ABSTRACT AND SPECIFIC AIMS

This project is designed to monitor the physiological and behavioral changes associated with arctic ground squirrel (AGS) reproduction. Specifically, results from this study will elucidate the timing and hormonal correlates of reproduction with the goal of establishing thresholds of reproductive hormone concentrations that could be used as markers for successful captive breeding in the future. There are four specific aims of this project: 1) genotype adult AGS currently at UAA in order to paternity test captive-bred pups; 2) monitor changes in behavior and circulating hormone levels (testosterone, estradiol, progesterone) in AGS upon emergence from hibernation; 3) pair animals for breeding immediately after emergence; and 4) monitor changes in vaginal cytology during all stages of reproduction in female AGS.

The overarching goal of this project is to identify changes in behavior and physiology related to AGS reproduction in order to identify physiological markers of reproduction. Results from this investigation will be of relevance to both the basic and applied arenas of biology by increasing our knowledge of comparative reproductive biology and using that information to increase the success of captive breeding of AGS. Having a dependable supply of AGS through successful captive breeding would help to sustain research work on climate change, complex biological processes, and biomedical research which all have the potential to transform the way in which we live, work, and see the world.

INTRODUCTION

The arctic ground squirrel (AGS, *Urocitellus parryii*) is a model organism for which complex biological processes are studied. Environmental studies have used AGS to investigate how the animals interact with their environment, particularly looking at how much influence biological cues versus environmental cues have on timing of the reproductive cycle (Sheriff et al., 2010; Buck and Barnes, 2003). AGS have also been used in biomedical studies, as the mechanisms involved in hibernation have been evaluated as a potential therapy for a range of serious medical emergencies for which the body cannot meet the demand for oxygen and energy (Drew et al., 2007; Storey K.B., 2009). The purpose of my study is to track changes in physiology and behavior associated with AGS during their breeding season in order to identify specific physiological markers that can be used to establish a successful breeding colony of AGS held in captivity at UAA. It will also develop the use of microsatellite markers to create pedigrees for pups that are born from successful matings. Ultimately, the goal of this study is to provide researchers with a dependable supply of laboratory animals through captive breeding of AGS. This would help sustain research involving climate change; complex biological processes; and biomedical

research that have the potential to change the way the medical field works, in particular the way in which medical emergencies are handled.

AGS are an excellent model organism due to their extreme and complex interactions with their arctic environment. The reproductive cycle of AGS is a good example of this interaction. Sheriff et al. reported that after a seven month long hibernation season, AGS reproductive cycle begins with males emerging and regenerating their testes for a period of three weeks. After this time, females emerge from hibernation and are ready to mate within a period of one to seven days. Sheriff et al. (2010) showed that while animals within a particular area exhibit a similar reproductive cycle, even over a small distance of 20 kilometers, animals can exhibit markedly different reproductive dates. These results indicate that AGS can adapt to changes within their environment through phenotypic plasticity; however, what environmental and biological cues these animals receive and which have the greatest impact on the timing of the reproductive cycle has yet to be determined. One way in which AGS reproduction can be further understood is through captive breeding of AGS, during which researchers have direct access to the animals and can monitor their physiology, behavior, and morphology throughout the experiment. In this way, researchers would gain insight into the complex reproductive process of AGS, as well as help disentangle the relative influence of environmental cues and circannual timing especially as it relates to this animal's reproductive cycle.

Captive bred AGS could be used in biomedical research which has the potential to transform the way the medical field works, particularly the way in which medical emergencies are handled. Hibernating arctic ground squirrels have the ability to reduce their metabolic rate to $\leq$ 2% of basal metabolic rate (Buck and Barnes, 2000) and reduce core body temperatures to as low as -2.9°C (Barnes, 1989). Medical researchers have taken an interest in how hibernators regulate this process, what prevents them from sustaining damage to their brain and organs during this time of hypothermia, and how this could transform the way in which medical emergencies are handled (Drew et al., 2007; Storey, K.B., 2009). The research is directed at ways in which biological mechanisms or drugs designed to mimic hibernator's biology could be used to treat medical emergencies such as cardiac arrest, stroke, and organ transplant. During trauma, the brain, heart, and other organs are at risk of damage due to the lack of oxygen or excessive energy demands that cannot be met by the body due to compromised oxygen delivery. If researchers could find a way to induce a torpor-like state in patients, damage to the brain, heart, or other organs could be prevented. This kind of work is of utmost importance in a society in which 72,628 people are waiting for organ transplants, 795,000 people every year experience a stroke, and 1,255,000 people experience a coronary attack (United Network for Organ Sharing, 2012; American Heart Association,

2012). All of these medical emergencies could be treated with methods that are being investigated by biomedical researchers studying hibernators like the AGS. By studying individual components of the hibernator's complex biological processes, researchers can transform the way in which the medical field handles these emergencies.

The purpose of my study is to track changes in physiology and behavior associated with AGS during their breeding season in order to identify specific physiological markers that can be used to establish a successful breeding colony of AGS held in captivity at UAA. It will also develop the use of microsatellite markers to create pedigrees for pups that are born from successful matings. By identifying physiological changes associated with AGS reproduction, the ability to successfully breed captive AGS would ensure a consistent and dependable supply of animals for research. This in turn will help sustain research on climate change, complex biological processes, and biomedical research which all have the potential to transform the way in which we live, work, and see the world.

EXPERIMENTAL DESIGN

The experimental design for this project involves five major parts: 1) identification of microsatellite markers in adult AGS currently at UAA, 2) tracking of behavioral and physiological changes in animals from emergence of hibernation through the breeding season, 3) captive breeding of four pairs of generically distinct AGS during their breeding season, 4) monitoring vaginal cytology in female AGS to identify post-ovulation receptivity, and 5) the creation of pedigree records through paternity testing for all captive-bred pups that result from successful matings.

In order to genotype adult AGS currently at UAA, we will extract DNA from blood samples, amplify microsatellite markers using PCR, and determine the particular alleles that are present in each squirrel through fragment analysis (preformed at Yale). Should fragment analysis fail, we plan to examine potential microsatellite markers using electrophoresis until satisfactory results are obtained. Approximately 1ml of blood will be drawn via cardiac puncture from adult AGS that are housed at the UAA vivarium. Blood will be collected, centrifuged, and white blood cells isolated for DNA extraction. DNA will be extracted and purified using a Qiagen DNeasy Blood and Tissue Kit. DNA will be amplified by PCR with primers and reaction conditions identified by Stevens et al. (1997). Methods will be similar to those used by Stevens et al. (1997) and May et al. (1997). The results will allow us to determine which males and females are genetically distinct from each other and can thus be used in complex breeding experiments (described below).

If there are not eight genetically distinct AGS – four males and four females – at UAA, then we will employ one of several options. With only two males and one female that are genetically distinct, we can evaluate the timing of the greatest reproductive success with these animals. The other AGS can be bred in conditions without ambiguity, with only one male being placed with one female. In this way, we will know the pedigree of the pups that are born and the hormone levels of the parents can still be evaluated. For the three or more AGS that are genetically distinct, we can evaluate the timing of the most successful breeding by varying when the males are with a particular female.

To conduct the breeding experiment, male AGS will be aroused from hibernation three weeks prior to when the female AGS are expected to end hibernation; males require a period of twenty one days of euthermy in order to become reproductively mature (Sheriff et al. 2010). After 21 days, females will be aroused temporally near their expected dates of ending heterothermy and subsequent emergence. This protocol ensures that all animals are reproductively mature at the correct time and follows the methods set out in Vaughan et al. (2006). Once animals are euthermic, ½ ml blood samples will be collected from all animals via cardiac puncture (Buck and Barnes, 2003) twice weekly for approximately eight weeks. At the same time that females are being sampled for blood, we will conduct vaginal lavages (Buck and Barnes, 1999) for later analysis of vaginal cytology (details below). This sampling regime will enable us to examine different aspects of AGS reproduction throughout reproductive development, mating, and gestation. If a female is not impregnated, this sampling regime will allow us to determine the rhythmicity of estrous. Blood samples will be centrifuged and plasma will be separated and stored at -80 C° until assayed for circulating hormone levels. Once all samples are collected, commercially available assay kits will be used to determine the concentration of the circulating reproductive hormones estradiol, progesterone and testosterone (Buck and Barnes, 2003).

AGS will be selectively paired for breeding beginning approximately two days after the female has become euthermic and twenty one days after the male has become euthermic (Sheriff et al., 2010). If there are genetically distinct males that can be bred to a particular female, then they will be placed with the female at different times, in order to evaluate reproductive success that corresponds to different hormone levels at that time. The rotation will begin on the second day post-emergence for females: a male will be placed with the female for a period of 12 hours, followed by a 24 hour rest, and then a different male will be placed with that female for 12 hours, followed by another 24 hour rest. Rotations will continue for approximately seven days. For animals that are not genetically distinct, only one male will be placed with a female, with the same schedule: the pair will be placed together for 12 hours the first day and 12 hours the third day, with a break in between, up to seven days.

Flow cytometry of the vaginal lavage samples will be implemented to evaluate the ratio of epithelial to leukocytes cells (Buck and Barnes, 1999). Female AGS are induced ovulators, yet they undergo vaginal estrus that is characterized by an increased ratio of epithelial to leucocyte cell number. We aim to test the hypothesis that vaginal estrus is associated with increased receptivity and propensity to become impregnated. Flow cytometry is a powerful tool that we will use to evaluate the ratio of epithelial cell to leukocytes number as well as the types of white cells present; this final assessment is conducted through the use of dyes specific to different cell types.

Captive-bred pups that result from successful matings will be paternity tested, if the parents were genetically distinct, in order to correlate the hormone levels in the males and females at the time with the successful mating. Pups from successful matings between non-distinct parents can also be linked to circulating hormone concentrations during the breeding season using dates, with conception being approximately 25 days before birth (Sheriff et al., 2010).

ANTICIPATED RESULTS

From the results of this study, we expect to be able to identify a set of behavioral and physiological markers that can be used in the future to mark the time of greatest reproductive success. This is expected because of the large quantity of data that can be collected and evaluated.

It is anticipated that an adequate number of male and female AGS currently at UAA will be genetically distinct. This assumption is based on the fact that we will be analyzing six different genomic locations (Stevens et al., 1997) in ten different squirrels and each of these loci are expected to reveal up to two different versions of the gene per squirrel. Only one of these six loci needs to be unique to a specific squirrel in order to make it distinct.

It is also expected that there will be changes in hormone levels in both male and female AGS and changes in vaginal cytology in the female AGS reproductive tract post-emergence (Buck and Barnes, 1999, 2003). We expect to see increased concentrations of hormones that regulate reproduction, such as testosterone, progesterone and estradiol, immediately following emergence from hibernation. In non-impregnated females, we expect that the ratio of epithelial to leukocytes will vary depending on the stage of estrus. It is currently accepted that leukocyte cells decline during female receptiveness while epithelial cells increase (Laren et al., 1977; Buck and Barnes, 1999). We expect our results to show a trend in the

ratio of epithelial to leukocyte cells that could be used to determine receptiveness in order to further evaluate the reproductive cycle in AGS.

It is not known whether there will be successful breedings during this experiment, as there is a narrow window of reproductive activity for AGS. The timing of reproduction in captive AGS is further complicated by the timing of emergence from hibernation, which may or may not match other AGS when they are held in captivity. However, we anticipate that the large quantity of data compiled will increase our understanding of the system such that at the very least the experiment can be modified to achieve successful breedings in the next season. For example, the conditions and timing set out in the current methods can be evaluated, and changed in order to find the correct timing of reproduction. Due to the number of animals used in this experiment, we do anticipate successful breedings, which will be used to evaluate the physiological and behavioral changes that accompanied the conception of the pups. These changes will be used as markers in future captive breeding experiments to increase reproductive success of captive AGS.

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PROJECT BUDGET

Items	Requested	Existing OURs Grant	Totals
Microsatellite Genotyping	---	---	---
DNA extraction buffers	---	$659.61	---
Oligonucleotide primers (Fluorescently-labeled)	---	$630.00	---
Shipping of Ologonucleotide Primers	---	$61.95	
PCR Reagents (including Taq, x)	$179.39	$179.39	---
PCR Reaction tubes (x4)	$155.68 ea.	$155.68	---
Fragment analysis at Yale	*$1/sample* (340 samples)	$1/sample (80 samples)	---
Shipping to Yale	$79.62	$50	---
Total	**$1,221.73**	**$1,816.63**	**$3,038.36**
Animal Care	---	---	---
Feed, Bedding, Space Rental	$500.00	---	---
Total	**$500.00**	---	**$500**
Hormone Kits	---	---	---
Testosterone (x2)	$350 ea	---	---
Progesterone (x2)	$516.63	$183.37	---
Estradiol (x2)	$350 ea	---	---
Shipping of Hormone Kits	$250.00	---	
Total	**$2,166.63**	**$183.37**	**$2,350.00**
Cytology	---	---	---
Dyes for staining	$500.00	---	---
Shipping for Dyes	$50.00	---	
Total	**$550.00**	---	**$550.00**
Total	**$4,438.36**	**$2,000.00**	**$6,438.36**

BUDGET JUSTIFICATION

This project is a comprehensive study of AGS reproduction, with many associated costs. Although I have already received some funding for this project, we have decided to expand the project and have had to change some of the methods that we had originally planned on using. The goal of this project is to identify specific physiological markers that can be used in the future, and such scientific work requires some trial and error; however, we believe that this project will succeed, giving us a more complete view of arctic ground squirrel reproduction.

Animal care costs are a necessary item in order to properly feed and care for the animals. Quality feed and bedding will be provided to all animals in order to ensure their health and well being throughout the experiment.

The molecular and genotyping supplies are necessary in order to carry out the methods of this experiment. The goal of this project is to identify physiological markers that can be used in the future to successfully breed AGS. It is therefore necessary to have a way to identify the timing of the greatest reproductive success. This will be done by breeding genetically distinct males with a particular female at different times. We can trace female receptiveness and reproductive success to the male with the greatest number of pups through paternity testing. Although this part of the project is costly, the supplies come in quantities that will provide UAA with materials to carry out multiple experiments in the future. If this particular experiment should fail in one part, due to timing of emergence or other unexpected problems, there will be usable supplies for future experiments.

Hormone kits and stains for vaginal cytology are also necessary. This project is a comprehensive study of AGS reproduction, and therefore, many aspects of biology must be examined. Reproduction is a complex process which requires evaluation of multiple factors that influence it. Therefore, these items are necessary expenses.

Any cost exceeding the allotted $5000 will be covered by existing grants provided by my mentor, Dr. Loren Buck through existing grants awarded by NIH, NSF and DoD.

PROJECT TIMELINE

Project Task	1/12	2/12	3/12	4/12	5/12
Microsatellite Identification: Adult AGS					
DNA Extraction					
PCR amplification					
Identify Breeding Pairs					
Captive Breeding and Sample Collection					
Collect Blood for Hormone Assays					
Attempt Captive Breeding					
Vaginal Lavages					
Paternity Test AGS Pups					
Take Blood Samples from Pups					
Extract DNA					
PCR Amplification					
Process Samples					
Run Hormone Assays					
Flow Cytometry					
Grant Requirements					
Presentation					
Budget Expenditures					
Final Written Report					