained results confirm that it can, indeed, be considered a proceptive behavior, since it started happening about two weeks prior to the first copulation seen, meaning it is not necessarily a signal of receptivity (Beach 1976). Our evidence confirms that proceptive behavior by the female usually calls the male's attention to the genital area and is followed by mount attempts and mounting, therefore increasing the chances of mating. It can be inferred that the female's sexual interest and proceptive behavior are important to reproductive success – the average frequency of this behavior in the pairs with litters was 5.5 times higher than for pairs without pups.

Frequencies of courtship behaviors during proestrus and estrus were compared. Different approaches to estimating dates that might separate proestrus from estrus were attempted, to increase the number of cases for analysis. The conservative approach of using only first day of copulation to define the change from proestrus to estrus revealed only one significant behavioral difference (Male Urine Mark) and two trends (Female Urinate and Male Sniff Urine) towards a higher frequency of these behaviors during proestrus. However, some females gave birth to pups but were not seen copulating, which confirmed that the observation periods did not capture all copulations.

Using dates of first Mount, as well as first Copulation, to increase the number of pairs analyzed, resulted in the behaviors that had approached significance in the other analyses (FU and MSU), becoming significantly different between proestrus and estrus. Mount Attempt approached significance, and Mount became significant; in some pairs, Mount was only observed on one day. FU, MSU and MUM happened more often during proestrus, while MM and MMA had higher frequencies during estrus.

Estimating time of conception for females that gave birth to pups allowed inclusion of data from pairs that had not been observed mating. Nevertheless, results did not change substantively. Due to the intrinsic variability of gestation lengths, plus multiple mating dates and sperm longevity in this species, these results may not accurately represent the true inflection point between proestrus and estrus. The estimated gestation length most commonly used for Mexican gray wolves has been about 62 days, although individual variability is expected, as with other species. Published values for generic gray wolves range from 56 to 70 days (Hayssen et al 1993), which spans two weeks. These values are all based on observed mating dates, not on known days of ovulation or of conception. Estimation of gestation length also is confounded by the long period of estrus accompanied by mating in wolves. There have been no attempts to determine whether conception is more likely early or late in this mating period. Calculating gestation length from the time of artificial insemination should yield a more accurate measure, especially if there was only one insemination date. Data from insemination of two Mexican wolves (one inseminated twice, two days apart, and the other once) yielded gestation lengths of 64 or 66 days (Asa et al 2006). Even this measure does not account for the potential for sperm to live multiple days in the female tract, which could mean that conception actually occurred one to several days after insemination, in which case gestation length would be less than 64 or 66 days. There is no equivalent data for wolves, but in domestic dogs, sperm has been estimated to survive up to six days after copulation (Concannon et al 1983), although two days would be more consistent with data from other mammalian species (Gomendio and Roldan 1993; Asa et al 2006).

From the comparison between pairs with and without litters, it is possible to infer that the quantity of behaviors displayed has a positive relationship with reproductive success. On average, pairs that exhibited higher frequencies of FU, MUM, MUMO, FTD, MSLG, MSU and MMA were the ones that did have litters. These behavioral

comparisons also suggest that the females that did not produce pups either did not come into estrus or did not accept the male they were paired with as an acceptable mate.
Most of these behaviors are either directly or indirectly related with olfactory communication, which is known to have a crucial part in courtship, pair bonding and reproduction. Failure to bond is associated with failure to reproduce (Rothman and Mech 1979). Previous studies showed that the olfactory sense is extremely important, especially for naïve males, to successfully breed, because not only is copulatory behavior (mounting, intromission and ejaculation) stimulated by olfactory investigation of estrus vaginal secretions, but that detection of those secretions may actually be a prerequisite. Intact males generally exhibit intense interest in proestrus' urine and on the genital area of estrus females, but sexually naive anosmic males showed no interest in proestrus or estrus females. On a study by Asa et al (1986), the sexually inexperienced anosmic wolves did not sniff or lick urine or vaginal secretions of the female partner and did not respond to the female's solicitations to mate. Besides, these males failed to recognize and respond to visual and tactile cues from their estrus partners.

Mean duration of estrus calculated for this study was 2.9 days (range one-seven), which is shorter than the typically reported for gray wolves and for domestic dogs. There may have been more days with copulation, if the behavior occurred outside the four hours of observation. It has been reported (Packard, unpublished data *in* Mech and Boitani 2003) that experienced captive wolves may present estrus shorter than a week, and that single copulations were successful in producing litters.

In this study, the average number of days between first detected Mount and first Copulatory Tie was three days. This adds information to describe reproductive behavior and courtship sequence for this species (and can likely be explained by what was already said about mounts and mount attempts – many are not successful). Mountings in the days or hours preceding the first copulatory tie might have been missed though, due to the four hour/day observation period.

In a comparison between pre- and post-conception periods, some significant differences were found. Female Urination, Male Urine Marking and Male Sniff Urine were more frequent in the seven days before estimated conception. During proestrus there was an increase in female urination frequency, which agrees with the literature (Mech and

Boitani 2003). The female may urinate more frequently as a means of signaling her reproductive status to the male during this period. Males increase urine-marking in advance of the breeding season, probably for territory defense (Mech and Boitani 2003). As for MSU, our result is in agreement with Packard (2003) and also with the hypothesis formulated by Asa (2015). The first states that, during proestrus, adult males usually become especially attentive to odors in female urine. The second hypothesis refers that during proestrus the males seem to pay more attention to the urine than to the female herself, then his interest shifts to her genitals with the onset of estrus. The results for MM show that there is significantly more MM in the post-conception period (seven days after estimated conception). One possible explanation could be that throughout most of the pre-conception period the female was probably non-receptive, consequently not tolerating mounts/copulation. Since mating can continue daily during estrus (receptive phase), mounts would happen more often on the post conception. The MSLG results were not significantly different before and after estimated conception, although they showed a tendency to higher frequencies post-conception.

Looking at the general patterns presented in the graphics depicting behaviors around first Copulatory Tie, Female Urination was often more frequent before the first copulatory tie. During the first day a Copulatory Tie was observed, the most frequent behaviors were sniffing/licking genitals, female tail deflection, mount attempts and mounts. Of course, mounting is part of copulation. Inexperienced males may direct mounting to the head or side of the female before learning to mount properly from behind, which is one possible explanation for the number of mount attempts by some males.

One aspect that should be noted is that what is here considered as day of first CopTie is in fact the first day a CopTie was recorded – the possibility that copulation happened before, during the time the wolves were not being observed, has to be acknowledged. As previously said, males may respond to the females' visual and olfactory stimuli by licking her genitals, then mounting (Kreeger 2003; Packard 2003). It is likewise established that positive sexual behavior towards the male may include reflex deviation of the tail to one side and permitting mounting (Kreeger 2003; Packard 2003), so it seems appropriate that there were more FTD concomitantly to more MM.

The graphics featuring Progesterone, Mounting and Copulatory Ties showed, for the most part, that the hormone profiles obtained and timing of endocrine changes compared with the behaviors displayed, were in accordance to the literature. That is, a progesterone peak (associated with the onset of estrus) often coincided with the occurrence of more mounts and copulatory ties, which indicates receptive behavior, typical of females in estrus. The estimated conception date (based on the 62 days of pregnancy) was compared with hormone and behavioral data from the graphics. It was possible to confirm that the predictions were generally correct, regarding the week of conception and, sometimes, probably even the day itself. The graphics featured in the Results (Anna's 2008 and Abby's 2011) can be representative of what was just described.

On most of the hormone graphics (featuring progesterone and estrogen or only progesterone, and done using females not paired), it is possible to see an estrogen peak between a day and a week before the first notable progesterone peak. Progesterone was low and started rising slowly around the same time as estrogen values also began increasing. It showed several peaks throughout the study period but one was usually higher. From these graphics it is, however, not possible to determine the general timing of this most notable peak, because it varied amongst females. Comparing these graphics with the most representative ones from females paired with a male and which had pups, the hormone dynamics were the same and the graphics appear very similar. Mean progesterone levels are similar in pregnant and non-pregnant wolves following ovulation (Kreeger 2003; Packard 2003). In bitches it has been shown (Concannon 1975, Concannon 2009, Concannon 2011) that estrogen starts rising around 10 days before the LH surge, and peaks one/two days before the LH surge. After this, with the onset of estrus, it subsequently decreases. There is a progesterone rise concomitant to the LH surge in 95% of the bitches, so we can infer that estrogen peaks around two days before progesterone starts increasing. In bitches progesterone starts decreasing around 40 days after it begins rising. The present data on Mexican gray wolves show that this hormone began decreasing between 10 to 30 days after its first rise, which is in accordance to the mentioned literature about dogs. In general, not all the timings of the dynamics reported for domestic dogs are in agreement with our results in wolves. However, the general patterns seem similar in the two species: an estrogen peak followed by a progesterone peak, with this last hormone remaining elevated for 10 to 30 days following its first rise.

In future studies, it might be interesting to measure indices of association and maintenance of proximity to determine whether they influence the occurrence of copulation. The first is an index of the extent to which two individuals associate with each other. The maintenance of proximity is one important measure of two individuals' relationship and expresses the extent to which their proximity is due to the movements of one or the other member of the dyad (Bateson et al 2007). It is known that pair-bonding is a very important part of the reproductive life of the wolves. These measurements provide some indication of the compatibility of a certain pair, which has management implications and can influence reproductive success.

Conclusions

To the best of our knowledge, this is a pioneer study on hormonal profiling and courtship behavior of Mexican gray wolves.

Regarding activity levels, no significant differences were found between morning and afternoon observations.

Several behaviors were correlated: Female Urinating with Male Urine Mark Over and Male Sniff Urine; Female Tail Deflect with Male Sniff/Lick Genitals, Male Mount Attempt and Male Mount; Male Sniff/Lick Genitals with Male Mount Attempt and Male Mount; Male Sniff Urine with Male Urine Mark Over; and Male Mount with Male Mount Attempt.

Comparing behavior frequencies in the pre- and post-conception periods, Female Urinating, Male Urine Mark, Male Sniff Urine and Male Mount showed significant differences in the seven days before and after estimated conception. Female Urinating, Male Urine Mark and Male Sniff Urine were more frequent pre-conception; and Male Mount was more frequent post-conception.

The average number of days between first detected Mount and first Copulatory Tie was approximately three days, and the mean duration of estrus was approximately three days as well. Parturition occurred an average of 63.4 days (range 57-73) after the last or only copulatory tie.

Female Tail Deflect was a reliable example of female proceptive behavior, and was positively related to reproductive success (defined as successfully giving birth). Successful pairs also showed higher frequencies of Male Urine Marking, Mounting and Mount Attempts.

Pairs that were together for the first time showed higher rates of Male Urine Mark Over, a behavior important for establishing and maintaining pair bonds.

Rate of Female Urination was often higher before the first copulatory tie. Of the behaviors expressed in the studied pairs, male sniff/lick genitals, female tail deflect, male mount attempt and male mount were the most frequent ones, especially on the first day of copulation.

A progesterone peak, associated with the onset of estrus, often coincided with the occurrence of Mounts and Copulatory Ties, as reported for domestic dogs. Estimations of conception dates were mostly in agreement with the hormonal data.

Summarizing the general progression of courtship and mating behavior of Mexican wolves: there was an increase in the frequency of female urination in the week prior to the first copulation. During this time, the male also began to urine mark and to sniff the female's urine more often. Sniffing/licking the female's genitals tended to occur more frequently during estrus than proestrus. On average, about three days after the first mount, copulation occurs. On the first day a copulatory tie occurs, sniffing the female's genitals, tail deflection by the female and mounting (successful or not) are the most frequent behaviors. During the post-conception period, there is a higher frequency of mounting and lower frequency of female urination, male sniffing her urine and male urine marking.

These observations may be useful in cases when artificial insemination (AI) is planned, because they can allow a better estimate of the correct timing for doing this procedure. They also add to knowledge of reproductive physiology of this keystone species.

We hope this study contributes to the knowledge about Mexican gray wolves, for it is only by scientific research and investigation that we can truly understand how to find a balance between wolf welfare/conservation, and human interests. Not only the wolf itself but actually the whole environment, of which people's legitimate rights are part of, have to be considered in order to achieve a proper conservation strategy. It is necessary to find a compromise between wolf and human requirements.

History has shown the incredible adaptability and survival skills of these animals – they have been wiped out of large areas relatively easily, but showed outstanding resilience and impressive ability to recover where and whenever given a chance.

"(…) humans have the technical power to decide the fate of the wolf. Currently these are good times for the wolves, and probably those good times will continue in the near future. However, the key to long term wolf conservation is the degree of tolerance and rationality that humans will be able to muster. Tolerance may mean accepting that wolves are totally protected in some areas, forbidden in others, and controlled in still others (…) The wolf knows how to handle all this; humans still have to learn."

(Mech and Boitani 2003; page 340)

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ATTACHMENTS

Annex I

Endangered Wolf Center Ethogram

MEXICAN WOLF BEHAVIOR STUDY: BEHAVIOR DEFINITIONS

Female defecate (♀ Def): female defecation [be sure to indicate location of feces on map]

Female urinate (♀ Urine): female squats to urinate

Male urine mark (♂ Mark): male raises leg to urine mark

Male urine mark-over (♂ Mark-Over): male urine-marks over female urine spot

Male sniff/lick genitals (♂ S/L Gen): male sniffs or licks under tail of female

Male sniff/lick urine (♂ S/L Urn): male sniffs or licks female urine spot on ground

Female tail-deflect (♀ Tail-D): female holds tail to one side

Male mount attempt (♂ Mount-A): male tries to mount but female moves away

Male mount (♂ Mount): male mounts female who stands

Copulatory lock begins (Cop-B): male and female turned end-to-end after copulation

Copulatory lock ends (Cop-E): male and female separate

Endangered Wolf Center – Observations' Sheet

MEXICAN WOLF BEHAVIOR STUDY

DATE ________ WOLF IDS ______________________________ WEATHER ______________

OBSERVER ______________ TIME START ________________ TIME END ______________

TIME (hr:min)	BEHAVIOR	TIME (hr:min)	BEHAVIOR

Demography		
Current Population Size (N)	248 (120.128)	
Specimens Excluded from Analyses	33(9.24)	
Target Population Size	300	
Mean Generation Time (T, in years)	5.8	
Projected Population Growth Rate	1.064	
Genetics		
	Current	**Potential**
Number of Founders	7	7 (0 additional)
Founder Genome Equivalents (FGE)	3.00	4.59
Gene Diversity (GD) Retained (%)	83.36	89.11
Population Mean Kinship (MK)	0.1664	
Mean Inbreeding (F)	0.1279	
% Pedigree Known	100	
N_e/N	.1266	
Years to 90%	Below	
Gene Diversity at 100 years from Present	65.17	

A

nnex II

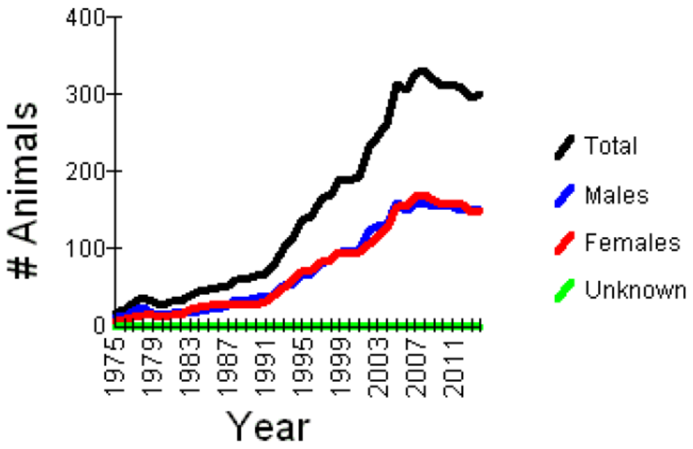

Figure 1. Census of Mexican wolves in the SSP.

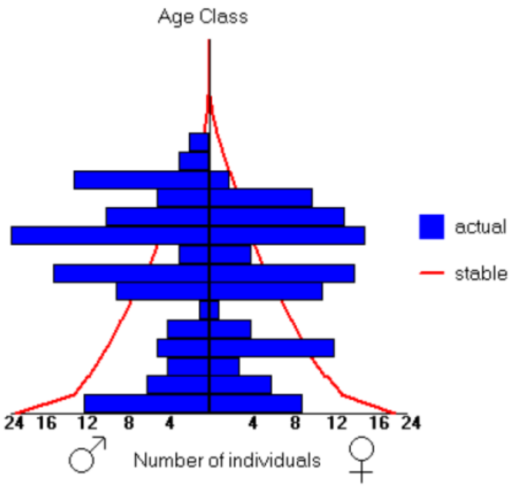

Figure 2. Age structure of Mexican wolf SSP captive population (does not include released or neuter animals).

Annex III

Protocol for Endocrine Determinations

Fecal Steroid Extraction Procedure

1. Collect fecal sample as fresh as possible. Freeze at -70ºC if not to be extracted immediately.
2. Thaw samples.
3. Record weight of empty 20 mL scintillation vial (plastic preferred). Add 0.5 g wet feces (± 0.1 g). Avoid obvious foreign material (straw, bone, sand, whole kernels of corn, etc.). If sample size permits, take sample from inside center. Break pellet into small pieces with spatula.
4. Add 5.0 mL working Extraction Buffer (50% Fecal Extraction Buffer/50% Methanol (Sigma M 1775)) and cap very tightly.
5. Vortex until sample is well dispersed.
6. Shake overnight (about 16 hours) at 200 RPM.
7. Allow to settle about 1 hour, then decant liquid from vial into 12 x 75 polypropylene tube.
8. Centrifuge 1 hour at 4,000 RPM.
9. Decant supernatant into clean cryovial and freeze at -70ºC until day of assay.
10. If a large pellet remains in the centrifuge tube, add about 0.5 cc of extraction buffer to centrifuge tube and vortex to resuspend solid matter; pour back into scintillation vial. Repeat until no visible solid material remains in tube. Can omit this step if amount of residue is very small.
11. Dry vials overnight in vented oven at 100ºC.
12. Allow to come to room temperature, then weigh. Record weight of vial + dried feces. Subtract weight of empty vial to determine weight of dry feces, used in calculation of hormone levels.

Fecal Steroid Extraction Buffer

(Phosphate/Methanol)

(Modifies Extraction Buffer from Sheidler et al. by increasing final methanol concentration from 20% to 50%; also adds sodium azide to inhibit bacterial growth. Demonstrated to increase yields of estradiol, progesterone and testosterone from feces of fennec fox, snow leopard, tiger, Speke=s gazelle, okapi and Eld=s deer by factors of 2-5.)

1. To approximately 700 mL d. H_2O add the following and stir until dissolved:

- 8.75 g NaCl Sodium chloride (Sigma S 9888)

- 5.55 g $NaH_2PO_4H_2O$ Sodium phosphate monobasic, monohydrate (Sigma S 9638)

- 8.87 g Na_2HPO_4 Sodium phosphate dibasic anhydrous (Sigma S 0876)

- 1.00 g Sodium Azide (Sigma S 2002)

2. Add 0.5 mL Tween 20 (Sigma P 1579). Stir gently until mixed.
3. pH to 7.0, then add d. H_2O to final volume of 1 L.
4. Add 1.0 g BSA Bovine Serum Albumin RIA Grade (Sigma A7888) to surface of solution; allow to dissolve slowly.
5. This stock solution may be stored at 4ºC for up to 1 year.
6. Warm stock to room temperature and mix with equal volume methanol to make Working Extraction Buffer. Stable for a few days at room temp. Discard if any cloudiness is observed (MeOH eventually precipitates BSA & salts.)[10]

<u>Time Line: Fecal Extraction</u>

- Do ahead: Number vials, weigh and record weights on computer in appropriate file. If vials are numbered but not weighed, tape sign on top warning that they still need to be weighed. Number one 12 x 75 and 1 cryotube with same number as each vial.
- Day before: OK to move samples from freezer to refrigerator to thaw.
- Day One: Aliquot samples into vials, add extraction buffer, mix, put on shaker.
- Day Two: Stop shaker and let settle for at least an hour.

Pour samples into 12 x 75 tubes; balance in carriers.

Spin 1 hour 4,000 RPM.

Pour supernatant into cryotubes. Freeze until assay.

Put vials into 100 degree drying oven overnight.

- Day Three (or later): Cool and weigh vials containing dry fecal residue. Record and save data.

[10] References:
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Annex IV

Table 1. Pups' Birthdays and Estimated Conception Dates

FEMALE	NAME	YEAR	PUP BIRTH DATE	EST CONCEPTION	HORMONES MEASURED	SEEN COPULATING
58	JUANITA	1998	2 MAY 1998	MARCH 1	E2, P4	NO
192	SAGUARO	2005	29 APRIL 2005	FEB 26	E2, P4	NO VALUES
204	FRIJOLE	2001	19 APRIL 2001	FEB 16	NO VALUES	YES
204	FRIJOLE	2002	25 APRIL 2002	FEB 22	E2, P4	YES
204	FRIJOLE	2003	28 APRIL 2003	FEB 25	E2, P4	YES
204	FRIJOLE	2005	1 MAY 2005	FEB 28	E2, P4	YES
204	FRIJOLE	2006	approx. 26 APRIL 2006	FEB 23	E2, P4	NO
431	SPORT	2002	22 MAY 2002	MARCH 21	E2, P4 (ONLY ONE VALUE)	NO
547	TANAMARA	2001	22 APRIL 2001	FEB 19	NO VALUES	YES
547	TANAMARA	2003	30 APRIL 2003	FEB 27	E2, P4	YES
547	TANAMARA	2004	24 APRIL 2004	FEB 22	E2, P4	YES
547	TANAMARA	2005	10 APRIL 2005	FEB 7	E2, P4	YES
658	NAKOMIS	2005	4 MAY 2005	MARCH 3	E2, P4	NO VALUES
685	ANNA	2003	25 APRIL 2003	FEB 22	E2, P4	YES
685	ANNA	2004	11 APRIL 2004	FEB 9	E2, P4	NO
685	ANNA	2005	9 APRIL 2005	FEB 6	E2, P4	YES
685	ANNA	2008	6 APRIL 2008	FEB 4	E2, P4	YES
797	MADRE	2013	20 MAY 2013	MARCH 19	E2, P4	YES
882	ABBY	2010	2 MAY 2010	MARCH 1	E2, P4	NO
882	ABBY	2011	1 MAY 2011	FEB 28	E2, P4	YES
1266	SIBI	2015	17 APRIL 2015	FEB 15	P4	YES

Wolf ID	Year	Mornings *1	Afternoons *2	Total nr of Behaviors	Days taken into account	Morning/Total	Afternoon/Total
58 JUANITA	1998	845	426	1271	45	0,664830842	0,335169158
204 FRIJOLE	2001	382	529	911	22	0,419319429	0,580680571
204 FRIJOLE	2002	561	178	739	26	0,759133965	0,240866035
204 FRIJOLE	2003	592	610	1202	44	0,492512479	0,507487521
204 FRIJOLE	2005	583	601	1184	45	0,492398649	0,507601351
204 FRIJOLE	2006	216	255	471	43	0,458598726	0,541401274
431 SPORT	2002	213	127	340	41	0,626470588	0,373529412
547 TANAMARA	2001	340	499	839	19	0,405244338	0,594755662
547 TANAMARA	2003	673	800	1473	35	0,456890699	0,543109301
547 TANAMARA	2004	447	841	1288	33	0,347049689	0,652950311
547 TANAMARA	2005	322	367	689	24	0,467343977	0,532656023
685 ANNA	2003	367	502	869	39	0,422324511	0,577675489
685 ANNA	2004	471	624	1095	43	0,430136986	0,569863014
685 ANNA	2005	376	305	681	28	0,552129222	0,447870778
685 ANNA	2008	386	473	859	21	0,449359721	0,550640279
797 MADRE	2013	270	212	482	56	0,560165975	0,439834025
882 ABBY	2010	361	330	691	41	0,522431259	0,477568741
882 ABBY	2011	226	230	456	33	0,495614035	0,504385965
1266 SIBI	2015	145	124	269	21	0,539033457	0,460966543

Table 2. Behaviors' Frequency Rates in the Mornings vs Afternoons

Notes:

*1: Total nr. of Behaviors in all the mornings

*2: Total nr. of Behaviors in all the afternoons

- Total nr of behaviors is the total from all the days

Mean value (mornings)	0,5
Mean value (afternoons)	0,5

FEMALE TAIL DEFLECT

Table 3. Number days between first FTD and first Copulatory Tie

Wolf ID	Year	Nr days between first FTD and first CopTie
204 FRIJOLE	2001	13
204 FRIJOLE	2002	17
204 FRIJOLE	2003	26
204 FRIJOLE	2005	8
547 TANAMARA	2001	7
547 TANAMARA	2003	28
547 TANAMARA	2004	17
547 TANAMARA	2005	13
685 ANNA	2003	28
685 ANNA	2005	12
685 ANNA	2008	6
797 MADRE	2013	15
882 ABBY	2011	11
1266 SIBI	2015	3

Mean value: 14,57

Pairs in which NO Mounting or Copulatory Ties where observed but had litters that same year: Only one case – Sport (431) in 2002

Table 4. DAYS BETWEEN 1st MOUNT AND 1st COPULATORY TIE[11]

FEMALE	Year	Nr. of Days
58 (Juanita)	1999	0
204 (Frijole)	2001	0
	2003	0
	2004	**3**
	2005	**4**
547 (Tanamara)	2000	0
	2001	**1** (first Cop, then Mount!)
	2003	0
	2004	**15**
	2005	0
685 (Anna)	2003	**1** (first Cop, then Mount!)
	2005	0
	2008	**4**
797 (Madre)	2013	**21**
882 (Abby)	2009	**5**
	2011	0
972 (Cedar)	2010	0
	2011	0
	2012	0
	2013	0
1266 (Sibi)	2015	**2** (first Cop, then Mount!)

[11] Only for the pairs in which both behaviors where observed

Male Urine Mark Over

Table 5. Couples with Litters

Juanita (58) and Cheech (72)

Year	Number of days observed	Number of MUMOs (total)	Average/ MUMO rate per day
1998	45	164	3.64

Frijole (204) and Alano (105)

Year	Number of days observed	Number of MUMOs (total)	Average/ MUMO rate per day
2001	22	21	0.95
2002	27	31	1.45
2003	45	33	0.73
2005	45	9	0.2
2006	44	14	0.32

Tanamara (547) and Santa Ana (412)

Year	Number of days observed	Number of MUMOs (total)	Average/ MUMO rate per day
2001	22	13	0.59

Tanamara (547) and Picaron (520)

Year	Number of days observed	Number of MUMOs (total)	Average/ MUMO rate per day
2003	39	223	5.72
2004	36	101	2.81
2005	24	42	1.75

Anna (685) and Prietito (536)

Year	Number of days observed	Number of MUMOs (total)	Average/ MUMO rate per day
2003	41	8	0.2
2004	46	24	0.52

Anna (685) and Dude (572)

Year	Number of days observed	Number of MUMOs (total)	Average/ MUMO rate per day
2005	45	11	0.24
2008	22	196	8.91

Madre (797) and Perkins (950)

Year	Number of days observed	Number of MUMOs (total)	Average/ MUMO rate per day
2013	56	26	0.46

Abby (882) and Perkins (950)

Year	Number of days observed	Number of MUMOs (total)	Average/ MUMO rate per day
2010	47	213	4.53
2011	38	99	2.61

Sibi (1266) and Lazarus (1177)

Year	Number of days observed	Number of MUMOs (total)	Average/ MUMO rate per day
2015	22	17	0.77

Table 6. Couples without Litters

Juanita (58) and Cheech (72)

Year	Number of days observed	Number of MUMOs (total)	Average/ MUMO rate per day
1999	45	174	3.87

Frijole (204) and Alano (105)

Year	Number of days observed	Number of MUMOs (total)	Average/ MUMO rate per day
2004	42	19	0.45

Tanamara (547) and Saric (284)

Year	Number of days observed	Number of MUMOs (total)	Average/ MUMO rate per day
2000	37	25	0.68

Madre (797) and Lazarus (1177)

Year	Number of days observed	Number of MUMOs (total)	Average/ MUMO rate per day
2014	50	14	0.28

Abby (882) and Rocky (681)

Year	Number of days observed	Number of MUMOs (total)	Average/ MUMO rate per day
2006	34	0	0
2008	20	1	0.05
2009	46	1	0.022

Corazon (886) and Apache (546)

Year	Number of days observed	Number of MUMOs (total)	Average/ MUMO rate per day
2006	36	0	0

Cedar (972) and Rocky (681)

Year	Number of days observed	Number of MUMOs (total)	Average/ MUMO rate per day
2010	45	6	0.13
2011	45	14	0.31
2012	52	7	0.13
2013	52	3	0.06

Rogue (1300) & Male 1297

Year	Number of days observed	Number of MUMOs (total)	Average/ MUMO rate per day
2015	52	3	0.06

Table 7. No Reproductive Activity Couples

(Couples that didn't have any Copulatory Ties or Mountings)

Desert Song (139) and Saric (284)

Year	Number of days observed	Number of MUMOs (total)	Average/ MUMO rate per day
1998	58	0	0
1999	57	16	0.28

Saguaro (192) and Apache (546)

Year	Number of days observed	Number of MUMOs (total)	Average/ MUMO rate per day
2002	38	253	6.66

Saguaro (192) and Chico (76)

Year	Number of days observed	Number of MUMOs (total)	Average/ MUMO rate per day
2003	21	12	0.57

Frijole (204) and Santa Ana (412)

Year	Number of days observed	Number of MUMOs (total)	Average/ MUMO rate per day
1999	57	43	0.75

Frijole (204) and Santa Ana (412)

Year	Number of days observed	Number of MUMOs (total)	Average/ MUMO rate per day
1998	57	3	0.05
2000	43	28	0.65

Frijole (204) and Alano (105)

Year	Number of days observed	Number of MUMOs (total)	Average/ MUMO rate per day
2007	26	2	0.08

Sport (431) and 573

Year	Number of days observed	Number of MUMOs (total)	Average/ MUMO rate per day
2001	37	15	0.41

Sport (431) and Prietito (536)

Year	Number of days observed	Number of MUMOs (total)	Average/ MUMO rate per day
2002	41	19	0.46

Nakomis (658) and Apache (546)

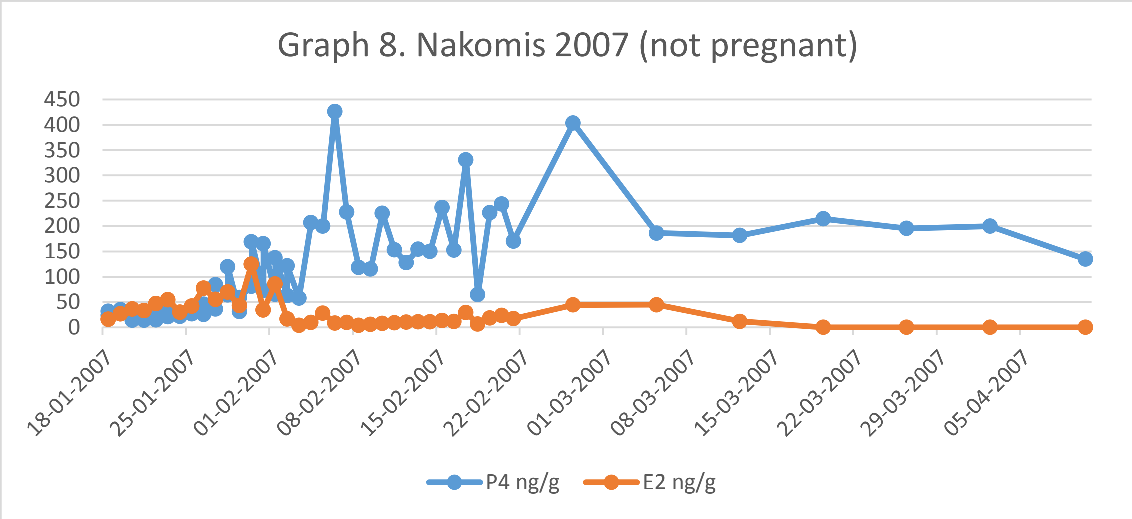
Year	Number of days observed	Number of MUMOs (total)	Average/ MUMO rate per day
2003	44	87	1.98

Annex V

Representative Graphics “P4 & E2”

658 Nakomis (2007) – not pregnant

Date	P4 ng/g	E2 ng/g
18/01/2007	23,33333	15,91667
18/01/2007	31,66667	15,91667
19/01/2007	30	26,875
19/01/2007	35	26,875
20/01/2007	14	36,26
20/01/2007	24	36,26
21/01/2007	14,28571	33,11429
21/01/2007	20	33,11429
22/01/2007	14,51613	46,8871
22/01/2007	24,19355	46,8871
23/01/2007	21,21212	54,57576
23/01/2007	37,87879	54,57576
24/01/2007	21,73913	29,93478
24/01/2007	28,26087	29,93478
25/01/2007	27,08333	42,10417
25/01/2007	35,41667	42,10417
26/01/2007	25,55556	77,16667
26/01/2007	45,55556	77,16667
27/01/2007	36,2069	55,06897
27/01/2007	84,48276	55,06897
28/01/2007	64	69,66
28/01/2007	120	69,66
29/01/2007	31,48148	42,96296
29/01/2007	59,25926	42,96296
30/01/2007	81,03448	125,0172
30/01/2007	168,9655	125,0172
31/01/2007	72,72727	34,45455
31/01/2007	165,1515	34,45455
01/02/2007	65,625	85,65625
01/02/2007	137,5	85,65625
02/02/2007	62,5	16,9375
02/02/2007	121,875	16,9375
03/02/2007	57,5	4,3125
04/02/2007	207,1429	9,928571
05/02/2007	200	27,77419
06/02/2007	426,3889	8,472222
07/02/2007	227,7778	9,814815
08/02/2007	118,75	3,875
09/02/2007	115	5,875
10/02/2007	225	7,770833
11/02/2007	153,125	9,25
12/02/2007	127,7778	10,5
13/02/2007	154,7619	10,69048
14/02/2007	150	10,71875
15/02/2007	236,3636	13,5
16/02/2007	152,7778	11,91667
17/02/2007	330,7692	29,5
18/02/2007	64,81481	6,481481
19/02/2007	226,4706	18,5
20/02/2007	243,75	23,875
21/02/2007	170,5882	17,29412
26/02/2007	403,5714	44,25
05/03/2007	186,3636	44,59091
12/03/2007	181,5789	11,69737
19/03/2007	214,2857	0
26/03/2007	195,4545	0
02/04/2007	200	0
10/04/2007	134,6154	0



685 Anna (2009) – not pregnant

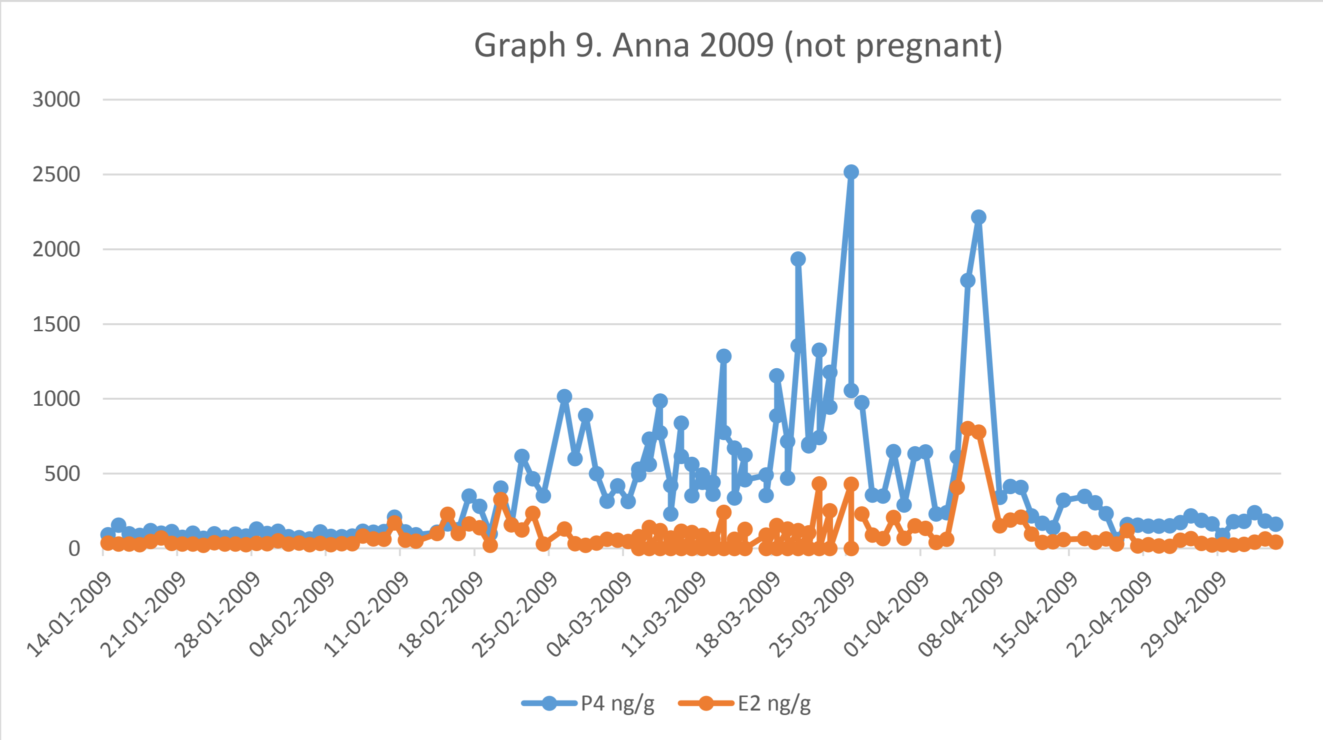
Date	P4 ng/g	E2 ng/g
14/01/2009	91,17647	34,55882
15/01/2009	154,5455	29,04545
16/01/2009	97,05882	28,44118
17/01/2009	86,95652	25,32609
18/01/2009	118,1818	45,15909
19/01/2009	101,6667	69,56667
20/01/2009	111,7647	32,38235
21/01/2009	73,07692	29,34615
22/01/2009	102,6316	28,52632
23/01/2009	66,66667	20,83333
24/01/2009	97,72727	38
25/01/2009	73,91304	28,47826
26/01/2009	95,2381	29,07143
27/01/2009	81,57895	23,78947
28/01/2009	130	33,1
29/01/2009	100	31,95
30/01/2009	114,7059	50,94118
31/01/2009	79,16667	29,97917
01/02/2009	71,66667	35,48333
02/02/2009	64	24,98
03/02/2009	110	33,36667
04/02/2009	79,41176	24,26471
05/02/2009	77,77778	31,07407
06/02/2009	83,33333	30,77778
07/02/2009	114	83,02
08/02/2009	107,8947	63,78947
09/02/2009	113,1579	62,05263
10/02/2009	209,0909	170
11/02/2009	110	55
12/02/2009	90,47619	47,33333

14/02/2009	107,8947	98,73684
15/02/2009	163,6364	227,0909
16/02/2009	125	100,4063
17/02/2009	350	164,7222
18/02/2009	281,25	137,3438
19/02/2009	97,82609	21,08696
20/02/2009	402,7778	325,4167
21/02/2009	159,6154	157,7692
22/02/2009	615,625	123,0313
23/02/2009	465,2174	234,913
24/02/2009	352,9412	28,82353
26/02/2009	1014,583	129,8854
27/02/2009	600	31,06667
28/02/2009	888,2353	20,23529
01/03/2009	500	35,92308
02/03/2009	316,0714	60,23214
03/03/2009	417,6471	55
04/03/2009	313,8889	46,33333
05/03/2009	530	78,8
05/03/2009	493,75	0
06/03/2009	730	139,425
06/03/2009	562,5	0
07/03/2009	985	118
07/03/2009	772,4138	0
08/03/2009	420	68,675
08/03/2009	230,4348	0

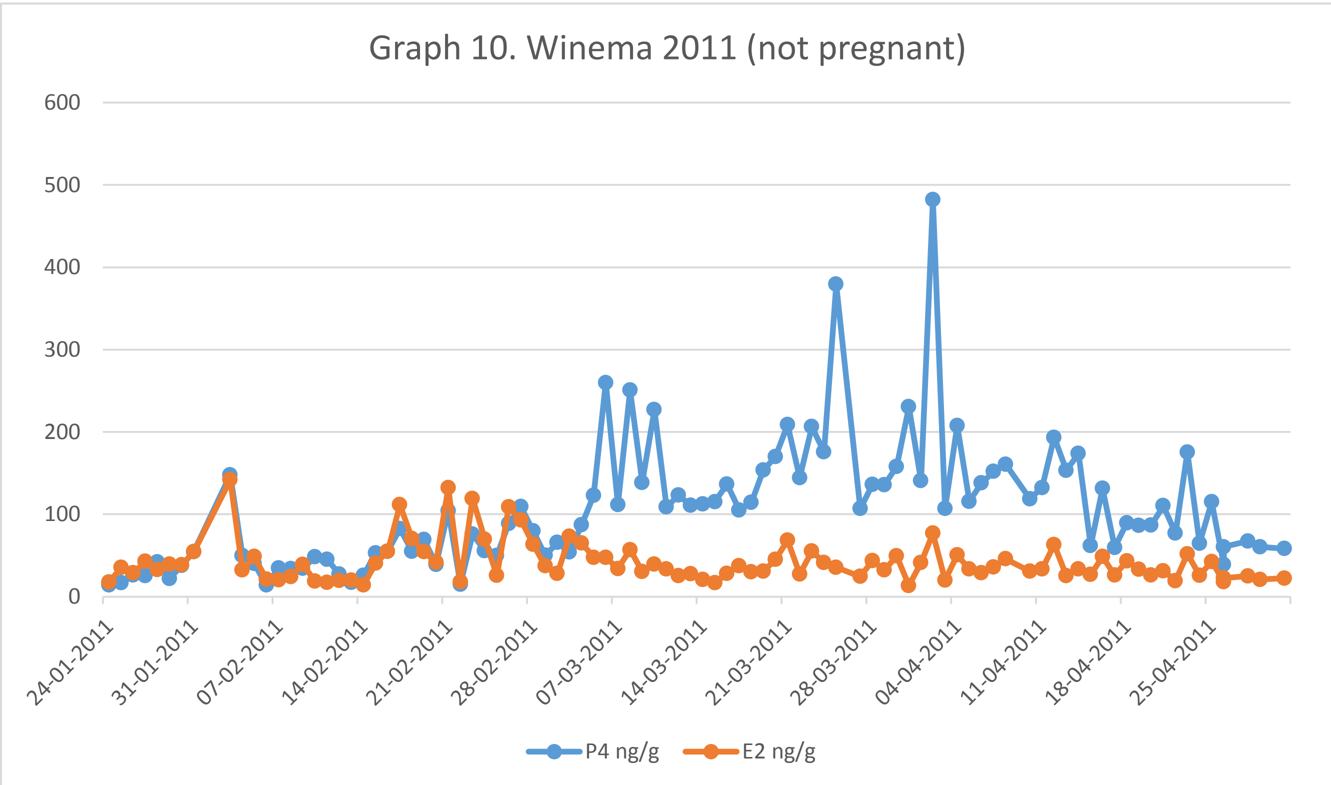
09/03/2009	837,5	113,775
09/03/2009	614	0
10/03/2009	562,5	105,15
10/03/2009	352,381	0
11/03/2009	490	87
11/03/2009	441,6667	0
12/03/2009	362,5	61,55
12/03/2009	442,5	0
13/03/2009	1285	240,625
13/03/2009	776,3158	0
14/03/2009	670	60,65
14/03/2009	336,3636	0
15/03/2009	622,5	128,475
15/03/2009	458,3333	0
17/03/2009	490	89,25
17/03/2009	354	0
18/03/2009	887,5	153,45
18/03/2009	1154	0
19/03/2009	715	129,325
19/03/2009	469,0476	0
20/03/2009	1355	118,875
20/03/2009	1934,286	0
21/03/2009	697,5	104,675
21/03/2009	684,7826	0
22/03/2009	1325	431,9
22/03/2009	741,6667	0

23/03/2009	1177,5	251,375
23/03/2009	944,1176	0
25/03/2009	2515	429,075
25/03/2009	1055,263	0
26/03/2009		
26/03/2009	975	229,1667
27/03/2009	355,8824	88,44118
28/03/2009	350	65,31818
29/03/2009	646,4286	207,3571
30/03/2009	290,625	68,03125
31/03/2009	631,5789	151,0263
01/04/2009	645,4545	134
02/04/2009	229,4118	39,91176
03/04/2009	238,4615	62,19231
04/04/2009	611,5385	407,6923
05/04/2009	1790	801,6
06/04/2009	2214,286	777,1429
08/04/2009	340,9091	150,9545
09/04/2009	414,2857	189,9286
10/04/2009	406,6667	208,8333
11/04/2009	218,1818	94,86364
12/04/2009	167,2414	40,74138

13/04/2009	139,1304	43,15217
14/04/2009	321,0526	59,39474
16/04/2009	347,619	65,38095
17/04/2009	304,3478	39,45652
18/04/2009	231,8182	62,52273
19/04/2009	59,72222	28,25
20/04/2009	160,3448	118,931
21/04/2009	155	15,35
22/04/2009	148,2759	24,98276
23/04/2009	148,4375	16,10938
24/04/2009	150	13,85185
25/04/2009	172	55,54667
26/04/2009	216,2791	66,22093
27/04/2009	188,0952	33
28/04/2009	163,4615	21,75
29/04/2009	87,5	23,96591
30/04/2009	179,1667	21,72917
01/05/2009	181,6667	27,61667
02/05/2009	238,8889	41,66667
03/05/2009	182,1429	62,84524
04/05/2009	162,2449	41,66327



938 Winema (2011) – not pregnant



Date	P4 ng/g	E2 ng/g
24/01/2011	13,88889	17,35296
25/01/2011	16,66667	35,22807
26/01/2011	26,1166	28,94475
27/01/2011	25,27632	42,58575
28/01/2011	42,13692	32,62237
29/01/2011	21,89944	39,20333
30/01/2011	37,69724	38,48405
31/01/2011	54,52783	54,27093
03/02/2011	147,8235	141,8611
04/02/2011	49,72013	32,13365
05/02/2011	39,98264	48,53581
06/02/2011	13,95375	20,94262
07/02/2011	34,55092	20,04222
08/02/2011	33,78094	24,06916
09/02/2011	34,13267	38,82281
10/02/2011	48,36347	18,83865
11/02/2011	45,26579	17,22369
12/02/2011	27,40488	19,47993
13/02/2011	17,23987	19,80411
14/02/2011	25,60592	13,89896
15/02/2011	53,02632	40,28643
16/02/2011	54,84338	54,88246
17/02/2011	82,52421	111,5214
18/02/2011	54,96955	70,26377
19/02/2011	69,20875	54,25085
20/02/2011	39,00661	41,74041
21/02/2011	104,0559	132,0797
22/02/2011	14,94885	17,76709
23/02/2011	75,95963	119,0109
24/02/2011	55,43719	69,7556
25/02/2011	49,92556	25,75368
26/02/2011	88,8365	108,6946
27/02/2011	109,1218	93,04126
28/02/2011	79,88488	63,39316
01/03/2011	50,03855	37,15793
02/03/2011	65,69528	27,92821
03/03/2011	53,87922	73,2337
04/03/2011	87,17982	65,02138
05/03/2011	122,7076	47,52836
06/03/2011	259,8063	47,52992
07/03/2011	111,6408	34,01332
08/03/2011	250,6627	56,94471
09/03/2011	138,4739	30,38833
10/03/2011	227,0326	39,28667
11/03/2011	108,8236	33,54668
12/03/2011	123,1547	25,33087
13/03/2011	110,8221	27,4333
14/03/2011	112,4903	20,56809
15/03/2011	115,2327	16,5773
16/03/2011	136,6374	28,19169
17/03/2011	105,0161	37,35453
18/03/2011	114,1156	29,91602
19/03/2011	153,4264	30,61471
20/03/2011	169,7764	45,04784
21/03/2011	208,6639	68,31073
22/03/2011	144,2388	27,28002
23/03/2011	206,4047	55,0698
24/03/2011	175,623	41,36539
25/03/2011	379,2343	35,39869
27/03/2011	106,8296	24,32507
28/03/2011	135,9922	43,50925
29/03/2011	135,8598	32,35486
30/03/2011	157,9101	49,282
31/03/2011	230,4064	13,23222
01/04/2011	140,6384	41,20018
02/04/2011	482,027	76,7895
03/04/2011	106,8466	19,86917
04/04/2011	207,6775	50,40121
05/04/2011	115,5311	33,33277
06/04/2011	137,9143	28,85442
07/04/2011	151,8743	35,95307
08/04/2011	160,5273	45,79407
10/04/2011	118,592	30,65066
11/04/2011	132,2664	33,43463
12/04/2011	193,2635	63,09333
13/04/2011	153,3263	25,26877
14/04/2011	173,6295	33,53549
15/04/2011	61,69537	26,82722
16/04/2011	131,2878	48,55573
17/04/2011	59,53397	26,02852
18/04/2011	89,50681	43,28125
19/04/2011	86,38583	33,00217
20/04/2011	86,69362	26,13327
21/04/2011	110,2413	31,25186
22/04/2011	76,9605	19,14294
23/04/2011	175,4888	51,84985
24/04/2011	64,68735	25,75859
25/04/2011	115,0257	42,39659
26/04/2011	38,85933	17,93772

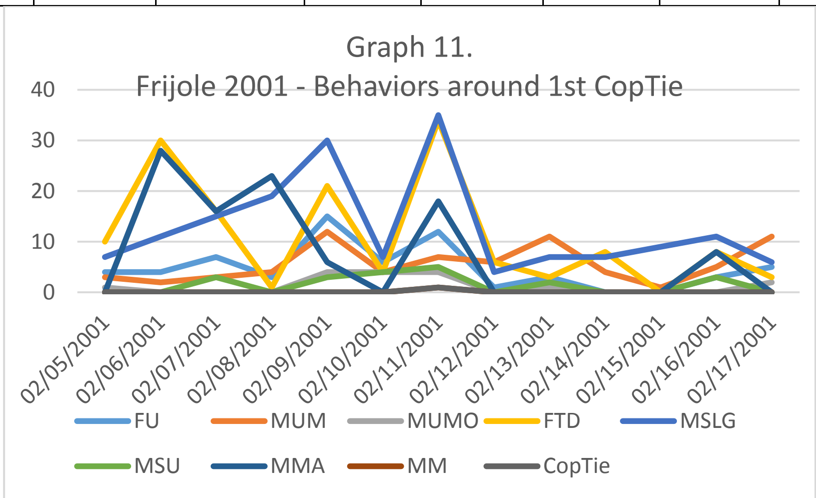
26/04/2011	60,37567	22,42165
28/04/2011	67,32237	24,92458

29/04/2011	60,45734	20,66815
01/05/2011	58,18574	22,27655

Behaviors around first CopTie

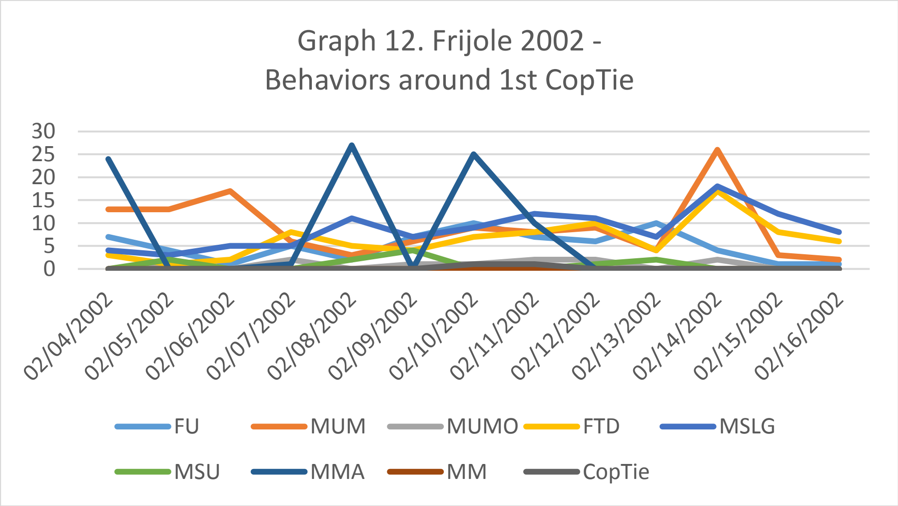
204 Frijole (2001)

Year, Date	FU	MUM	MUMO	FTD	MSLG	MSU	MMA	MM	CopTie
02/05/2001	4	3	1	10	7	0	0	0	0
02/06/2001	4	2	0	30	11	0	28	0	0
02/07/2001	7	3	0	16	15	3	16	0	0
02/08/2001	3	4	0	1	19	0	23	0	0
02/09/2001	15	12	4	21	30	3	6	0	0
02/10/2001	6	4	4	4	7	4	0	0	0
02/11/2001	**12**	**7**	**4**	**34**	**35**	**5**	**18**	**1**	**1**
02/12/2001	1	6	0	6	4	0	0	0	0
02/13/2001	3	11	1	3	7	2	0	0	0
02/14/2001	0	4	0	8	7	0	0	0	0
02/15/2001	0	1	0	0	9	0	0	0	0
02/16/2001	3	5	0	8	11	3	8	0	0
02/17/2001	5	11	2	3	6	0	0	0	0



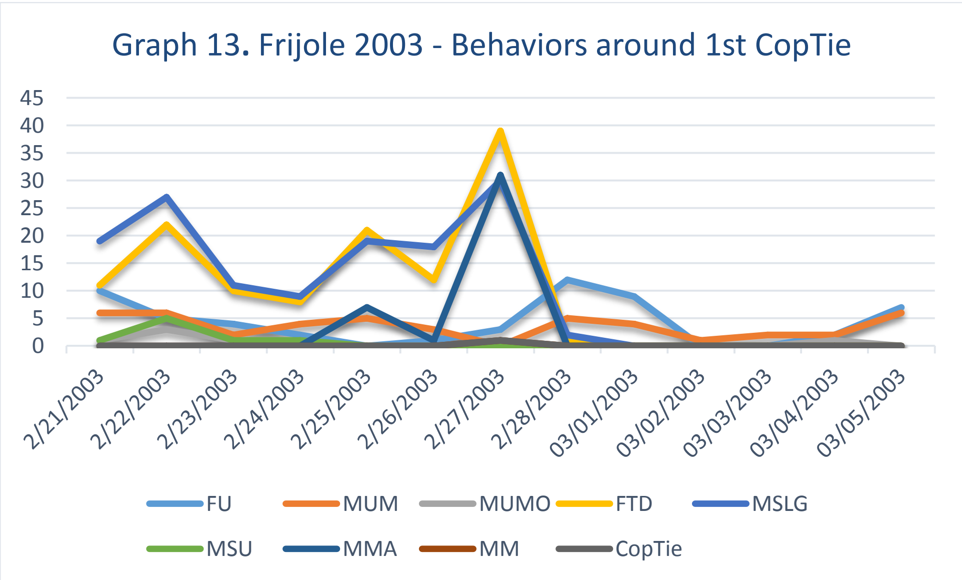
204 Frijole (2002)

Year, Date	FU	MUM	MUMO	FTD	MSLG	MSU	MMA	MM	CopTie
02/04/2002	7	13	0	3	4	0	24	0	0
02/05/2002	4	13	1	1	3	2	0	0	0
02/06/2002	1	17	0	2	5	0	0	0	0
02/07/2002	5	6	2	8	5	0	1	0	0
02/08/2002	2	3	0	5	11	2	27	0	0
02/09/2002	7	6	1	4	7	4	0	0	0
02/10/2002	**10**	**9**	**1**	**7**	**9**	**0**	**25**	**0**	**1**
02/11/2002	7	8	2	8	12	0	10	0	1
02/12/2002	6	9	2	10	11	1	0	0	0
02/13/2002	10	4	0	4	7	2	0	0	0
02/14/2002	4	26	2	17	18	0	0	0	0
02/15/2002	1	3	0	8	12	0	0	0	0
02/16/2002	1	2	0	6	8	0	0	0	0



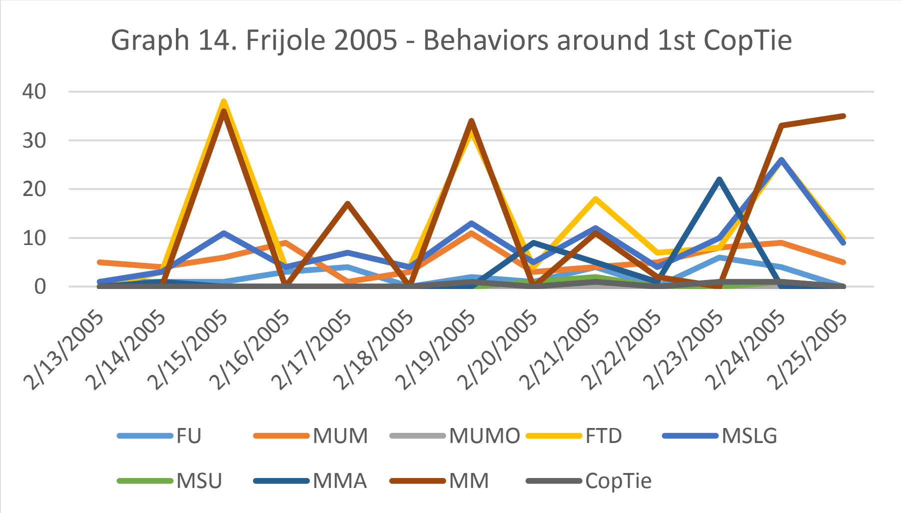
204 Frijole (2003)

Year, Date	FU	MUM	MUMO	FTD	MSLG	MSU	MMA	MM	CopTie
2/21/2003	10	6	1	11	19	1	0	0	0
2/22/2003	5	6	3	22	27	5	0	0	0
2/23/2003	4	2	1	10	11	1	0	0	0
2/24/2003	2	4	0	8	9	1	0	0	0
2/25/2003	0	5	0	21	19	0	7	0	0
2/26/2003	1	3	0	12	18	0	1	0	0
2/27/2003	**3**	**0**	**1**	**39**	**30**	**0**	**31**	**1**	**1**
2/28/2003	12	5	0	1	2	0	0	0	0
03/01/2003	9	4	0	0	0	0	0	0	0
03/02/2003	0	1	0	0	0	0	0	0	0
03/03/2003	0	2	0	0	0	0	0	0	0
03/04/2003	2	2	1	0	0	0	0	0	0
03/05/2003	7	6	0	0	0	0	0	0	0



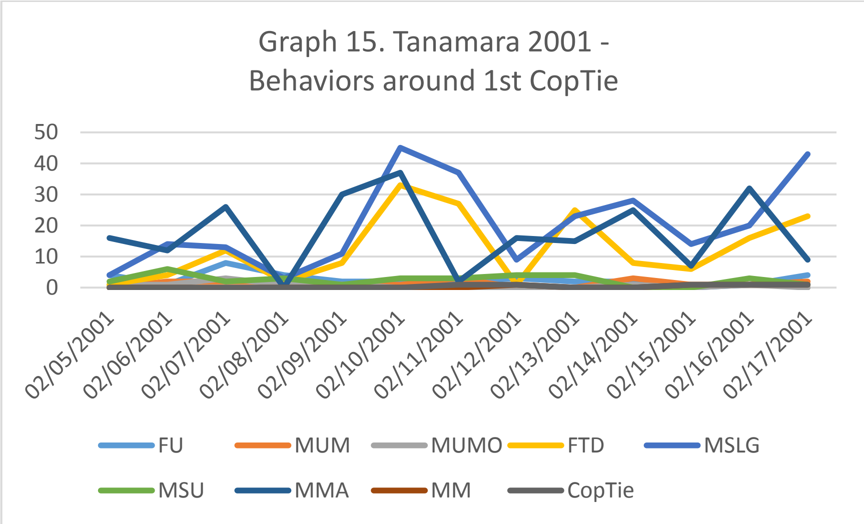
204 Frijole (2005)

Year, Date	FU	MUM	MUMO	FTD	MSLG	MSU	MMA	MM	CopTie
2/13/2005	1	5	0	0	1	0	0	0	0
2/14/2005	1	4	0	3	3	0	1	0	0
2/15/2005	1	6	0	38	11	0	0	36	0
2/16/2005	3	9	0	4	4	0	0	0	0
2/17/2005	4	1	0	7	7	0	0	17	0
2/18/2005	0	3	0	4	4	0	0	0	0
2/19/2005	**2**	**11**	**0**	**32**	**13**	**0**	**0**	**34**	**1**
2/20/2005	1	3	0	4	5	1	9	0	0
2/21/2005	4	4	0	18	12	2	5	11	1
2/22/2005	0	5	0	7	4	0	1	2	0
2/23/2005	6	8	0	8	10	0	22	0	1
2/24/2005	4	9	0	26	26	1	0	33	1
2/25/2005	0	5	0	10	9	0	0	35	0



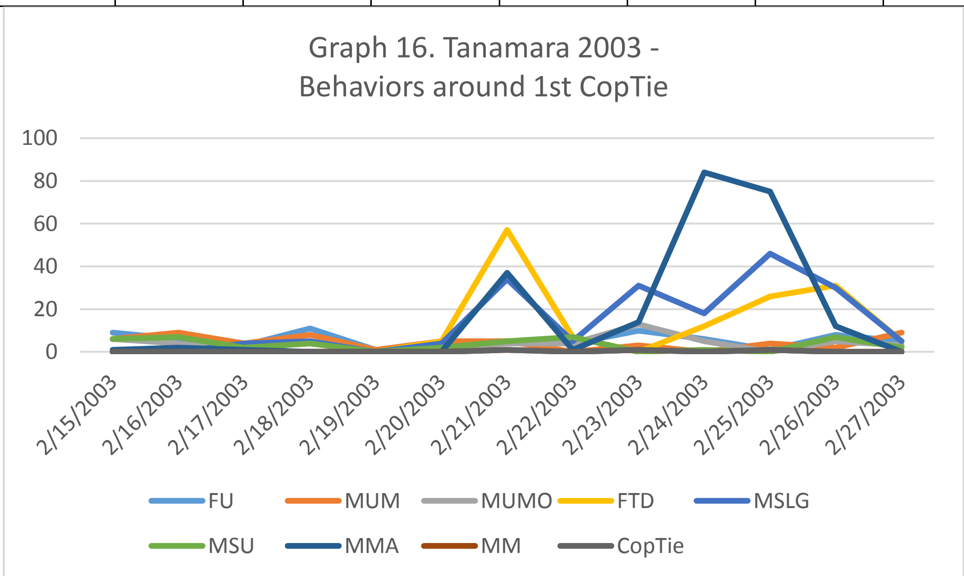
547 Tanamara (2001)

Year, Date	FU	MUM	MUMO	FTD	MSLG	MSU	MMA	MM	CopTie
02/05/2001	4	0	1	1	4	2	16	0	0
02/06/2001	1	2	1	4	14	6	12	0	0
02/07/2001	8	2	3	12	13	2	26	0	0
02/08/2001	4	2	1	2	3	3	0	0	0
02/09/2001	2	1	1	8	11	1	30	0	0
02/10/2001	2	1	0	33	45	3	37	0	0
02/11/2001	**2**	**3**	**0**	**27**	**37**	**3**	**2**	**0**	**1**
02/12/2001	3	0	0	1	9	4	16	1	1
02/13/2001	2	0	0	25	23	4	15	0	0
02/14/2001	2	3	1	8	28	0	25	0	0
02/15/2001	0	1	0	6	14	0	7	1	1
02/16/2001	1	1	1	16	20	3	32	1	1
02/17/2001	4	2	0	23	43	1	9	1	1



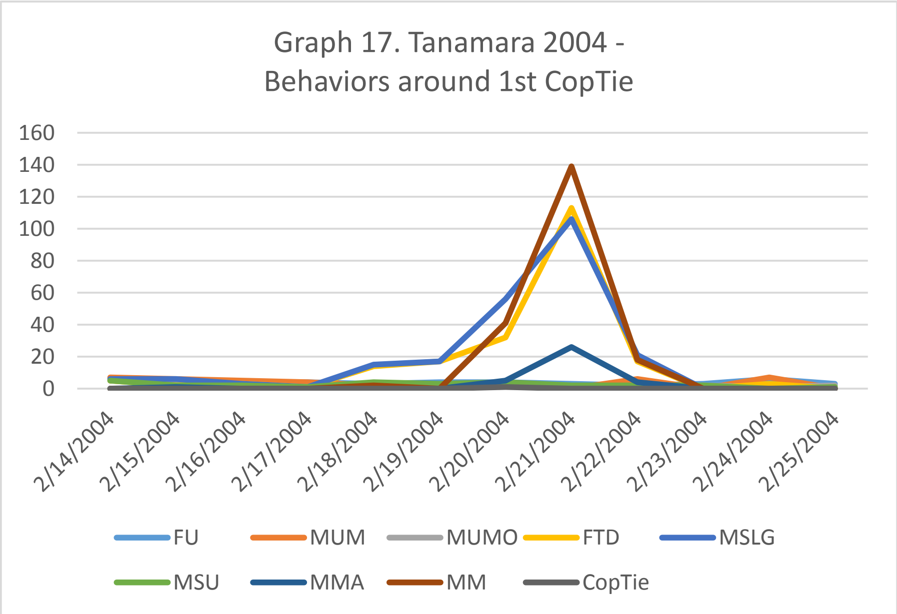
547 Tanamara (2003)

Year, Date	FU	MUM	MUMO	FTD	MSLG	MSU	MMA	MM	CopTie
2/15/2003	9	6	6	0	0	6	1	0	0
2/16/2003	6	9	4	0	0	7	2	0	0
2/17/2003	3	4	0	3	4	2	1	0	0
2/18/2003	11	8	5	5	5	4	0	0	0
2/19/2003	1	1	0	0	0	0	0	0	0
2/20/2003	3	5	1	5	4	2	0	0	0
2/21/2003	**5**	**5**	**4**	**57**	**34**	**5**	**37**	**1**	**1**
2/22/2003	4	0	4	7	5	7	1	0	0
2/23/2003	10	3	13	0	31	0	14	1	1
2/24/2003	6	0	5	12	18	1	84	0	0
2/25/2003	1	4	0	26	46	0	75	1	1
2/26/2003	8	2	5	31	30	7	12	0	0
2/27/2003	5	9	3	5	5	2	0	0	0



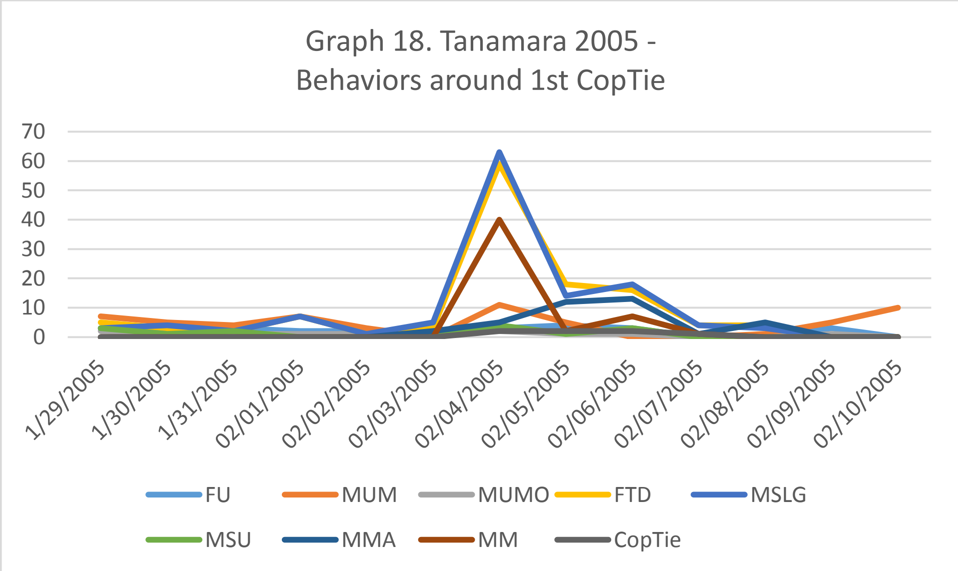
547 Tanamara (2004)

Year, Date	FU	MUM	MUMO	FTD	MSLG	MSU	MMA	MM	CopTie
2/14/2004	6	7	5	5	6	5	0	0	0
2/15/2004	4	6	3	2	6	2	1	0	0
2/16/2004	4	5	3	3	3	2	0	0	0
2/17/2004	4	4	1	1	1	1	0	0	0
2/18/2004	3	2	2	14	15	4	0	2	0
2/19/2004	4	1	2	17	17	3	0	0	0
2/20/2004	**4**	**4**	**3**	**32**	**56**	**4**	**5**	**41**	**1**
2/21/2004	3	0	1	113	106	2	26	139	0
2/22/2004	2	6	1	17	21	2	4	18	0
2/23/2004	3	0	2	0	0	2	0	0	0
2/24/2004	6	7	1	3	0	0	0	0	0
2/25/2004	3	0	2	0	0	1	0	0	0



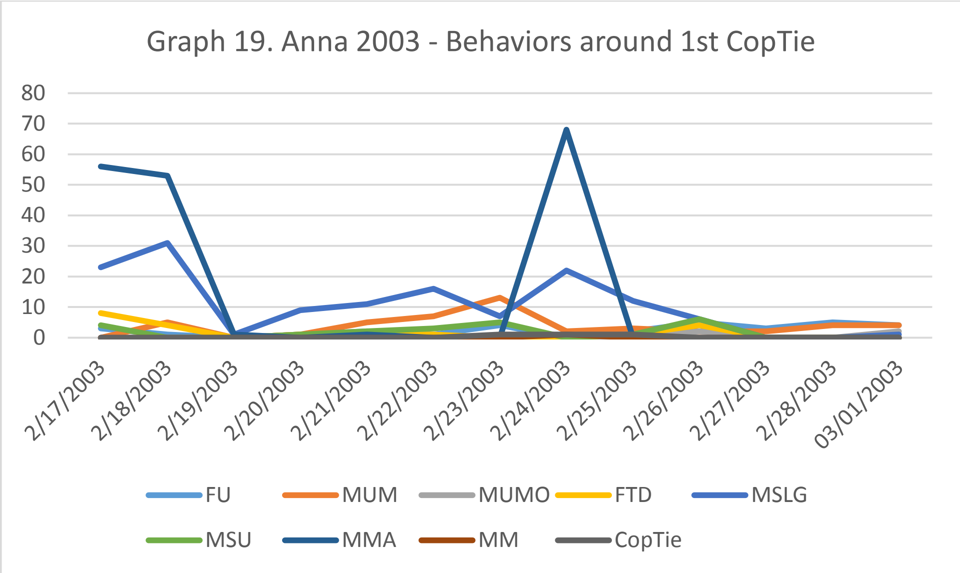
547 Tanamara (2005)

Year, Date	FU	MUM	MUMO	FTD	MSLG	MSU	MMA	MM	CopTie
1/29/2005	3	7	2	5	3	3	0	0	0
1/30/2005	2	5	1	3	4	1	0	0	0
1/31/2005	3	4	1	2	2	2	0	0	0
02/01/2005	2	7	1	7	7	0	0	0	0
02/02/2005	2	3	1	1	1	0	0	0	0
02/03/2005	2	0	2	3	5	1	2	0	0
02/04/2005	**3**	**11**	**2**	**59**	**63**	**4**	**5**	**40**	**2**
02/05/2005	4	5	1	18	14	1	12	2	2
02/06/2005	3	0	1	16	18	3	13	7	2
02/07/2005	0	0	0	4	4	0	1	1	1
02/08/2005	0	1	0	4	3	0	5	0	0
02/09/2005	3	5	1	0	0	0	0	0	0
02/10/2005	0	10	0	0	0	0	0	0	0



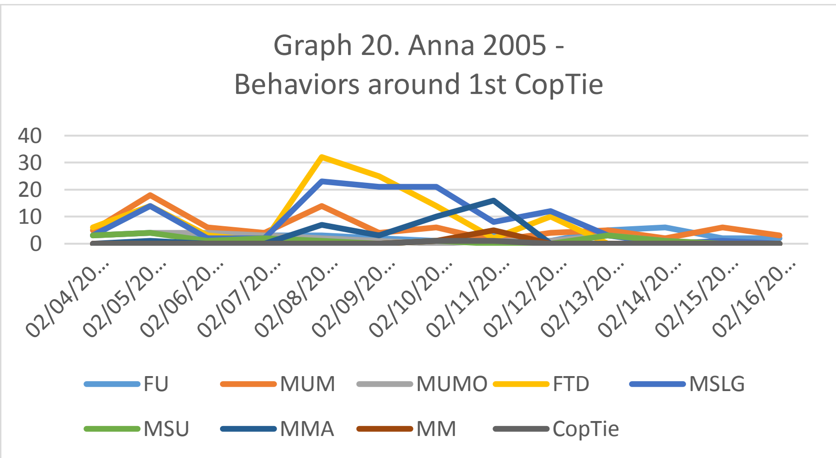
685 Anna (2003)

Year, Date	FU	MUM	MUMO	FTD	MSLG	MSU	MMA	MM	CopTie
2/17/2003	3	0	0	8	23	4	56	0	0
2/18/2003	1	5	0	4	31	0	53	0	0
2/19/2003	0	0	0	0	1	0	1	0	0
2/20/2003	1	1	0	1	9	1	0	0	0
2/21/2003	2	5	0	1	11	2	1	0	0
2/22/2003	1	7	0	1	16	3	0	0	0
2/23/2003	**4**	**13**	**0**	**0**	**7**	**5**	**0**	**0**	**1**
2/24/2003	0	2	0	0	22	0	68	1	1
2/25/2003	2	3	0	1	12	1	0	0	1
2/26/2003	5	2	2	4	6	6	0	0	0
2/27/2003	3	2	0	0	0	0	0	0	0
2/28/2003	5	4	0	0	0	0	0	0	0
03/01/2003	4	4	2	0	1	0	0	0	0



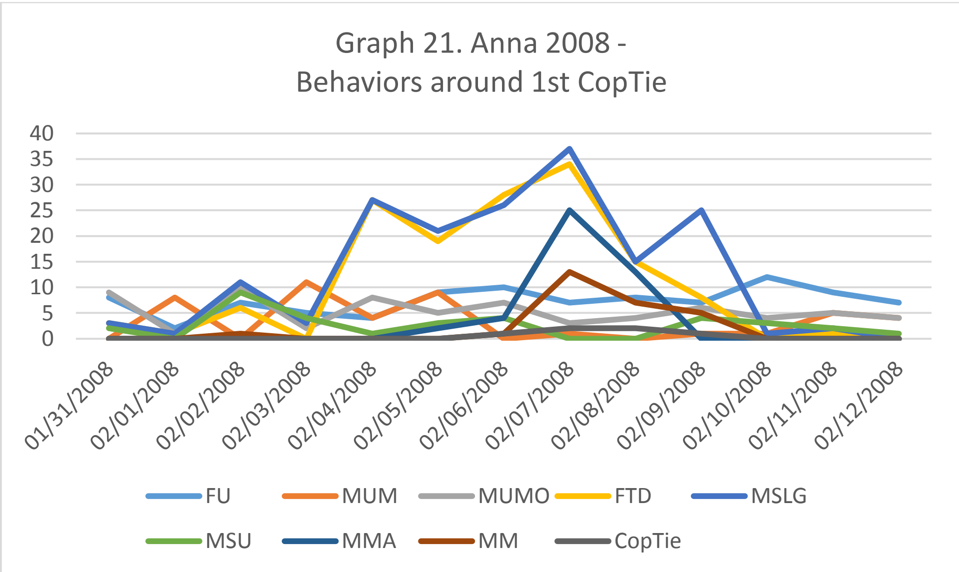
685 Anna (2005)

Year, Date	FU	MUM	MUMO	FTD	MSLG	MSU	MMA	MM	CopTie
02/04/2005	3	5	3	6	3	3	0	0	0
02/05/2005	4	18	4	14	14	4	1	0	0
02/06/2005	2	6	4	3	2	1	0	0	0
02/07/2005	3	4	3	1	2	2	0	0	0
02/08/2005	3	14	2	32	23	1	7	0	0
02/09/2005	2	4	1	25	21	0	3	0	0
02/10/2005	**1**	**6**	**0**	**14**	**21**	**1**	**10**	**1**	**1**
02/11/2005	3	1	1	2	8	0	16	5	1
02/12/2005	1	4	1	10	12	0	0	0	0
02/13/2005	5	5	3	0	3	3	0	0	0
02/14/2005	6	2	1	0	0	1	0	0	0
02/15/2005	2	6	0	0	1	0	0	0	0
02/16/2005	2	3	0	0	0	0	0	0	0



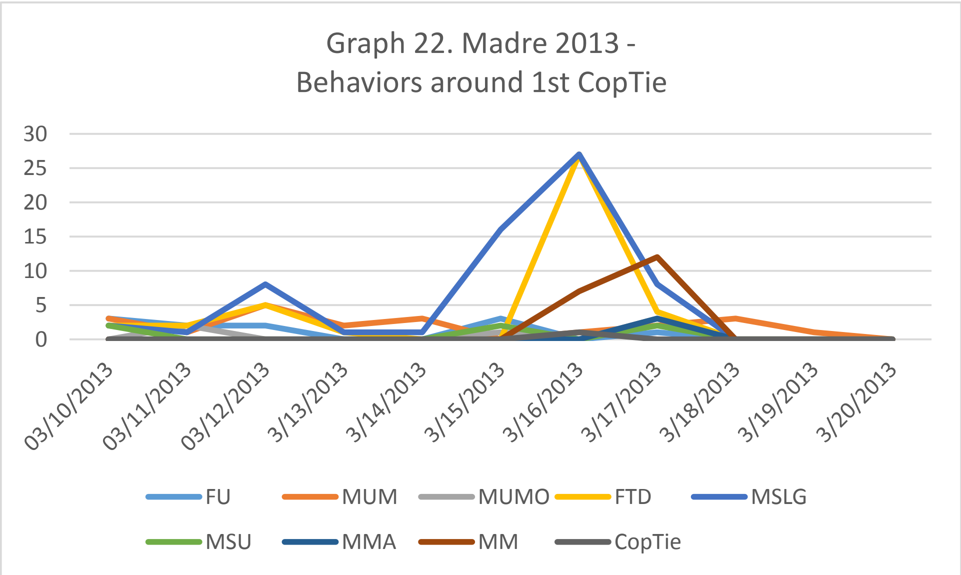
685 Anna (2008)

Year, Date	FU	MUM	MUMO	FTD	MSLG	MSU	MMA	MM	CopTie
01/31/2008	8	0	9	3	3	2	0	0	0
02/01/2008	2	8	1	1	1	0	0	0	0
02/02/2008	7	0	10	6	11	9	0	1	0
02/03/2008	5	11	2	0	3	4	0	0	0
02/04/2008	4	4	8	27	27	1	0	0	0
02/05/2008	9	9	5	19	21	3	2	0	0
02/06/2008	**10**	**0**	**7**	**28**	**26**	**4**	**4**	**1**	**1**
02/07/2008	7	1	3	34	37	0	25	13	2
02/08/2008	8	0	4	15	15	0	13	7	2
02/09/2008	7	1	6	8	25	4	0	5	1
02/10/2008	12	1	4	0	1	3	0	0	0
02/11/2008	9	5	5	1	2	2	0	0	0
02/12/2008	7	4	4	0	0	1	0	0	0



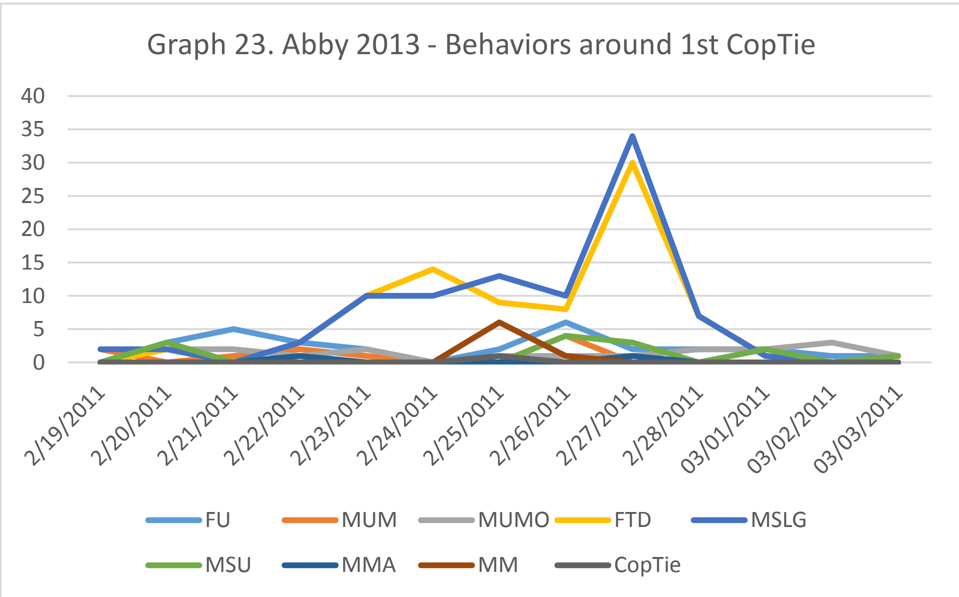
797 Madre (2013)

Year, Date	FU	MUM	MUMO	FTD	MSLG	MSU	MMA	MM	CopTie
03/10/2013	3	3	0	2	2	2	0	0	0
03/11/2013	2	1	2	2	1	0	0	0	0
03/12/2013	2	5	0	5	8	0	0	0	0
3/13/2013	0	2	0	1	1	0	0	0	0
3/14/2013	0	3	0	0	1	0	0	0	0
3/15/2013	3	0	1	0	16	2	0	0	0
3/16/2013	0	**1**	**0**	**27**	**27**	**0**	**0**	**7**	**1**
3/17/2013	1	2	3	4	8	2	3	12	0
3/18/2013	0	3	0	0	0	0	0	0	0
3/19/2013	0	1	0	0	0	0	0	0	0
3/20/2013	0	0	0	0	0	0	0	0	0



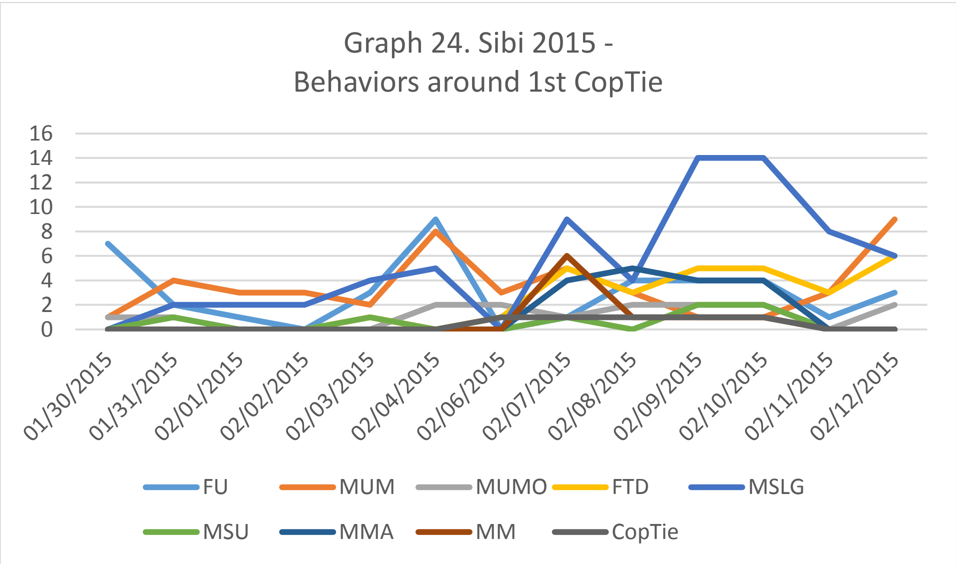
882 Abby (2013)

Year, Date	FU	MUM	MUMO	FTD	MSLG	MSU	MMA	MM	CopTie
2/19/2011	0	2	0	0	2	0	0	0	0
2/20/2011	3	0	2	2	2	3	0	0	0
2/21/2011	5	1	2	0	0	0	0	0	0
2/22/2011	3	2	1	3	3	1	1	0	0
2/23/2011	2	1	2	10	10	0	0	0	0
2/24/2011	0	0	0	14	10	0	0	0	0
2/25/2011	**2**	**0**	**1**	**9**	**13**	**0**	**0**	**6**	**1**
2/26/2011	6	4	1	8	10	4	0	1	0
2/27/2011	2	0	1	30	34	3	1	0	0
2/28/2011	2	0	2	7	7	0	0	0	0
03/01/2011	2	0	2	1	1	2	0	0	0
03/02/2011	1	0	3	0	0	0	0	0	0
03/03/2011	1	0	1	0	0	1	0	0	0



1266 Sibi (2015)

Year, Date	FU	MUM	MUMO	FTD	MSLG	MSU	MMA	MM	CopTie
01/30/2015	7	1	1	0	0	0	0	0	0
01/31/2015	2	4	1	0	2	1	0	0	0
02/01/2015	1	3	0	0	2	0	0	0	0
02/02/2015	0	3	0	0	2	0	0	0	0
02/03/2015	3	2	0	1	4	1	0	0	0
02/04/2015	9	8	2	0	5	0	0	0	0
02/06/2015	**0**	**3**	**2**	**1**	**0**	**0**	**0**	**0**	**1**
02/07/2015	1	5	1	5	9	1	4	6	1
02/08/2015	4	3	2	3	4	0	5	1	1
02/09/2015	4	1	2	5	14	2	4	1	1
02/10/2015	4	1	2	5	14	2	4	1	1
02/11/2015	1	3	0	3	8	0	0	0	0
02/12/2015	3	9	2	6	6	0	0	0	0



Values for the Graphics "P4, MM & CopTie"

204 Frijole (2005)

Year, Date	MM	CopTie	P4 ng/g	E2 ng/g
2/13/2005	0	0	60,0	47,13889
2/14/2005	0	0	58,0	39,83333
2/15/2005	36	0	66,7	79,625
2/16/2005	0	0		
2/17/2005	17	0	55,3	89,34211
2/18/2005	0	0	65,6	73,53125
2/19/2005	**34**	**1**	91,7	89,30556
2/20/2005	0	0	111,5	81,92308
2/21/2005	11	1	252,9	105,6176
2/22/2005	2	0	**483,3**	263,1667
2/23/2005	0	1	186,7	111,1333
2/24/2005	33	1	184,1	51,38636
2/25/2005	35	0	220,6	72,14706

547 Tanamara (2003)

Year, Date	MM	CopTie	P4 ng/g	E2 ng/g
2/15/2003	0	0	36,36364	18,45455
2/16/2003	0	0		
2/17/2003	0	0		
2/18/2003	0	0	100	34,71429
2/19/2003	0	0		
2/20/2003	0	0		
2/21/2003	**1**	**1**	177,4194	53,41935
2/22/2003	0	0	129,1667	30,6875
2/23/2003	1	1		
2/24/2003	0	0		
2/25/2003	1	1		
2/26/2003	0	0	145,9459	14,71622
2/27/2003	0	0	158,8235	11,67647

547 Tanamara (2004)

Year, Date	MM	CopTie	P4 ng/g
2/14/2004	0	0	260
2/15/2004	0	0	
2/16/2004	0	0	
2/17/2004	0	0	218,4211
2/18/2004	2	0	
2/19/2004	0	0	313,8889
2/20/2004	**41**	**1**	285,7143
2/21/2004	139	0	
2/22/2004	18	0	
2/23/2004	0	0	227,5
2/24/2004	0	0	863,8889
2/25/2004	0	0	

685 Anna (2003)

Year, Date	MM	CopTie	P4 ng/g	E2 ng/g
2/17/2003	0	0		
2/18/2003	0	0		
2/19/2003	0	0		
2/20/2003	0	0		
2/21/2003	0	0		
2/22/2003	0	0	36,2069	18,91379
2/23/2003	**0**	**1**	159,6154	14
2/24/2003	1	1	28,125	7,703125
2/25/2003	0	1		
2/26/2003	0	0		
2/27/2003	0	0		
2/28/2003	0	0		
03/01/2003	0	0		

685 Anna (2008)

Year, Date	MM	CopTie	P4 ng/g	E2 ng/g
01/31/2008	0	0	102,5	84,075
02/01/2008	0	0		
02/02/2008	1	0	102,9412	25,79412
02/03/2008	0	0	128,125	38,625
02/04/2008	0	0	261,3636	159,4545
02/05/2008	0	0	200	25,34211
02/06/2008	**1**	**1**	446,1538	31
02/07/2008	13	2	162,5	26,3125
02/08/2008	7	2	528,5714	20,19048
02/09/2008	5	1	336,6667	15,66667
02/10/2008	0	0	543,75	13,4375
02/11/2008	0	0	422,2222	22,94444
02/12/2008	0	0	411,5385	10,15385

797 Madre (2013)

Year, Date	MM	CopTie	P4 ng/g
03/10/2013	0	0	
03/11/2013	0	0	32,27
03/12/2013	0	0	25,38
3/13/2013	0	0	30,91
3/14/2013	0	0	
3/15/2013	0	0	37,3
3/16/2013	**7**	**1**	75,7
3/17/2013	12	0	
3/18/2013	0	0	
3/19/2013	0	0	72,1
3/20/2013	0	0	85,0

882 Abby (2011)

Year, Date	MM	CopTie	P4 ng/g	E2 ng/g
2/19/2011	0	0	241,3445	281,8086
2/20/2011	0	0	237,5808	182,0066
2/21/2011	0	0	95,74533	146,5989
2/22/2011	0	0	120,3981	318,6687
2/23/2011	0	0	187,4234	221,2531
2/24/2011	0	0	75,22183	**607,1515**
2/25/2011	**6**	**1**	**317,1433**	189,7167
2/26/2011	1	0	151,482	144,2082
2/27/2011	0	0	204,2615	124,1031
2/28/2011	0	0	320,3573	44,80807
03/01/2011	0	0	215,2139	53,14107
03/02/2011	0	0	204,5138	184,1769
03/03/2011	0	0	715,9304	33,12638

1266 Sibi (2015)

Year, Date	MM	CopTie	P4 ng/g
01/30/2015	0	0	
01/31/2015	0	0	
02/01/2015	0	0	199,1
02/02/2015	0	0	27,8
02/03/2015	0	0	25,7
02/04/2015	0	0	
02/06/2015	**0**	**1**	
02/07/2015	6	1	
02/08/2015	1	1	146,3
02/09/2015	1	1	432,6
02/10/2015	1	1	472,2
02/11/2015	0	0	513,8
02/12/2015	0	0	