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Captive Breeding, Anti-Predator Behavior and Reintroduction of the Pacific Pocket Mouse
(*Perognathus Longimembris Pacificus*)
2014-2016

By

Debra Shier, Ph.D., Samantha Leivers Ph.D., Shauna King, Rachel Chock and JP Montagne

Final Report
To

State of California
Department of Fish and Wildlife
South Coast Region
3883 Ruffin Road
San Diego, CA 92123

Captive Breeding, Anti-Predator Behavior and Reintroduction of the Pacific Pocket Mouse (*Perognathus Longimembris Pacificus*)

For the period
July 1, 2014 – November 30, 2016

Debra M. Shier, Ph.D., Dr. Samantha Leivers, Ph.D., Shauna King, Rachel Chock and JP Montagne
San Diego Zoo
Institute for Conservation Research
Division of Applied Animal Ecology
Escondido, CA 92027

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ABSTRACT

The primary goal of this project was to use captive breeding techniques to increase numbers of the endangered pacific pocket mouse (*Perognathus longimembris pacificus;* PPM), learn about the species behavioral ecology as it related to their reproduction and survival skills, genetics and stress, prepare naïve animals for reintroduction to the wild, and reintroduce them to sites within their historic range from which they have previously been extirpated. During Phase 1, we established a captive breeding facility, collected 30 founders from the wild, began breeding mice and conducting research on behavioral ecology, stress and genetics. During Phase 2, we used the results from Phase 1 to design and complete additional studies and grow our captive population of PPM to 110 by fall of 2015. In response to this colony growth and species specific population modeling we were able to prepare for and reintroduce our first group of 50 mice into Laguna Coast Wilderness Park in early summer of 2016. Preparation included, site coordination, permitting, trapping, installing a predator exclusion/dispersal dampening fence and acclimation cages, release cohort selection, diet transition, health checks, marked with P-chips, etc. And, because our research indicated that captive-born mice behave differently than wild-caught mice when exposure to predators and because wild-caught PPM antipredator behavior eroded over time in captivity, we conducted antipredator training for all animals in the release cohort and those on the alternate list. Once mice were reintroduced, we conducted post-release monitoring at 1 and 3 months to assess release success. As of the end of summer 2016, we are cautiously optimistic regarding the release population, but given low numbers of PPM on the site, we propose to supplement with approximately 25 mice in late spring of 2017.

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[1] Shier, D.M. et al. 2016. Captive Breeding, Anti-Predator Behavior and Reintroduction of the Pacific Pocket Mouse (*Perognathus longimembris pacificus*), 2014-2016. California Department of Fish and Wildlife, Wildlife Management, Nongame Wildlife Unit Report, 2016-10. Sacramento, CA 87 pp + Appendices.

INTRODUCTION

The pacific pocket mouse (*Perognathus longimembris pacificus; PPM*) is endangered and only 3 extant populations are known to exist. The largest remaining population, the Santa Margarita population, is located in the Oscar One and Edson training areas on Marine Corps Base Camp Pendleton. PPM populations expand and contract in response to rainfall patterns. Southern California experienced low levels of rainfall for several years in a row (70% of normal mean precipitation 2005-2006, 52% of normal mean precipitation 2006-2007, and 39.5% of normal mean precipitation 2007-2008 with 2013 being the driest on record for decades. In 2006 we initiated a translocation program for PPM with the goal of developing translocation methods for the species. However, population surveys for PPM conducted on the Santa Margarita population between 2006 and 2008 indicated that this population had contracted significantly (Shier 2008, 2009). Between 2006 and 2008, PPM numbers in the Santa Margarita population were too low to conduct a translocation. Thus, in 2008, we submitted a proposal to captively breed and reintroduce PPM.

A conservation breeding program was initiated by the San Diego Zoo Institute for Conservation Research in 2012. This report covers Phase 2 of the program.

Objectives

The primary goal of this project is to use non-invasive captive breeding techniques to increase numbers of PPM, prepare naïve animals for reintroduction to the wild, and reintroduce them to sites within their historic range from which they have previously been extirpated. The long term goal is to establish several additional wild populations across the historic range of the species. In the process we are learning about the species behavioral ecology, physiology and genetics. In particular, we have designed experiments to examine the species antipredator behavior, mating behavior, foraging behavior, interspecific interactions, stress and genetics. Specifically, during Phase 2 we addressed the following objectives:

Objective 1: Collect additional founders, if needed

Objective 2: Provide ongoing husbandry of captive PPM and continue the captive breeding program to increase numbers of PPM within the captive population

Objective 3: Conduct research on the species' reproductive behavior

Objective 4: Conduct research on the species' anti-predator behavior and provide predator training, if needed, to prepare captive bred animals for reintroduction to the wild

Objective 5: Conduct research on the species' foraging behavior

Objective 6: Conduct endocrinology research to understand the effects of stress in the captive breeding, reintroduction, and translocation process

Objective 7: Genotype new founders and offspring

Objective 8: Develop a disease risk assessment plan and health criterion for PPM to be released

Objective 9: Upon the production of 200 PPM in captivity, reintroduce PPM to sites within its historic range to facilitate species recovery

Objective 10: Measure reintroduction success

Personnel

The following people conducted research on PPM associated with this project: Dr. Debra Shier designed, conducted and supervised field and captive research. Amaranta Kozuch, Melanie LaCava, Jamie Chang, Shauna King and Samantha Leivers conducted captive research and daily husbandry. Shauna King, Samantha Leivers, Jamie Chang and Rachel Chock conducted field research. Asako Navarro conducted genetic analyses. Jamie Ivy developed the species specific genetic model.

COLLECTION OF FOUNDERS

Our goal was to bring 30 breeding founders into captivity to begin the PPM breeding program. These individuals were to come from the 3 remaining extant populations: 1). Dana Point (DP); 2). Santa Margarita (SM); and 3) South San Mateo (SSM). The established target number was N = 10 from each population. For captive breeding to be successful, it is important to have both experienced breeders (adults) and young animals (young of the year; YOY) that will survive in captivity long enough to produce multiple litters. Thus, of the 10 animals from each population, we planned to bring in 2 adult males: 2 adult females: 3 (YOY) males: 3 YOY females constituting no more than 10% of adults or 20% of juveniles in each population to minimize impacts to the wild populations. As of the beginning of Phase 2, we had collected 10 from Dana Point, 6 from SSM and 14 from SM. However a subset of these animals had bred by summer of 2016. As of August 2016, we had achieved breeding by 3 of the 10 DP animals, 5 of the 6 SSM animals and 9 of the 14 SM animals. Thus, we proposed to collect additional animals in September of 2016. We chose not to attempt collection from SM in 2016 as the United States Geological Survey (USGS) surveys that had been conducted in 2016 indicated that this population size was relatively low in 2016. We attempted collection at SSM and DP.

METHODS

General trapping protocol

There were 2-4 people in the field team. We opened no more than 200 traps each night. On all sites we placed flags in high quality PPM habitat (sandy areas with open vegetation) and placed 2 traps at each flag. Flags were spaced >10m apart to maximize the probability of collecting unrelated animals. We used 9-inch Sherman traps with shortened doors to prevent tail severance. We baited traps with white millet that was cooked for 1 minute in a microwave to prevent germination. Traps were set just before sunset (18:30-20:00) and checked at midnight and dawn unless otherwise noted. If we found a trap with ants inside or within 6 inches of it, we closed the trap for the night. We took a GPS location at every trap in which we captured a PPM.

Processing

All PPM captured were aged (adults = tawny brown pelage; YOY = pelage grey or partially grey with molt line), sexed and assessed for reproductive condition. If a female was obviously pregnant, she was released. All animals were inspected for physical condition (e.g. pelage condition and ectoparasites). If the animal captured was selected as a founder for the captive facility, it was transferred to a holding cage (19 X 30 X 20 cm) for transport that contained 5 cm of clean sand, millet and a 6 inch section of 1inch PVC tubing. A fecal sample was taken to assess stress.

Dana Point

There have been no surveys of the Dana Point population over the last few years. Thus, we set out traps and intended to use a "pay as you go" strategy to ensure that we did not collect more than 10 percent of the adults and 20 percent of YOY. On September 12, 2016, we set out 60 flags in open sandy patches and placed 2 9-inch Sherman traps with shortened doors at each flag (Figure 1). We avoided areas of sensitivity for coastal California gnatcatchers (*Polioptila californica*). We opened and set traps between 18:30 and 20:00 hours baited with autoclaved millet. We checked traps at midnight and again at 02:00 and closed all traps at the 02:00 trap check.

South San Mateo

South San Mateo (SSM) is located in the northern part of Marine Corps Base Camp Pendleton (Figure 2.). During our spring/summer 2016 PPM field research at SSM, we documented 30 unique PPM. By July 25th USGS had documented PPM presence at 6 additional track tube grids. To date, there is insufficient data to assess the relationship between track tube detections and number of PPM. Thus, in consultation with U.S. Fish and Wildlife Service (FWS), we decided to err on the conservative side and count a single PPM at each USGS track tube grid (Figure 4) that contained < 10 PPM detections and 2 PPM at each USGS track tube grid that contained ≥ 10 PPM detections. Using this approach for the track tube data along with the numbers of individual PPM captured by USGS, 15 individual PPM were documented by USGS. To document additional individuals, we trapped the SSM population from July 17 to August 16, 2012. We surveyed SSM with assistance from Will Miller from FWS. We identified 15 additional areas off of the USGS grids to target for trapping (Figure 3). We trapped each of the 15 sites plus 5 USGS track tube grids for 3-5 consecutive nights for a total of 3290 trap nights.

Figure 1. United States Geological Survey Topographic map of the Dana Point population. The location of the population is shown with a red box.

Figure 2. United States Geological Survey Topographic map showing the location of the South San Mateo population (marked with the red square).
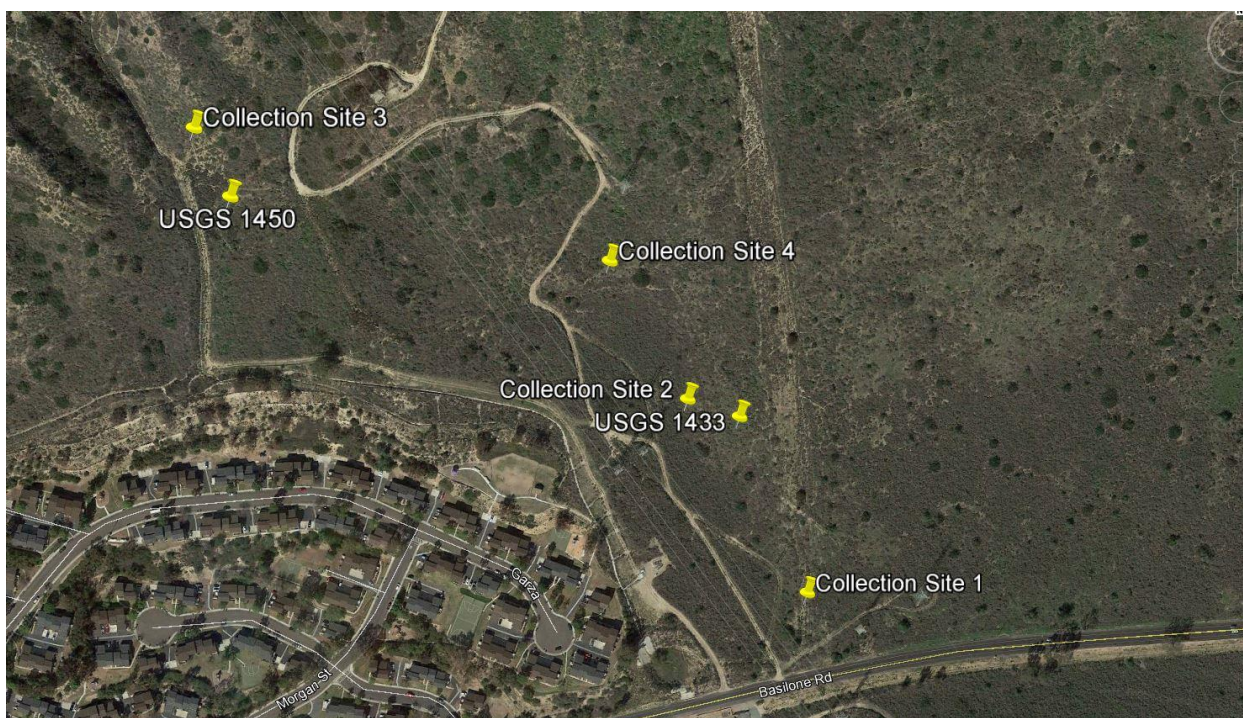
Figure 3. Satellite map showing 2016 trapping sites within the SSM population on Marine Corps Base Camp Pendleton.

RESULTS and DISCUSSION

Dana Point

We did not capture any PPM and surmise that the animals at this site had already begun to aestivate. We plan to return to DP in May/June of 2017 to conduct a complete site survey and attempt to collect 7 additional founders.

South San Mateo

We captured a total of 5 PPM and brought 4 (2 females: 2 males) into captivity. Table 1 provides details for each animal captured. No ectoparasites were observed on any of the PPM captured.

Table 1. PPM Captures at Santa Margarita population Marine Corps Base Camp Pendleton

Date	Site	Latitude	Longitude	ID	Sex	Age	Weight (g)	Estrous Swelling	Nipples	Scrotal	Released/Removed
9/7/2016	2	33°23'26.18"N	117° 33'45.89"W		Male	Adult				Non scrotal	Removed
9/7/2016	3	33°23'32.35"N	117° 34'00.57"W		Male	Adult				Non scrotal	Removed
9/7/2016	3	33°23'31.92"N	117° 34'00.92"W		Female	Adult		1	Not visible		Removed
9/7/2016	3	33°23'32.12"N	117° 34'01.01"W		Male	Adult	7.3			Non scrotal	Released
9/7/2016	4	33°23'29.39"N	117° 33'49.03"W	22	Female	Adult	6.4	1	Not visible		Removed

Quarantine

The 4 PPM founders collected in September were quarantined for 14 days at the PPM facility. All PPM were inspected upon entry into quarantine and a bodyweight taken. Weekly

bodyweights were obtained. A single fecal ova and parasite (o/p) exam was done and the results were negative. During the quarantine period an inspection was done by a veterinarian. All PPM appeared to be in good health.

HUSBANDRY METHODS

Housing

The mice were housed in the renovated PPM facility. The facility contained 3 animal rooms, a room for behavioral experiments, a bathroom, and a separate space for food preparation, sample storage and computer work. A central air conditioning/heating unit was installed to maintain facility temperature between 68 and 75˚F. Each animal room contained skylights to maintain natural lighting conditions. Target humidity (50 - 60%) was achieved using tanks of water and/or humidifiers. Shelving was installed in each room to increase the capacity of the facility. Each room contained 3 wooden shelves along the perimeter of the room and in the 2 larger rooms, 2 additional shelves were built in the center of the room to function as tables or accommodate expansion as numbers of PPM increase. In total, the facility was designed to hold as many as 275 PPM. Detailed information on captive diet and enrichment food items is provided in Appendix B, Feeding and Enrichment Schedule.

Social Cages

At the end of the quarantine period, animals were moved to social cages in the PPM facility. Social cages are clear, acrylic boxes divided into 2-6 compartments (each compartment measuring 30 x 12 x 30 cm) which share a long side. Mice were separated by a clear, acrylic barrier such that 2 to 6 mice were housed individually in a single social cage unit. The number of units in a box was determined by the length of the wall in each room. To allow olfactory and some tactile contact between neighboring animals, the clear barriers have 1 cm wide slots every 3 cm along the bottom third of the divider and 5 mm holes in the top third. In addition, each unit has 5 mm holes in the top 1/3 in the exterior walls of the short end so that animals in adjacent units will be able to smell each other. These cages have been shown to facilitate socialization and thus maintain estrous cycling in other heteromyid rodents (Yoerg 1999, Yoerg and Shier 2000). Males had only female neighbors and vice versa. Each mouse was provided an artificial nest chamber.   In the social cages, a 12 cm piece of 2.5 cm white PVC pipe joined to a T-section formed an entrance/exit "burrow" to the nest jar.

Data Collection

Weights

All animals were weighed weekly for the first 4-6 weeks after being brought into captivity to assess their physical condition. Thereafter, females were weighed weekly and males were weighed every 2 weeks.

Estrus

Reproductive condition was assessed for all females every 1-3 days during non-estrous periods. To assess estrous condition, females were scored according to the scale in Table 2.

Table 2. Scoring System for Estrous Condition in Heteromyids after Villablanca, unpublished

Genitals		
	1	Clitoris large, vulva not swollen
	2	Vulva slightly risen, diameter greater than clitoris
	3	Vulva noticeable risen, longer than wide
	4	Vulva large, top flat, edges round
	5	Vulva taut, top flat, edges straight
Discharge		
	1	None
	2	Dry white crust
	3	Stiated cast
	4	Plug (mucous, black, post-copulatory noted)
	5	Fresh or dried blood

Estrus cycling

Table 3 shows the number of females who had at least one estrus cycle during 2014 (from 1st July onwards), 2015 and 2016. A female is designated as having a complete estrus cycle if she was successfully mated or had a mucosal plug, regardless of her level of estrus swelling.

Table 3: Female estrus condition data between July 2014-2016.

	Date of first estrus cycle	Date of last estrus cycle	# total estrus cycles	# Adult cycling females	#YOY cycling females	#Total cycling females	Range of cycles (min-max)	Mean cycles per cycling female
July 2014-Dec 2014	N/A	9/22/2014	21	8(20) 40%	0(10) 0%	8(30) 27%	1-5	1.9
2015	2/16/2015	9/2/2015	170	26(30) 87%	7(20) 35%	33(50) 66%	1-12	5.15
2016	2/8/2016	9/21/2016	146	37(45[a]) 82%				
			77	15(20) 75%	8(21) 38%	23(41) 56%	1-7	3.21

[a]Denotes the number of cycling adult females between Feb-June 2016, prior to the June 2016 release of 25 adult females.

The first estrus cycle of the year tends to be in the beginning/middle of February with the final estrus cycle of the year occurring sometime in September. Through 2015 and 2016, 80-87% of adult females had at least one complete estrus cycle, with some females having up to 12 estrus cycles across the course of the breeding season. This means that average cycle length is extremely variable between mice, with some mice coming into estrus once during an entire breeding season and others cycling approximately every 13.5 days.

In 2015, 88% (14 out of 16) wild caught females cycled, whilst a similar 86% (12 out of 14) of captive born females cycled. In 2016, 60% (6 out of 10) wild caught females cycled, whilst 90% (9 out of 10) captive born females cycled. Statistical analysis of these data shows that there is no significant difference in the percentage of wild caught mice and the percentage of captive-born mice that cycled at least once during a breeding season (2015: Mann Whitney U test, $N= 26$, $U = 110.0$, $p = .951$, 2016: Mann Whitney U test, $N=15$, $U = 35.0$, $p = .280$). However, in 2015, captive born mice had significantly more estrus cycles (*Mdn*= 9) than wild caught mice (*Mdn*= 3), (Mann Whitney U test, $N= 26$, $U = 138.5$, $p = .004$), although this trend was not evident in the 2016 data (Mann Whitney U test, $N=15$, $U = 33.5$, $p = 0.456$).

The number of females that begin cycling in their year of birth has increased from none in 2014 to 35% and 38% respectively in 2015 and 2016. Indeed, in 2016, we recorded our earliest ever YOY estrus cycle, with a female having a full physical estrus cycle at 37 days of age. Of those YOY who come into estrus, the mean age of first estrus is 88.9 days of age ($N= 15$, *Mdn* = 88 days). YOY females who begin cycling tend to be born at the beginning of the breeding season, with 93% (14 out of 15) of cycling YOY being born between the seasons of March and May.

Male reproductive condition

Males are checked for reproductive condition once a week all year round. Males are categorized as either not scrotal (NS), partially scrotal (PS), or scrotal (S) dependent upon the extent to which the testes have descended into the scrotum. Only males that are partially scrotal or scrotal are used in mating trials with estrus females.

Data from male pups born since July 2014 shows that 97% of males (70 of 72) become at least partially scrotal within their year of birth, taking an average of 42.1 days (*Mdn*= 37, *Min*= 20, *Max*= 114) to become scrotal or partially scrotal. It is very rare for adult males to not become at least partially scrotal during the breeding season, although in 2016, three adult males remained non-scrotal throughout the year.

Male reproductive physiology

Through collaboration with the Institute of Conservation Research Reproductive Physiology Department, we were able to obtain data and images of a deceased male's testicles and sperm, which were collected posthumously.
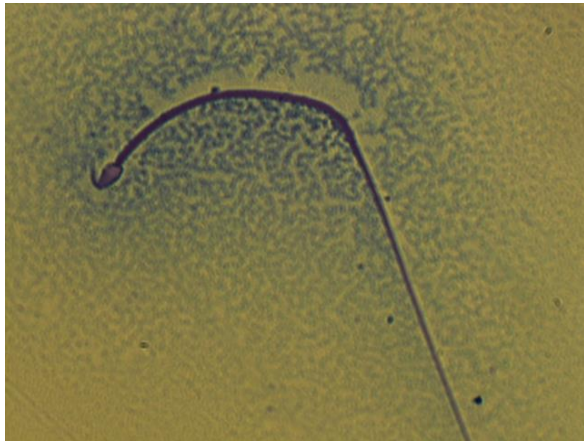
Figure 4. Image of Pacific pocket mouse sperm from deceased #17.

The sperm in the sample taken (Figure 4) had a motility of 69% and the morphology was similar to that of other mouse species, constituting a relatively long tail, a large mid-piece and an apical hook on the sperm head. The testes weighed between 0.0186-0.0136 grams, were between 3.8-4.3mm in length and 2.65-3.00mm in width. Certain characteristics seen in pocket mouse sperm have been shown to be selected for via sperm competition in other rodent species (e.g. variation in apical hook length and curvature, Sandera et al. 2013)but significant cross-species comparisons and further samples would need to be analyzed before we can make conclusions regarding the extent to which pocket mouse sperm morphology may inform us about pocket mouse reproductive ecology.

Mate pairings and copulations

We paired males with females during peak estrus when the female was perforate. 66.8% of the time, the female had an estrus swelling of at least a 2/3, but in 33.2% of cases perforate females had an estrus swelling of between 1/2 and 2. Mate pairings were chosen based on a number of factors, including relatedness, male reproductive condition and current genetic representation in the captive population.

Introduction of pairs occurred in a standard set-up using a 30 x 50 x 100cm breeding arena (Figure 5). The female was provided with two units to explore and the male was provided with one unit, with a plastic divider between the two sections of the arena to allow some visual, olfactory and tactile contact between mice. The divider was placed approximately 1 inch into the sand so that the mice had to dig under the barrier to access each other.

Figure 5. Typical set-up in the breeding arena between a female (#65) and male (#129).

All breeding events were carefully monitored by researchers to ensure the health and safety of the animals and if any aggressive behavior that resulted in contact was observed (biting, wrestling or aggressive chasing/darting), the event was ended and provided she was uninjured and still perforate, the female would be paired with another male. Affiliative and aggressive reproductive behaviors are outlined in Table 4.

Table 4. Ethogram of aggressive and affiliative reproductive behaviors.

Behavior	Description
Aggressive	
Lunge threat	A sudden strong movement forward with feet or teeth directed toward the other animal
Fight	Contact that includes hitting with feet, biting, or bodies locked and rolling over
Jump/avoid	One animal jumps upwards and back while facing the other
Chase	Rapid movement directed toward the other animal as it moves away
Flee	Rapid movement away from the other animal
Affiliative	
Circle	Male/Female pair make oral/genital contact and move in circle
Nose-to-nose	Male and female touch noses
Grooming	When the animal licks or nuzzles an area of the body, or uses the forelimbs to scratch or otherwise manipulate another part of the body.
Sandbathing	Rubbing side or ventrum against the sand (Randall 1981)
Mount	Male climbs on back of female
Lordosis	Female lays down in sand on her side and raises one back leg
Intromission	Male mounts female when she is in lordosis for at least 4 seconds

Copulation was deemed to have occurred if the animals performed a 'locked' intromission, whereby the male mounts the female and the animals twist into a locked position. Once intromission is complete, the animals separate and groom and a rapid behavioral change can be observed, with both animals performing aggressive behavior towards the other. Since July 2014, we have attempted to mate females during 186 estrus cycles for a total of 630 breeding events (with females being paired with multiple males during any one estrus cycle). This means that, when a female came into estrus, we had a 43.0% (80 of 186) success rate of mating her during her estrus cycle. Table 5 shows descriptive statistics that compare breeding events that ended in copulations and those that did not. The number of intromissions in a breeding event that resulted in copulation ranged from 1 to 15 and the duration of the longest intromissions was between 8 and 15 seconds (*Mean* = 10.4 seconds).

Table 5. Descriptive statistics for breeding events that did and did not result in copulation. Ns are provided for individual variables due to missing data.

	Copulated (80 breeding events)	Did not copulate (550 breeding events)
Male weight (grams)	*N*= 80 *Mean* = 7.8 *Mdn*= 7.7	*N*= 550 *Mean*= 7.5 *Mdn*= 7.4
Female weight (grams)	*N*= 80 *Mean*= 6.9 *Mdn*= 6.9	*N*= 550 *Mean*= 6.7 *Mdn*= 6.7
Female age (months)[a]	*N*= 53 *Mean*= 14.7 *Mdn*= 12	*N*= 434 *Mean*= 13.4 *Mdn*= 11
Male age (months)[a]	*N*= 37 *Mean*= 13.7 *Mdn*= 12	*N*= 313 *Mean*= 16.22 *Mdn*= 13
Male reproductive status (scrotal or partially scrotal)	*N*= 80 Scrotal = 52.5% Partially scrotal= 47.5%	*N*= 549 Scrotal= 52% Partially scrotal= 48%
Estrus swelling (% females per category)	*N*= 80 *1/2*= 0% *2*= 23.8% *2/3*= 33.8% *3*= 32.5% *3/4*= 6.3 % *4*= 3.8%	*N*= 550 *1/2*= 0.9% *2*= 33.6% *2/3*= 29.3% *3*= 28.5% *3/4*= 4.2% *4*= 3.5%
Estrus discharge (% females per category)	*N*= 66 *Clear*= 50.0% *Crust*= 36.4% *Crust/Blood*= 4.5% *Blood*= 5.1%	*N*= 444 *Clear*= 45.9% *Crust*= 32.7% *Crust/Blood*= 2.0% *Blood*= 19.4%
Female origin	*N*= 80 *Wild caught* = 33.8% *Captive born*= 66.3%	*N*= 550 *Wild caught* = 33.8% *Captive born*= 66.3%
Male origin	*N*= 80 *Wild caught* = 53.8% *Captive born*= 46.3%	*N*= 550 *Wild caught* = 33.8% *Captive born*= 66.3%

[a]For captive born mice only as the age of wild caught mice is unknown.

Pregnancies and litters

Of the 80 breeding events that resulted in copulations, 32 resulted in a litter of pups (40.0%). Since July 2014, the mean length of gestation was 23.3 days (*Mdn*= 23), with an average of 3.8 (*Mdn*= 4) pups to a litter and a pup mortality ($\leq$ 30 days old) of 3.4% and average juvenile mortality (31-365 days old) of 4.6% as of November 2016. Table 6 shows these values for individual years.

Table 6. Pregnancy and litter data between July 2014 and November 2016.

	Mean (Mdn) gestation in days	# litters born	Total # pups born	Mean (Mdn) litter size	Pup mortality (≤ 30 days old)	Juvenile mortality (31-365 days old)
July 2014-Dec 2014	22.7 (22)	3[a]	$N= 12$[b] ♀= 6 ♂= 5	4 (5) Min= 2 Max= 5	8.3%	16.7%
2015	23.4 (23)	16	$N= 59$[b] ♀= 21 ♂= 33	3.7 (4) Min= 2 Max= 7	10.2%	5.6%
2016	23.1 (23)	15[c]	$N= 58$ ♀= 22 ♂= 36	3.9 (4) Min= 2 Max= 5	0%	3.5%[d]

[a] Two litters were conceived prior to July 2014

[b] Some pups died prior to being sexed

[c] One litter of 5 pups was not included due to still birth from a Caesarian section, please see "Health and Disease".

[d] As of November 2016.

In the wild, female pocket mice have been observed given birth to their own litter within the same breeding season as their own birth (Shier 2008a) . Although female YOY come into estrus in their first year of birth captivity (see above), we did not run any successful breeding events with female YOY that ended in copulation prior to June 2016. Between June and September 2016, a total of 18 breeding events were run with YOY female mice (for a total of 9 females who had between 1 and 4 estrus cycles each). Of these breeding events, 6 resulted in copulations (for a total of 5 mice, one of whom was successfully mated twice). These 6 copulations resulted in the birth of 3 litters from 3 different YOY females for a total of 11 pups. Pup mortality for these litters was 0%, confirming that captive YOY females can successfully birth and raise pups in the same breeding season in which they themselves were born.

Data analysis

In order to investigate factors related to successful breeding events, whether a copulation resulted in a litter being born, and litter size, we combined data from both the Phase 1 report and the Phase 2 data, for a total of 881 breeding events.

To investigate the factors that influenced whether or not a breeding event resulted in a copulation, we began by examining the relationship among predictor variables using a combination of t-tests and Pearson's rank correlation. Male weight and female weight were correlated ($r= .092$, $p= .007$), suggesting that mating trails were run with animals of similar size. There was also a main effect of origin (wild caught or captive born) on male weight ($t_{876}= 12.63$, $p< .001$) and female weight ($t_{879}= 13.08$, $p< .001$). In order to address this multicollinearity, a weight ratio variable was computed by dividing male weight by female weight. We then ran

General linear mixed models (GLMM) analysis with a binary dependent variable (Copulated: Yes/No), controlling for male and female ID as crossed random effects, and entering the following variables as fixed factors: estrous swelling, weight ratio, male reproductive status, female origin and male origin. None of these predictor variables significantly predicted whether or not a breeding event resulted in a copulation.

To investigate the factors that influenced whether or not a copulation resulted in pregnancy, we ran a GLMM mixed models analysis with a binary dependent variable (Gave birth: Yes/No), controlling for male and female ID as random crossed effects, and entering the following variables as fixed factors: estrus swelling, weight ratio, male reproductive status, number of intromissions, duration of longest intromission, presence of copulatory plug, male origin and female origin. There was a main effect of longest intromission, $z= 2.15$, $p= .032$, with longer intromissions more likely to result in a litter of pups being born. There was also a marginally significantly effect of female origin, $z= 1.93$, $p= .053$ which, when analyzed using omnibus contrast analysis, became significant, $\chi^2= 4.43$, $p= .035$. This result suggests that captive born females who successfully copulate are more likely to give birth to a litter than wild-caught females who successfully copulate.

To investigate factors influencing litter size, we ran a GLMM analysis with dependent variable 'pups born', controlling for male and female ID as random crossed effects, and entering the following variables as fixed factors: weight ratio, duration of longest intromission, presence of copulatory plug, and female origin. Due to the relatively small number of observations (38 litters born), a smaller number of predictor variables were chosen for the analysis based upon their significance in the previous analyses. The random effects (male ID and female ID) resulted in an estimated covariance of 0 and so they were removed from the model and a GLMM analysis was run with dependent variable "pups born" and entering the following variables as predictors: weight ratio, duration of longest intromission, presence of copulatory plug, and female origin. None of these predictor variables significantly predicted litter size.

RESULTS and DISCUSSION

Whether or not a breeding event resulted in copulation or not was not predicted by any of our variables. Although there has been some research into mate choice in Heteromyids (Randall 1991; Roest 1991; Yoerg and Shier 2000), this literature is focused on kangaroo rats and the extent to which mate choice influences reproduction and the potential genotypic, phenotypic and behavioral traits that might influence mate choice in pocket mice is scarce. Some research on mate choice in the captive PPM population is discussed below, but further research must be carried out to determine the role of intersexual selection in pocket mice.

Whether or not a breeding event resulted in a litter being born was significantly influenced by the duration of the longest intromission, with successfully mated females more likely to give birth if they had a longer intromission with their mate. Whether or not a female gave birth was not influenced by any other variables in the model, including the presence or absence of a copulatory plug. This is an interesting finding as the role of the copulatory plug in PPM and across species is still unknown. Research on rodents speculates that copulatory plugs may aid in sperm release, sperm transport, coital stimulation and female chastity (by reducing the chances of the female mating with another male) (Schneider et al. 2016). 66.1% of breeding events that resulted in copulation also resulted in a copulatory plug. Of those successful breeding events that resulted in

a pregnancy, 60.9% of females had a copulatory plug present after mating. However, in those females who mated but did not go on to give birth, a copulatory plug after mating was still present after copulation 69.6% of the time. This suggests that the presence of a copulatory plug after mating does not influence pregnancy.

Our analysis did not determine any factors related to litter size in PPM. However, due to a relatively small number of observations, the number of predicator variables that could be entered into the analysis was limited and thus potential significant predicators may have been left out of the model. As we collect further data on breeding events and litter size over the course of the captive breeding project, we will continue to investigate factors related to litter size in PPM. Furthermore, reproductive success can be determined by a number of outcomes other than litter size, including pup survival and pup weight on weaning. In future analyses, we may be able to run these variables as proxies of reproductive success as we collect more data.

REPRODUCTIVE EXPERIMENTS

QUESTION 1: DO CAPTIVE FEMALE PPM COMMUNICATE ESTRUS THROUGH SANDBATHING?

Olfaction is the dominant sensory modality in mammalian reproduction. For males olfactory signaling can be seen as a handicap because it may be costly (e.g. may alert predators to the signaler (Gosling and Roberts 2001)). Evidence for condition dependence in the expression of olfactory signals especially in rodents, suggests that these costs are prohibitive for poor-quality males. Territorial or dominant males signal at higher rates (Gosling and Roberts 2001; Gosling et al. 2000). Not surprisingly, females choose between males using such olfactory cues and females prefer males whose patterns or frequencies of scent marking indicate that they are of high quality (Clark et al. 1992; Fisher et al. 2003; Rich and Hurst 1999) and/or whose frequency of scent marking renders them more familiar (Swaisgood et al. 2000; Tang-Martinez et al. 1993).

For females, scent marking can be used to communicate reproductive receptivity. Sandbathing is a form of scent marking and occurs in most Heteromyid species, including *Perognathus longmembris pacificus* (Eisenberg 1963b; Shier pers. obs.). The behavior consists of digging in the substrate (typically sand), followed by rubbing the ventral or lateral portion of the body on the substrate, either once, or several times in rapid succession (For a more detailed description, see Methods below).

Both the dorsal sebaceous gland and the perineal region contain scents or oils that are transferred to the substrate during sandbathing (Eisenberg 1963a). These oils not only contribute to maintaining a healthy pelage (Eisenberg 1963, Randall 1993), but are also used for olfactory communication (Eisenberg 1963ab, Randall 1991, 1994a). Female but not male, Merriam's kangaroo rats (*Dipodomys merriami*) respond to the dorsal gland secretions of males, and prefer secretions taken from familiar males to those taken from unfamiliar males (Randall, 1991). In the field, males of this species sandbathe near the burrows of estrous females; sandbathing sites may function to establish and maintain familiarity between potential mating partners (Randall, 1991).

Research on Heermann's kangaroo rat (*D. heermanni*) suggests that females may use sandbathing to communicate reproductive condition to males (Shier, unpublished data). Estrous but not anestrous female, *D. heermanni*, sandbathed more in male scented sand than in self-scented sand. The reproductive condition of females may influence the communicative signals they produce as well as their own responses to signals of conspecifics. Estrous females often emit odors that are more attractive to males than those of females in anestrous (Johnston, 1979, 1983; Brown, 1985; Huck et. al. 1989; Ziegler et al. 1993). In a complementary fashion, other studies have shown that estrous females prefer male odors (Johnston 1983; Ferkin, 1995). Because sandbathing in this family has both a functional role of maintaining pelage and a communicatory role, conveying estrus information through sandbathing would be adaptive by increasing potential reproductive output without increasing energy expenditure. Therefore, communication through sandbathing would be a particularly important during the mating season when the communication of individual identity to gain mates is most important.

Pocket mice are solitary territorial rodents. The ability to coordinating mating while minimizing costly physical interactions may require some form of communication at a distance. The goal of this study is to determine if sandbathing is used by captive PPM females to communicate reproductive condition.

METHODS

Subjects were n=20 adult female PPM. A standard introduction arena (36 x 24 x 12in) divided into 3 equal compartments served as the test arena. We covered the bottom 5 cm of the arena with sand. Tests were 10 minutes in duration. All tests were videotaped from above and watched via remote computer in another room. We tested females in and out of estrus in the presence of 3 scent types [unscented (control), female scented or male scented]. Donor scent was acquired by collecting ½ cup of sand from the donor individual's cage and sprinkling it into the appropriate compartment in the testing arena. Position of sand type in the arena was counterbalanced. The top layer of sand was removed between each test. We measured the amount of time focal females spend in each scent type, and the frequency and duration of sandbathing and digging across scent treatments. This experiment was conducted in the spring during the peak period of females estrous cycling.

Data Analysis

We used GLMM to model the effects of reproductive condition (anestrus vs estrus), history (wild-caught vs captive-born) and scent type (clean, male or female) on female PPM behavior. We conducted all data analyses in Stata version 14.

RESULTS and DISCUSSION

Results of this experiment suggest that female PPM may be communicating their reproductive condition through sandbathing. There is a significant interaction between scent type and female estrous condition (investigate: $z = 2.76$, $p = 0.006$; Figure 6). Females are spending more time investigating male scented sand compared to female scented sand when they are in estrus but not while they are out of estrus. Females are also sandbathing more in male scented sand, but again, only when they are in estrus. There was no difference in the responses of wild-caught and

captive-born females to scent exposure (z = -0.01, p = .990), indicating that captive female PPM exhibit wild-type behavior. These results are in agreement with those from wild PPM experiments and serve to bolster our understanding of sandbathing behavior in PPM and suggest that during mate pairings in captivity, PPM females that sandbathe are communicating reproductive condition to males with whom they are paired. Thus, pairings which do not result in sandbathing are unlikely to result in copulation.
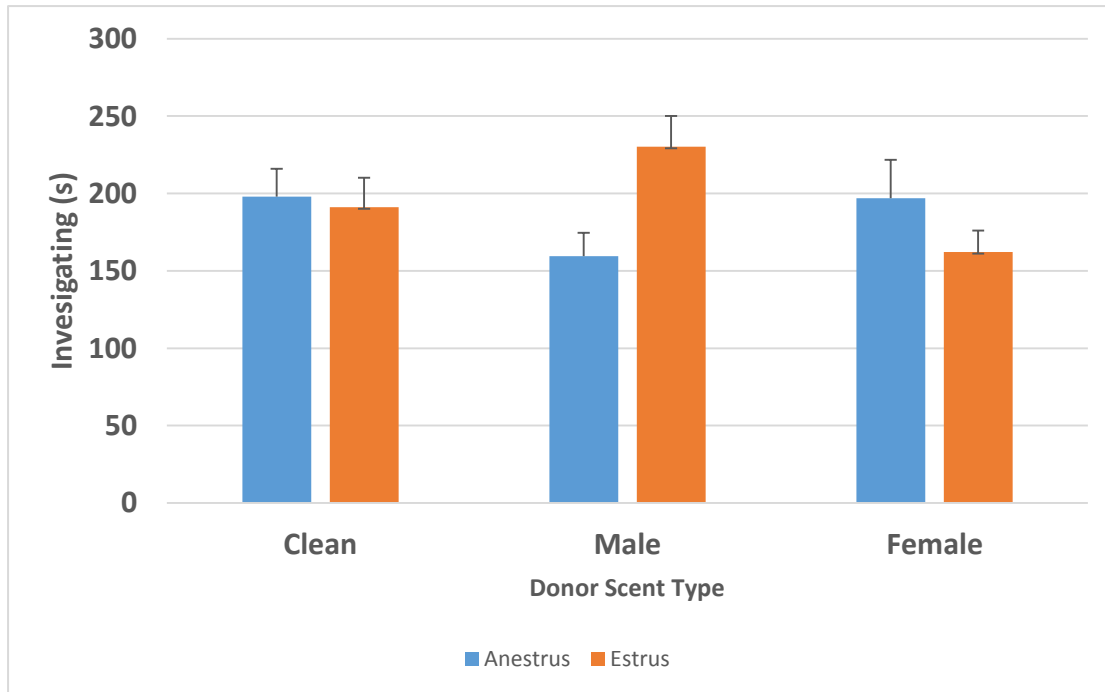

Figure 6. Time spent investigating by females in and out of estrus

QUESTION 2: DO FEMALE PPM PREFER TO MATE WITH FAMILIAR MALES?

Mating behavior may be best viewed as the product of individual reproductive strategies that can change as conditions for reproductive success change (Austad 1984; Gross 1996; Kirkpatrick 1987). Because animals may use mixed strategies as ways of dealing with uncertainty (Flaxman 2000; Haccou and Iwasa 1995), mating tactics should reflect opportunistic responses to changes in social and ecological characteristics of the environment that vary in space and time (Austad 1984). Seasonal shifts in the resource base or operational sex ratio may lead to facultative shifts in mating behavior as potential mates become either spatially or temporally clumped or more spread out and difficult to find (Kvarnemo and Ahnesjo 1996). Alternative mating strategies may take many forms that depend on previous behavior, physiological state, scope of available alternative tactics and density of competitors (Lucas et al. 1996).

Familiarity is a factor that affects mating behavior and interactions of sexually reproducing mammals (Patris and Baudoin 1998; Tang-Martinez et al. 1993). In species that must avoid inbreeding, familiarity may be a mechanism for avoidance and inhibition of mating (Kuester et al. 1994; Pusey and Wolf 1996).

In solitary species in which unrelated individuals maintain adjacent territories, however, familiarity may be an important mechanism of neighbor recognition and mate choice. Familiarity minimizes aggression and increases tolerance among neighbors (Temeles 1994). Females that are less aggressive

towards familiar males may allow them to approach and initiate sexual behavior (Daly 1977; DeVries et al. 1997; Huck et al. 1989; Newman and Halpin 1988; Patris and Baudoin 1998; Randall 1991, 1991b; Shapiro et al. 1986; Tang-Martinez et al. 1993)

METHODS

We tested n= 20 adult female PPM in and out of estrus with familiar and unfamiliar male scent. A standard introduction arena (24 x 24 x 12in) divided into 2 equal compartments served as the test arena. We covered the bottom 5 cm of the arena with sand. Tests were 10 minutes in duration.   All tests were videotaped from above and watched via remote computer in another room. We tested females in the presence of familiar and unfamiliar male scent. Donors for scent trials were opposite sex mice housed either adjacent to or away from focal females. Donor scent was acquired by collecting ½ cup of sand from the donor individual's cage and sprinkling it into the appropriate compartment in the testing arena. We measured the amount of time focal females spend in each scent type, and the frequency and duration of sandbathing and digging across scent treatments. This experiment was conducted in the spring when females were cycling.

We used GLMM to model the effects of familiarity and estrus on female behavior. We conducted all data analyses in Stata version 14.

RESULTS and DISCUSSION

Familiarity with a male did not affect female behavior regardless of estrous condition (Figure 5). Females both in and out of estrus allocated approximately the same amount of time to sandbathing, digging and avoidance behavior (in burrow) when paired with familiar or unfamiliar males (sandbathing: $z = -0.21$, $p = 0.831$; digging: $z = -0.71$, $p = 0.477$; in refuge: $z = 0.15$, $p = 0.881$; Figure 7). While these data suggest that familiarity plays no role in precopulatory mate choice, female may have behaved differently if they were paired with familiar and unfamiliar males as opposed to just being exposed to their scent. Thus, further tests will examine whether familiarity affects mate choice by pairing females with familiar and unfamiliar males.
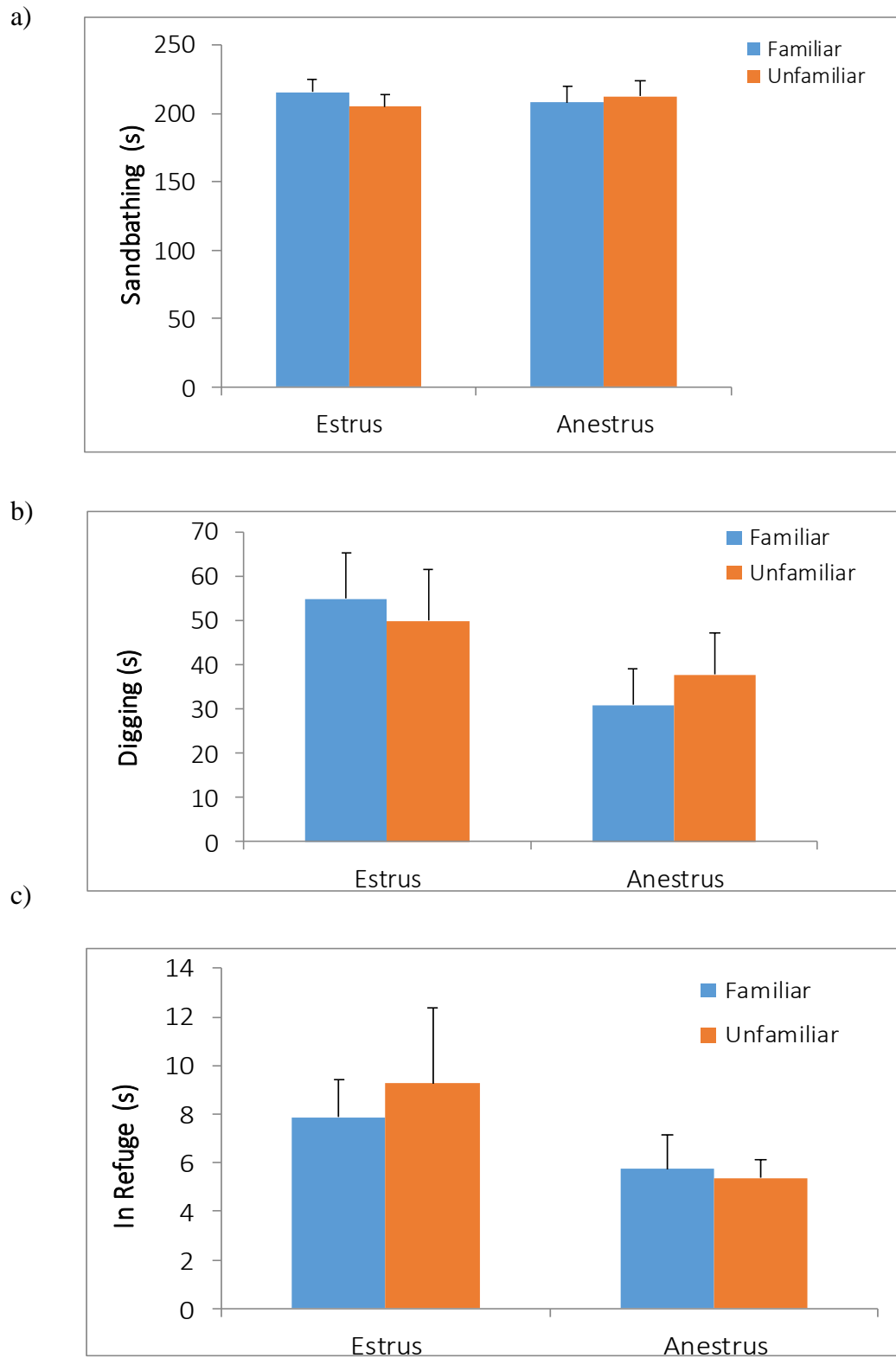
a)

b)

c)

Figure 7. Effect of familiarity and estrus on time spent: a) sandbathing, b) digging and c) in refuge.

ANTIPREDATOR EXPERIMENTS

Numerous studies have shown that once a program has established sufficient breeding, the challenge is to successfully release captive-born offspring to the wild. Perhaps most problematic is the fact that captive environments often fail to provide the experiences necessary to ensure survival upon release of captive born young into native habitat (Beck 1995; Beck et al. 1994). Numerous studies have shown that captive-born animals have a higher mortality rate than wild-caught animals after release in the wild (Beck et al. 1994; Ginsberg 1994; Griffith et al. 1989; Miller et al. 1994a). The survival skills of wild-caught individuals may also erode while in captivity (Yoerg and Shier 1997). In several species, the increased mortality has been linked to ineffective antipredator behavior (Biggins et al. 1999; Fischer and Lindenmayer 2000; Frantzen et al. 2001; Wallace 1994; Yoerg and Shier 2000).

Perhaps surprisingly, predator recognition in many animals depends upon experience (reviewed by Griffin et al. 2000). Anti-predator behavior often must be functional when a predator is first encountered, but animals can improve their responses with experience (Shriner 1995). A substantial empirical literature demonstrates that animals that initially show no recognition of fear can be conditioned to respond to live and model predators (Griffin et al. 2000). The type (habituation, social learning or facilitation etc.) and specificity (how specific to the target predators is the enhanced response?) of learning in nature will elucidate the factors that affect the development of survival skills, and therefore play important roles in the development of training protocols.

To date, predator training research has been conducted across several taxa [(fish; Brown and Leland 2003), (birds; Maloney and McLean 1995; McLean et al. 1999), (mammals; Griffin and Evans 2003; Griffin et al. 2001; McLean et al. 2000; Miller 1990; Miller et al. 1994b; Mineka and Cook 1988)] and recent research indicates that these training programs can be effective in terms of long term post-release survival (Shier and Owings 2006, 2007). In rodents, predator training research has shown that the social environment may play a critical role in predator training protocols. For prairie dogs, pairing alarm vocalizations with predator exposure can enhance training (Shier and Owings 2006) and juveniles trained in the presence of experienced adult demonstrators were more wary with predators than control juveniles (Shier and Owings 2007). Perhaps most interesting and pertinent to this research was a study conducted on Heermann's kangaroo rat. Yoerg and Shier showed that despite being solitary, young kangaroo rats learn antipredator behavior from their mothers (Yoerg and Shier 1997). Young kangaroo rat pups shadow their mothers in the presence of a snake, but not in tests without the snake present (Yoerg and Shier 1997).

We began antipredator experiments in Phase 1 to assess wild-caught PPM antipredator behavior and establish a baseline. In Phase 2, we examined: 1) if wild-caught PPM behavior eroded in response to time in captivity; 2) whether captive born adults showed similar behavior to wild-caught adults; and 3) whether antipredator behavior of captive born mice changes during ontogeny. Results from these experiments provided the information needed to determine whether mice slated for release would need to be trained with predators in order to facilitate post-release survival. In addition, we reexamined the antipredator behavior of wild-caught founders to determine if their antipredator behavior eroded in captivity. If wild-caught behavior changes

in captivity, we can use this information to make changes to PPM captive environment and experience in order to minimize these behavioral losses in the captive population.

GENERAL METHODS

We followed the same antipredator experiment protocol described in Phase I for both the Owl and Snake experiments.

Owl Experiments

Mice were tested 3 treatments: 1) Owl (mounted owl swooped down over testing area on pulley system); 2) PVC (control treatment in which a PVC pipe approximating the size of the Owl model is swooped down over the testing arena); and 3) Control (sham control in which the mouse is placed in the arena, but nothing is swooped down over the arena). For the owl model we used a taxidermically mounted Barn Owl (*Tyto alba*) with its wings out and talons extended during all tests.   All tests occurred in a testing arena. The clear acrylic testing arena (48 x 24 x 12 in) rested on the floor of the room and was filled with 5 cm of sand. The test arena was divided into four equal-sized quadrants delineated on the side of the cage for the observers (Quadrant 4 nearest the Owl; Quadrant 1 was furthest away.). A small spring of Buckwheat (*Eriogonum fasciculatum)* was placed in the center of each quadrant to provide cover. A pulley system was mounted to the walls and ceiling of the testing room such that the owl or PVC pipe control could "sit" high in the room against a wall and a curtain drawn in order that the test mouse could not view the stimulus when placed into the arena. The room was illuminated from the ceiling with a single 100watt red light bulb. A video camera was mounted on the ceiling to record all tests for later transcription in JWatcher. The behaviors documented are listed in Table 9.

Prior to a test, the owl or PVC pipe was pulled up into ready position above the arena and the curtain closed. A PPM was then carried from its home cage to the testing room in its nest jar and placed in the center of the testing arena. An observer stood quietly against the wall to manipulate the curtain and pulley system. Animals were given 5 minutes to acclimate to the testing arena. The test was 7.5 minutes divided into three 2.5 minute periods (pre-stimulus control during which the stimulus was behind a curtain; sit, during which the curtain was removed to reveal the stationary PVC or owl; and swoop, during which the stimulus, PVC or owl, was released to swoop down over the top of the focal subject). At the end of the test, the PPM was removed from the test arena in its nest jar and returned to its home cage. Before another mouse was tested, the sand in the arena was thoroughly sifted and mixed and any feces or urine was removed. A cup of clean sand and 1 teaspoon (per quadrant) of seeds were sprinkled on top before each test. No animal was tested in different treatments on one night. Control tests were identical to the Owl or PVC tests except that no stimulus was present. The treatment order was counterbalanced to avoid order effects. Tests were conducted between dusk and 11 pm. Females were anestrus during tests.

Snake Experiments

Each individual mouse was tested in 3 treatments: 1) Snake (presentation of king snake, *Lampropeltis getula californiae,* into the stimulus compartment of the arena; 2) Stick (presentation of stick approximately the same size and shape of the snake when elongated into

the stimulus compartment of the arena; and 3) Empty (empty compartment control). The stick condition served to as a control used examine the effect of a visual stimulus similar approximating the size and shape of the snake. We used two king snakes during snake tests to reduce effects of pseudoreplication. All tests occurred in the same testing arena as described previously except that the pulley system was not used during snake trials. Instead, a clear perforated barrier was placed between the stimulus compartment and the mouse compartments which allowed for visual and olfactory cues between the snake and focal mouse. In addition, we placed an artificial burrow (6 inch long PVC tube) into each quadrant for cover.

Prior to a test, we sifted the sand in the arena and sprinkled 1 cup of clean sand on top of the existing sand. We placed the snake or stick into the stimulus compartment and slid a black opaque cardboard between the stimulus compartment and the mouse quadrants. In the snake trials, we allowed the snake to acclimate to the arena for 10 minutes prior to introducing the mouse. A PPM was then carried from its home cage to the testing room in its nest jar and placed in the center of the testing arena for a 5 minute acclimation period. The test was 10 minutes in duration. After the first 5 minutes (pre-visual stimulus), we removed the black cardboard to expose the stimulus compartment (visual stimulus). At the end of the test, the PPM was removed from the test arena in its nest jar and returned to its home cage. Before another mouse was tested, the sand in the arena was thoroughly sifted and mixed and any feces or urine was removed. A cup of clean sand and 1 teaspoon of seeds (per quadrant) was sprinkled on top before each test. Control tests were identical to the Snake or Stick tests except that no stimulus was present. Each focal subject was tested in a single treatment each night and all 3 tests were completed over the course of 3-5 nights. Treatment order was counterbalanced to avoid order effects. Tests were conducted between dusk and 11 pm. Females were anestrus during tests.

Table 9. Ethogram of behaviors in anti-predator experiment.

Behavior	Description
Digging	movement of sand either with fore or hind feet
Sand Bathing	rubbing side or ventrum against the sand
Grooming	scratching with fore or hind feet and or licking their fur
Running	rapid movement
Walking	slowly moving; not running
Foraging	collection of seeds into cheek pouches and/or caches and/or eating
Freeze/Still	does not move, frozen in one spot
Scanning/ Looking	head up (eye shine visible) moving head around with all four feet on ground
Rearing up/ Standing	stretching up on rear feet and looking around
Climbing	crawling up buckwheat vegetation within enclosure
Out of Sight	cannot see animal or distinguish the behavior

Data Analysis

Three composite variables were used for data analysis: 1) vigilance = freeze/still, scanning/looking, rearing up/standing; 2) locomotion = running/walking; and 3) maintenance = digging, sandbathing, grooming and foraging.

For each composite variable, we used a fractional response logistic regression model to handle proportional data. We used AIC (Akaike Information Criterion) to assess model fit. Once the top models were identified, we examined contrasts for each predicator and ran posthoc comparisons. We conducted all data analyses in Stata version 14.

QUESTION 1: EFFECT OF CAPTIVITY ON WILD-CAUGHT PPM ANTIPREDATOR BEHAVIOR IN RESPONSE TO OWLS AND SNAKES

OWL EXPERIMENT METHODS

We tested (n=25) wild-caught mice both within 2 months of being brought into the captive facility (short-term captive) and again after 1 year in captivity (long-term captive).

RESULTS and DISCUSSION

Results indicate that wild-caught PPM behavior changed in response to time in captivity. A mouse's history (recently transferred from wild to captivity vs. housed in captivity for >1 year) was strongly predictive of both vigilance (Wald $\chi^2 = 36.97$, p <0.0001; Figure 8) and maintenance (Wald $\chi^2 = 34.28$, p <0.0001). Wild-caught mice exhibited higher rates of vigilance and lower rates of maintenance behaviors when recently brought into captivity compared to after they were in captivity for over 1 year. These results indicate that wild-type behavior erodes in captivity and a schedule of antipredator training should be instituted in order to reduce the effects of captivity on wild-caught individuals. Sex and population of origin provided no additional explanatory power.
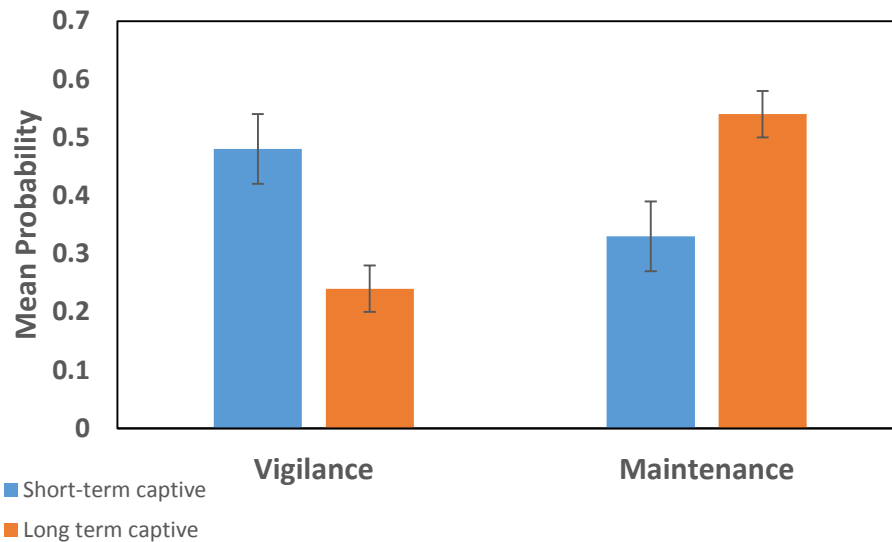
Figure 8. Effect of time in captivity on antipredator behavior in the presence of owls.

SNAKE EXPERIMENT METHODS

We tested (n=25) wild-caught mice both within 2 months of being brought into the captive facility (short-term captive) and again after 1 year in captivity (long-term captive).

RESULTS and DISCUSSION

Results indicate that wild-caught PPM behavior changed in response to time in captivity in the presence of snakes as well. A mouse's history (recently transferred from wild to captivity vs. housed in captivity for >1 year) was strongly predictive of both locomotion (Wald $\chi2 = 6.33$, p <0.012; Figure 9) and maintenance (Wald $\chi2 = 13.51$, p <0.0002) but not vigilance (Wald $\chi2 = 0.36$, p = 0.551). Wild-caught mice exhibited higher rates of maintenance behaviors when recently brought into captivity compared to after they were in captivity for over 1 year. These results combine with those from the owls tests indicate that wild-caught mice become less wary over time in captivity and confirm that occasional antipredator training should be instituted in order to reduce the effects of captivity on wild-caught individuals. Sex and population of origin provided no additional explanatory power.
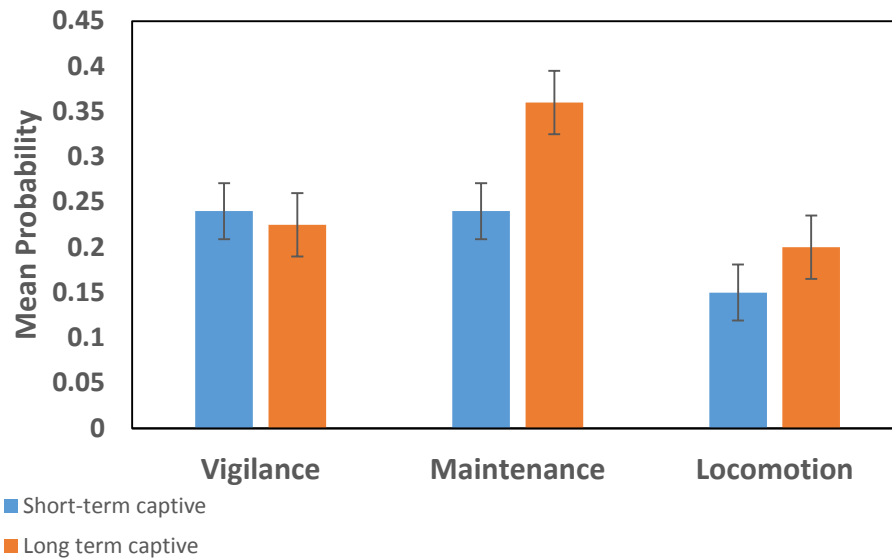
Figure 9. Effect of time in captivity on antipredator behavior in the presence of snakes.

QUESTION 2: DOES CAPTIVE-BORN PPM ANTIPREDATOR BEHAVIOR CHANGE DURING DEVELOPMENT IN THE ABSENCE OF TRAINING?

OWL EXPERIMENT METHODS

We tested n= 23 captive-born PPM at weaning (30days) and again as adults (>1 year).

RESULTS and DISCUSSION

Captive born PPM antipredator behavior in the presence of owls changes over development. Captive born PPM were more wary in the presence of owls as newly weaned mice than as adults (vigilance: z = 3.160; p = 0.002; maintenance z = -2.880; p = 0.004; Figure 10). There was no difference in the amount of time mice spent locomoting as juveniles vs adults.
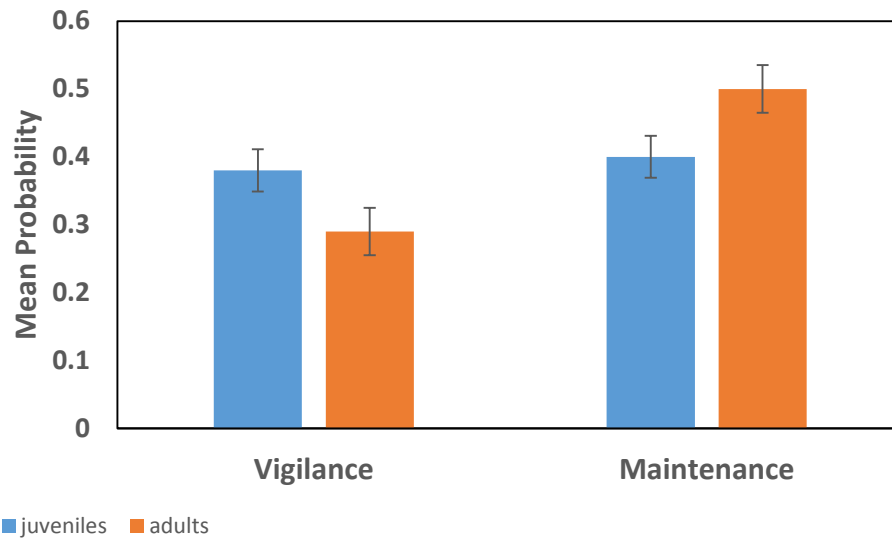
juveniles ■ adults

Figure 10. Effect of development on PPM antipredator behavior with owls.

SNAKE EXPERIMENT METHODS

Subjects were 27 wild-caught PPM and 24 captive-born PPM. All mice were tested as adults (>1 year of age).

RESULTS and DISCUSSION

Results from this experiment indicate that captive-born adult PPM behave differently than wild-caught PPM when tested with snakes. Though age did not influence vigilance behavior in the presence of snakes, juvenile mice were significantly less likely than captive-born mice to exhibit maintenance behaviors during exposure to snakes (Wald $\chi2 = 13.15$, $p < 0.0003$; Figure 11) and mice were more likely to locomote during snake tests when they were newly weaned compared to when they were adults. These results indicate that captivity is having a taming effect on the mice and highlights the need for regular exposure to antipredator challenges to maintain survival skills.
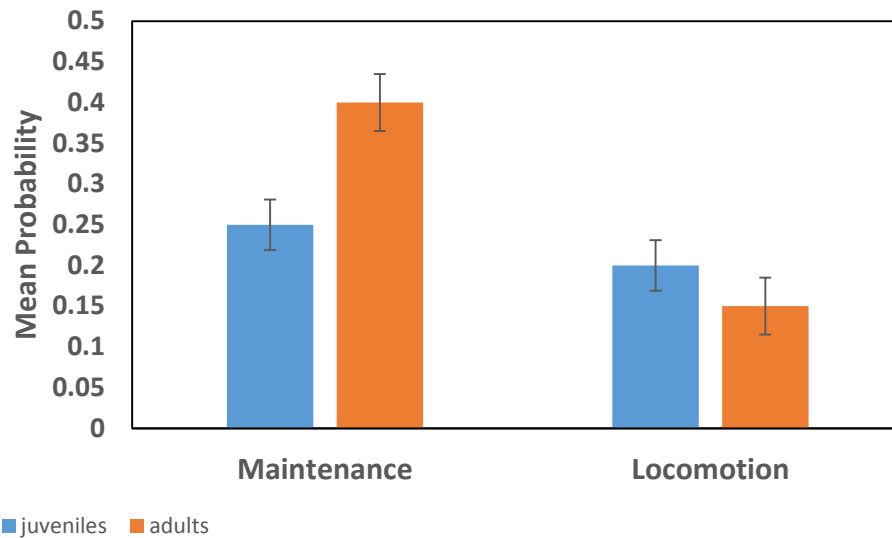
Figure 11. Effect of development on PPM antipredator behavior with snakes.

QUESTION 3: DO CAPTIVE-BORN PPM RESPOND TO PREDATORS LIKE THEIR WILD-CAUGHT COUNTERPARTS?

OWL EXPERIMENT METHODS

Subjects were 27 wild-caught PPM and 24 captive-born PPM. All mice were tested as adults (>1 year of age).

RESULTS and DISCUSSION

Results from this experiment indicate that captive-born adult PPM behave differently than wild-caught PPM when tested with owls. Wild-caught PPM were more wary in the presence of an owl model and allocated significantly more time to vigilance (Wald $\chi2 = 15.20$, p <0.0001; Figure 10) and maintenance (Wald $\chi2 = 16.18$, p <0.0001; Figure 12) compared to their captive born counterparts. There was no difference in the amount of time PPM of different origin (wild-caught vs. captive-born) allocated to locomotion.
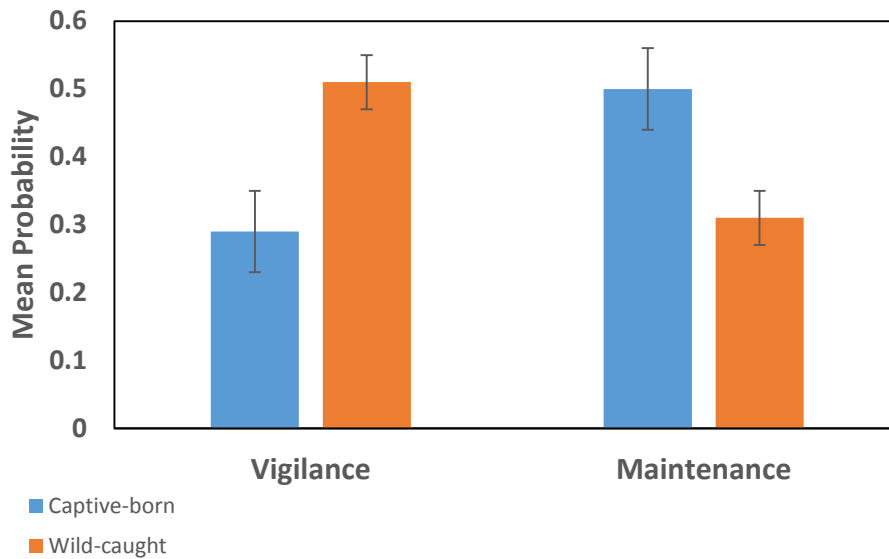
Figure 12. Effect of origin on vigilance of PPM in response to owls.

SNAKE EXPERIMENT METHODS

Subjects were 27 wild-caught PPM and 24 captive-born PPM. All mice were tested as adults (>1 year of age).

RESULTS and DISCUSSION

Results from this experiment indicate that captive-born adult PPM behave differently than wild-caught PPM when tested with snakes. Though history did not influence vigilance behavior in the presence of snakes, wild-caught mice were significantly less likely than captive-born mice to exhibit maintenance behaviors during exposure to snakes (Wald χ^2 = 13.15, p <0.0003; Figure 13).

Taken together with results from the previous experiments, these results indicate that captive born adult PPM are not behaving appropriately in the presence of aerial and snake predators and thus predator training is required to facilitate the development of effective survival skills in mice slated for reintroduction to the wild.
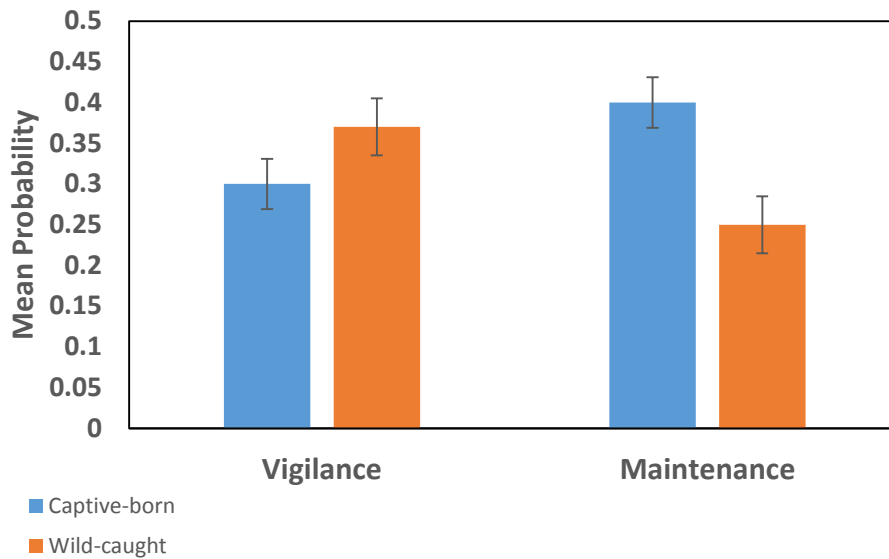
Figure 13. Effect of origin on vigilance of PPM in response to snakes.

Predator training

One of the critical components of survival following reintroduction is avoiding predators. The ability to recognize and detect predators through attention to sensory cues, remaining vigilant, seeking and effectively utilizing cover, and responding to predator presence when appropriate are all important aspects of behavioral competency (Chizar et al. 1993). Because ineffective antipredator behavior often results in death, historically, it was thought to develop in the absence of experience. Certainly, it is the case that many young animals perform species-typical antipredator behaviors without prior experience. The C-start or fast-start startle response in fish (Eaton et al. 1977) or freezing behavior exhibited by three-spinned sticklebacks, *Gasterosteus aculeatus*, in response to a looming stimulus are classic examples of a comparatively innate antipredator behavior (Giles 1984). Yet, a growing literature shows that antipredator behavior is complex, the development of effective antipredator skills can require some form of experience and animals that initially exhibit ineffective antipredator skills can be trained to recognize and respond to live or model predators (see reviews in Brown and Laland 2001; Griffin et al. 2000) or predator cues (Brown and Smith 1998; Mirza and Chivers 2000; Wisenden et al. 2004).

Predator training typically involves some form of associative learning in which animals are purposely exposed to predators or cues of predator presence (CS; conditioned stimulus, predator or predator model) paired with an aversive stimulus (US; Unconditioned Stimulus, e.g. spray of water). The outcome of which is a learned association between the predator and a negative experience. This type of predator training teaches animals to recognize and to avoid predators. Some may consider antipredator training through exposure to predators to contradict welfare of captive animals because predator training often requires subjecting them to stressful stimuli (Teixeira et al. 2007). However exposure to predators in a training context is a short term acute stressor and thus not likely to lead to distress (defined as a biological cost of stress that causes the animal to divert energy away from normal biological functions such as the immune system; Moberg 2000). By contrast, nature contains stressors that can enhance cognitive processes and hone animals' adaptive behavioral responses (Reading et al. 2013; Teixeira et al. 2007) and without exposure, animals slated for release will likely experience distress arising from release

into a novel wild habitat for which the animal has no adaptive response (Swaisgood 2010; Teixeira et al. 2007; Zidon et al. 2009). Thus, predator training may reduce the negative effects of distress experienced by releases and improve welfare. Predator avoidance training is increasingly being implemented in recovery programs that require captive breeding and reintroduction (reviewed in Shier 2016)

Developing a training protocol to modify a target species behavior requires an understanding of several aspects of the target species: the species natural history, in particular, its habitat type, lifestyle, the length of time isolated from predators (generations or duration in captivity), social behavior, the range and type of predators (e.g. native vs invasive, mammalian vs raptorial) that the target species encounters in the wild, the variation and form of antipredator behavior wild-born individuals exhibit (e.g. freeze, flee, concealment, return to refuge, evasive maneuvers, vigilance, etc.), and the extent to which the target species interacts with potential competitors. Understanding these aspects of the target species will allow the researcher to determine: 1) the number of predator types with which to train the target species; 2) the type of training that may be most effective; 3) the number of training events required to modify behavior; and 4) appropriate competency goals.

In light of the results of the antipredator experiments which showed that captive born PPM were significantly less wary of both predator types when compared to wild-caught mice and that wild-caught behavior eroded in just one year of captive living, we developed a predator training protocol for PPM which was then used during the spring of 2016 to prepare the mice in the release cohort for reintroduction (see Reintroduction Section below and Appendix F - Reintroduction Plan for details about the 2016 reintroduction).

METHODS

We chose to train PPM to both owls and snakes. However, as owls prey from above and looming stimuli have been shown to elicit overhead fright responses in the absence of experience with predators, we chose to present the owl following the same protocol that we use for owl antipredator tests. Because snakes are ambush predators, we designed a training protocol that utilized learning theory and paired the presentation of the snake with a multimodel aversive stimulus, a playback of a PPM distress call combined with a physical thump. We conducted predator training trials in a standard 20x48 inch testing arena (Compartments 1-4), filled with 5 cm of sand with the individual mouse's artificial burrow and California buckwheat sprigs for shelter.

A training event consisted of a 5 minute exposure to the snake during which the mouse received 3 training exposures (playback of distress call + thump) following an approach to within 2 inches of the snake compartment (a clear perforated barrier separated the snake and the mouse). The thump was delivered using a device installed beneath the testing arena. The thumping device was attached to a lever that allowed the trainer to observe the trial and deliver the thump immediately following the approach of the mouse. If less than 3 trainings were achieved the focal mouse was retrained at least 3 days later.

Training Protocol

1) Sift training arena thoroughly prior to placing focal subject (all except the snake compartment).
2) Setup below cage thumper in middle of arena (horizontally) and right under the divider between Compartments 1 (snake) and 2 (mouse).
3) Place charged Bluetooth speaker in middle of Compartment 1 but against edge facing into the mouse portion of the arena. Make sure distress calls are loaded on device and ready to play.
4) Set up 6 springs of Buckwheat (2 per compartment)
5) Sprinkle ½ tsp of finch seed across arena sand.
6) Place snake (one of the kingsnake exemplars chosen randomly) into Compartment 1. Leave in for 10 minutes before first trial.** if running multiple trainings in a row, no additional period of snake and no mouse needed.
7) Turn on the video recorder.
8) Transfer the first focal subject from its cage in a PVC tube (45 degree angle from barrier) and place it into the middle of the arena
9) Quietly move to sit behind the barrier and watch the trial. When the mouse approaches to within 2 inches of the barrier, hit the thumper and then immediately play the distress call. The trial ends after 3 training events + 30 seconds or at 5:00 minutes. ***DO NOT "TRAIN" a focal mouse more than 3 times as the mouse could acclimate to the procedure.
10) At the end of the training, turn off the video recorder and return the focal subject to its home cage.
11) Sift training arena (all except snake compartment).
12) Repeat steps 5-10 for remaining focal subjects.

FORAGING EXPERIMENTS

The foraging behavior of PPM has been little studied. Meserve (1976) found PPM showed diet specialization on grass and forb seeds. Relatively little utilization of shrubs was documented with the exception of one occasionally important shrub, California Buckwheat (Meserve 1976a; Meserve 1976b). However, the study was limited in several ways: 1) the results are based on preferences of only 2 to 5 individuals; 2) the study was conducted in the dry season when most flowers and fruits of perennials shrubs were not available; and 3) PPM did not complete excretion of lab provided foods resulting in discarded early samples. Because preferred food items are likely to be eaten first, the current data on diet preferences need to be interpreted with caution.

We are in the process of conducting a series of experiments on foraging in PPM. We examined foraging decisions under predation pressure in Phase I of the project and plan to examine diet preferences and foraging efficiency in the next phase of the project. We will examine whether these behaviors vary across founder population. Our goal with these experiments is to develop an understanding of the species foraging behavior, cues and/or food types that are required for captive born young to develop effective foraging skills prior to release.

Diet Preference

In the wild PPM has been shown to have a strong preference for native and nonnative grass seed and little to no preference for shrubs or most forbs (with the exception of California buckwheat during the autumn; (Meserve 1976a). But species diet preferences are shaped by availability and thus may vary across sites. We examined diet preferences in captivity and determine whether these preferences vary across founder population. We used Giving Up Density (GUD) to quantify preference. The GUD is an effective tool to look at foraging behavior, which estimates the quitting harvest rate (Brown 1988). The assumption with the GUD is that if there was no perceived threat, the animal would forage until just before the density of food is so low that it would take as much energy to search as it would gain from the food. If there is a perceived threat, then the animal will forage in the patch less and there will be more food left over. If the foraging patches follow a gradient from most impacted to least impacted the threat should be reflected in the GUD of the patches.

METHODS

We tested 36 adult PPM [n=26 wild caught (Dana Point: n=8; Santa Margarita: n= 12 and South San Mateo: n=6) and n= 10 captive born] with 7 seed types (California aster, croton, purple needlegrass, saltgrass, California sagebrush, California buckwheat, and white sage) to determine preferred seed types. We presented seeds in plastic containers following the "Cafeteria Method" (Drozdz 1966)(Figure 14). We used a double wide arena the floor of which was covered in 3 inches of sand.

At dusk we removed the home jars and any other cache pockets from each focal subject's home cage. We temporarily stored seeds taken in a labeled baby food jar. We removed seed from each mouse for 2 hours prior to the start of their preference trial. We spread ½ cup of sand from the focal subject's home cage evenly throughout its designated arena. We then arranged the 7 plastic containers in a circle equidistant from the center of the arena, as shown in Figure 14 below. We then filled the center of each plastic container with one of the seven seed types, randomizing the location of each seed in each trial. Once the arena was setup, we collected the focal subject and placed them gently into the testing arena.

Trials were 2 hours in duration and were recorded using a video camera mounted overhead. At the end of the trial, the focal subject was returned to its home cage and uneaten plant/seed material was collected, sorted by type, counted, and reweighed.

This experiment was conducted during the spring and again in the fall to determine if there is variation in diet preferences of PPM across season.
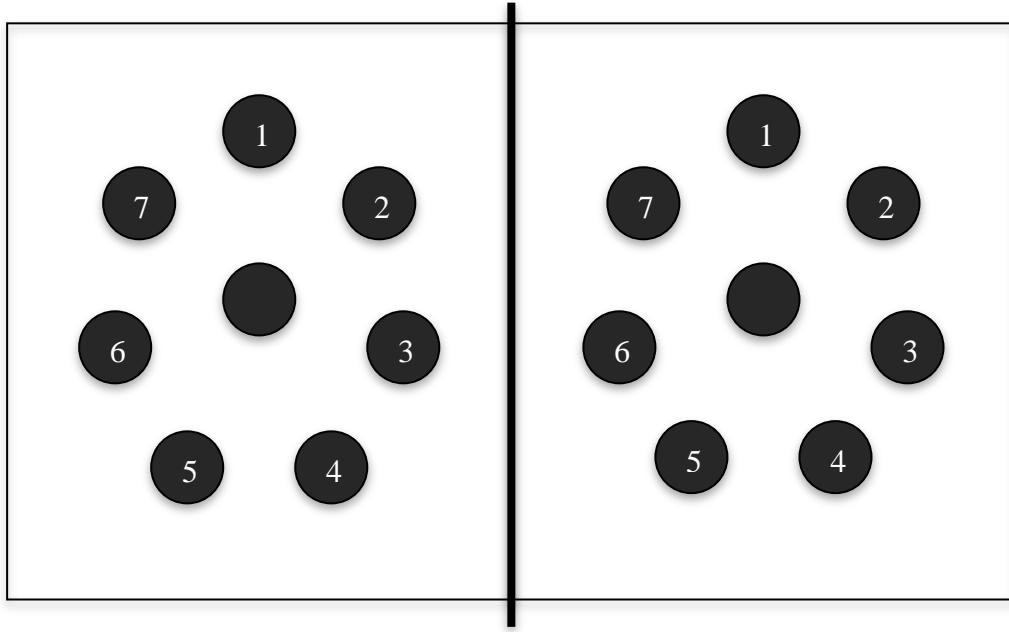
Figure 14. Testing arena. A darkened barrier separates two arenas with seven elevated stoppers arranged in a circle surrounding one unaltered stopper level with the sand.

RESULTS and DISCUSSION

The results showed significant differences in the seed preferences exhibited by wild-caught PPM captured from the 3 different source populations (Figure 15). Wild-caught PPM from Dana Point collected significantly more California croton than PPM sourced from either Santa Margarita or South San Mateo (GLM; $p < 0.001$), while PPM from South San Mateo collected significantly more white sage during trials (GLM; $p < 0.001$). Wild-caught PPM sourced from the Santa Margarita population showed no preferences between the seed types presented.
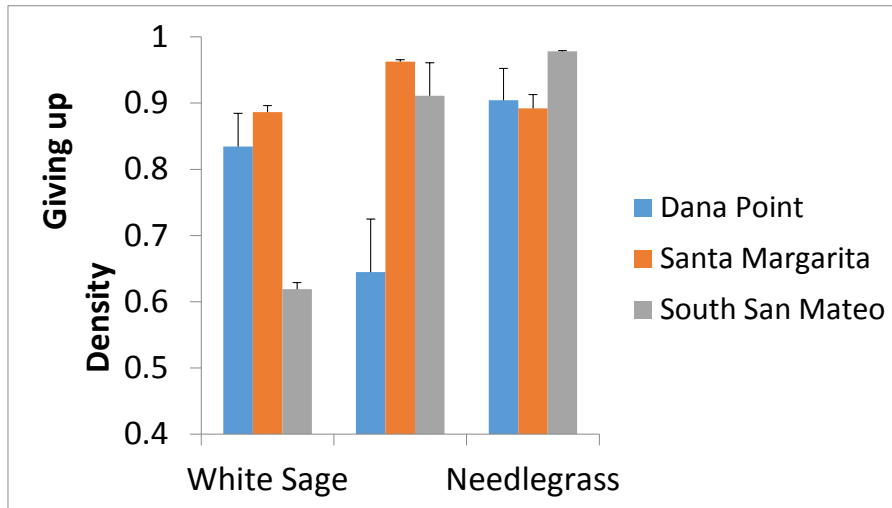
Figure 15. Effect of population origin on seed preferences.

In addition, wild-caught and captive born PPM collected different seed types. While overall wild-caught PPM preferred white sage and croton over needlegrass, captive born PPM preferred needlegrass seeds over the other two seed types (Figure 16). These results suggest that captive-born mice require exposure to native seeds in order to inculcate a preference for particular seed types. Thus, for PPM slated for reintroduction, exposure to seed types that are present at the release site may be critical for effective forage selection following release.
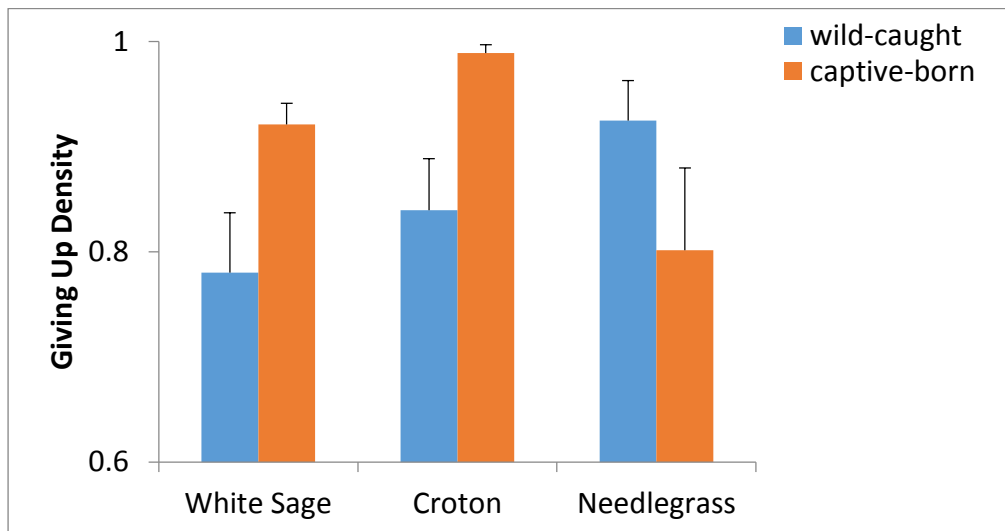

Figure 16. Effect of PPM origin on seed preferences (wild-caught vs captive-born).

The standard diet of captive PPM is a finch seed mix which is supplemented by native species enrichment (California buckwheat). We compared the GUD of seed types that the captive-born

PPM were exposed to during development compared to those that they were not exposed to during development. The results show a significant preference for the seeds that they were exposed to during development (Figure 17).
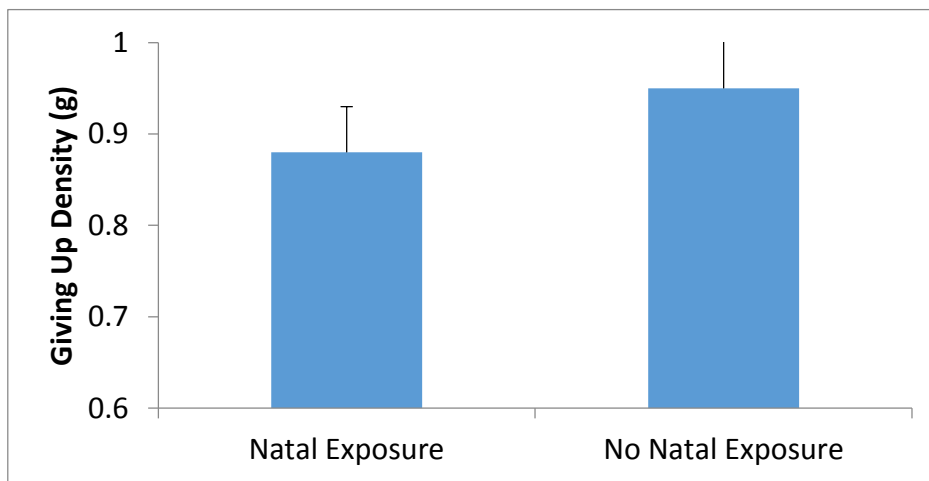

Figure 17. Effect of exposure to seed types on the development of preferences.

Wild-caught PPM prefer white sage and California croton seeds over needlegrass. Captive-born mice show the opposite preferences. Because source population predicts seed preferences, wild PPM collected from each of the remaining extant sites showed different seed preferences. This is likely due to exposure to these species during their time in the wild. Captive –born mice were raised on a standard non-native diet because limited nutritional information is available for native species. However, because the goal of the program is reintroduction to the wild, we have been providing native seeds as enrichment to allow for exposure. These results indicate that without that exposure, preferences for some native seeds are not developed. Thus, in response to these experiments, we have modified the diet of captive PPM to include several additional native seed types as enrichments (Appendix B). Further research is needed to determine if there is a sensitive period for this exposure to native seed types or if we can inculcate a preference even after the mice are adults.

Caching Behavior of PPM in the Wild and Captivity

Food storage is a necessary adaptation for many animals to withstand variable or unpredictable food availability (Giannoni et al. 2001; Vander Wall & Jenkins 2003). Caching and cache pilfering have been well documented in multiple heteromyid communities (e.g. Price et al. 2000; Leaver & Daly 2001; Swartz et al. 2010), suggesting this behavior plays an essential role for survival in habitats that vary temporally in seed production. PPM are no exception; they utilize facultative torpor and remain in their burrows, mostly inactive, for up to 8 months during the year (Shier 2008). During this time of year when plants are not producing seeds, they are thought to be primarily reliant upon their seed caches in their burrows (Kenagy 1973). Thus, the behavior of collecting and caching seeds is critical to PPM survival in the wild.

Experiment 1. Comparing caching of wild to captive PPM

We conducted a study of caching behavior comparing wild animals, wild-born captive animals, and captive-born captive animals. Subjects in the wild were 10 PPM (6 adult males and 4 adult

females) provisioned with seeds as part of an interspecific cache pilfering study. Each individual was placed in a bottomless plexiglass arena (24 x 24 x 24 in) on the sand by its burrow. In the arena was a seed tray with 5g hulled millet. Natural vegetation cover was present in the arena, or sprigs of vegetation were added to provide cover. Individuals were able to dig under the edge of the arena and remove seeds to their burrow or pit caches. Trials lasted as long as the PPM took to remove all the seeds from the tray, or if the animal did not return after 30min.

Subjects in captivity were 20 PPM (5 adult males, 5 YOY males, 5 adult females, 5 YOY females). Each individual was tested once in the fall and again in the spring following a reduced diet during the winter. One wild-born PPM died during the winter so our sample size for spring was 9 wild-born PPM. All tests occurred in a testing arena that consisted of a plywood box (30 x 30 x 18 in) lined with a tarp and filled with 10 cm of sand. The same Plexiglas arena used in the field was placed inside the testing arena. In the arena was a seed tray with 5g hulled millet and a small pile of CareFresh bedding for cover. The individual's nest jar was placed outside the Plexiglas arena, with the t-tube under the edge of the wall, providing access to its burrow for caching. Prior to beginning each trial, the sand in the testing arena was mixed and raked and 0.75 cups of sand from the animal's home cage were sifted into the Plexiglas arena to provide olfactory cues of its home territory. A one hour observation began after the 15 minute acclimation period, or as soon as the animal began foraging, whichever occurred first. The test ended after an hour of observation, or when all seeds were removed from the tray, whichever occurred first.

Of the 10 individuals in the wild, 9 of them took all or some of the seed from the tray and cached it. During the first trial in captivity in the fall of 2013, only 2 out of 20 animals (one wild-born, one captive-born) took any seed from the tray. When we repeated the trials in captivity during spring 2014, 3 out of 9 wild-born animals cached seeds and again only 1 in 10 captive-born individuals cached any seeds. Overall there was a clear reduction in caching between wild and captive animals (Figure 18). This is likely due to the abundance of food that captive animals received, and they spent more of their time exploring, digging, and sand bathing in the new arena than wild animals did. As predicted, we did see an increase in caching in the spring following winter months with reduced food availability. Only wild-born animals exhibited this behavioral shift. However, we regularly observed captive PPM caching seeds in their nest jars and the sand of their home cages. Thus we designed a second experiment to study caching behavior of captive PPM over the course of a week rather than an hour.
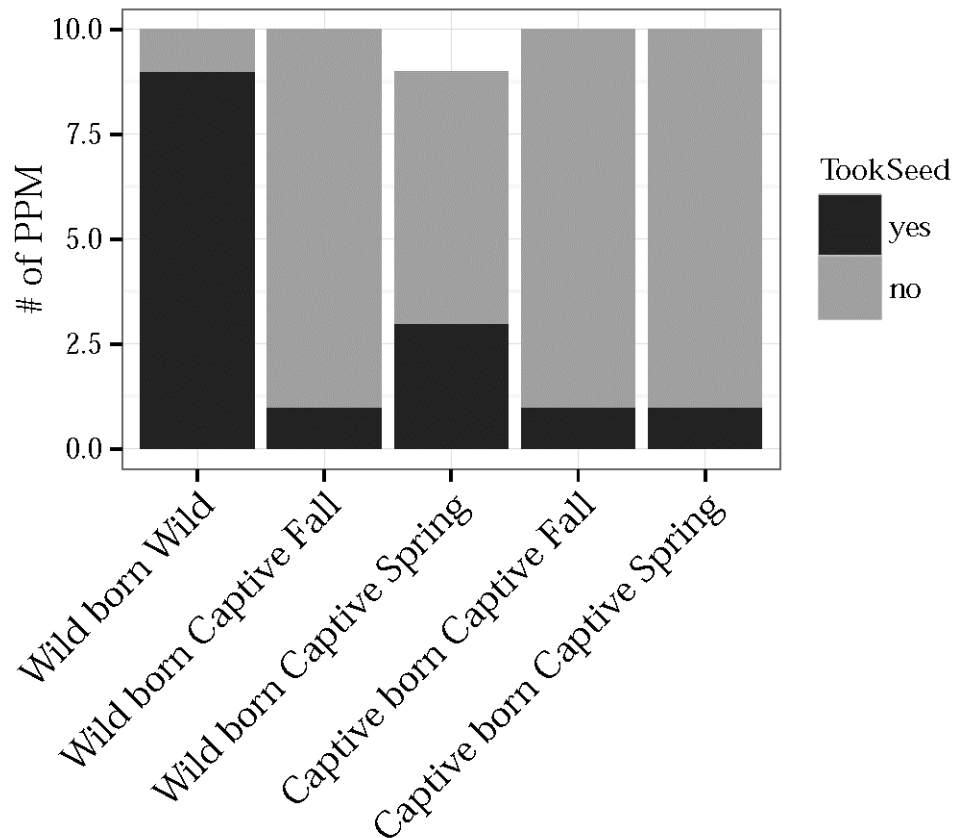
Figure 18. Caching in wild vs. captive PPM.

Experiment 2. Cache placement of wild-born vs. captive-born PPM in captivity

We conducted a weeklong caching study of captive PPM in their home cages in September 2016. There is disagreement in the literature whether *P. longimembris* primarily larder hoards or scatter hoards seeds (Jenkins & Breck 1998; Price et al. 2000). The aim of this study was to better understand the seed caching behavior of PPM and quantify the proportion of seeds they ate, larder hoarded, scatter hoarded, or left on the soil surface. We used both wild-born and captive-born individuals to test whether the groups differ in caching behavior.

Subjects in captivity were 10 wild-born PPM and 10 captive-born PPM. Prior to the study we removed all sand and seeds from each home cage and removed larder hoards from nest jars, which we stored and returned at the end of the study. We refilled cages with clean sand, the original nest jars, t-tubes, bedding material, and a petri dish where we placed all feed for the 7-day duration. We pre-weighed 14g of their normal diet of finch seed mix and 2g was fed daily in addition to their normal lettuce or spinach for water. PPM were left undisturbed for the duration of the trial and allowed to cache and re-cache their seeds for a week. After the 7th day we removed each PPM from its home cage and separately collected seeds that remained in the feeding tray, on the surface of the soil, from the nest jar, and those buried in the sand. We sifted each segment separately and weighed the seeds to quantify the proportion in each location. The

difference in the initial weight and the remaining weight of seeds in all the sections of the cage gave us the total weight eaten in the 7 days.

Our preliminary results indicate that captive PPM both cache seeds in their nest jars (larder hoard) and also cache seeds in the sand of their enclosures (scatter hoard). This matches observations in the field that animals both scatter hoard and larder hoard seeds. Very few seeds were left untouched in the feeding dish or on the surface of any cages, indicating that both wild-born and captive-born eat or cache the majority of their seeds in captivity.

GENETICS

In Phase 1 of the PPM conservation breeding and reintroduction program we focused on establishing a microsatellite library to characterize genetic diversity in wild populations of PPM. Earlier analyses have shown that the extant populations in the wild represent three genetically distinct clusters (Dana Point, South San Mateo, Camp Pendleton), and the initial 22 founders of the captive population assigned predictably with the genetic cluster from which they were taken.

From July 2014 to December 2016, we continued the genetic assessment of PPM in the captive breeding program. The retention or loss of genetic diversity in the captive population was evaluated by calculating several genetic indices using microsatellite genotype data. In addition, relatedness analysis was performed for every additional founder brought in from the wild to supplement the captive population. This information was used to direct breeding efforts, by determining closely related founders that should not be recommended to breed. The effects of losing 50 individuals from the captive population to establish a new population at Laguna Coast Wilderness Park (LCWP) was also assessed by comparing genetic diversity estimates for the captive population in 2016 with the inclusion and exclusion of the 50 chosen individuals. The genetic diversity of the new, reintroduced LCWP population was also examined. We anticipate having to perform a parentage analysis in 2017, with the sampling of the first cohort of offspring from the reintroduced population. The exclusionary power of the 15 existing microsatellite loci was tested using two methods of parentage analysis, categorical assignment and pedigree reconstruction. We were interested in comparing the ability of each approach to identify parent pairs of known trios in order to determine the better method.

Additionally, unprocessed samples from Camp Pendleton collected between 2002-2008 were genotyped and sequenced to further look for any potential genetic structure within the site. Phylogenetic analysis was updated with the inclusion of these newly processed wild Camp Pendleton individuals. Effort was also undertaken to develop a genetic test for sex determination in PPM with the intent of clarifying sexes for the captive breeding program, and to resolve the karyotype of this species through identification of autosomal and sex chromosomes.

METHODS

Sample collection

During the time period of this report, the Genetics division processed an additional 201 PPM samples consisting of 45 wild PPM from Camp Pendleton that were collected between 2002-2008, eight new founders, and 148 captive born PPM. The standard ear snip protocol (Alexander

and Riddle 2005; Loew et al. 2005; Metcalf et al. 2001; Waser et al. 2006) was used to obtain genetic samples. Ear snips can be as small as a pencil point and still provide ample genetic data for analysis of parentage, genetic relationships and dispersal (Waser et al. 2006). Ear snips were obtained by sterilizing scissors with 70% ethanol, holding the scissors on a tangent from the edge of the pinna, and snipping a sliver (~0.5mm) off the edge of the pinna. Tissue samples were then transferred to and stored in a vial with 95% ethanol. Scissors were sterilized between animals. DNA was extracted from ear snips using the QIAamp DNA Mini Kit (Qiagen Inc.) following the manufacturer's protocol. DNA samples were stored at -20°C post-extraction.

Mitochondrial control region sequencing

A 756-bp fragment of the mitochondrial D-Loop control region was sequenced using a modified version of primers L16007 and H00651 (Kocher et al. 1989). Amplification was verified on a 1.5% TBE agarose gel, and products were purified using ExoSAP-IT® (Affymetrix). Cycle sequencing was performed using BigDye^{TM} 3.1 and sequencing products were sequenced bi-directionally on an ABI 3130 Genetic Analyzer (Life Technologies). Contigs were assembled and edited using SEQUENCHER 5.1 (Gene Codes Corp.) and sequences were aligned using the built-in version of CLUSTAL W.

Microsatellite genotyping and evaluation

Seventeen microsatellite markers specifically designed for PPM were used in this study. PCRs were performed using the Qiagen Multiplex PCR Kit (Qiagen Inc.) and organized into seven multiplex schemes. Amplification products were verified on a 1.5% TBE agarose gel. Fragment analysis was performed using capillary electrophoresis on an ABI 3130 genetic analyzer (Applied Biosystems) and alleles were scored relative to an internal size standard (500 ROX) using GENEMAPPER 3.0 (Applied Biosystems). These data were used in several analyses, which are summarized below.

Microsatellite markers were evaluated for deviations from Hardy-Weinberg equilibrium and signs of linkage disequilibrium at the population level using the software GENEPOP 4.1 (Rousset 2008). Significance at an initial p-value of 0.05 was corrected for multiple tests using the B-Y False Discovery Rate method (Benjamini and Yekutieli 2001). We found cases of significant deviation from Hardy-Weinberg equilibrium and signs of linkage disequilibrium at some loci; however, they were not consistent across all populations. All 17 loci were included in population structure analyses, but two loci (Plp55 and Plp86) that exhibited some signs of allelic dropout were excluded from diversity and relatedness analyses, which is more sensitive to the effects of allelic dropout. Even with the reduced number of 15 loci, the probability of two individuals in the captive population having the same genotype (PI) is 9.8×10^{-17} and probability of two full siblings having the same genotype (PI_{SIB}) is 6.0×10^{-7}.

D-loop sequencing analysis

To date, a total of 100 individuals made up of 31 wild founders from the captive population and 69 PPM sampled in the wild between 2002-2008, have been sequenced at the mitochondrial D-loop region. Almost half of the sequences came from Camp Pendleton (52), while there are 27 from Dana Point and 21 from South San Mateo.

The software TCS 1.21 (Clement et al. 2000) was used to create a haplotype network depicting the genealogical relationships between the various D-loop haplotypes. D-loop haplotypes from five subspecies of *P. longimembris* (*bangsi, brevinasus, internationalis, longimembris, pacificus)* were used to generate a Bayesian inference tree using **MRBAYES** (Ronquist and Huelsenbeck 2003).

Genetic differentiation and structure

The computer program GenoDive (Meirmans and Van Teinderen 2004) was used to calculate pairwise F_{ST} for the wild population and founders for each sample site. Significance for pairwise differentiation for F_{ST} was determined with 9,999 permutations. To evaluate hierarchical population genetic structure, a Bayesian clustering analysis was performed using the computer program Structure 2.3.2 (Pritchard et al. 2000). This analysis is used to objectively determine the number of genetic clusters that exist among a set of individuals using their multilocus genotype data, without any preconceived notions regarding possible population structure. We ran the analysis with typical default settings, including an admixture model with correlated allele frequencies. Bayesian simulations were run for each population number (K) ranging from 1–10. For each value of K, we ran 10 independent replicates with a burn-in period of 250,000 steps followed by 750,000 iterations, thereby assuring convergence on the inferred likelihood values. We follow the protocol of Evanno et al. (2005), wherein the true value of K is inferred using an ad hoc statistic, ΔK, based on the rate of change in the log probability of data between successive K values ($\Delta K = m(|L''K|) / s[L(K)]$). An additional 69 captive born PPM were included in the structure analysis: 2013 (n=18); 2014 (n=33); and 2015 (n=18).

Genetic Diversity Calculations

The computer program **GENALEX** 6.5 (Peakall and Smouse 2012) was used to calculate the total number of alleles, observed heterozygosity and expected heterozygosity. Allelic richness was calculated using HP-RARE (Kalinowski 2005), which takes into account sample size differences by rarefaction. Inbreeding coefficients per population were estimated using FSTAT 2.9.3 (Goudet 2001).

The PPM populations for Dana Point, Camp Pendleton, and South San Mateo designated as "wild" include wild caught founders from both the captive population and PPM sampled from the wild between 2002 and 2008. PPM populations designated as "founder" for Dana Point, Camp Pendleton, and South San Mateo represent only the wild caught founders for the captive population. The population "Wild (All)" represents all samples from the wild from all three locations, combined. Captive populations representing each year since the inception of the captive breeding program are designated "F_0" (2012) for the founders, "F_1" (2013), "F_2" (2014), "F_3" (2015), and "F_4" (2016). These populations include all surviving individuals that were included as potential breeders at the end of the designated year. In 2016, 50 individuals were chosen from the captive population to establish the newly reintroduced population at Laguna Coast Wilderness Park, which is designated as "R_0". The F_4 population was assessed for genetic diversity indices prior to the pulling of the 50 reintroduced individuals (F_4-pre) as well as after (F_4-post). Populations were organized in this way to make direct gene diversity comparisons.

Founder relatedness

Relatedness analysis was performed for all new PPM founders brought in from the wild to establish or supplement the captive population. Breeding recommendations were made for these founders to assist captive breeding efforts which aim to reduce inbreeding and maximizing genetic diversity in the captive population. The computer program COANCESTRY 1.0 (Wang 2011) was used to estimate relatedness using three different estimators: Wang; Queller and Goodnight; and Lynch and Ritland. Simulated genotypes across four relationship categories (parent-offspring, full sibling, half sibling, and unrelated) were generated using allele frequencies among wild individuals (including wild founders) for each population. Averages and variances were calculated at each relatedness category for each relatedness estimator. The relatedness estimator with the smallest variance across most relatedness categories for the given population (Dana Point, Camp Pendleton, South San Mateo) was chosen as the estimator to calculate relatedness among founders for that population. Empirical relatedness values for each possible pairing of founders within each founder population were calculated using the computer program SPAGeDi (Hardy and Vekemans 2002). Breeding recommendations were made to avoid mating pairs identified as exceeding relatedness values of half siblings. A total of 15 loci were used for the relatedness analysis. It is important to note that due to small sample sizes, relatedness estimates have a wide range of error and breeding recommendations should be received with caution.

Paternity analysis

We evaluated the effectiveness of the 15 microsatellite loci in detecting true parent pairs from a set of candidate parents by performing two different methods of parentage analysis: categorical assignment and sibship reconstruction. Next year we hope to use parentage analysis to assess which founders bred to produce cohorts within the newly released population at Laguna Coast Wilderness Park. The goal this year was to test which method of analysis performs better in detecting the true parent pair from a pool of candidate individuals. Parentage for a total of 58 PPM born in captivity in 2015 were validated from a pool of 81 candidate parents of known sexes (47 males and 34 females) that were founders or captive born PPM prior to 2015.

The program CERVUS (Kalinowski et al. 2007) was used to calculate exclusion probabilities as well as the likelihood of each putative parent-offspring pair given their multilocus genotype and allelic frequencies based on the categorical assignment method. The sibship reconstruction method using COLONY (Jones and Wang 2009), on the other hand, infers sibship and parentage among individuals using a full-pedigree likelihood method, assigning offspring to family clusters.

Genetic sexing

Dr. Cynthia Steiner designed multiple primers with the goal of genetically sexing PPM to aid the captive breeding program and to resolve the karyotype of this species via identification of autosomal and sex chromosomes. Much of the literature surrounding genetic sexing in rodents focuses on the family Muridae, which has made it a challenge to sex PPM. The primer pair UbaE18/UbaE19, which targets an intronic region of the Uba gene, successfully differentiated the sexes via gel electrophoresis. Sex determination was performed on PPM cell culture lines that were established from pups of unknown gender, and the cytogenetics team is currently in the

process of resolving PPM karyotypes using this new information for the proper identification of autosomal chromosomes. The Genetics division tested the primers on various Heteromyidae species in the Frozen Zoo®: four *Dipodomys* species; four *Perognathus* subspecies; and two *Chaetodipus* species.

RESULTS and DISCUSSION

Phylogenetics

A total of eight unique mitochondrial haplotypes were found in the D-loop among 100 individuals, represented by 13 variable sites. Figure 19 shows a geographical map of the three sites (Dana Point, Camp Pendleton, and South San Mateo) with a pie chart representing the different haplotypes found at each site. Dana Point samples are strictly comprised of Haplotype 1. South San Mateo also had a unique haplotype (Haplotype 7) as well as two shared haplotypes with Camp Pendleton (Haplotypes 4 and 5). Camp Pendleton had the greatest diversity of haplotypes, however, it is important to note that a great majority of individuals were sampled from that site compared to Dana Point and South San Mateo. Haplotype 1, which was found to be unique to Dana Point, was the most genetically distant of all haplotypes with three mutation steps from other haplotypes. Overall, haplotypes were highly connected with few mutational differences as shown by the haplotype network (Figure 20).

Results from the Bayesian inference analysis showed subspecies *bangsi* and *internationalis* form a strongly supported cluster, while there is little genetic structure observed in the remaining subspecies (Figure 21). Within PPM (*pacificus*), all haplotypes with the exception of Haplotype 1 which is unique to Dana Point form a strongly supported cluster (0.997). There are some phylogenetic relationships consistent with findings using a cytochrome b marker by Swei et al. 2003. Though many of the phylogenetic relationships they found were weakly supported, they also showed that many haplotypes from *bangsi* and *internationalis* have an association at the base of the tree. They also found more basal haplotypes within the subspecies *pacificus* that originated from Dana Point. This marker may not be the most appropriate marker for high resolution of phylogenetic relationships among *P. longimembris pacificus* due to the low number of variable sites.
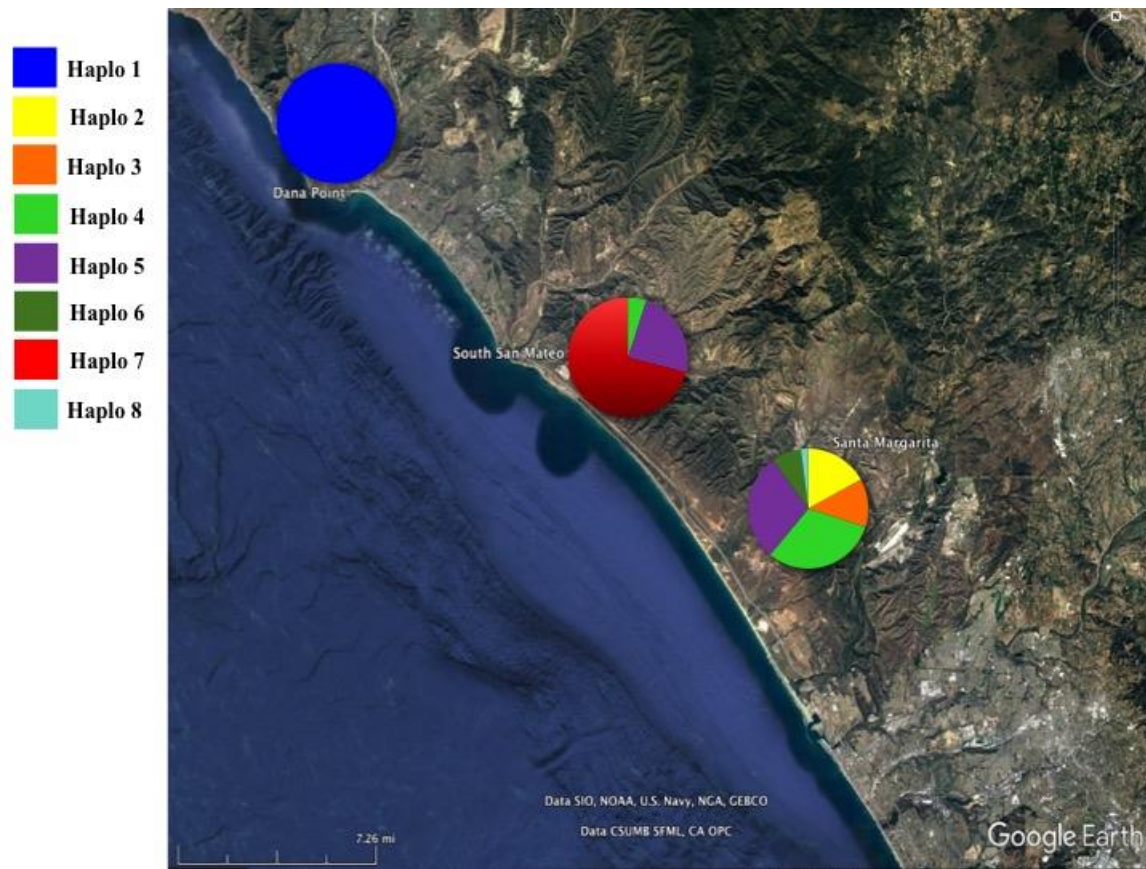
Figure 19. Map representing the haplotype diversity and frequency observed at each site: Dana Point, South San Mateo, and Santa Margarita.
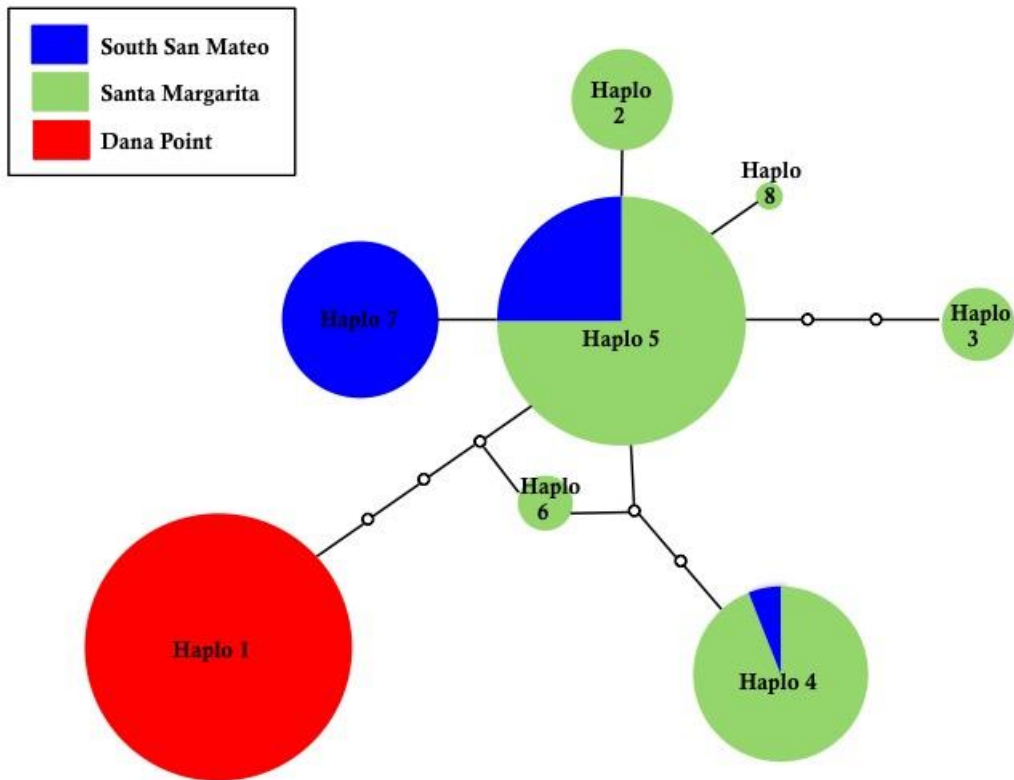
Figure 20. A statistical parsimony network depicting mutational relationships between PPM D-loop haplotypes. Each haplotype contains the percent contribution from each population, which is designated by a unique color. The areas of the circles representing each haplotype correspond to its frequency. Each line represents a single mutational event, and each dot represents a putative nucleotide change. A 95% connection limit was applied.

OR4490_bangsi_
OR4492_bangsi_
1   OR4488_internationalis
OR4489_internationalis_
OR4496_bangsi

pacificus-(5) shared
pacificus-(8) Oscar
pacificus-(2) Oscar
0.997   pacificus-(7) SSM
pacificus-(3) Oscar
pacificus-(4) shared
0.5217
pacificus-(6) Oscar
0.7655
0.5003   pacificus-(1) DP
OR4486_brevinasus_
0.9987
OR4493_brevinasus_
0.51   OR4499_longimembris_
OR4495_longimembris
1   OR4494_brevinasus_
OR4498_longimembris

0.0030

Figure 21. A Bayesian inference consensus tree generated from *P. longimembris* haplotypes using a GTR model gamma distributed with Invariant Sites (GTR+I+G). Posterior probabilities are marked at the nodes and each branch is designated by the haplotype ID and subspecies. The scale bar represents the number of substitutions per site.

Genetic differentiation and structure

Pairwise Fst values show that the founders and wild individuals from the same sample site display very little genetic differentiation to each other (Table 10), as we might have expected. When comparing the three wild populations, Dana Point seems to be the most genetically differentiated as reflected by the high pairwise Fst values with South San Mateo and Camp Pendleton.

Results from the structure analysis show that the founders group predictably with the genetic cluster from which they were sampled (Figure 22). There was an equal likelihood of seven genetic clusters (K=7) versus three (K=3), possibly due to additional clusters that were a result of the over-representation of certain captive born PPM forming new clusters. Therefore, three clusters were chosen as the most likely true K value. There seems to be particularly strong representation in the captive population from the South San Mateo cluster in 2014, however, 2015 shows increased representation from Dana Point and Santa Margarita.

Table 10. Genetic differentiation at 17 microsatellite loci for PPM from Dana Point (DP), Santa Margarita (SM), and South San Mateo (SSM). F_{ST} values that are statistically significant are bolded ($p < 0.05$).

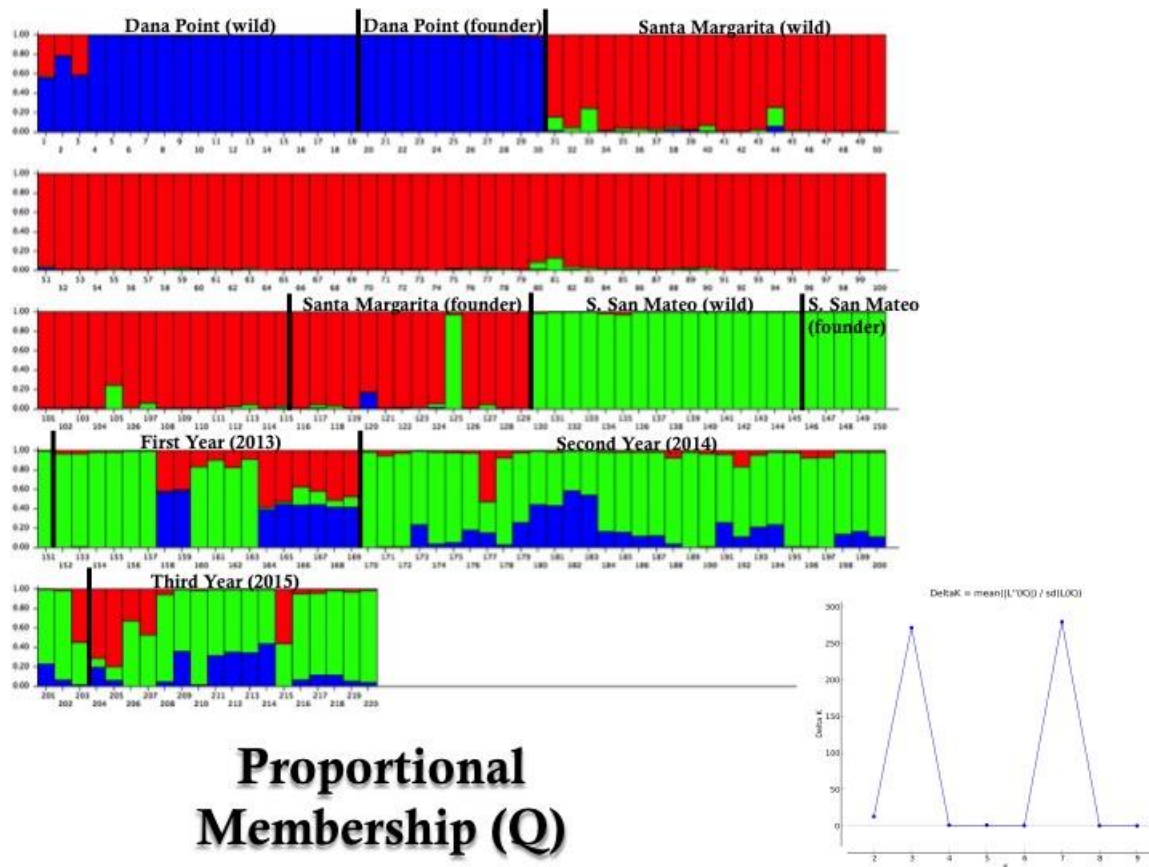|	DP wild	DP founder	SM wild	SM founder	SSM wild
DP founder	-0.010				
SM wild	**0.288**	**0.309**			
SM founder	**0.322**	**0.344**	0.003		
SSM wild	**0.443**	**0.481**	**0.193**	**0.212**	
SSM founder	**0.442**	**0.502**	**0.196**	**0.206**	**0.033**

Figure 22. Structure bar plot (K=3) showing the proportional membership of each individual (y-axis) to a given genetic cluster as represented by a unique color.

Genetic diversity

Table 11 shows genetic diversity estimates for wild and founder populations of Dana Point, Camp Pendleton, and South San Mateo. These comparisons were made to observe the genetic diversity captured in the founder population in relation to that observed in the wild. As expected, the total number of alleles is lower in the founder populations than that in the wild population for each location. However, allelic richness, heterozygosities and inbreeding coefficients in the founder populations are comparable to that seen in the wild.

Comparisons were also made for the combined wild populations, combined captive founder population (F_0) established in 2012, and the newly reintroduced population at Laguna Coast Wilderness Park (R_0) as shown in Table 12. The founder population is shown to capture 68% of the total alleles observed in the wild, and the R_0 population at Laguna Coast Wilderness Park (LCWP) represents about 97% of the total alleles present in the captive founder population. Allelic richness is slightly lower in the R_0 population; however, heterozygosity values are high indicating high genetic diversity. The high inbreeding values for the combined wild and founder populations can most likely be attributed to the Wahlund effect where there exists population structure representing groups of individuals of varying allele frequencies, which results in what seems like heterozygote deficiency (Hedrick 2013).

Table 11. Summary statistics from microsatellite data among the wild and founder populations for Dana Point (DP), Santa Margarita (SM), and South San Mateo (SSM): total sample size (N); total number of alleles in each population (A); allelic richness (AR); observed heterozygosity (H_O); expected heterozygosity (H_E); and inbreeding coefficient (F_{IS}).

	DP wild	DP founder	SM wild	SM founder	SSM wild	SSM founder
N	30	11	99	14	26	10
A	48	31	133	102	62	52
$AR*$	2.4	2.0	6.2	6.2	3.7	3.5
H_O	0.30	0.30	0.71	0.70	0.55	0.53
H_E	0.32	0.28	0.75	0.73	0.57	0.55
F_{IS}	0.07	-0.05	0.07	0.08	0.06	0.07

* *AR* based on a minimum sample size of 10 diploid individuals

Table 12. Summary statistics from microsatellite data among the combined wild (Wild- All) and combined founder populations (F_0) as well as the reintroduced population at Laguna Coast Wilderness Park (R_0): total sample size (N); total number of alleles in each population (A); allelic richness (AR); observed heterozygosity (H_O); expected heterozygosity (H_E); and inbreeding coefficient (F_{IS}).

	Wild (All)	F_0 (2012)	R_0 (2016)
N	155	21	50
A	155	106	104
$AR*$	8.1	7.1	6.1
H_O	0.601	0.492	0.687
H_E	0.765	0.672	0.671
F_{IS}	0.217	0.291	-0.013

* *AR* based on a minimum sample size of 21 diploid individuals

The last set of comparisons looks at temporal changes in genetic diversity of the captive population from its inception in 2012 to the present (Table 13). The supplementation of new founders in certain years appear to increase allelic diversity as reflected by the increased total number of alleles for that year with the exception of 2016. This year a Camp Pendleton founder that possessed four unique alleles died without breeding and the new South San Mateo founders added two unique alleles to the captive population. The allelic diversity as represented by the total number of alleles is currently higher in the captive population than in the initial founder population, though allelic richness is slightly lower. Genetic diversity seems to be relatively unchanged even after the loss of 50 individuals to the release site. Heterozygosity values have steadily increased over the span of four years, and the inbreeding coefficient has steadily decreased to near zero.

Table 13. Summary statistics from microsatellite data for the captive population from 2012 to 2016: total sample size (N); sample size of newly added founders for the year (N_F); total number of alleles in each population (A); allelic richness (AR); observed heterozygosity (H_O); expected heterozygosity (H_E); and inbreeding coefficient (F_{IS}).

	F_0 (2012)	F_1 (2013)	F_2 (2014)	F_3 (2015)	$F_{4\text{-pre}}$ (2016)	$F_{1\text{-post}}$ (2016)
N	21	42	66	110	167	117
N_F	---	5	4	0	4	4
A	106	113	122	122	120	119
AR*	7.1	6.8	6.8	6.6	6.4	6.5
H_O	0.492	0.598	0.651	0.692	0.705	0.713
H_E	0.672	0.690	0.691	0.700	0.708	0.717
F_{IS}	0.291	0.144	0.066	0.016	0.007	0.01

* AR based on a minimum sample size of 21 diploid individuals

Founder relatedness

Figure 23 shows a scatterplot of relatedness values from all possible founder pairs for a given population. A table of mean relatedness coefficients and variances for each relatedness category is given, along with a table of dyads that represent a relatedness level of half siblings or higher.

a) Dana Point
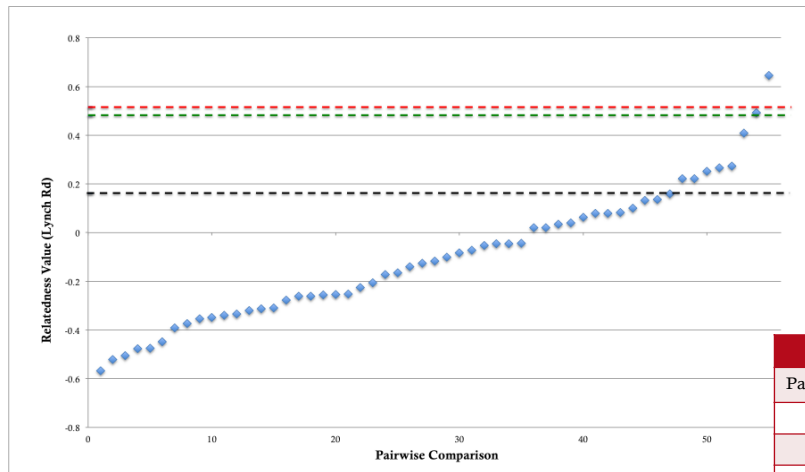
Mean relatedness coefficients and variances

Unrelated	Half sibling	Full sibling	Parent-offspring
-0.002 (0.053)	0.162 (0.063)	0.515 (0.072)	0.481 (0.061)

Relationship Level	Pair
Parent-offspring/Full-sib	8 x 9
Full-sib	4 x 5
Half-sib	1 x 3
Half-sib	2 x 7
Half-sib	2 x 24
Half-sib	4 x 7
Half-sib	5 x 7
Half-sib	10 x 11

b) Santa Margarita
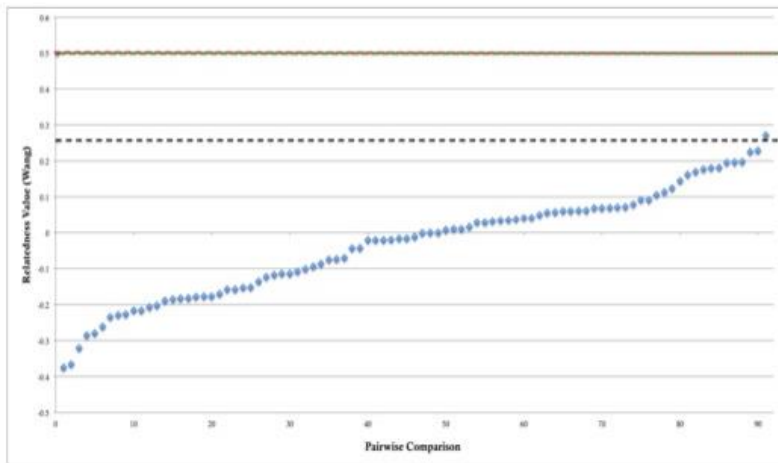
Figure 23. Pairwise

Mean relatedness coefficients and variances

Unrelated	Half sibling	Full sibling	Parent-offspring
0.0003 (0.018)	0.258 (0.016)	0.495 (0.016)	0.495 (0.004)

Relationship Level	Pair
Half-sib	13 x 20

c) South San Mateo
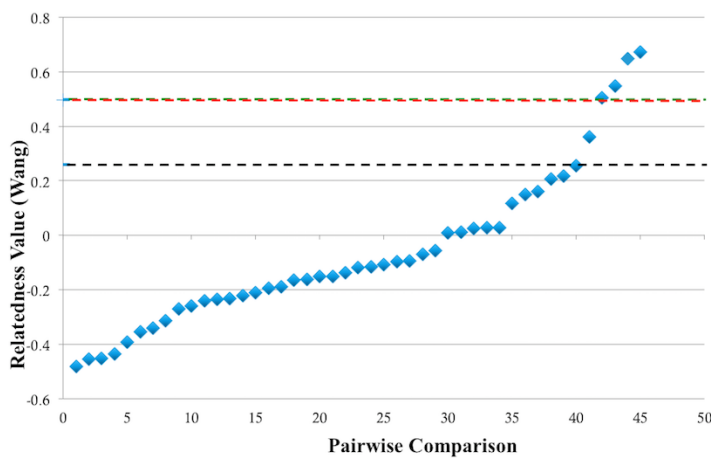
Mean relatedness coefficients and variances

Unrelated	Half sibling	Full sibling	Parent-offspring
-0.007 (0.040)	0.259 (0.031)	0.496 (0.027)	0.498 (0.009)

Relationship Level	Pair
Parent-offspring/Full-sib	22 x 23
Parent-offspring/Full-sib	25 x 27
Parent-offspring/Full-sib	26 x 27
Parent-offspring/Full-sib	209 x 210
Half-sib	25 x 26

Figure 23. Pairwise relatedness coefficients of founders from (a) Dana Point (b) Camp Pendleton (c) South San Mateo. Cutoffs between half siblings (black), full siblings (red), and parent-offspring (green) are indicated by dashed lines. The estimator chosen for the population is indicated on the y-axis. The mean and variance for each of the four relatedness categories are shown in the blue table. Pairs of individuals that exceed the cutoff for half siblings are show in the red table so as to avoid breeding.

The following conclusions were made:

- For the Dana Point population there were two pairs, 8x9 and 4x5, that exhibited high relatedness at the level of parent-offspring or full sibling. There were also multiple dyads that exhibited relatedness at the level of half siblings: 1x3; 2x7; 2x24; 4x7; 5x7; and 10x11. Recommendations have been made to avoid mating these pairs.
- For the Camp Pendleton population there is a pair of individuals, 13x20, that exhibited relatedness at the level of half siblings. Recommendations were made to avoid mating this pair.
- For the South San Mateo population there were four pairs that exhibited relatively high relatedness at the full sibling and parent-offspring level: 22x23; 25x27; 26x27; and 209x210. Dyad 25x26 exhibited relatedness at the level of half siblings. Recommendations were made to avoid mating these pairs.

Paternity analysis

Both methods performed similarly at detecting the true parent pairs. Of the 58 offspring, CERVUS correctly assigned the true parent pairs for 54 individuals and COLONY assigned both parent pairs correctly for 52 individuals. In both cases, the same three discrepancies were observed in offspring from the same litter where the wrong Dana Point male founder was inferred as the sire. This is most likely due to the lower genetic diversity observed in PPM from Dana Point. In another case, both methods inferred the incorrect dam, however, the true dam and inferred dam were offspring from the same parent pair though they came from different litters. The additional two cases in which COLONY inferred the wrong parent pair occurred because the software inferred a putative, unsampled male as the true sire. In both cases, the true sire was inferred as the second most likely possibility. While COLONY may have an advantage when there are higher numbers of unsampled sires and dams, CERVUS was the more accurate for two cases, therefore, we will use both methods next year for the paternity analysis (Harrison et al. 2013).

Genetic sexing

The newly designed sexing primers successfully differentiated sexes in the four *Perognathus* subspecies. Several cases were resolved in which sexes of deceased pups from the captive population were unknown.

STUDBOOK PEDIGREE

The studbook pedigree indicates the current captive-born portion of the population is descended from 10 founders with 15 potential founders still remaining (Figure 24/Table 14). The gene diversity of the captive-born portion of the population is 92.38%, which is equivalent to that found in approximately 6 or 7 unrelated animals (FGE = 6.56).
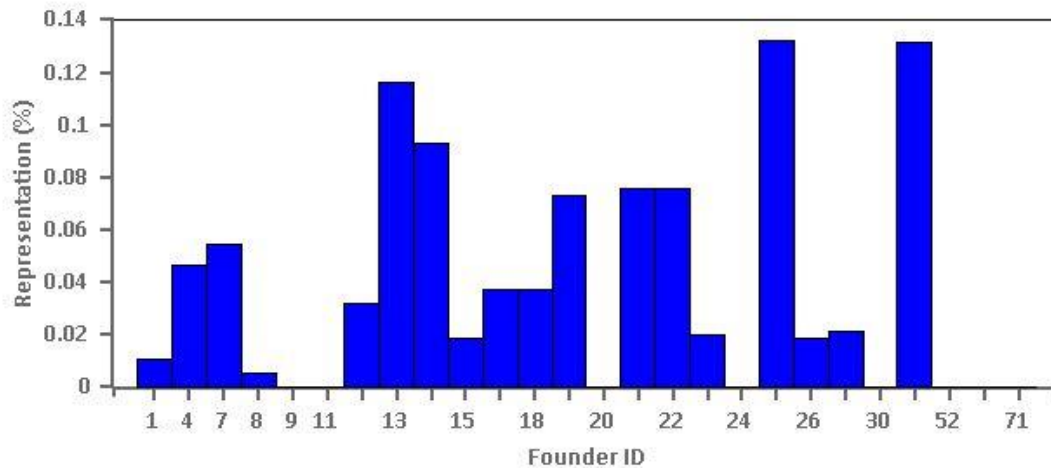
Figure 24. Graph illustrating the distribution of founder representations in the captive-born portion of the PPM population.

Table 14. Captive population genetics summary from studbook – definitions of terms are included in Appendix E.

	2016	**Current Potential**
Number of Founders	18	12 additional
Founder Genome Equivalents (FGE)	7.03	28.27
Gene Diversity Retained (%)	92.89	98.23
Population Mean Kinship	0.0711	-----
Mean Inbreeding (F)	0.01	-----
% Known Pedigree	100%	-----
% Ancestry Certain	92%	-----

These data combined with Studbook Pedigree information were used to decide on breeding priorities for 2017.

ENDOCRINOLOGY

Much stress physiology research, particularly in non-human animals, has focused on refining species-specific standards for measuring the activation of the hypothalmic-pituitary-adrenal cortex (HPA) axis via glucocorticoid (GC) production (Moberg 2000). Traditionally, adrenal cortex activity has been measured through analysis of GC concentrations in plasma, but blood sampling has drawbacks (Harper and Austad 2000; Keay et al. 2006; Lane 2006), including the increase in GCs that the sampling procedure itself may cause (Cook et al. 2000).

An alternative approach is to analyze GCs and their metabolites in feces (Touma et al. 2004). In this case, the collection method is non-invasive and hence less likely to affect GC production, and the timing of sample retrieval is more flexible than other methods (which is useful when direct access to animals is limited or not possible). In addition, fecal GC (FGC) levels represent an aggregate of GCs and their metabolites over a period of time (Keay et al. 2006).

Therefore, FGC levels can be useful in understanding how persistent stressors can affect animals and their welfare. This use extends to assessing stress in animals that are part of conservation programs as these typically involve some combination of stressors, such as captivity, marking, monitoring, transport and handling, in addition to environmental and social disturbance. Mitigating stress responses to these procedures may ultimately be important for the success of conservation efforts (Dickens et al. 2010; Teixeira et al. 2007). As a result, conservation researchers have begun to investigate the effect on FGC levels of common conservation practices (e.g., trapping, Harper and Austad 2001; transport, Millspaugh et al. 2007; radio transmitters, Pereira et al. 2009; Wells et al. 2003; captivity, Rothschild et al. 2008).

Many wildlife conservation projects in recent years involve the translocation of free-ranging animals. However, translocation as a practice has been historically associated with a high animal mortality rate (Griffith et al. 1989). As typical translocations involve at least all of the stressors aforementioned, a few studies have examined FGC levels in response to translocation events (Dickens et al. 2009a, 2009b; Franceschini 2008; Pinter-Wollman 2009; Viljoen et al. 2008). While FGCs and their metabolites have been examined in a growing number of species of conservation concern, the biological relevance of the technique has been validated in only a small percentage of the species (Touma and Palme 2005). Because of the high variability in GC metabolism and excretion within and across species, validation steps are crucial to reliably assess adrenocortical activity for a given species (Hunt et al. 2004).

In Phase 1 of this program, we collected fecal samples across time periods and contexts to bank for assaying. We began by determining if PPM showed a diurnal cycle in their stress hormones. We found is no clear pattern of diurnal activity of cortisol for male or female PPM.

In Phase 2, we attempted both behavioral and pharmacological challenges meant to modulate adrenocortical activity. Pharmacological tests have proved useful for validation. However, as validation results may not be straightforward, it is recommended to take advantage of biologically relevant stressors (e.g., restraint, agonistic interactions, predator stimuli) to validate assay methods as well (Touma and Palme, 2005). As a behavioral challenge, we presented PPM with a naturally occurring stressor, predator urine (fox). Exposure to predator urine has been shown to stimulate adrenocortical activity in other animals (Harris et al. 2012; Masini et al. 2005; Monclús et al. 2006).

PHARMACOLOGICAL EXPERIMENT

In 2014, we began the pharmacological challenge experiment ant to modulate adrenocortical activity. The pharmacological tests included the administration of adrenocorticotropic hormone (ACTH) and dexamethasone (DEX). ACTH is secreted by the anterior pituitary gland as part of the stress response by the HPA axis. ACTH, in turn, promotes a sharp increase in GC secretion from the adrenal cortex, which has been detected in fecal assays for a variety of species after the injection of ACTH (Palme et al., 1998; Wasser et al. 1997). DEX is a synthetic GC; injection of

this compound activates the negative feedback loop of the HPA axis, thus reducing the level of endogenous GCs secreted into the bloodstream. After DEX injection a marked dip in the concentration of GCs and/or their metabolites has been observed in the feces in a range of vertebrate species (Sheriff et al. 2010; Touma et al. 2005; but see Dehnhard et al. 2003).

METHODS

We tested n=20 captive PPM (10 males: 10 females) in the ACTH challenge in fall of 2014 and replicated the experiments with n=20 wild-caught PPM founders (10 males: 10 females) in fall of 2015. Each PPM was assigned to one of four treatment groups: 1) ACTH treatment; 2) DEX treatment; 3) saline treatment; or 4) undisturbed treatment. The saline treatment group was needed so we could distinguish between the effects of the injection itself and the true pharmacological responses to ACTH and DEX. Injections were administered twice, 20 minutes apart. Fecal samples were collected every 2-4 hours for 48 hours (Table 15; Touma et al. 2003; Touma and Palme 2005) for a total of 19 fecal collections per individual starting at time 0 which immediately proceeded the injection. We used the following dosage for ACTH 0.1µg/gm, which will meet the 2 IU/kg dose recommended by Wasser and colleagues (S. Wasser personal communication, 2012). Dexamethasone (Dexamethasone sodium phosphate 4mg/g) was injected at doses of 1µg/g and replicated with a higher dose 8µg/g. The saline group was injected with normal saline (0.9% weight/volume NaCl). For injections, one person carefully scruffed the animal as another stabilized the legs. Dr. Jack Allen, veterinarian at the Zoo, conduct all injections. The injection took no more than 1 minute. Following injections, animals were placed immediately into PVC burrows and returned to their home cage.

Fecal samples were assayed for glucocorticoid metabolites using standard methods developed in the ICR endocrinology lab, which have been conducted successfully for dozens of species and were discussed in detail in our Phase 1 report. Immunoassays (enzyme immunoassay and/or radioactive immunoassay) specific for cortisol and corticosterone was tested for cross reactivity to determine the most effective measure of corticoid metabolites. *High-performance liquid chromatography* (HPLC) separation was used to identify peak immunoreactivity as compared to known standards.

Data Analysis

We used multilevel modeling to examine the relationship between history (wild-caught vs. captive-born), treatment (Control, Saline, ACTH or DEX) and time.

Table 15. Fecal collection regime over 48 hours.

Collection	Time Period	Time of Day
Baseline	0	0740-0900
1	B+4h	1200-1300
2	B+8h	1600-1700
3	B+(8-12h)	1600-2100
4	B+(12-14h)	2000-2300
5	B+(14-16h)	2200-0100
6	B+(16-18h)	0000-0300
7	B+(18-20h)	0200-500
8	B+(20-22h)	0400-0700
9	B+(22-24h)	0600-0900
10	B+(28h)	1200-1300
11	B+(32h)	1600-1700
12	B+(32-36h	1600-2100
13	B+(36-38h)	2000-2300
14	B+(38-40h)	2200-0100
15	B+(40-42h)	0000-0300
16	B+(42-44h)	0200-0500
17	B+(44-46h)	0400-0700
18	B+(48h)	0600-0900

RESULTS and DISCUSSION

Results from the pharmacological validation experiment indicate that fecal corticoids can be used to assess stress in wild-caught PPM. Results show the predicted responses in wild-caught PPM but not captive-born PPM. There was a significant three-way interaction effect indicating that the mouse's history (captive-born or wild-caught) was predictive of how they responded to the pharmacological treatments over time (GLMM: χ^2 = 215.66, p< 0.001). For wild-caught mice, ACTH elevated fecal cortisol, peaked 14-16 hours after administration (collection time period 5: 2200-0100 hours: z= 2.17, p = 0.03; Figure 25) while DEX suppressed fecal cortisol levels with the lowest levels detected during the same time frame relative to control mice (z= -3.05, p = 0.002; Figure 26). Captive-born mice showed a significantly different response to the pharmacological treatments over time with significantly lower cortisol levels overall (20-80 ng/g compared to 20-300 ng/g for wild-caught mice). Sex did not provide any useful explanatory information.
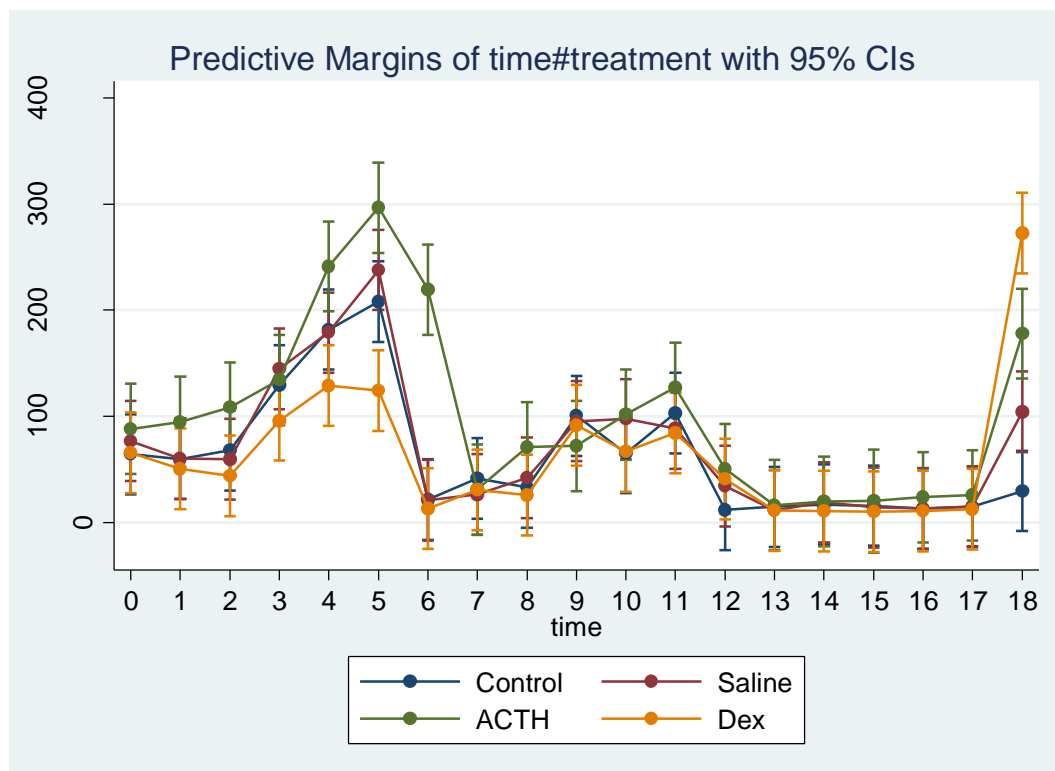
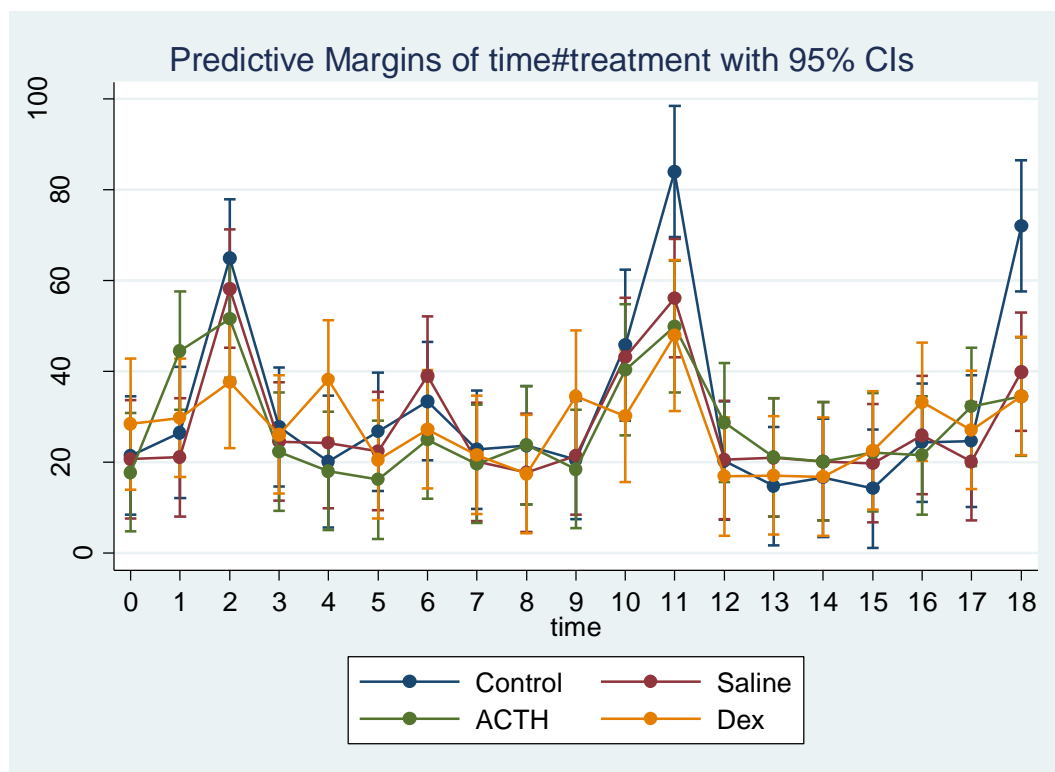Figure 25. Effects of pharmacological treatment on wild-caught PPM over 48 hours.

Figure 26. Effects of pharmacological treatment on captive-born PPM over 48 hours.

Our results show that we have developed a radioimmunoassay that is specific for cortisol in PPM rat fecal extract. Furthermore, the increase in fecal cortisol concentrations in PPM after exposure to predator urine suggests that fecal cortisol concentrations are indicative of adrenocortical activity (Cockrem and Silverin, 2002; Harris et al., 2012; Masini et al., 2005; Monclús et al., 2006).

Our results indicate that FC is a reliable indicator of adrenal activity, being detected by our cortisol antibody. However, we do not presume that cortisol is the most prevalent circulating form of glucocorticoid. Restrictions in our protocol prevented us from taking blood samples from the pocket mice, but previous research on Merriam's kangaroo rat suggests that both cortisol and corticosterone are present in circulation (Preston 2001). The lack of specificity of the two corticosterone antibodies relative to the cortisol antibody is likely due to the complexity of the extract and the origin of the antibodies. Fecal extract contains a mixture of parent compounds, the products of endogenous GC metabolism and microbial degradation, and artifacts of the extraction procedure. The polyclonal corticosterone antibodies reacted similarly with numerous HPLC fractions, limiting the utility of either antibody for our purposes. On the other hand, the monoclonal antibody showed a reasonable degree of specificity for HPLC fractions corresponding to the retention time of cortisol. Although these results alone do not confirm cortisol as the immunoreactive analyte, when taken together with the increasing concentration following exposure to predator urine, they indicate that we have a valid measure of adrenal activity.

PREDATOR URINE EXPERIMENT

METHODS

We tested each of our n=20 captive PPM in 2 treatments: 1) exposed to predator urine (n=10; 5M:5F) and 2) control (water; n=10; 5M:5F). PPM were tested in our standard testing arena (24 x 24 inches). We purchased fox urine from predator pee.com.   Twenty-four hours prior to testing, we cleaned the cage of the focal subject being sure to remove all fecal pellets. On the night of the test, we removed 2-6 fecal samples from the cage to serve as a baseline for that subject. In each treatment, 1 ounce of predator urine was be placed in a baby food jar lid filled with clean sand and placed into the center of the arena. We then transferred the focal subject from its cage in a PVC tube and placed it into the arena. At the end of the 10 minute test, the focal subject was returned to its home cage. Sample collection began 2 hours after the completion of the test (Table 16). Fecal samples were collected at 2-4 hour increments for 48 hours following testing. We regarded all pellets collected from an individual for a given time window or during a collection event as a single sample. We stored fecal samples upon collection in a conventional freezer (set at -23º C) at the captive breeding facility and transferred them to -80º C freezers prior to assaying.

Table 16. Time points for fecal collection.

Collection	Code	Time of Day
Baseline	B0	2015-2230
1	B+2	2230-0030
2	B+4	0030-0230
3	B+6	0230-0430
4	B+8	0430-0645
5	B+10	0645-0830
6	B+(14-16)	1030-1230
7	B+(18-22)	1430-1630
8	B+(22-24)	1830-2030
9	B+24	2030-2230
10	B+26	2230-0030
11	B+28	0030-0230
12	B+30	0230-0430
13	B+32	0430-0630
14	B+34	0630-0830
15	B+(38-42)	1030-1230
16	B+(44-46)	1430-1630
17	B+(46-48)	1830-2030
18	B+48	2030-2230
19	B+50	2230-0030

RESULTS and DISCUSSION

Results from the predator urine experiment indicate that fox urine is not a sufficient stressor for cortisol to show up reliably in PPM fecal pellets. There was no effect of treatment (fox urine vs control; z=0.56, p = 0.577), history (captive born vs wild caught; z = 0.61, p = 0.545) or sex (z=-0.53, p = 0.599) on fecal corticoids. Given the results from the pharmacological validation experiment, if exposure to fox urine was a stressor for PPM, we would see the effects of the stressor show up in the fecal pellets during collection period 6 (14-16 hours following exposure). We did not (Figure 27). These results show that exposure to fox urine is not a stressor for PPM.
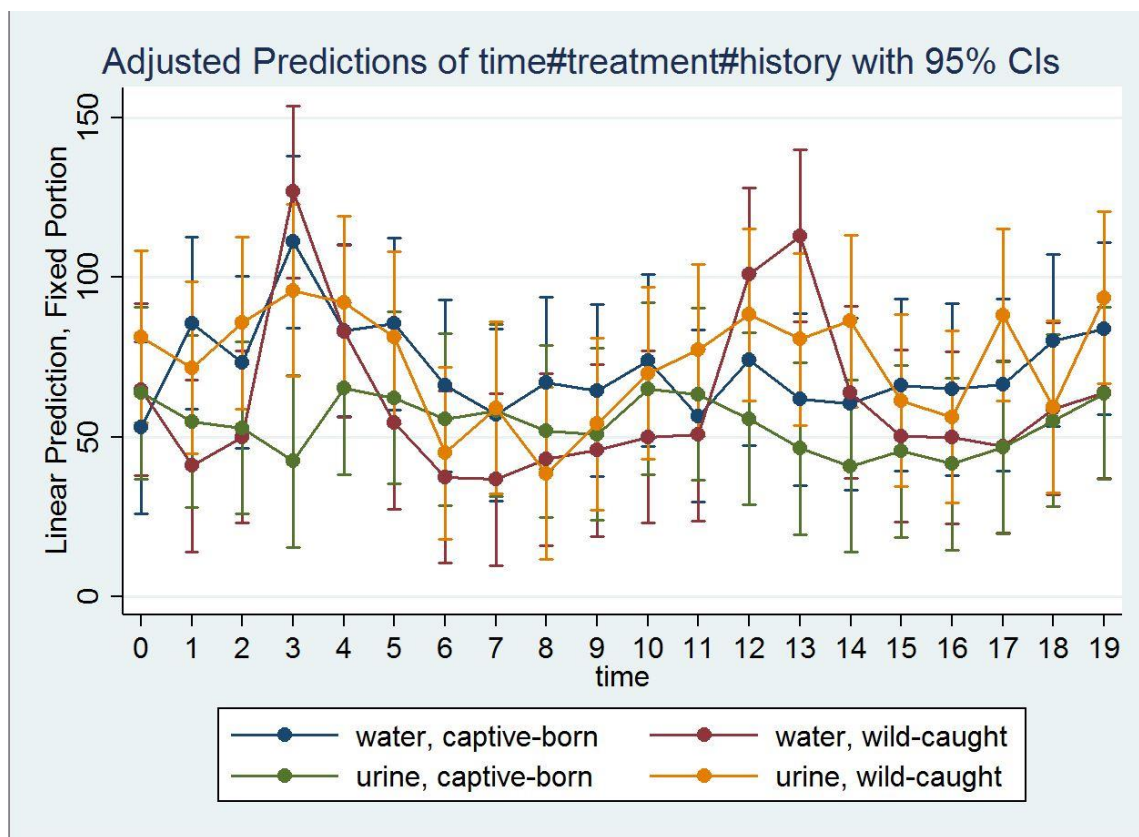
Figure 27. Effect of predator urine on PPM fecalcorticoids over a 48 hour period.

HEALTH AND DISEASE

Very little is known about disease in PPM. In Phase 1, we began to gather information to develop a disease risk assessment and mitigation plan for PPM by conducting health assessments. Upon capture of founders we documented the physical condition of captured PPM, and our veterinarians conducted health assessments upon entry into quarantine. During Phase 2, we updated our Disease Risk Management Plan (Appendix C) based on all health information gathered to date from mice in the captive colony and the information we have gathered on known viruses and bacterial pathogens found in the species, the species assemblage present at the source and potential release sites, and the PPM captive diet.

Assessing the health of wild-founders

For each founder, we assessed physical condition via inspection of the animals in the wild. We assessed the following: weight; pelage condition; external body condition; and ectoparasite load. Founders were combed systematically and any ectoparasites (e.g., fleas, ticks, lice) were collected into ethanol to be counted. A fecal sample was taken from each wild-caught founder and a fecal o/p test was run to examine individuals for endoparasites. The PPM fecal samples were soaked in saline and placed into a zinc sulfate solution for a flotation technique to increase the yield of any ova in the sample. All 4 PPM (2 males and 2 females) were healthy at intake and none had ectoparasites (ticks) upon capture (Appendix A). All fecal assays for endoparasites

were negative. The newly captured wild PPM from South San Mateo were judged to be healthy and were incorporated into our captive population.

Colony health

The PPM program maintains a healthy population of captive mice. Since the inception of the program, very few mice have presented with health issues. During Phase 1, we had the manifestation of nasal crust which is not contagious but continues to affect approximately 10% of our captive population including both wild-caught and captive-born animals. There has been no serious disease or damage from this chronic intermittent crusty material as the mice continue to behave normally and there are no associated physical symptoms (high morbidity, zero mortality). After Phase 2, it appears to be a part of PPM captive living. We also continued to see a small number of cases of kyphosis, a spinal deformity visible at birth that leads to premature death (see Appendix D for a list of cases).

In early May of 2016, mice began exhibiting clinical signs of ocular discharge, nasal discharge, and some facial swelling. Thirteen mice were affected. The onset of signs occurred about a week after a change in the seed in the diet from a primarily nonnative seed mixed to a native seed mix in order to transition the release cohort to a wild diet prior to reintroduction. Treatments included nonsteroidal anti-inflammatory drugs and antibiotics (administered per os) for pain management and a presumed bacterial problem. The small size of the patient will not allow for common ante-mortem diagnostics (blood testing, etc).

Diagnostic testing consisted of bacterial culture, but a specific cause was never identified. Ten mice immediately recovered but 3 were managed in isolation (separate bags, gloves, foot baths) for approximately one month. Six of the affected mice were in the release cohort.

Possible causes of this illness are unknown; differentials include trauma from seed, bacteria, viruses, other; no etiologies have been ruled in or out.   If this was infectious, it could have represented 1) an agent already present in the mice (though not previously causing clinical signs), or 2) something introduced indirectly via seed, cages (sitting outside after cleaning), or another unknown source. Based on an internal discussion among the program leads, veterinarians and pathologists, the decision was made to remove the 6 previously symptomatic mice from the release cohort. The decision was based on the fact that this was an unknown entity and though it appeared to be resolved, there was a small risk of ongoing disease / poor survival in released mice, or of disease introduction to other native rodents in the release site (deer mice, kangaroo rats, etc). One caveat: if this was an infectious agent, there was also a possibility that all the mice had been exposed. Thus, even removing these 6 recovered animals from the cohort did not entirely remove risk related to this disease.

California Department of Fish and Wildlife (CDFW) and USFWS staff were briefed on the issue and together it was decided that the despite the low risk, alternate animals would replace all mice in the release cohort that showed any signs of the eye illness and the reintroduction would proceed.

In response to this illness, we implemented new biosecurity measures to minimize the risk of exposure and spread from mouse to mouse. These included using individual bags for each mouse for weighing, reproductive condition checks and health checks. All individual bags are replaced with damaged or soiled. The use of gloves and hand sanitation via hand washing or gel

in between handling of mice. Disinfect the scales between each use with PPM.   These new biosecurity measures were in addition to twice yearly complete cleaning of all cages including removal of all sand and bleaching of all surfaces. The new emphasis on biosecurity and handling has played an important role in managing the health of the PPM. Since May 2016 we have not seen any new cases.
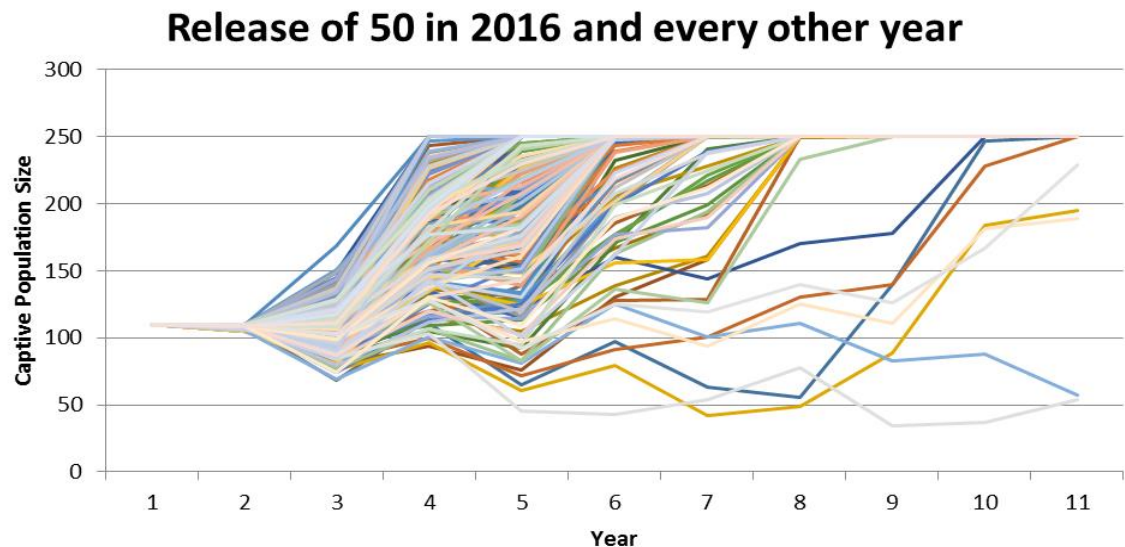
REINTRODUCTION

Genetic Modeling for Release

In 2014, we reevaluated our Genetic Management Plan to determine if with data from the breeding facility, we would be able to reintroduce mice in 2015. The captive population was aging and reintroduction research on other species has shown that reintroductions are more successful if animals are held in captivity for fewer generations and/or shorter periods of time. To develop our genetic management plan, we used the software program, Vortex. However, this program did not allow us to take into account some of the important aspects of PPM reproduction phenology such as mate preferences and the fact that young of the year can reproduce in the year that they are born. Thus, in 2015, our Population Geneticist, Jamie Ivy, developed a species specific model for PPM. We incorporated the data from the captive facility and modeled the captive population viability and genetic diversity with removal of various numbers of PPM for reintroduction under several scenarios. The modeling indicated that we could release 50 mice in 2016 and every other year while maintaining captive population viability and genetic diversity (Figure 28a).

Alternatively, should we reintroduction 50 mice in 2016 but need to supplement the population to provide support during establishment, we could release 50 in 2016 and supplement with 25 in 2017 (Figure 28b). However, under this scenario, we would need to avoid releases in 2018 and 2019 in order to ensure that the captive population did not crash.

Several other scenarios were examined, but models indicated that all scenarios that either put more animals out or put them out more frequently would cause the captive population to crash (See Figure 28c for an example).

a)

b)

Release of 50 in 2016 and 25 in 2017

c)

Release 40 in 2016 and
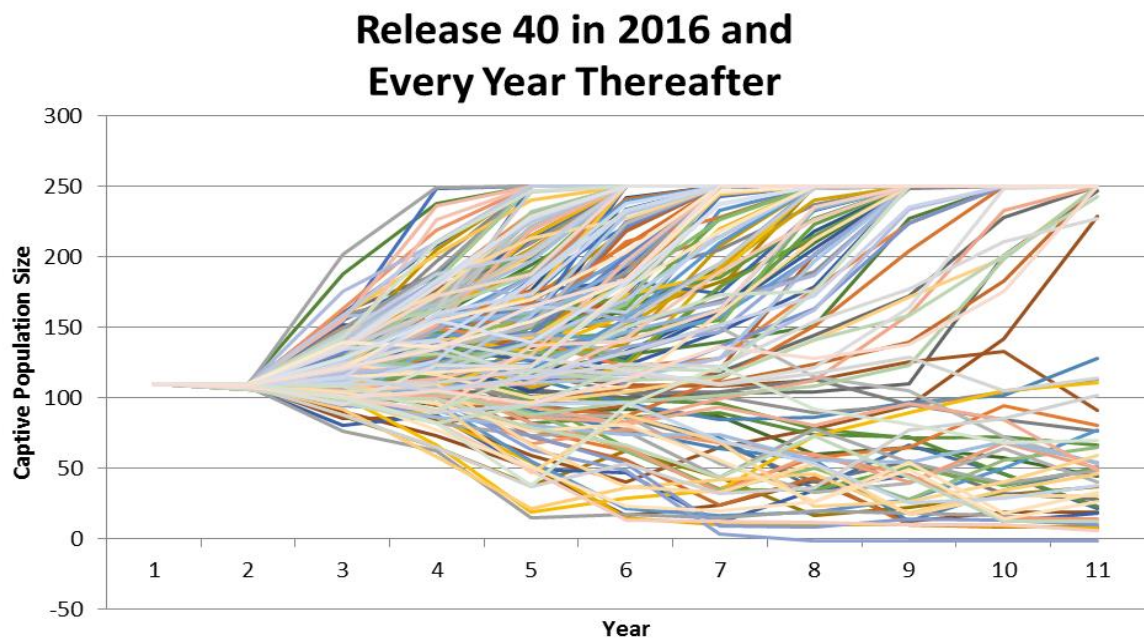Every Year Thereafter
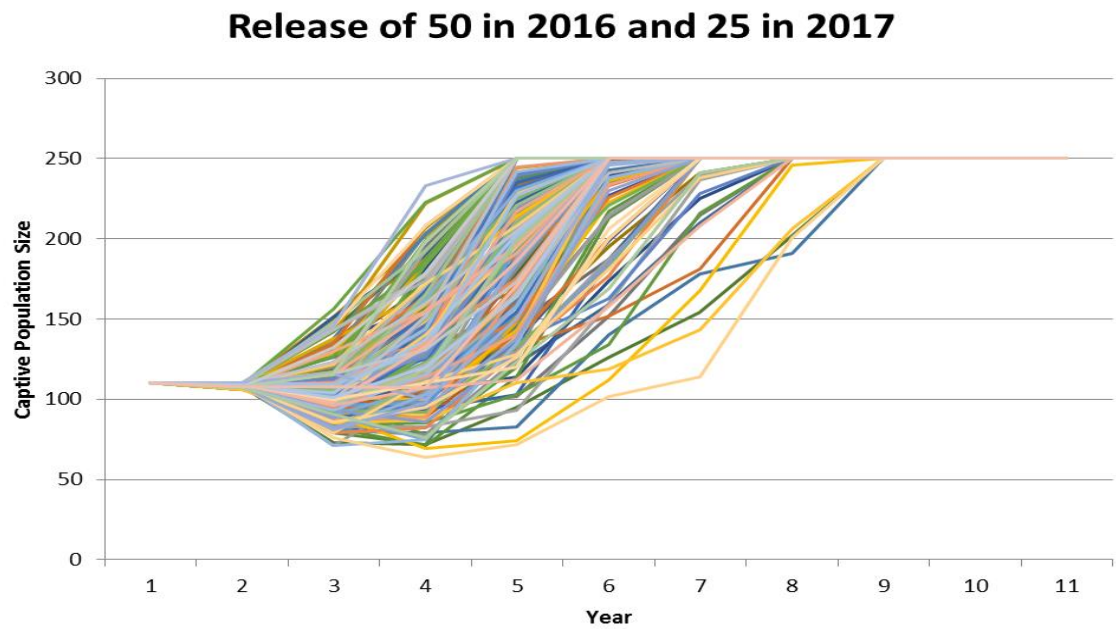
Figure 28. PPM genetic models.

By the end of the 2015 breeding season, there were 110 PPM in the captive breeding facility. Based on the results of the PPM specific model, we proposed to reintroduce our first release cohort of 50 mice in the spring or early summer of 2016.

METHODS

Release site permissions and preparation

All necessary permits, i.e. County, California Environmental Quality Act (CEQA), and State Historic Preservation Office (SHPO) were obtained. Archaeologists surveyed the reintroduction site, and did not find any cultural resources; however, as per the request of The Tongva Ancestral Territorial Tribal Nation, an archaeologists was present during the installation of the dispersal dampening/predator exclusion fence, and acclimation cages.

Habitat at the release site was assessed for the presence of sensitive plant and animal species prior to the introduction of PPM at the release site. During reconnaissance of the site in December of 2013 and October of 2015, no sensitive plant species were observed, but a seasonal depressional wetland with potential to support the western spadefoot toad (*Spea hammondii*) and a coastal California gnatcatcher (*Polioptila californica californica*) were observed near the edge of the proposed reintroduction site. Because the location of enclosure fencing and artificial burrows were not fixed, impacts to the seasonal wetland were avoided by leaving it outside the boundaries of the release site, and impacts to any sensitive plant species were avoided by aligning fencing and locating artificial burrows and human access routes away from sensitive plant locations. While impacts to the gnatcatcher could be avoided by locating the release site away from the gnatcatchers' territory(ies), surveys were performed concurrent with sensitive plant surveys to better understand habitat use by gnatcatchers in the project vicinity. Gnatcatcher surveys were conducted by William Miller of USFWS While there were gnatcatchers in the vicinity, none were currently nesting in the reintroduction site. Overall, adverse impacts to the gnatcatcher were avoided by taking gnatcatcher territorial habitat use into consideration when locating project fencing around the release site.

Habitat at the release site was proposed to be managed for suitability for PPM with a target of no more than 60% shrub cover and no more than 50% annual grass cover. Based on the low cover of shrubs and grasses observed at the site in October of 2015 and in the spring of 2016, no shrub or grass cover manipulation was necessary.

Upon observing small burrows (PPM size) at the site, there was a concern that there were PPM already present; therefore, we trapped for one night in May prior to the installation of the fence. No PPM were caught.

Once the fence was installed, the release site was trapped to determine nontarget small mammal densities. This information allowed us to determine where to site acclimation cages and if nontarget densities needed to be reduced prior to release of PPM. Research on interspecific competition in pocket mice not covered by this grant is underway (see Appendix G for details) and has shown that of the sympatric rodent species that PPM live among, PPM experience the highest levels of competition from the Dulzura kangaroo rat (*Dipodomys simulans*) and Deer mice (*Peromyscus Maniculatus*)(See Appendix G, Figures 6 and 7). Thus, to reduce potential competition following release, we assessed the densities of all sympatric rodents and removed

them in cases in which they were assessed as medium or high (see Appendix F: Reintroduction plan for density categories by species). Because the densities of Deer mice and Dulzura kangaroo rats were high (Table 17), we nonlethally reduced (i.e. moved outside the fence) the densities of these species to minimize competition with PPM once released.

To limit dispersal and allow pocket mice to acclimate to the new site (Germano 2001, Long et al. 2006), sites were prepared for "soft release". An acclimation cage was constructed for each pocket mouse (Figure 29). Acclimation cages consisted of an underground wood retention basket (15.2 x 15.2 x 7.6 cm) set 0.3m underground, two biodegradable cardboard mailing tubes (2.5 cm-diameter), which connected the underground chamber to the surface on either side, and an above-ground wire mesh retention cage (.2 x .2 x 0.2 m made of 0.635cm mesh; Figure 29). This design allowed for movement of pocket mice between the underground chamber and the above-ground retention cage, but deterred escape during the acclimation period (Long et al. 2006; Shier 2009; Shier and Swaisgood 2012a; Truett et al. 2001). Acclimation cages were placed approximately 5m apart (depending on the terrain) in a grid configuration with a 20-25m buffer around the perimeter of the grid allowing PPM to spread out and space for PPM density to increase within the enclosure (Figure 30). This spacing approximates medium to high burrow density in the wild (Shier, unpublished data). The grid and buffer zone encompassing 1.26-1.64 acres was temporarily surrounded by a 4-6 ft. fence buried 1 foot below the surface to dampen dispersal and deter predation attempts (Figure 30). We installed remote cameras within the release enclosure to monitor PPM activity during acclimation and following release.

Table 17. The number of California pocket mice (CHCA), Dulzura kangaroo rat (DKR), cactus mouse (PEER), and deer mice (PEMA) trapped and removed (non-lethally) from the inside of the predator exclusion/dispersal dampening fence.

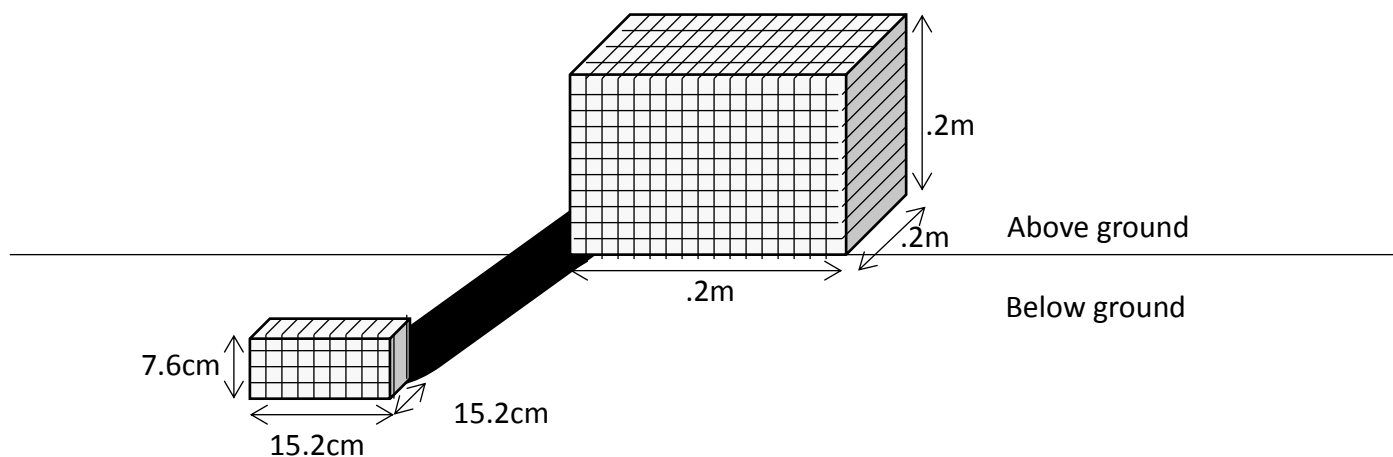|	CHCA	DKR	PEER	PEMA
Trapped	2	11	4	18
Removed	0	3	0	11
Total	**2**	**8**	**4**	**7**

Figure 29. Acclimation cage for PPM

Figure 30. Release site at Laguna Coast Wilderness Park

Selection of Release cohort

Using PMx, and maintaining the genetic diversity of both the captive and reintroduced populations, we selected 50 mice (25 males, and 25 females, and 10 alternates to make up the release cohort (Table 18). It was suggested that we might want to consider including a small percentage of wild-caught captive breeding founders in the release cohort in order to capitalize on differences in their survival skills following release. We took this recommendation into consideration and included n=5 founders (10%) in the release cohort.

Table 18. Release cohort.

PPM ID	Sex	Generation	Acc. Cage
2	Female	0	2
12	Male	0	1
13	Female	0	48
21	Male	0	49
22	Female	0	44
29	Female	1	26
38	Male	1	50
43	Female	1	4
48	Male	2	3
49	Male	2	5
62	Male	2	7
64	Female	2	6
65	Female	2	8
66	Male	2	9
67	Male	2	36
70	Female	2	30
73	Male	1	38
74	Male	2	40
79	Female	2	32
82	Female	2	46
86	Female	2	35
88	Female	2	37
91	Male	2	41
92	Female	2	39
95	Male	1	47
96	Male	2	34
99	Female	2	42
100	Male	2	31
101	Female	2	43
104	Female	1.5	45
108	Female	2	29
110	Female	2	33
112	Male	1	28

114	Female	1	23
115	Male	1	27
116	Female	1	21
117	Male	1	25
120	Female	2	18
121	Male	2	24
122	Female	2.5	17
124	Male	2.5	22
126	Male	2.5	20
129	Male	1	19
132	Female	2.5	14
135	Female	2	12
137	Male	2	16
143	Female	2.5	10
144	Male	2.5	15
145	Male	2.5	13
147	Male	2.5	11

Animal Preparation

Marking with unique IDs

We marked all mice with P-chips prior to release to the wild. These tiny microtransmitters were placed into their tail and are read with a laser scanner providing a reliable method for determining individual identification.

Diet transition

Our nutritionist created a plan to gradually (over six weeks) transition the diet for the mice in the release cohort from finch seed and greens (Romaine lettuce and spinach) to native seeds. A diet transition was also created for females that were to be released and on the lactating diet. These plans were implemented.

Survival skill training

All PPM in the release and alternate cohorts received snake and owl antipredator training (see Predator Training Protocol above). One mouse did not display the appropriate antipredator response. She was removed from the release cohort and replaced with an alternate.

Additionally, once the mice were transferred to their acclimation cages at the release site, a founder male failed to use the underground portion of his acclimation cage. After 2 days, we made the decision to bring this male back to the conservation breeding facility. He was quarantined and reintegrated into the captive colony. A mouse from the alternate cohort was then transferred to the release site to replace him.

Disease risk assessment

As 6 of the mice included in the release cohort had at one point exhibited minor ocular discharge and/or facial swelling, we had an internal discussion with our Wildlife Disease pathologists and Veterinarians regarding the risk of introducing a novel pathogen into the wild. We decided that even though the risk of introducing any pathogen into the wild was extremely low, we would replace all 6 mice that had had symptoms for any length of time with mice from our alternate list that were never symptomatic.

Additionally, after extensive research (Decker et al. 2001; Garcia-Prieto et al. 2008; Harkness et al. 2010; Mantooth et al. 2001; Parmenter et al. 1998; Zhao and Duszynski 2001), we could not identify any agents that would require active management once the PPM were reintroduced (see Disease Risk Assessment Section for full discussion of eye illness).

Health Checks

All animals in the final release cohort had a pre-ship medical examination (including a fecal analysis) by a veterinarian. All mice were reported as healthy and obtained a health certificate.

Transfer of PPM to the Release Site, Acclimation Period and Release

We transferred all 50 members of the release cohort to the acclimation cages at dusk on June 6, 2016. While in the acclimation cages, the mice were fed daily. They were held in cages for 7 days. On the evening of June 13, 2016, we released the mice by removing the above-ground portion of the acclimation cages.

Post-release Supplementation and Monitoring

Supplemental Feeding

We placed 50 supplemental feeding stations (PVC t-tubes) throughout the enclosure. The mice received locally sourced native seeds, autoclaved finch seeds, and lettuce. We provisioned the site three times a week for the first month post-release, and then reduced the feeding to weekly through September 30, 2016.

Behavioral Observations

On the night of the release and one week post-release, we conducted focal observations at the artificial burrows. Several natural behaviors such as foraging, digging, and sand bathing were observed. Numerous competitors (i.e. Cactus mouse, California pocket mouse, deer mouse, and Dulzura kangaroo rat) were observed as well.

Camera Trapping

We set up 20 remote cameras to collect images (photographs and videos) throughout the enclosure. The cameras were set facing acclimation cages, new small burrows, scorpion burrows, feeding stations, or open spaces. We saw PPM exhibit natural behaviors (e.g. foraging, grooming, and digging), and use the supplemental feeding stations (Figure 31), and the biodegradable underground portions of the acclimation cages (Figure 32).

Figure 31. A Pacific pocket mouse inside a supplemental feeding station at Laguna Coast Wilderness Park on August 3rd, 2016.

Figure 32. A Pacific pocket mouse entering the underground portion of the acclimation cage at Laguna Coast Wilderness Park on August 15th, 2016.

We used the program Adobe Bridge CC to categorize and process the images (photographs and videos) obtained (see Appendix H for Photo Organization Protocol).

Measuring Reintroduction Success

We conducted trapping to assess the short term release success of the PPM 1-month, and 3-month post-release, and to use the data to estimate survivorship, and population size or density of PPM and heterospecifics. Competitor populations were also reduced if the number of individuals was above the low range. Two hundred and thirty-seven traps (5 m spacing) were checked twice a night for four consecutive nights. Table 19 shows the individuals caught and removed at the 1-month trap check:

Table 19. Non-target removal 1- month post release

Species	Individuals	Removed	Remaining
CHCA	2	0	2
DKR	8	1	7
PEER	2	0	2
PEMA	9	3	6
PPM	22	0	22

As of mid-July 2016, a minimum of 24 PPM were known to be alive (48%; 10 males, and 14 females) from the trapping efforts. The PPM appeared healthy, and had good weights. Additionally, males were scrotal, and females were showing signs of cycling.

Table 20. Non-target removal 3- month post release

Species	Individuals	Removed	Remaining
CHCA	2	0	2
DKR	11	2	9
PEER	2	0	2
PEMA	4	0	4
PPM	11	0	11

As of mid-September, a minimum of 11 PPM were known to be alive (22%; 6 males, and 5 females) from the trapping efforts. All of the PPM caught were born in either 2014 or 2015. The PPM appeared healthy, and had good weights. One female showed signs of lactating. It is possible that PPM had begun to aestivate at this site as they had on South San Mateo and Dana Point, in which case the number of PPM surviving on the site would have been higher than the minimum estimate.

Fecal Collection

We attempted to collect fecal samples at all stages of the release (i.e. pre-release, during acclimation, 1-month post-release, and 3-months post-release). We successfully obtained fecal samples at all stages with the exception of the acclimation period. These samples will be assayed to determine levels of corticosterone, which will inform us on stress levels during the reintroduction process.

CONCLUSION

During Phase 2 we continued captive breeding of the endangered Pacific Pocket Mouse. We brought an additional 4 founders into the program. Within the captive breeding facility, the health of all of the collected founders was assessed and each individual was genotyped. Behavioral research continued to provide direction for captive management and reintroduction. Perhaps most importantly, we successfully produced a sufficient number of healthy PPM to conduct our first reintroduction back into the wild. Early results of this reintroduction effort are promising but given the small number of individuals, we propose to supplement the release site in 2017 with a second group of founders.

INCIDENTAL DEATHS

We had 27 PPM mortalities associated with this research during Phase 2.

Death of adult founders:

1. Female 3, a founder from Dana Point, was brought into captivity on 6/21/2012 as an adult. She was found dead in her cage on December 8, 2014. She had a long history of nasal infection and had been treated with antibiotics off and on for several months.
2. Male 4, a founder from Dana Point, was brought into captivity on 6/21/2012 as an adult. He was found dead in his cage on December 8, 2014.
3. Male 23, a founder from South San Mateo, was brought into captivity on 8/17/2012 as an adult. He was found dead in his cage on 5/10/2015. He was found dead in his cage after being wounded during a pairing with a female during a breeding attempt.
4. Male 1, a founder from Dana Point, was brought into captivity on 6/21/2012 as an adult. He was found dead in his cage on June 28, 2015.
5. Male 17, a founder from Santa Margarita, was brought into captivity on 7/2/2012 as an adult. He was found dead in his cage on February 2, 2016.

Death of PPM produced in captivity:

1. Pups #75-77 were all from the same litter. Their mother, wild-caught female 71 suffered a prolapsed uterus during delivery and neglected the pups after birth. All were found dead on 7/2/2014.
2. Pup #84 was born on 7/14/2014 to Female 34 and Male 46. It was found dead on 8/4/2014 with a partially torn stomach.
3. Pup #81, female was born on 7/14/2014 to Female 34 and Male 46 with a spinal deformity, Kyphosis. She was found dead on 11/8/2014 and cause of death was the spinal deformity.
4. PPM #32 female was born on 7/5/2013 to Female 22 and Male 21. She was found dead on 12/16/2014.
5. PPM #31 male was born on 7/5/2013 to Female 22 and Male 21. He was found dead on 3/24/2015.
6. PPM #68 male was born on 5/23/2014 to Female 33 and Male 44. He was found dead in his cage on 4/29/2015.
7. Pup #98, female was born on 4/21/2015 to captive born PPM Female 33 and Male 44. She was found dead on 6/6/2015. She was the runt of the litter.
8. Pup #111 was born on 5/25/2015 to captive born female 82 and wild-caught male 12. It was found dead on 6/15/2015. It was the runt of the litter.
9. PPM #78 was born on 7/14/2014 to captive born female 34 and male 46. He was found dead on 6/23/2015.
10. PPM #34 female was born on 7/5/2013 to Female 22 and Male 21. She was found dead on 7/15/2015.
11. Pup #107, male was born on 5/25/2015 to Female 82 and Male 12 with a spinal deformity, Kyphosis. He was found dead on 7/17/2015 and cause of death was the spinal deformity.

12. Pup # 138 was born on 7/31/2015 to female 86 and male 72. It was found dead on 8/16/2015 outside of its nest jar.
13. Pup #133, male was born on 7/31/2015 to female 86 and male 72 with a spinal deformity, Kyphosis. He was found dead on 9/2/2015 and cause of death was the spinal deformity.
14. Pups #139-140 were all from the same litter to female 64 and male 73. They were found dead on 9/9/2015 to infanticide by the mother.
15. Pup #146, male was born on 8/31/2015 to Female 87 and Male 73 with a spinal deformity, Kyphosis. He was found dead on 9/27/2015 and cause of death was the spinal deformity.
16. PPM #90 female was born on 3/31/2015. She became pregnant in early 2016 but surpassed her due date. We decided that a c-section was required to attempt to save the life of the female. A c-section was performed and the 5 unborn pups were dead in the womb and removed. The female survived for 3 days but was found dead on 4/20/2016 likely due to complications from the c-section.
17. PPM #182 female was born on 5/20/2016 to Female 106 and Male 16. She was found dead on 6/26/2016. She was the runt of the litter.
18. PPM #153 female was born on 3/28/2016 to Female 132 and Male 115. She was found dead on 7/17/2016.
19. PPM #33 female was born on 7/5/2013 to Female 22 and Male 21. She was found dead on 9/13/2016.

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APPENDIX A - HEALTH STATUS OF FOUNDERS DURING QUARANTINE

All newly captured PPM founders were quarantined for 14 days. All PPM were inspected upon entry into quarantine and a bodyweight taken. Weekly bodyweights were obtained. A single fecal o/p exam was done and results were negative. A visual exam was done during the quarantine period by a veterinarian. All PPM appeared to be in good health.

PPM #207   Male   #616496: none

PPM #208   Female   #616497: none

PPM #209   Male   #616498: none

PPM #210   Female   #616499: none

APPENDIX B – FEEDING AND ENRICHMENT SCHEDULE

Table 1. Feeding schedule for <u>males and non-lactating females (Feb 1st – Sept 30th)</u>

Sunday	Monday	Tuesday	Wednesday	Thursday	Friday	Saturday
1/2 tsp. seed mix	1/2 tsp. seed mix	1/2 tsp. seed mix	1/2 tsp. seed mix	1/2 tsp. seed mix	1/2 tsp. seed mix	1/2 tsp. seed mix
Romaine Lettuce (2" x 2")	1 mealworm	Spinach (1 leaf)	***Enrichment item	Romaine lettuce (2"x 2")	1 mealworm	Spinach (1 leaf) ***Enrichment item

Table 2. Feeding schedule for <u>males and non-lactating females (October 1st – Jan 31st)</u>

Sunday	Monday	Tuesday	Wednesday	Thursday	Friday	Saturday
1/2 tsp. seed mix		1/2 tsp. seed mix		1/2 tsp. seed mix		1/2 tsp. seed mix
1 mealworm		Spinach (1 leaf) ***Enrichment item		Romaine lettuce (2"x 2") 1 meal worm		Spinach (1 leaf) ***Enrichment item

Table 3. Feeding schedule for lactating females

Sunday	Monday	Tuesday	Wednesday	Thursday	Friday	Saturday
1/2 tsp. seed mix	1/2 tsp. seed mix	1/2 tsp. seed mix	1/2 tsp. seed mix	1/2 tsp. seed mix	1/2 tsp. seed mix	1/2 tsp. seed mix
Romaine lettuce (2"x 2")	Spinach (1 leaf)	Romaine lettuce (2"x 2")	Spinach (1 leaf)	Romaine lettuce (2"x 2")	Spinach (1 leaf)	Romaine lettuce (2"x 2")
¼ tsp. powdered milk	1 mealworm	¼ tsp. powdered milk	***Enrichment item	¼ tsp. powdered milk	1 mealworm	***Enrichment item

Enrichment

- When available, four California native seeds types are used for enrichment (*Croton californicus, Eriogonum fasciculatum, Salvia* apiana, and *Stipa* pulchra) and black oiled sunflower seeds
- Half of a paper towel roll will be provided as extra cover and for chewing as well at all times

Distribution:

- A pinch of seeds is sprinkled throughout the entire cage, to encourage foraging

Table 4. The list and order of enrichment seeds offered

Enrichment
Stipa pulchra
Eriogonum fasciculatum
Salvia apiana
Black oil sunflower seed
Croton californicus

APPENDIX C – DISEASE RISK MANAGEMENT PLAN

The San Diego Zoo Institute for Conservation Research has conducted a disease risk assessment for the species prior to reintroduction.

Potential Hazard Identification

The list of known potential pathogens in Heteromyids is short, including oxyurids and other nematodes, *Eimeria* sp., cestodes, acanthocephalans, and Hantavirus (Decker et al. 2001; Garcia-Prieto et al. 2008; Harkness et al. 2010; Mantooth et al. 2001; Parmenter et al. 1998; Zhao and Duszynski 2001). However, these are considered endemic agents, which means they are either nonpathogenic to the host or lack epizootic potential. Other plausible agents include *Salmonella* sp., *Yersinia* sp., and *Coxiella* sp. These agents are similarly considered endemic opportunists lacking epizootic potential (Harkness et al. 2010).

Disease Risk

The risk of significant disease introduction or spread is low, as no significant hazards have been identified. The fact that translocations and reintroductions are being limited to historic range in close proximity to current range (i.e., within the same ecological zone for any known or unknown pathogens) also minimizes disease risk, including the theoretical risks posed by unknown pathogens that might be present.

Risk Mitigation

No agents requiring active surveillance have been identified. Recommendations for risk mitigation are therefore limited to best practices for population management and reintroduction and translocation planning. These include routine risk matching for translocations and reintroductions (matching characteristics of source and destination populations/locations by staying within historic range), population health assessments by a veterinarian prior to animal movements, and population-level disease surveillance through postmortem examinations on the captive and wild populations.

Quarantine of Mice Brought into Captivity

Wild-caught animals will be quarantined for a minimum of 14-30 days in the Harter Veterinary Medical Clinic or in the PPM facility (each population held in separate rooms) at the Safari Park. Each population will be "all in, all out" quarantine meaning the 14-30 day time period starts on the day the last animal arrives. During the quarantine period, each animal will be examined by a veterinarian for assessment of overall health and presence of ectoparasties, have body weights obtained at least twice during this time frame, and have feces examined for parasites or ova. Quarantine will allow the identification of any health issue so that the wild-caught animals can be treated and ensure that no infectious or contagious agents are brought into the captive population.

Prevention of infectious or contagious agent spread within the captive colony

In response to the eye illness observed in the late spring of 2016, we recommend biosecurity measures to minimize the risk of exposure and spread of any infectious or contagious agent from mouse to mouse. These included the following: 1) Use of individual bags for each mouse for weighing, reproductive condition checks and health checks. All individual bags to be replaced with damaged or soiled; 2) The use of gloves and hand sanitation via hand washing or gel in between handling of mice; 3) Disinfect the scales between each use with PPM; and 4) Twice yearly complete cleaning of all cages including removal of all sand and bleaching of all surfaces.

Preparation for Reintroduction of Mice

Comprehensive veterinary health examinations will be carried out on all mice when animals are tween two and four weeks prior to translocation. Only those individuals assessed as healthy by a wildlife veterinarian will be translocated to Laguna Coast Wilderness Park (LCWP). Health checks will include:

- Thorough physical examination,
- Faecal parasitology

Mice will be excluded from the release if health problems are found. Data will be recorded on each animal's individual profile document for future reference.

APPENDIX D – PHASE 2 SUMMARY OF NECROPSY FINDINGS

11-18-16
Pathology Summary Report
Species: Pacific pocket mouse (*Perognathus longimembris pacificus*)
Site: Institute for Conservation Research Breeding Colony
Contact: Debra Shier (dshier@sandiegozoo.org; dmshier@g.ucla.edu)

The Wildlife Disease Laboratories received 32 Pacific pocket mice mortalities between 7-2-14 and 9-13-16. All animals had been housed in the captive breeding colony at the Institute for Conservation Research, San Diego Zoo Global. Of 33 mice received, 8 were not examined via necropsy (at the request of the program manager), so the cause of death was not determined. The necropsy findings, including gross and histologic examination, for the remaining 25 animals are summarized below.

1. Fetuses / neonates

Complete necropsies were performed on 8 neonatal pocket mice. One litter of five was stillborn following a Caesarean section on the dam (615088, #90), who was reported to be 6 days overdue and who was not responsive to medical therapy (per history, submission form). The cause of stillbirth was not determined in these five animals, but there was no evidence of underlying infectious or inflammatory disease. One litter of three pups (#75, #76, #77) died at approximately 5 days of age with no evidence of recent nursing (attributed to maternal neglect or lactation failure, per history on submission form).

2. Juveniles and young of the year

Necropsies were requested and performed on six Pacific pocket mice categorized as juveniles or young adults in the time range specified above. These ranged in age from 16 days to 112 days.

Three of the six animals had varying degrees of skeletal malformation affecting the sternum and/or spine. One of these was euthanized (615207; #107; necropsy #58486) due to the severity of malformation of the sternum and spine (*pectus carinatum*, characterized by a convex deformity of the sternum, and kyphosis, a dorsal deviation of the spine). This animal was also in poor body condition, which was secondary to skeletal abnormalities, and had evidence of mild, chronic hemorrhage in the lung. Scoliosis, or lateral spinal deviation, was identified at necropsy in animal 616321; #182 (necropsy number 59547). That animal was reported to be chronically underweight, with continued weight loss post-weaning, and very mild hemorrhage was identified in the lung on histologic examination. Mouse 615116; #98 (necropsy number 58343) had sternal malformation, bony remodeling, and fibrosis of the bone. That animal also had hemorrhage in the lung that likely contributed to death.

Death was attributed to poor body condition / inadequate fat stores in one animal (616128; #153 necropsy number 59619), but the underlying cause could not be determined due to post mortem autolysis.

Two animals housed with conspecifics had possible histories of trauma, though this could not be confirmed on necropsy in either case (614386 #84, necropsy number 57362; and, 615211; #111, necropsy number 58371). Both of these animals had evidence of hemorrhage in the lung, and in the latter case this was severe enough to cause death. The cause was not definitively determined.

Note: An additional six juveniles or young of the year were received but necropsies were not requested (#138, #139, #140). Three of these had a history of spinal malformation, as reported on the necropsy submission form (#81, #133, #146).

3. Adults

Eleven adult Pacific pocket mice were examined by necropsy in the time period specified. Causes of death were variable, with no one disease issue predominating. Animals ranged in age from 341 days to several years (wild caught animals were of unknown age).

In three animals, poor body condition (an absence of fat stores) was the most significant finding, and an underlying cause was not identified; two of these (612245; #1; necropsy number 58420; and 612248 #4, necropsy number 57828) were wild caught and of unknown age.

Infection (presumptive bacterial infection) contributed to death in two cases. In one, there was incisor fracture with secondary infection of the soft tissues of the head (614250; #68, necropsy 58196). Another case had aspiration pneumonia (614367; #78, necropsy number 58407), and also intestinal parasites (spirurid nematodes) that may have been clinically significant. Spirurid nematode parasites were also present in the intestines of 612273; #17 (necropsy number 59186), a wild caught male. That animal also had multiple age-related changes including degenerative lesions in the spine, and mild, localized inflammation in the nasal passages.

Death in one adult female was attributed to complications of Caesarean section (615088 #90, necropsy number 59309).

One adult mouse had severe spinal malformation (613295; #34, necropsy number 58476), characterized by ventral spinal deviation (lordosis). In this case, there was associated acute and significant damage to the spinal cord that had occurred very shortly before death. Examination of the uterus on this female revealed a recent pregnancy with probable fetal death (abortion) or resorption.

Conspecific trauma was the cause of death in one adult male (612238; #23, necropsy number 58240).

Cause of death was not determined in two cases. In one case (613293; #32, necropsy number 57847), there was hemorrhage in the brain that suggested a possible cardiovascular event shortly before death. In the other case, lesions were suggestive of possible heart failure but this could not be confirmed (613292; #31, necropsy number 58098)

One additional adult mouse (#3; 612247) was received by the WDL but necropsy was not requested.

Comment:

Causes of death in the Pacific pocket mouse colony between July 2014 and September, 2016 varied somewhat with age class. Neonatal deaths were generally uncomplicated and associated

with maternal issues (stillbirth associated with C-section; failure to nurse). Of juvenile animals examined, skeletal malformations (typically spinal and/or sternal) were a significant contributor to death; severe spinal deformity was also identified in an adult during this time period, and has been documented in the colony in the past. Note that several animals for which necropsies were not requested also had a clinical history of spinal lesions. Underlying causes could include nutritional imbalances (calcium, phosphorous, vitamin D, or other factors), or the disease could have a genetic component. Other contributing factors may also play a role. Further investigation of the epidemiology and possible causes of skeletal deformities in this colony is warranted, especially because these cases often have associated secondary lesions that contribute to death. Since this disease appears to occur at birth or very early in life, complete necropsies on young animals will be a useful source of data.

Spirurid nematodes were identified in 2 animals at necropsy, and are of unknown clinical significance. The source of these nematodes is not known; spirurids usually have a multi-host life cycle with insects acting as intermediate hosts.

Remaining causes of death were variable and no overarching trend was identified. There was no evidence of significant infectious disease in the population.

Megan Jones, DVM, PhD, Dipl. ACVP
Wildlife Pathologist, mjones@sandiegozoo.org, 619-231-1515 ex. 4844

APPENDIX E -- DEFINITIONS OF GENETIC TERMS

Current Gene Diversity (GD) -- The proportional gene diversity (as a proportion of the source population) is the probability that two alleles from the same locus sampled at random from the population will not be identical by descent. Gene diversity is calculated from allele frequencies, and is the heterozygosity expected in progeny produced by random mating, and if the population were in Hardy-Weinberg equilibrium.

Founder – An individual obtained from a source population (often the wild) that has no known relationship to any individuals in the derived population (except for its own descendants).

Founder Genome Equivalents (FGE) – The number wild-caught individuals (founders) that would produce the same amount of gene diversity as does the population under study. The gene diversity of a population is 1 - 1 / (2 * FGE).

Founder Representation – Proportion of the genes in the living, descendant population that are derived from that founder. I.e., proportional Founder Contribution.

Inbreeding Coefficient (F) – Probability that the two alleles at a genetic locus are identical by descent from an ancestor common to both parents. The mean inbreeding coefficient of a population will be the proportional decrease in observed heterozygosity relative to the expected heterozygosity of the founder population.

Mean Kinship (MK) – The mean kinship coefficient between an animal and all animals (including itself) in the living, captive-born population. The mean kinship of a population is equal to the proportional loss of gene diversity of the descendant (captive-born) population relative to the founders and is also the mean inbreeding coefficient of progeny produced by random mating. Mean kinship is also the reciprocal of two times the founder genome equivalents: MK = 1 / (2 * FGE). MK = 1 - GD.

Percent Pedigree Certain – Percent of an animal's genome that is traceable to known Founders through specific ancestors (individuals with possible multiple sires [MULTS] not included). Percent certain represents a higher degree of knowledge than percent known, and is therefore always less than or equal to percent known.

Percent Pedigree Known – Percent of an animal's genome that is traceable to known Founders, through identification of either specific ancestors or all possible ancestors (MULTs included).

Release Plan for Reintroduction of Pacific Pocket Mice (Perognathus longimembris pacificus) to Laguna Coast Wilderness Park

INTRODUCTION

The Pacific pocket mouse (*Perognathus longimembris pacificus*, "PPM") is one of 16 currently recognized subspecies of the little pocket mouse (*Perognathus longimembris*); a widespread species that is distributed throughout arid regions of the western United States extending into the northern part of the Baja California peninsula and west central Sonora, Mexico (Williams et al. 1993). PPM is a small, burrowing rodent in the same family as kangaroo rats (Heteromyidae) that primarily feeds on seeds and is associated with fine grain, sandy substrates in coastal strand, coastal dunes, river alluvium and coastal sage scrub habitats within approximately 4 kilometers (2.5 miles) of the ocean in southern California. Historically, it was documented from near El Segundo in Los Angeles County to the vicinity of the Mexican border in San Diego County. Following 20 years with no reports of the subspecies, PPM was emergency listed by the U.S. Fish and Wildlife Service (USFWS) as federally endangered in February of 1994, following the rediscovery of a single population at the Dana Point Headlands in the City of Dana Point. Since its listing, the PPM has been found at three additional sites, all within the bounds of Marine Corps Base Camp Pendleton (Camp Pendleton). However, it is feared that one of the Camp Pendleton populations has become extirpated, and the other two Camp Pendleton populations fall within military training areas where they are threatened by troop training activities. All known PPM populations are endangered by habitat fragmentation and small size. Because coastal southern California is nearly completely urbanized, conservation and management of all remaining suitable habitat, particularly those areas with sandy substrates, is critical to the recovery of the subspecies.

Within Orange County, in addition to the Dana Point population, PPM is historically known from the San Joaquin Hills where University of California, Irvine graduate students studied the dynamics of a coastal sage scrub small mammal community from 1968-1971 (M'Closkey 1972, Meserve 1976a, Meserve 1976b). Although habitat at their study site was replaced by the Spyglass Hill residential development in the early 1970's, suitable habitat for PPM remains within portions of the San Joaquin Hills. The Dana Point population and presence of suitable habitat in the San Joaquin Hills prompted the County of Orange and Participating Landowners to formulate a conservation strategy for PPM as part of the Orange County Central and Coastal Subregions Natural Community Conservation Plan/Habitat Conservation Plan (NCCP/HCP) adopted in 1996. Accordingly, PPM is an NCCP/HCP "Identified Species", which authorizes NCCP/HCP Participating Landowners to "take" PPM in association with implementation of Planned Activities in exchange for implementation of conservation and habitat management actions of benefit to PPM.

Because the Dana Point population is likely to remain vulnerable to extirpation from its isolation and small size, the NCCP/HCP conservation strategy for PPM involves studying the Dana Point population, identifying suitable habitat elsewhere in the NCCP/HCP habitat reserve to relocate animals to, and, if deemed feasible, perform translocations to expand the species' range and improve its prospects for long-term survival. This is consistent with The Recovery Plan for the Pacific Pocket Mouse (*Perognathus longimembris pacificus*) (USFWS 1998) which calls for the protection and maintenance of 10 independently viable and stable populations of the subspecies. Such populations are proposed to be conserved through the protection of known and newly

discovered populations, and through implementation of measures to create additional populations.

Implementation of the NCCP/HCP conservation strategy for PPM is being performed within the context of the larger PPM recovery effort. The highest PPM Recovery Plan priorities (USFWS 1998) have been to protect, conserve, and appropriately manage the existing populations, and to search for unknown populations. Since listing, habitat supporting the Dana Point Headlands population has been permanently conserved and is actively being managed by the Center for Natural Lands Management (URS and CNLM 2005). Recent monitoring suggests this population has remained stable over the past few years and has grown and expanded since its discovery (CNLM 2013). On Camp Pendleton the northernmost discovered PPM population is on land managed by the California Department of Parks and Recreation (CDPR), where two habitat enhancement efforts have been performed to improve habitat conditions for PPM (Montgomery 2005; Riefner 2010). Despite these efforts, it is feared that this population may no longer remain extant. The remaining two populations occur on Camp Pendleton , where the USFWS has been coordinating with the Marine Corps to safeguard them.

Since its listing there have been over 80 surveys for PPM (2015 USFWS CFWO Survey Report Database) throughout its historic range, with no new population discoveries since 1995. In 2010 and 2011, trained scent dogs were used to perform extensive surveys throughout much of the most promising remaining habitat on Camp Pendleton and within the San Joaquin Hills (Brehme et al. 2012, Smith 2011). The failure to discover new populations during these and other surveys suggests that conservation of additional populations relies on population creation via translocation or captive propagation (Recovery Plan item 5.7 under Task 5, "Identify and Implement Measures to Create Additional Populations).

Efforts to identify reliable concentrations of individuals within the extant populations from which the 50 or more animals that are needed to support a meaningful translocation have proven unsuccessful (Shier 2008a, b). Because captive propagation can be scaled up and implemented over time, it has been determined to be the only logistically feasible strategy for obtaining the number of individuals needed for population creation. In 2011, consensus was reached among the USFWS, the California Department of Fish and Wildlife (CDFW) and species experts that PPM population parameters had reached a critical point that justified establishment of a captive breeding program. Captive breeding of PPM was initiated in 2012 at the San Diego Zoo Safari Park, where a total of 30 population founders from the three extant PPM populations were brought into captivity and are currently being bred. Captive breeding has been successful at growing the size of the captive population and population modelling now indicates we are ready to introduce our first group of PPM in the spring of 2016, without negatively impacting the continued viability or genetic diversity of the captive population. Consistent with the provisions of the NCCP/HCP, the first PPM introduction is proposed to be performed within the NCCP/HCP habitat reserve.

This document includes the proposed release plan for the staged introduction of up to 200 healthy PPM to Laguna Coast Wilderness Park (LCWP) in the NCCP/HCP coastal habitat reserve to establish a self-sustaining free-living PPM population. Mice will be selected on the basis of genetic recommendations by San Diego Zoo geneticists. Selected animals will be assessed for health and behavioural characteristics prior to translocation. The initial release will consist of up to 50 mice and is currently scheduled for Spring 2016. Because there is a risk of low survivorship when releasing captively bred animals, the decision to supplement the

introduced population with subsequent animal releases will be based on monitoring of the survivorship and reproduction of released mice. If first year monitoring of the introduced animals confirms that the site has appropriate habitat attributes for PPM but there was low survivorship, no evidence of population recruitment, or a need for more genetic diversity in the introduced population, then a subsequent release of 25-50 animals is proposed in year two. Over time, this proposal could involve the phased release of up to 200 captively bred individuals to achieve the goal of establishing a healthy self-sustaining population of PPM within LCWP. The initial introduction effort will be considered successful with the persistence of 30 or more animals at the introduction site and evidence of at least two consecutive years of reproduction within the population.

SITE SELECTION

Despite extensive efforts to locate additional populations of PPM since its rediscovery, as discussed above, PPM is only known to remain extant at the Dana Point Headlands and two locations on Camp Pendleton.   Efforts to locate additional populations have been guided in part by a habitat suitability model (Spencer et al. 2000) that was prepared to identify remaining potentially suitable habitat for PPM based on soil and vegetation characteristics. This model has identified remaining natural habitat in the San Joaquin Hills as one of the largest contiguous blocks of potentially suitable habitat for PPM within its historical range (Spencer et al. 2000). Habitat suitability rankings generated from this model have been used to target live trapping and scent dog surveys for PPM within habitat conserved in the San Joaquin Hills as part of the NCCP/HCP reserve system (Smith 2011, Miller 2011, Tremor 2013).   These surveys have covered portions of Upper Newport Back Bay, LCWP, Aliso and Wood Canyons Wilderness Park, Crystal Cove State Park, and the Irvine Open Space Preserve.   While these surveys have not been successful at locating PPM, they have provided fine scale soil and vegetation data that confirms the presence of several sites in the San Joaquin Hills with similar habitat attributes to PPM occupied habitat at Dana Point and Camp Pendleton.

Using this information, in the Fall of 2013, the PPM Working Group, comprised of PPM species experts and stakeholders, including individuals from USFWS, California Department of Fish and Wildlife (CDFW), San Diego Zoo Institute for Conservation Research, United States Geological Survey, Camp Pendleton, Center for Natural Lands Management, San Diego Natural History Museum, CDPR, along with representatives from OC Parks and the Irvine Ranch Conservancy, visited the sites identified as having appropriate habitat attributes for PPM to review and rank their suitability for reintroducing PPM to the San Joaquin Hills.   The highest ranked of the sites by the working group, and the site selected for this reintroduction is a site within LCWP. This site is located along Water Tank Road (Figure 1) in a remote area that is protected and buffered from human development, has a relatively low level of recreational trail use that is compatible with a reintroduction, and is in an area where there is sufficient space to sustain a PPM population.

ANIMAL SELECTION

The first release will consist of up to 50 mice. The exact identities will depend on breeding recommendations, health and behavioural suitability. An individual profile document for each

mouse chosen for release will be set up, including all pertinent information on the animal, ID photos, history of veterinary checks, weights, behavioural assessments, trapping history etc. Our goal will be to include a small percentage (up to 10%) of wild-caught PPM founders in the release.

ANIMAL PREPARATION

Disease Risk Assessment

The San Diego Zoo Institute for Conservation Research has conducted a disease risk assessment for the species prior to reintroduction.

Potential Hazard Identification

The list of known potential pathogens in Heteromyids is short, including oxyurids and other nematodes, *Eimeria* sp., cestodes, acanthocephalans, and Hantavirus (Decker et al. 2001; Garcia-Prieto et al. 2008; Harkness et al. 2010; Mantooth et al. 2001; Parmenter et al. 1998; Zhao and Duszynski 2001). However, these are considered endemic agents, which means they are either nonpathogenic to the host or lack epizootic potential. Other plausible agents include *Salmonella* sp., *Yersinia* sp., and *Coxiella* sp. These agents are similarly considered endemic opportunists lacking epizootic potential (Harkness et al. 2010).

Disease Risk

The risk of significant disease introduction or spread is low, as no significant hazards have been identified. The fact that translocations and reintroductions are being limited to historic range in close proximity to current range (i.e., within the same ecological zone for any known or unknown pathogens) also minimizes disease risk, including the theoretical risks posed by unknown pathogens that might be present.

Risk Mitigation

No agents requiring active surveillance have been identified. Recommendations for risk mitigation are therefore limited to best practices for population management and reintroduction and translocation planning. These include routine risk matching for translocations and reintroductions (matching characteristics of source and destination populations/locations by staying within historic range), population health assessments by a veterinarian prior to animal movements, and population-level disease surveillance through postmortem examinations on the captive and wild populations.

Health checks

Comprehensive veterinary health examinations will be carried out on all mice between two and four weeks prior to translocation. Only those individuals assessed as healthy by a wildlife veterinarian will be translocated to LCWP. Health checks will include:

- Thorough physical examination,
- Faecal parasitology

Mice will be excluded from the release if health problems are found. Data will be recorded on each animal's individual profile document for future reference.

Behavioural assessments

Behavioural assessments will be carried out on all mice one to two months prior to release. If survival skill (e.g. predator avoidance, foraging, etc.) training is required, we will conduct training trials in the month prior to release. All animals requiring skill training will be reassessed in the weeks prior to release to ensure behavioural competency. Acceptance or rejection of mice for release to LCWP will be determined on the basis of the researchers' expert opinions combined with results from the behavioural competency assessments. Animals will be considered competent if they exhibit similar behaviour to wild-caught adults. Animals not considered competent will be removed from the potential group to be released and may remain as breeders in the captive breeding facility. All animals slated for release will be individually marked prior to release using the current USFWS and CDFW approved method (VIE or P-chipping).

RELEASE STRATEGY

Release site preparation

Habitat at the release site will be assessed for the presence of sensitive plant and animal species prior to the introduction of PPM at the release site. During reconnaissance of the site in December of 2013 and October of 2015, no sensitive plant species were observed, but a seasonal depressional wetland with potential to support the western spadefoot toad (*Spea hammondii*) and a coastal California gnatcatcher (*Polioptila californica californica*) were observed near the edge of the proposed reintroduction site. Because the location of enclosure fencing and artificial burrows are not fixed, impacts to the seasonal wetland will be avoided by leaving it outside the boundaries of the release site, and impacts to any sensitive plant species that are discovered will be avoided by aligning fencing and locating artificial burrows and human access routes away from sensitive plant locations. While it is anticipated that impacts to the gnatcatcher can similarly be avoided by locating the release site away from the gnatcatchers' territory(ies), more surveys are needed and will be performed concurrent with sensitive plant surveys to better understand habitat use by gnatcatchers in the project vicinity.

Habitat at the release site is proposed to be managed for suitability for PPM with a target of no more than 60% shrub cover and no more than 50% annual grass cover. Based on the low cover of shrubs and grasses observed at the site in October of 2015, no shrub or grass cover manipulation is anticipated at this time. However, because grass cover has potential to change quickly in response to precipitation, grass cover will need to be re-evaluated in December of 2015 or January of 2016 to determine if reduction of annual grasses is required in advance of the introduction effort. Overall, it is anticipated that adverse impacts to the gnatcatcher will be avoided by limiting any necessary vegetation manipulation to the control of non-native annual grasses, and by taking gnatcatcher territorial habitat use into consideration when locating project fencing around the release site.

One to three months prior to the release of PPM, the release site will be trapped to determine nontarget small mammal densities. This information will allow us to determine where to site

acclimation cages and if nontarget densities must be reduced prior to release of PPM. Should densities of Western harvest mice (*Rethrodontomys megalotis*), Deer mice (*Peromyscus Maniculatus*), Cactus mice (*Peromyscus eremicus fratercculus*), California pocket mice (*Chaetodipus californicus*), Dulzura kangaroo rats (*Dipodomys simulans*), desert cottontails (*Sylvilagus audubonii*) and brush rabbits (*Sylvilagus bachmani*) be categorized as "medium" to "high" within the immediate area of the release, we will reduce the density of these species by trapping and relocating a subset of the individuals outside of the fenced site until densities are categorized as "low" (See Table 1 for density categories/species; Kelt et al. 2008; M'Closkey 1972; McClenaghan 1983; Meserve 1976c; Schwilk and Keeley 1998).

To limit dispersal and allow pocket mice to acclimate to the new site (Germano 2001, Long et al. 2006), sites will be prepared for "soft release". An acclimation cage will be constructed for each pocket mouse (Figure 2). Acclimation cages may consist of an underground wire, wood or cardboard retention basket (15.2 x 15.2 x 7.6 cm) set 0.3m underground, two biodegradable cardboard mailing tubes (2.5 cm-diameter), which will connect the basket to the surface on either side, and an above-ground wire mesh retention cage (.2 x .2 x 0.2 m made of 0.635cm mesh; Figure 2). This design will allow movement of pocket mice between the retention basket and the above-ground retention cage, but will deter escape during the acclimation period (Truett et al. 2001, Long et al. 2006). Acclimation cages will be placed approximately 5m apart (depending on the terrain) in a grid configuration with a 20-25m buffer around the perimeter of the grid allowing spread and space for PPM density to increase within the enclosure (Figure 3). This spacing approximates medium to high burrow density in the wild (Shier, unpublished data). The grid and buffer zone encompassing 1.26-1.64 acres will be temporarily surrounded by a 4-6 ft. fence buried 1-2 feet below the surface to dampen dispersal and deter predation attempts (Figure 4). We will install remote cameras within the release enclosure to monitor PPM activity during acclimation and following release.

Transportation of Animals Slated for Release

Pocket mice will be transported individually from the captive breeding facility at the Safari Park to the selected release site in standard rat cages containing their nest jar, nesting material, substrate from their home cage and seed. Total transit time will not exceed 3 hours. Animals will be transferred to acclimation-cages in the early evening to give them ample time to explore acclimation cages prior to sunrise. Because releases of kangaroo rats have shown that individuals released with neighbours have higher survival when compared to kangaroo rats released without neighbours (Shier and Swaisgood 2012b), acclimation cage location will be based on captive housing location. Once animals are transferred to cages, researchers will ensure that all PPM have found the artificial burrow and gone down into the below ground portion of the cage at least once prior to leaving the site.

Soft-Release

Animals will be held in the acclimation cages for no more than 7-10 days to allow acclimation to the release site and dampen dispersal. While in acclimation cages, pocket mice will be fed a combination of their standard captive diet (finch seed mix), native plant seeds present on the release site and used for enrichment in captivity and a fresh piece of lettuce every other day. All releases will occur before July 2016 to ensure that pocket mice have sufficient time to establish new burrows prior to aestivation. At the end of the acclimation period, the above-ground retention cages will be removed, but the boundary fence, underground retention boxes and

artificial burrows will be left in place to provide temporary shelter for pocket mice. Because the below ground portion of the cage is biodegradable, retention boxes and artificial burrows will not be removed after release. The enclosure/exclosure fence will be removed to return the site to its natural appearance once the mice have settled and reproduction of releasees has been documented. We anticipate reaching this point by January of 2017. However, should post-release monitoring indicate that PPM density at the site requires supplementation with additional captive bred individuals, we would leave the fence in place for an additional year.

Post-release Supplementation and Monitoring

Seed will be broadcast at the release site 3 evenings a week for the first month following release and one evening each week thereafter until the end of September. In subsequent years, precipitation levels and vegetation condition will be used to determine if supplementation is required. Seed will be microwaved for 1-3 minutes before it is broadcast at the release site to prevent seed germination and alteration of the native vegetation community.

We will measure reintroduction success directly through live capture and indirectly through behavioral and stress assessments. Immediately following release, we will conduct behavioural observations to examine the establishment process. We will observe the behaviour of pocket mice during the first two hours following release from the acclimation cages and document behaviours such as: sand-bathing, digging, foraging, and fighting. We will conduct a minimum of 20 focal animal observations during the remainder of the active season by placing a remote camera at the burrow of each animal.

We will attempt to re-trap all marked animals present at the release site and count emergent young at various time points following release. Typically, these are conducted at 1 and 3 month intervals, in the spring or summer following release and then annually thereafter for three to five years depending on funding. Time points for post-release monitoring during the reintroduction year will be determined based on the release date. For instance, if the release is in April 2016, then subsequent trapping will occur in May 2016 and again in early August 2016. During subsequent years, the timing of monitoring will be designed to occur in early to mid- May to assess overwinter survivorship, and in mid-July to look for evidence of reproduction.   To measure survivorship and track the capture histories of individual animals we will use the current USFWS approved method for permanent marking   and identification of each released animal to mark and follow released animals and their young. During each trapping bout, an effort will be made to determine the fate of all released animals and to estimate the size of the population. This will likely involve a combination of trapping until no new animals are detected and mark-recapture statistical methods. To assess stress within the reintroduced population,   fecal pellets produced by PPM during handling or that are found within traps containing PPM will be collected for analysis of stress hormone levels.

Post-release Site Remediation

Our plans for introduction of PPM to the release site involves removing the above ground portions of acclimation cages immediately following the acclimation period (i.e. within 7-10 days of animal release) and removing the enclosure/exclosure fencing within one year following animal releases if PPM readily take to the site, or within two years if supplementation with additional captive bred animals is warranted after the initial release effort. Although care will be taken to remove all evidence of the introduction effort, disturbance from the installation and

removal of fencing and acclimation cages, and the likely inadvertent creation of trails associated with gaining access to the site for monitoring and management activities could result in lasting habitat impacts if not remediated.   Therefore, a component of project implementation will be to minimize and remediate areas of soil disturbance and to plant any disturbed areas with appropriate native vegetation following project implementation. Plans for habitat restoration and enhancement will be coordinated in advance with OC Parks and the Natural Communities Coalition. To ensure that all habitat impacts are remediated, the Natural Communities Coalition is authorized by the U.S. Fish and Wildlife Service and California Department of Fish and Wildlife to use a portion or all of the $134,000 remaining in the Pacific Pocket Mouse Management Fund for remediation of the release site.

SIGNAGE

We propose to develop in close coordination with OC Parks temporary signage that can be erected at the location of the reintroduction site that informs park visitors the purpose of any visible evidence of the reintroduction effort (e.g., predator exclusion fencing, acclimation cages, traps). While the size, location and informational content of the signage is flexible, we anticipate attaching the signage to the predator exclusion fencing while it is up, and possibly on one or more posts adjoining nearby trails until all evidence of the reintroduction effort is removed. Suggested informational content will be to explain that the fencing and other materials are only temporarily in place in support of the effort to reintroduce the endangered Pacific pocket mouse to the San Joaquin Hills, and to request that park visitors stay on trails and away from habitat supporting the introduction effort.

PUBLIC OUTREACH

We propose to develop in close coordination with OC Parks an informational brochure that can be handed out and displayed at the Nix Nature Center and other appropriate LCWP staging areas and trailheads. The purpose of the brochure will be to inform the public of the plight of the Pacific pocket mouse, its history as a species that was once known from the San Joaquin Hills, and how the County of Orange is helping to support its conservation and recovery through support of the reintroduction effort and participation in the NCCP/HCP. The brochure will not identify the specific location of the introduction site but will advise park visitors what they may encounter and how they should react should they come across the introduction site. Based on the interest of OC Parks, Wildlife Agency and/or San Diego Zoo biologists will be available to provide talks on the reintroduction effort to friends groups, park volunteers and speakers forums. Other outreach will be performed via the San Diego Zoo's Blog on the captive breeding program (http://blogs.sandiegozoo.org/tag/pacific-pocket-mouse/).

COORDINATION WITH ORANGE COUNTY

As the landowner supporting reintroduction of PPM within LCWP, the County of Orange has an active role reviewing this reintroduction plan and advising the USFWS, CDFW and the San Diego Zoo on how best to integrate and maintain compatibility of the reintroduction effort with park operations. While the County is encouraged to contact USFWS, CDFW or Dr. Debra Shier

of the San Diego Zoo, at any time during reintroduction plan implementation with comments or concerns, upon initiation of the reintroduction effort quarterly status reports will be prepared and transmitted to OC Parks to facilitate communication and keep the County advised of the results and progress of the reintroduction effort.   These quarterly reports will include an updated implementation calendar outlining planned and completed introduction and management tasks, information on the numbers and status of animals that have been released, results from monitoring efforts and any issues encountered during plan implementation. Further input and approval will be sought in advance from OC Parks for the installation of any infrastructure (e.g. fencing or signage) or habitat management activities beyond that described in this plan.

USFWS Contact:

William B. Miller, Fish and Wildlife Biologist

2177 Salk Avenue, Suite 250

Carlsbad, California 92008

760-431-9440 Extension 206

William_B_Miller@fws.gov

CDFW Contact:

Nancy Frost, Senior Environmental Scientist (Mammal and Bird Specialist)

3883 Ruffin Road

San Diego, CA 92123

858-467-4208

nancy.frost@wildlife.ca.gov

San Diego Zoo Contact:

Debra M. Shier, Ph.D.

Brown Endowed Associate Director of Applied Animal Ecology

San Diego Zoo Institute for Conservation Research

15600 San Pasqual Valley Road

Escondido, CA 92027

Phone (310) 569-2486

dshier@sandiegozoo.org

dmshier@g.ucla.edu

Table 1. Density Categories for Potential Non-target Competitors

	Density per Hectare		
	Low	**Medium**	**High**
CHCA*	<7.26	7.26-13.52	>13.52
DKR	<12	12-18	>18
PEER	<4	4-6.4	>6.4
PEMA	<6.67	6.67-9.85	>9.85
REME	<10.97	10.97-21.94	>21.94

*due to lack of published data, SD pocket mouse was used as reference for density categories

Figure 1. Proposed Released Site Location at LCWP

Figure 1: Laguna Coast Wilderness Park Pacific Pocket Mouse Introduction Site

Figure 2. Acclimation cage for PPM

.2m

.2m

.2m

Above ground

Below ground

7.6cm

15.2cm
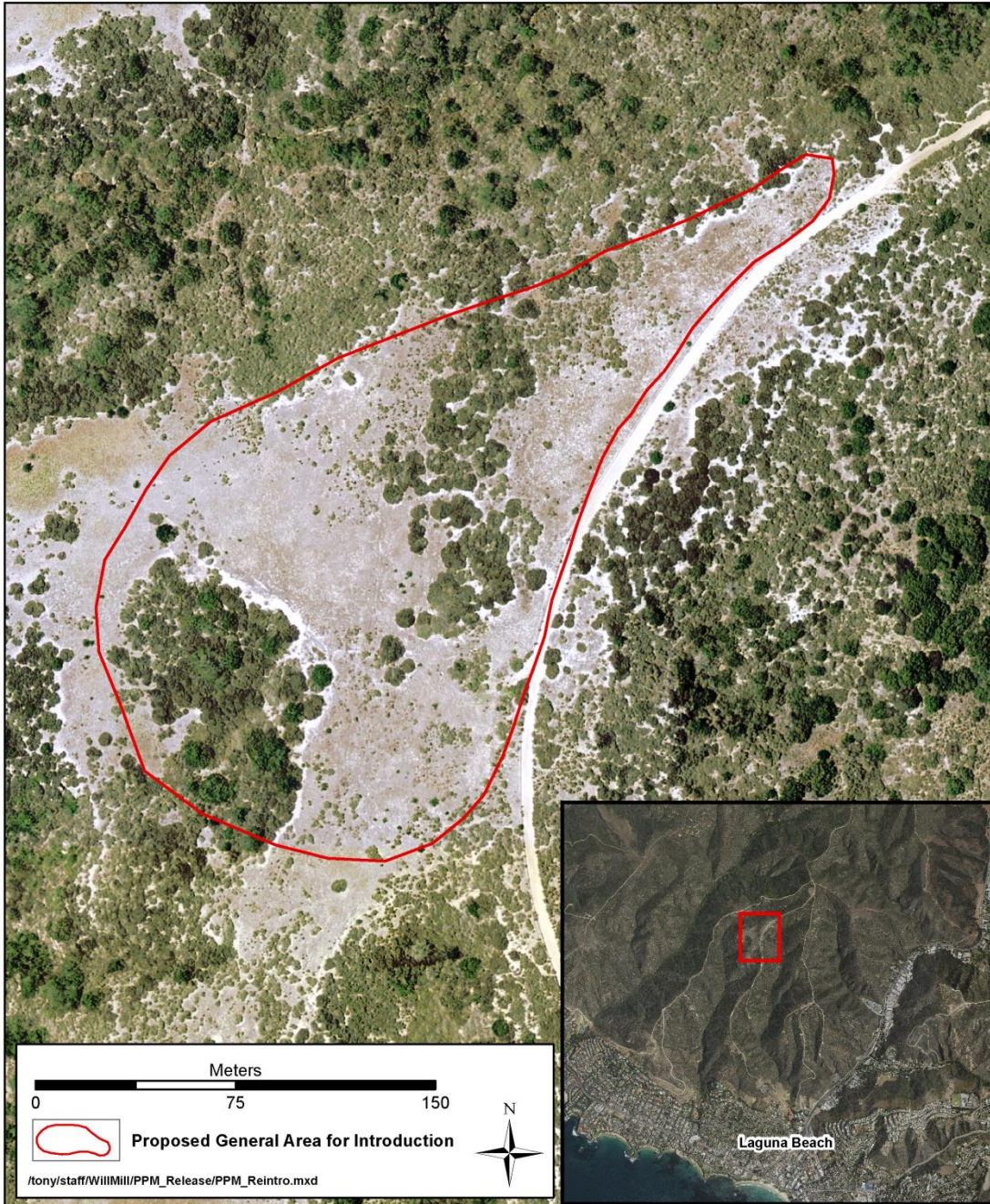
15.2cm
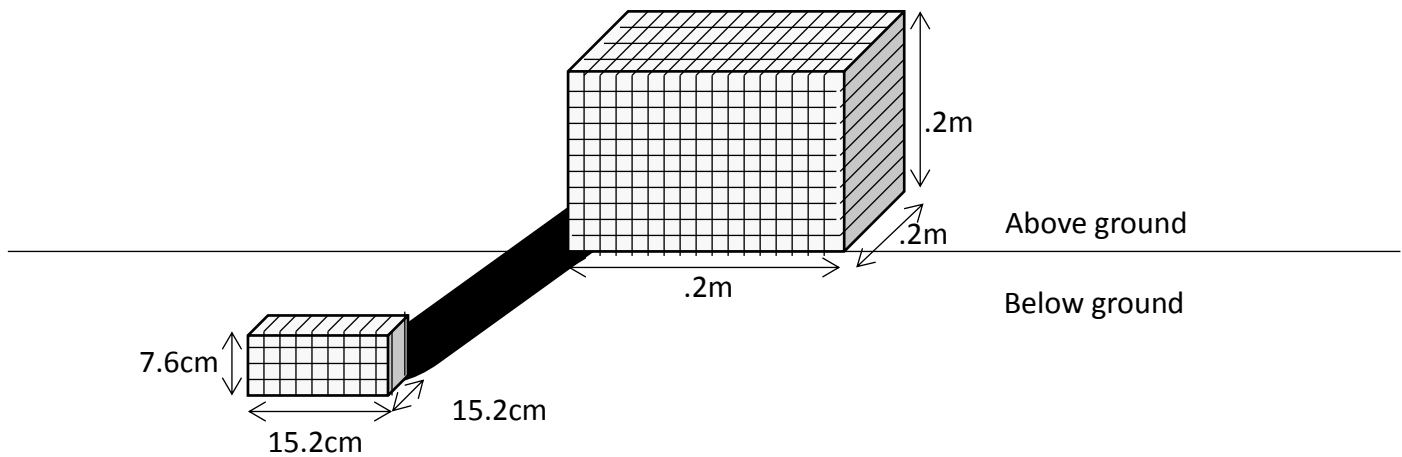
Figure 3. Example Release Site Grid

20-25m

5m

5m

20-25m

Acclimation cage

enclosure/exclosure fence
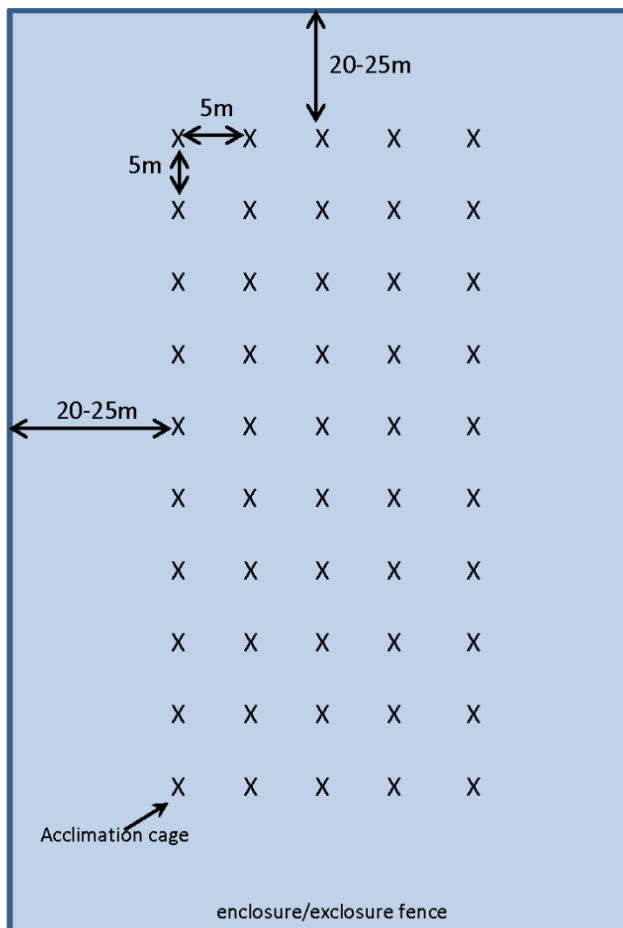
Figure 4. Example of enclosure/exclosure fencing
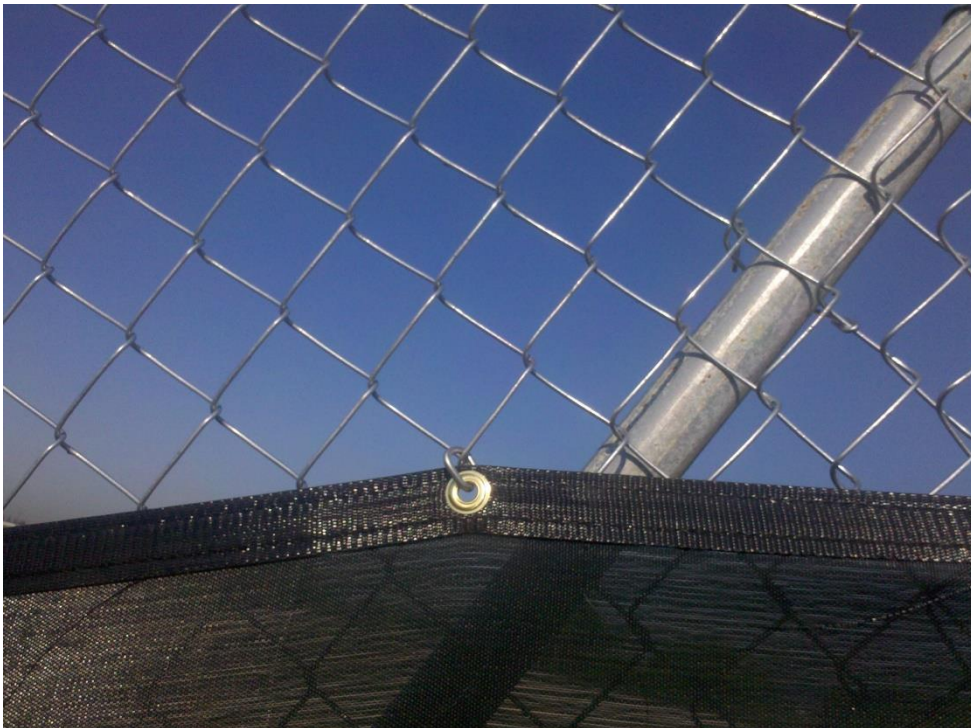
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APPENDIX G - INTERSPECIFIC COMPETITION EXPERIMENTS

Multiple species that occupy similar niches in the same community may compete either directly or indirectly for resources. The intensity and outcome of these interactions determines whether competitors are able to stably coexist, or if one will be driven to local extinction (Ziv et al. 1993). These processes have not previously been considered in reintroduction biology and have the potential to improve applied conservation science and management (Seddon et al. 2007). To achieve stable coexistence, there may be a difference in species' interference competition ability that counteracts a difference in species' exploitative competition ability (Amarasekare 2002). If one species in a pair were superior in both, competitive exclusion would be achieved (Ziv et al. 1993). Alternatively, species may partition their spatial or temporal niche to reduce competition. We are investigating how the Pacific pocket mouse (*Perognathus longimembris pacificus*, PPM), a small, specialist forager, maintains a stable population in a community of larger generalist competitors.

It is unclear why PPM are not expanding into additional suitable habitat, but competitive exclusion by heterospecifics has been identified as a possible factor inhibiting recolonization for both natural and reintroduced populations. The historical range of PPM overlaps broadly with other small rodent species that are potential competitors for food and burrows. The reintroduction sites for PPM also include kangaroo rats, which are absent from extant PPM communities. For this reason, we are also studying Los Angeles pocket mouse (*P. l. brevinasus*, LAPM) communities to understand the relationship between little pocket mice (*Perognathus longimembris spp.*, PELO) and a suite of 4 competitor species. We investigate direct competition through staged interactions between PELO and competitor species. Pilfering food from other individuals may be an important component of indirect competition, and we tested whether these species use heterospecific scent cues to find or avoid caches. We also conducted a yearlong monitoring effort of eight plots of PELO habitat to assess the spatial and temporal overlap of the species. Studying the mechanisms that lead to successful niche partitioning and coexistence in intact PELO communities will contribute to our knowledge of rodent community structure, and this research will aid in release site selection and long-term site management for the reintroduction of PPM.

Trapping

Trapping locations

Between July 2014 and September 2016 we conducted research in two LAPM communities and one PPM community (Figure 1). We worked in the San Felipe Valley Wildlife Area (Figure 2) from April-August 2014. We conducted research in the San Jacinto Wildlife Area (Figure 3) from May 2015-July 2016. We also carried out experiments at US Marine Corps Base Camp Pendleton in South San Mateo (Figure 4) during August 2016.

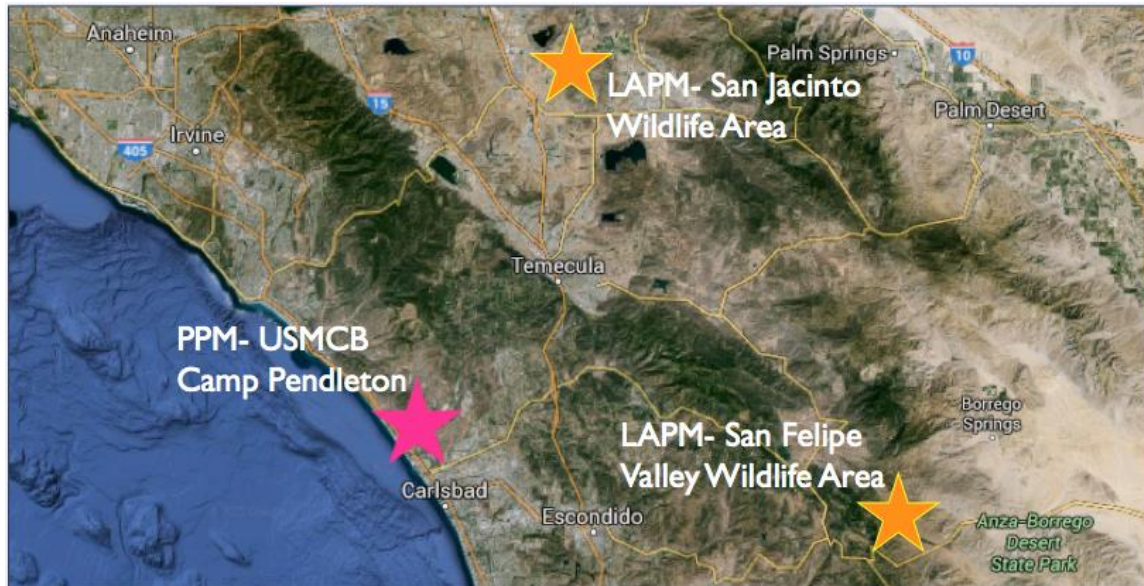Figure 1. Location of three interspecific competition research areas

Figure 2. Trapping locations in the Arkansas Canyon area of the San Felipe Valley Wildlife Area (West of S2- San Felipe Road).
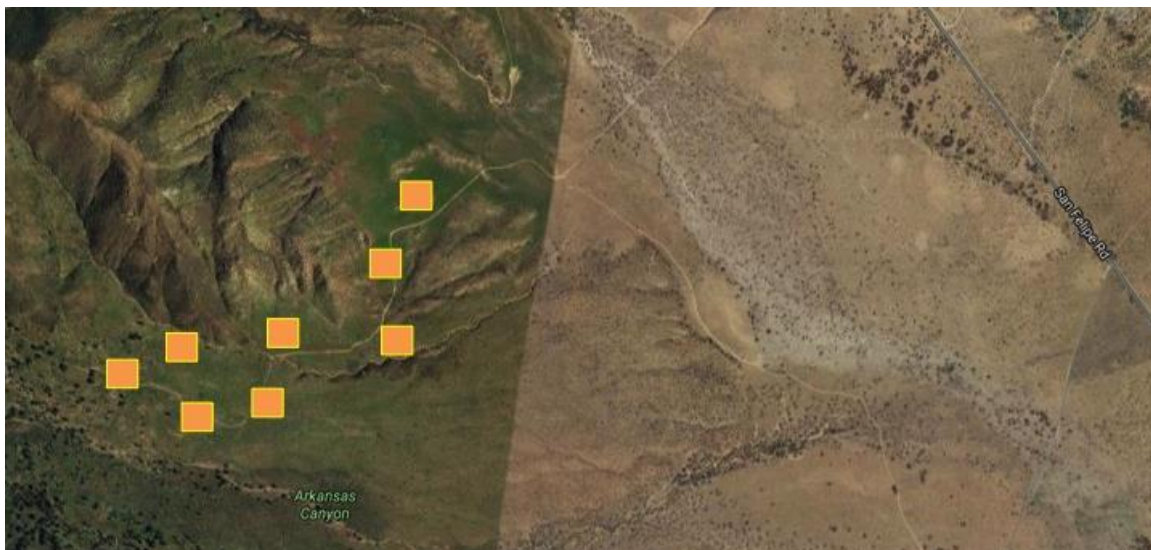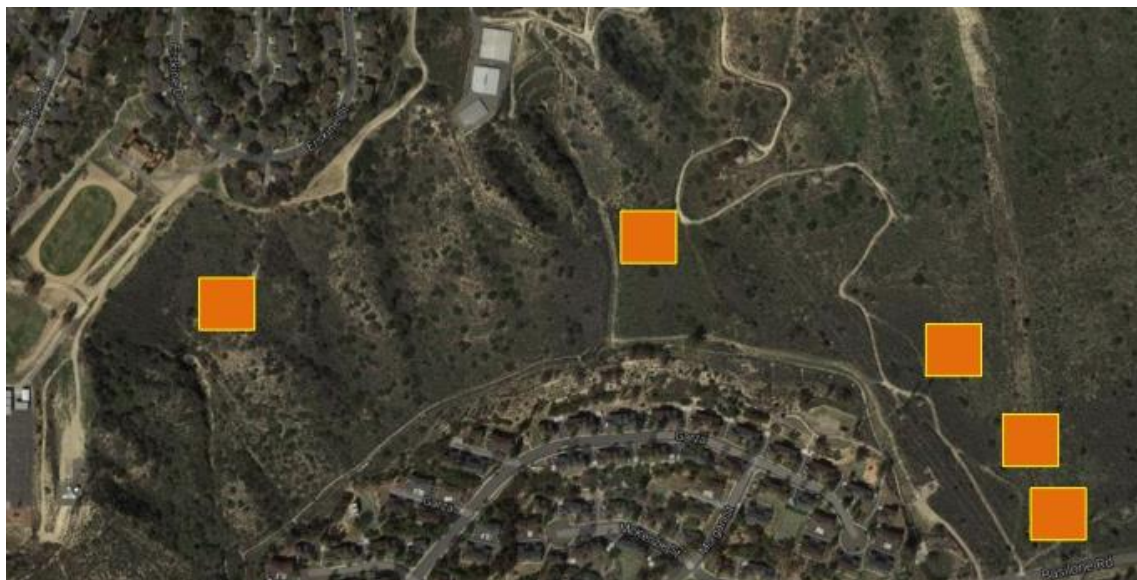
Figure 3. Location of trapping areas shown in orange on the Upland Game area of the San Jacinto Wildlife Area

Figure 4. Trapping locations north of Basilone Road in South San Mateo on USMCB Camp Pendleton. PPM were captured in all locations.

Trapping and tagging methods

We used 9" Sherman live-traps with modified shortened doors to avoid catching the tails of the larger species. In each year traps were spread across non-contiguous sites of 25-50 traps per site. Each trap was marked with a numbered pin flag and reflective tape. Traps were opened and baited with sterilized millet seed between 1800-2000 and checked twice during the night at 2200 and 0200. All traps were closed during the 0200 check. All animals were individually tagged for identification. We used uniquely numbered ear tags for *Dipodomys simulans* and *Peromyscus maniculatus* (Monel 1005-1, National Band and Tag Co., Newport, KY). For species with small ears (*P. l. pacificus*, *P. l. brevinasus*, *Chaetodipus fallax*, *Reithrodontomys megalotus*) we injected visible implant elastomer (VIE, Northwest Marine Technology, Inc., Shaw Island, WA) in unique color combinations just under the skin along the side of the tail. These permanent marks were visible under a black light on subsequent captures.

Direct Competition Experiment

Dominance rank can often be predicted by body size, where the larger animal (or species) dominates the smaller ones (Frye 1983; Rychlik and Zwolak 2006) There are, however, exceptions to this rule (Langkilde and Shine 2007), and wild pocket mice have been observed to chase larger competitors, suggesting that dominance may be determined by territory ownership. We staged paired encounters in the field to determine whether there are predictable dominance and territorial relationships between PELO and four putative competitors.

Staged encounters- Methods

We staged 5-minute dyadic encounters between PELO and DKR, CHFA, PEMA, and REME. All animals were trapped and marked for identification. Unique ID, species, sex, weight, and trap location was recorded. Adult males and anestrus but sexually mature females were used in dyadic encounters. For each pairing one species was randomly be chosen as the resident, and the encounter was staged where the resident animal was captured.

We used a clear Plexiglas open-bottomed, square interaction arena (24" x 24" x 24") in the field. Animals were on natural substrate and able to see their surroundings. A removable opaque partition initially split the arena into two sides. A PELO and an opponent were placed on separate sides to acclimate (indicated by commencement of foraging) before the trial began. At the start of the trial, the barrier was removed and the two individuals were allowed to interact and observed for 5 minutes. In the event of a locked battle, animals would be separated to prevent injury and the trial will be terminated. *No injuries were sustained or locked battles occurred in our 146 trials with LAPM or 20 trials with PPM.* Trials were recorded with an infrared camera for later transcription and analysis. Immediately following the interaction animals were returned to their location of capture and released.

For each focal individual we counted the number of aggressive behaviors, which included "approach" (oriented head and body and moved towards the other individual), "displace" (an approach that resulted in the other individual moving away), "chase" (pursuit of a fleeing individual), "lunge" (thrusting or throwing body towards other), "attack" (initiate sparring, biting, or locked battle), and "sandbathe" (rubbing side or ventrum against sand depositing scent). We also counted the number of submissive behaviors, which were "retreat" (movement away from opponent after initiating proximity), "displaced" (moves away from approaching

opponent), "flee" (rapid movement away from other individual following engagement), and "jump/avoid" (jumps upwards and back away from other animal).

Here we describe behavioral dominance and subordination in terms of the position of a species in competitive interactions with other species from the community, rather than the rank of an individual compared to conspecifics. By combining behavioral data from many individuals, we assume we are observing average interactions between a species pair. The agonistic behaviors we observed were almost instantaneous, thus we recorded counts rather than duration. We accepted that a species that was aggressive more often and submissive less often was dominant in a given interaction. To calculate a dominance index, we added all aggressive behaviors and subtracted all submissive behaviors, then divided by the total number of behaviors. In effect, each score indicates an animal's relative display of dominance-typical behavior to subordinate-typical behavior. The index ranged from -1 (always submissive) to 1 (always aggressive). A score of zero indicated the same number of aggressive and submissive behaviors.

Sample Size: We conducted 48 trials between LAPM and DKR (24 LAPM resident, 24 LAPM intruder); 48 trials between LAPM and CHFA, and 48 trials between LAPM and PEMA. REME were difficult to locate in LAPM habitat, thus we conducted 2 trials between LAPM and REME, and 20 trials between PPM and REME. Each individual was tested a maximum of 2 times (once as resident, once as intruder) if recaptured in separate trapping bout.

Results

We found DKR, CHFA, and PEMA were significantly higher than PELO on the dominance index (Figure 5; DKR V=1010, p>0.01; CHFA V=933.5, p>0.01; PEMA V=962, p>0.01). There was no difference between REME and PELO in overall dominance (V=86.5, p=0.7).

PELO is lower on the dominance index when paired with DKR than with either PEMA or REME, but there is no difference between PELO's interaction with DKR or CHFA. Residency status, sex, or sex of the opponent had no effect on PELO's dominance index score. REME are lower on the dominance index than DKR, CHFA, or PEMA. CHFA, PEMA, and DKR are not significantly different from one another in dominance. There are no effects of residency status, sex of the PELO, or sex of the competitor on the dominance index scores of each species.

Figure 5. Dominance index for each species pair by residency status is show. Boxplots of medians, interquartile ranges, and outliers show the spread of data. Values close to 1 are most dominant and values close to -1 are most subordinate. The greater difference between a competitor (grey bars) and a PELO (open bars) represents a greater the asymmetry in dominance.
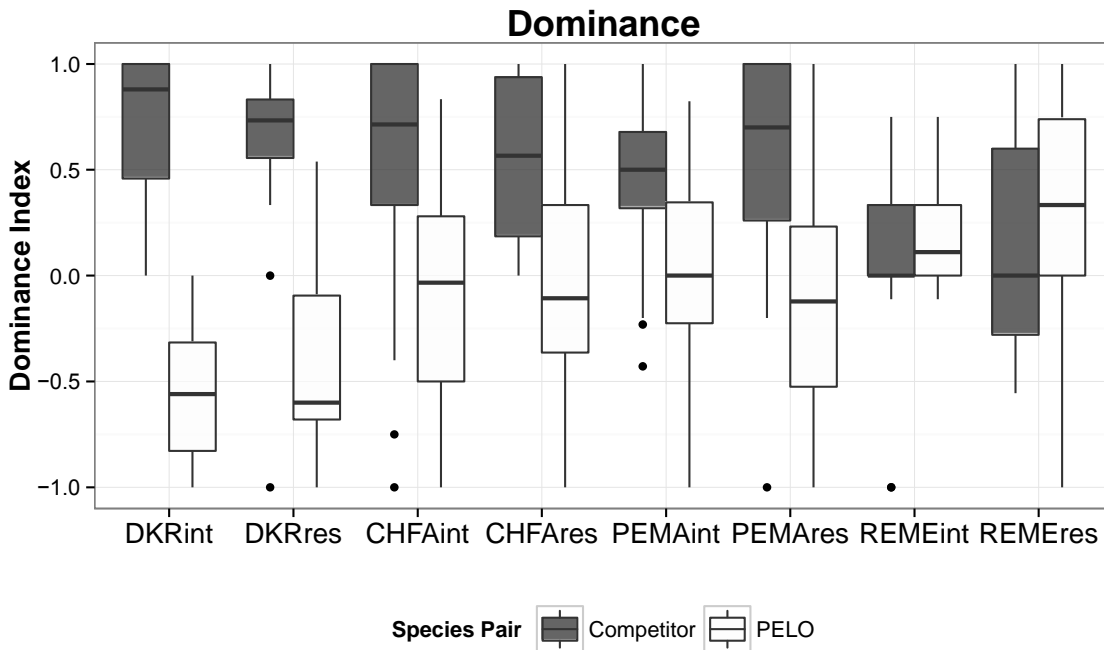

DKR is the only species that exhibited a higher frequency of aggressive behaviors than PELO (Figure 6; $V=913.5$, $p<0.01$). PELO was less aggressive when paired with DKR than when paired with CHFA or PEMA. PELO exhibited similar levels of aggression towards CHFA, PEMA, and REME, and these three competitor species had similar aggression levels as one another and as their PELO opponents.

Figure 6. The number of aggressive behaviors in each 5min trial is shown between each species pair by residency status. All competitor species are in grey and PELO aggressive behaviors are open bars.
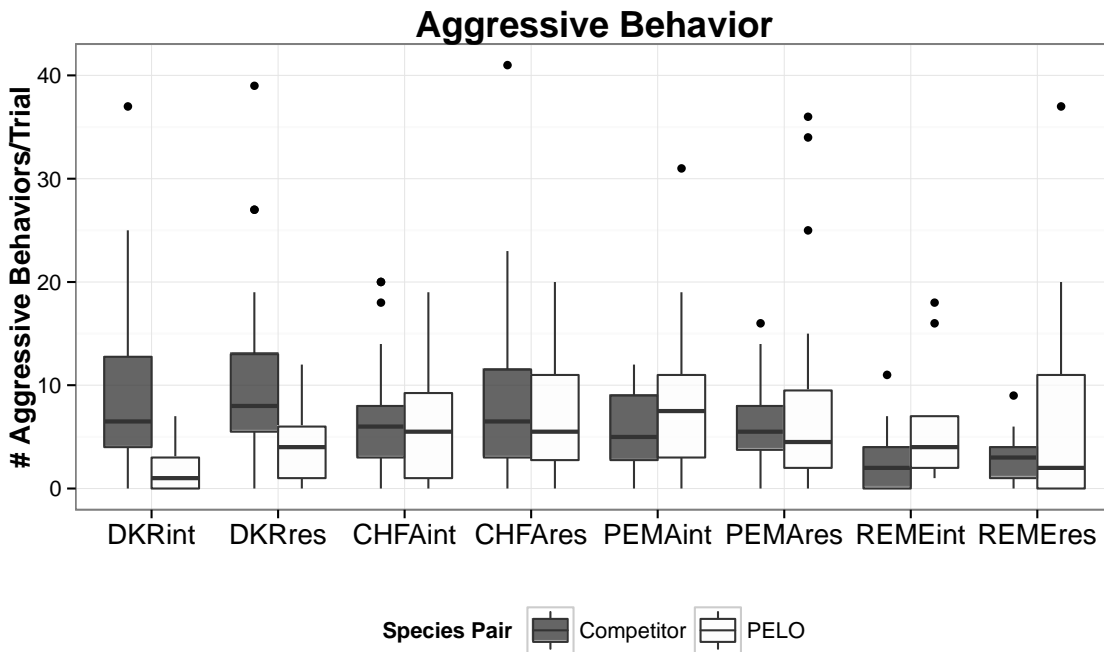
PELO exhibited more submissive behaviors than DKR, CHFA, or PEMA, but did not differ from REME (Figure 7). PELO displayed fewer submissive behaviors with REME than the other three competitors. There were no significant differences between any of the competitor species in number of submissive behaviors.

Figure 7. The number of submissive behaviors in each 5min trial is shown between each species pair by residency status.
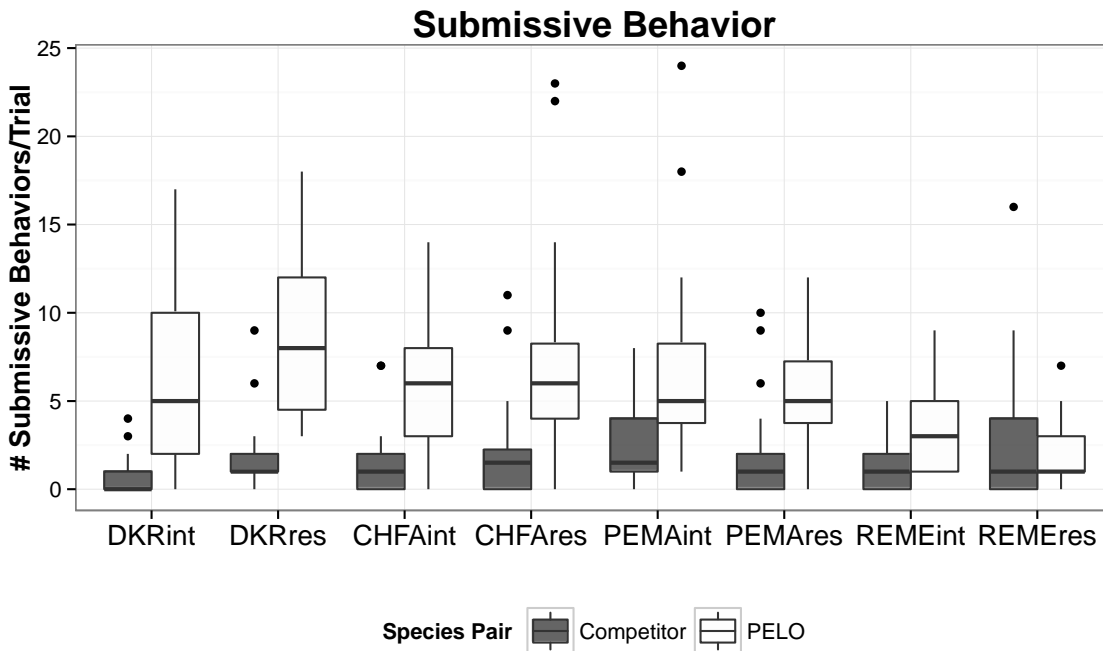
Our findings suggest that PELO are subordinate to all of the larger competitors (DKR, CHFA, and PEMA), and is neither dominant nor subordinate to REME. The greatest difference in dominance was with DKR, the largest competitor in the community. We also found that PELO are just as aggressive as their competitor species, with the exception of DKR, which are more aggressive than PELO. The difference in dominance scores for the species pairs primarily comes from PELO exhibiting more submissive behavior than all their larger competitors. Overall, PELO are likely most affected by DKR in direct interactions and are least affected by REME. There is no evidence that residency status affects PELO behavior, thus PELO are not interspecifically territorial.

Indirect Competition Experiment

Seed caching (food hoarding) is a strategy many rodents employ when a food source is not available year round (Andersson and Krebs 1978). Caches are susceptible to removal by an individual other than the cacher (Leaver and Daly 2001; Vander Wall and Jenkins 2003). PELO may be negatively impacted by pilfering, but may also benefit by pilfering from species that travel further while foraging. We studied whether each of the species uses scent cues of conspecifics or heterospecifics to either find or avoid the caches of other individuals.

Cache pilfering methods

We put one individual in a tank 4ft by 2ft with six artificial caches of 2g millet seed buried in sand scented from conspecifics, heterospecifics, and unscented (Figure 8). The animal was allowed to explore and dig up caches undisturbed for 3 hours. At the end of the trial the animal was released at the location where it was captured, and the artificial caches were sifted and the remaining seeds weighed. We conducted trials with LAPM with sand scented by CHFA (n=20), DKR (n=20) and PEMA (n=20). We also conducted trials with CHFA, DKR, and PEMA with sand scented by LAPM (n=20 for each species).

Figure 8. An LAPM (top center) in the cache pilfering experiment. Each cup contained an artificial cache of seeds buried in sand, with the scent treatment of each cup described. This individual has uncovered four of the caches and pit cached the seeds in the corner of the tank.
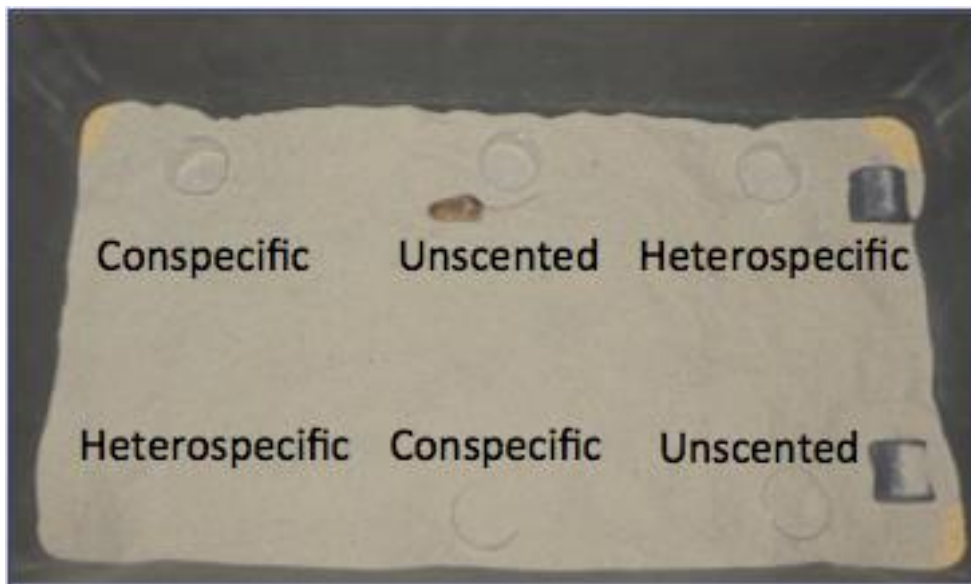
Results and Discussion

We found that LAPM take more seeds from conspecific scented than unscented caches (Wilcoxon p=0.01), but there was no difference in the amount of seed pilfered from conspecific versus heterospecific, or heterospecific and unscented caches. Of the other species we tested, only PEMA showed any preference between the scent treatment groups, and also took more seeds from conspecific than unscented caches (Wilcoxon p=0.03). There were no differences in PEMA and any of the other treatment groups. Neither CHFA nor DKR exhibited any preference or avoidance of any of the scent treatments.

Our results indicate pocket mice and competitors pilfer from each other at similarly low rates, and that heterospecific scent cues are not used to find or avoid surface caches. LAPM and PEMA may be attracted to conspecific scent, but overall the scent of the seeds may be the primary cue all of the species are using and cache pilfering may be more opportunistic than targeted.

Niche partitioning

In a community of species that are similar in their diet and habitat requirements, niche partitioning is a plausible mechanism of coexistence. Comparing multiple locations with varying abundances of pocket mice and heterospecifics can determine whether microhabitat use and peak activity times for pocket mice varies with competitive pressure.

Niche partitioning methods

We established 8 grids in the San Jacinto Wildlife Area (Figure 3) where we conducted our study of spatial and temporal niche partitioning. We selected locations where LAPM were present, along with different suites of heterospecifics. We placed flags 6.25m apart in a grid of 7x7, and placed a trap at each flag. We trapped 3 consecutive nights each month around the new moon between August 2015 and July 2016. We checked traps three times throughout the night; approximately 2hrs after sundown, the middle of the night, and approximately 2hrs before sunrise. The exact time of the checks varied seasonally, as the sunset and sunrise times changed.

We individually tagged each animal, and every month assessed weight and reproductive condition. During the spring of 2016 we conducted habitat surveys at every trap location, following the methods of Brehme et al. 2016.
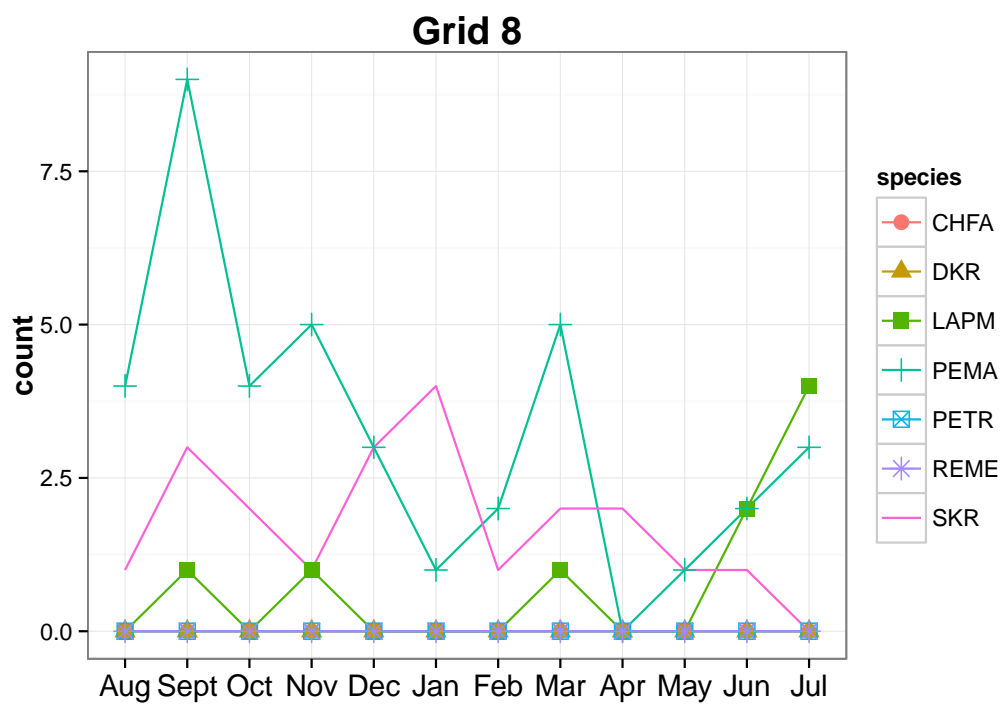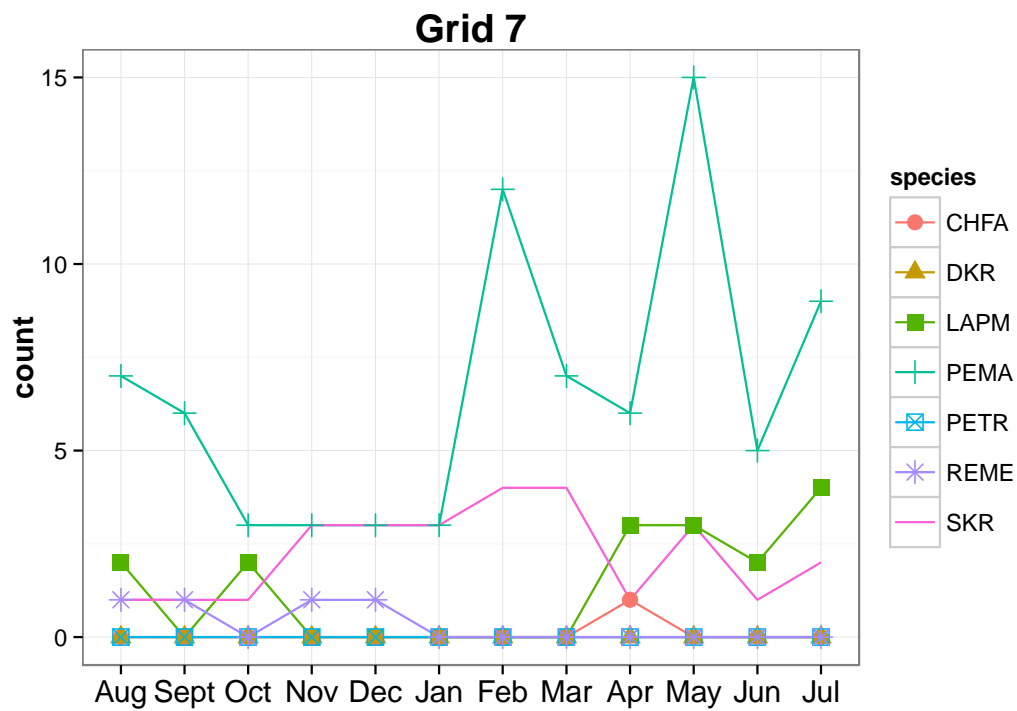
Preliminary results from year-round trapping

The number of unique individuals we trapped each month are displayed by trapping grid below. We are currently working to map home ranges and will assess overlap of spatial and temporal habitat use for each species.

Grid 1

Grid 2

Grid 3

Grid 4

Grid 5

Grid 6

Grid 7

Grid 8
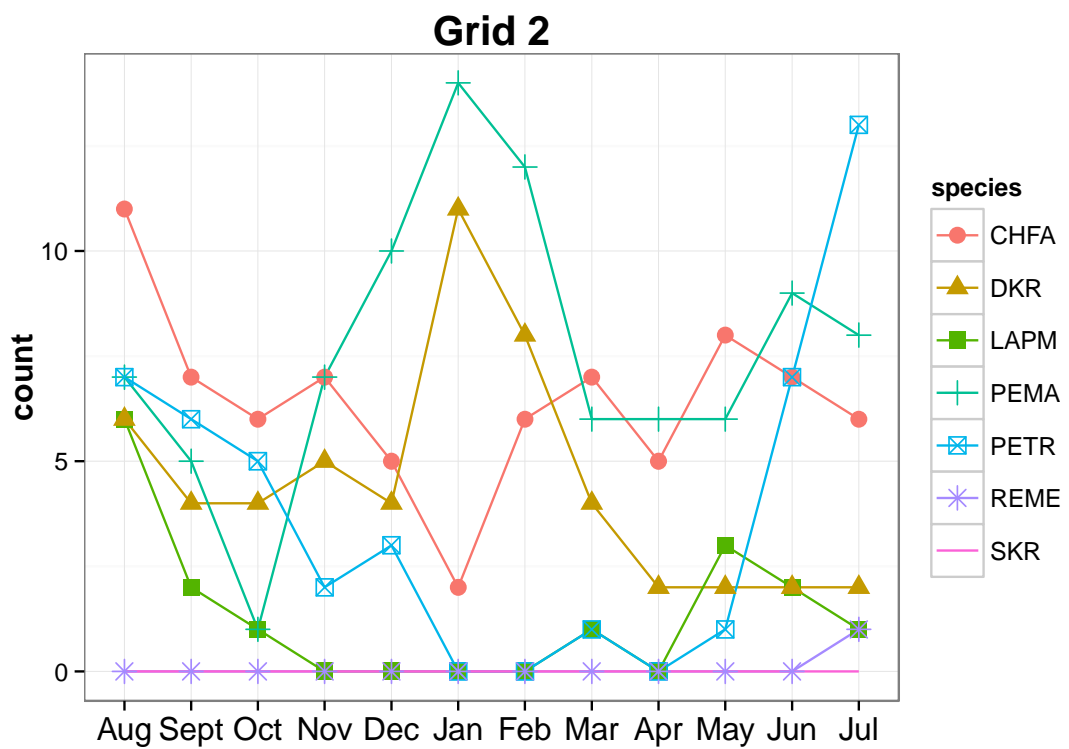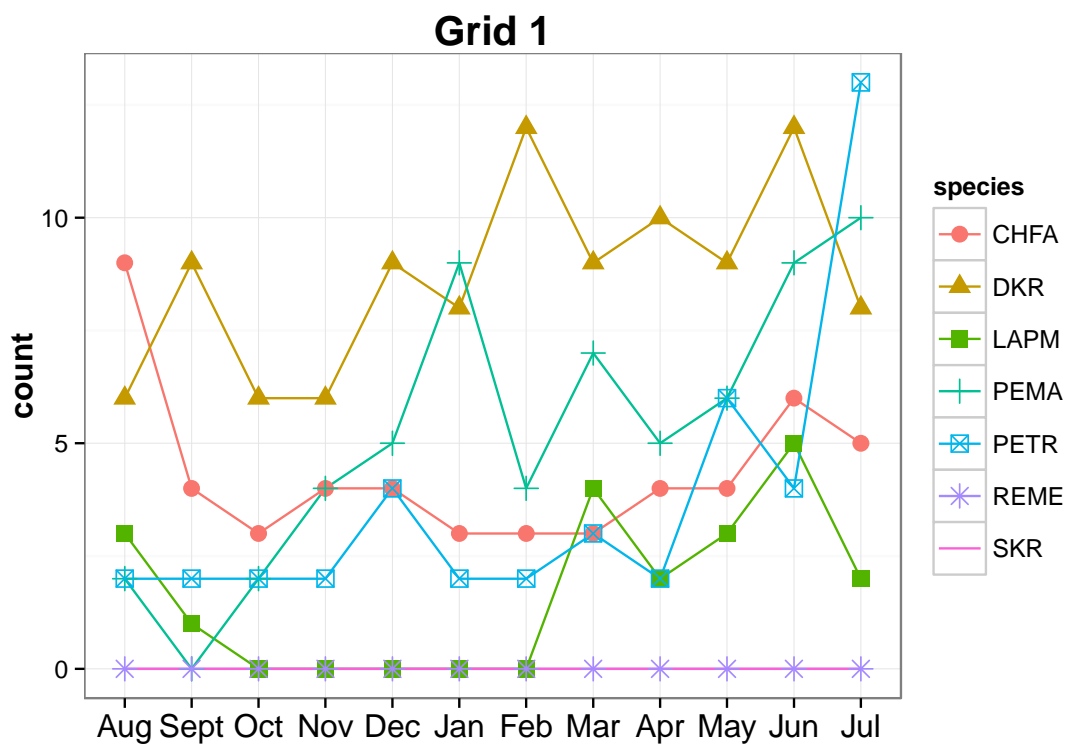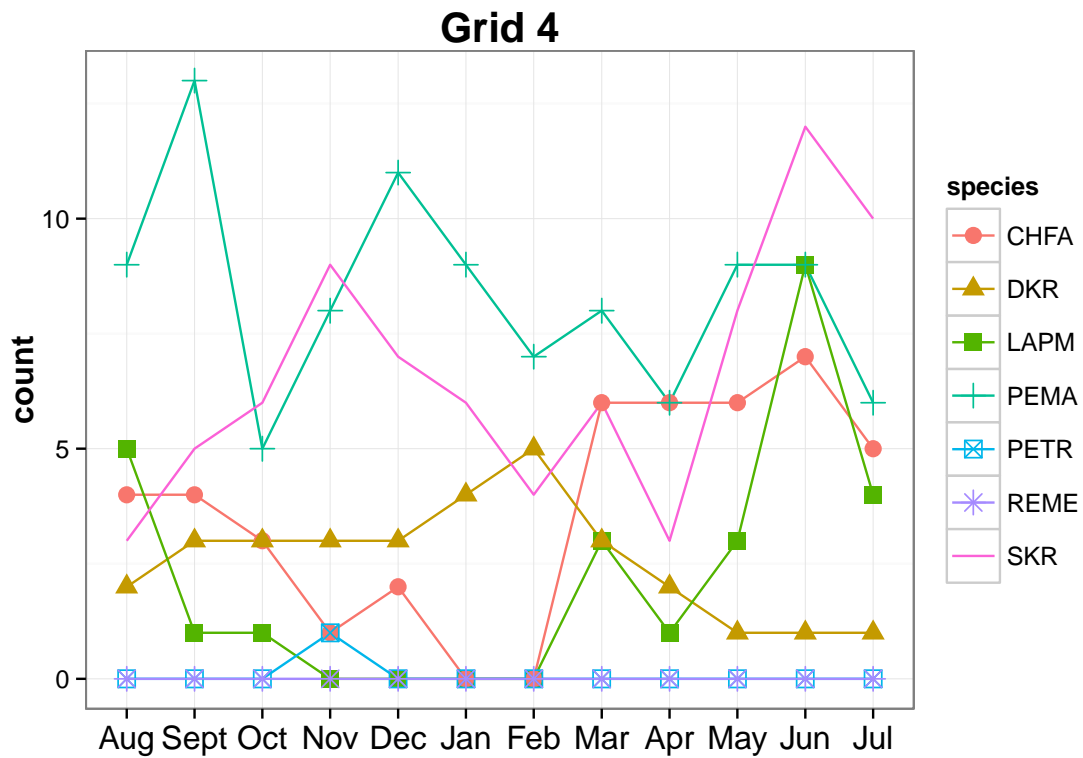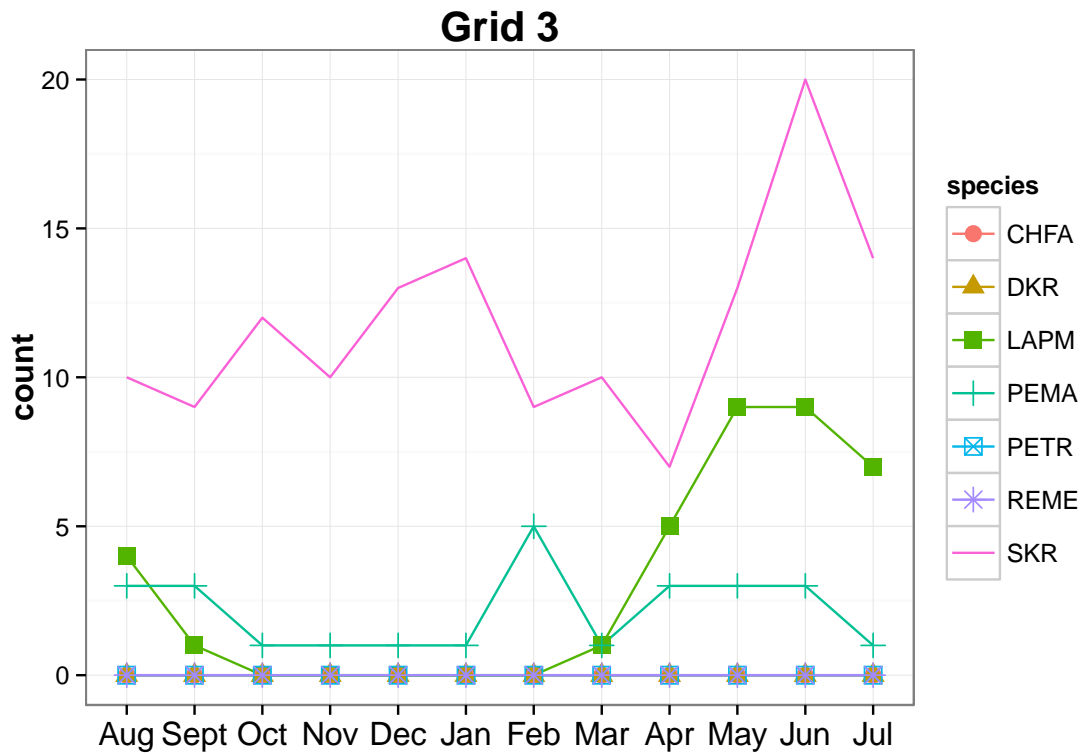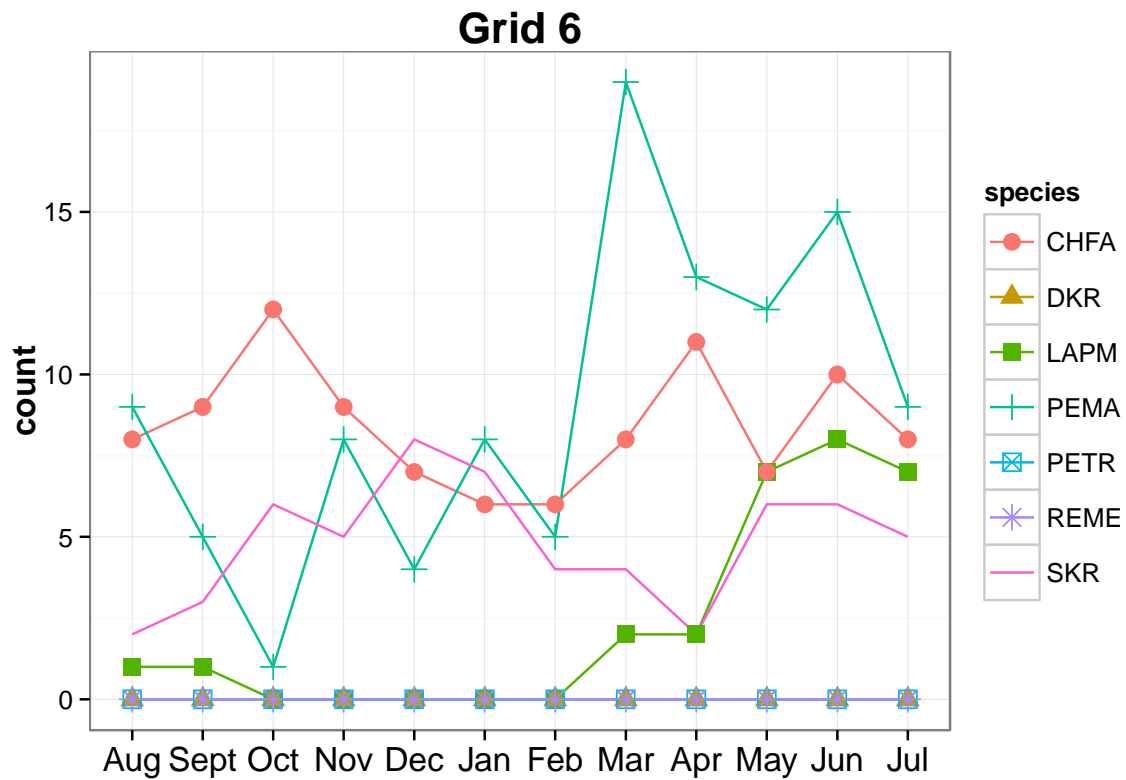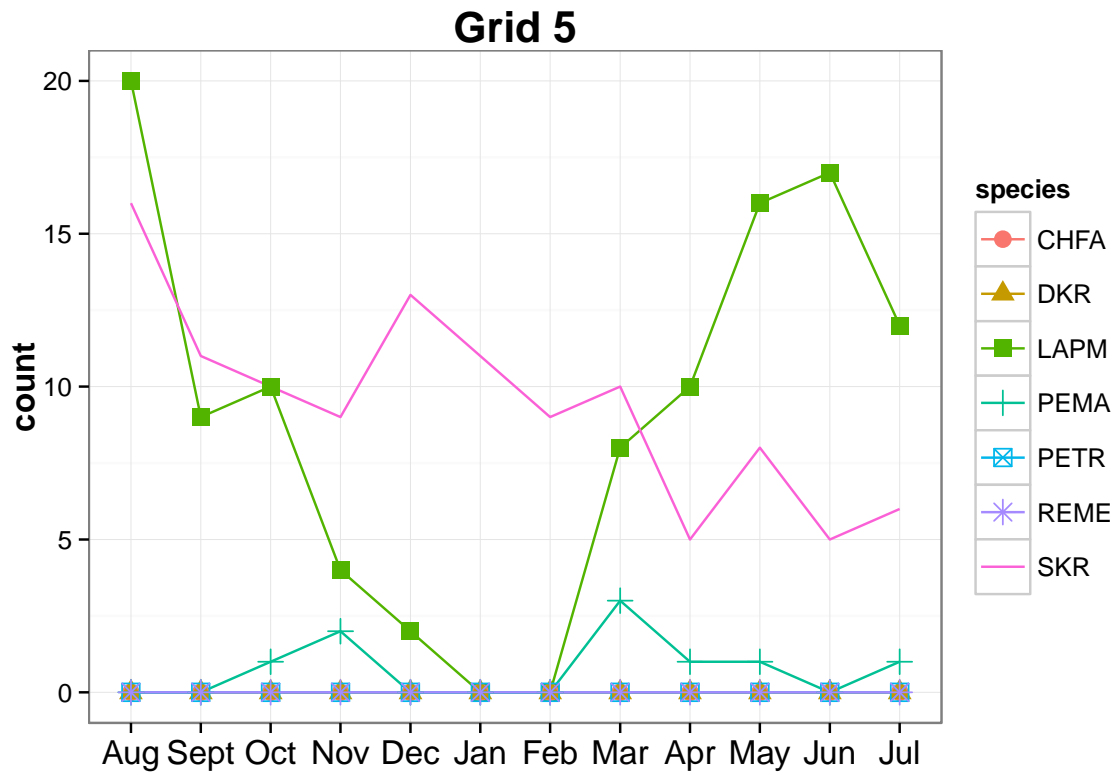
APPENDIX H - TRAIL CAMERA PHOTOGRAPH PROCESSING PROTOCOL AUGUST 2016

As part of the recovery effort to prevent the extinction of the Pacific pocket mouse (PPM), we released 50 PPM from our captive breeding facility (45 captive born, and 5 wild born that were brought into captivity in 2012) into the wild in order to establish the first of several new PPM populations in their historic range. The reintroduction took place in June 2016 at Laguna Coast Wilderness Park in Orange County. One of the methods we employed to monitor this new population is to set trail cameras around the reintroduction site. The camera uses a motion/heat sensor known as a Passive Infrared detector (PIR) that takes a picture when an animal enters its field of view by sensing a change in infrared light. We placed 20 cameras in various locations (i.e. acclimation cages, supplemental feeding stations, new burrows, and other places of interest), and changed locations every one to two weeks.

We have collected a large number of photographs from these trail cameras, and in order to utilize the information, we need to classify what is in each picture (e.g. species, behaviour, number of animals, etc.). The photos are saved on a high capacity external hard drive, and the photographs are organized by date (YYYYMMDD), camera number (1-20), and SD card (a or b).

Photo processing will be done with the program Adobe Bridge which is set up to easily navigate to the appropriate folder, view the photos, and tag each photo with keyword(s).

The Binder

There is a binder labeled "PPM Trail Camera Processing 2016" which contains the datasheets needed for photo processing.

- *Trail Camera Photograph Processing Protocol August 2016* – This document.

- *Volunteer Hours* – Please fill out the day and hours you worked.

- *Files Completed* – Please fill out each time you tag photos.
    - Completed – Check if you tagged the entire folder, or completed an unfinished folder
    - Folder Names – These are prefilled.

        -Date (YYYYMMDD-MMDD) the camera was in a particular location (e.g. June 15th-22nd, 2016 is 20160615-0622).

        -Camera number and SD card (e.g. 13b).

    - *Initials* – Write your initials beside the folder(s) you worked on.

    - *Date* – Write the date you worked on the folder(s).

- *Comments* – Write anything you think is of note (e.g. you only completed tagging photos 01-67, all the photos in the folder were bad due to poor angle).

- *Best of Photographs 2016* – If you tag a photo as "Best of " please record on this data sheet the date, folder names, file name, initials, description.

- *QC Shauna* – QC stands for quality control. Please fill out if you are uncertain of what is in the photograph, and you want Shauna to ensure you have tagged the correct keywords.

  - *Date* – Today's date

  - *Initials* – Your initials

  - *Folder names* – Date (YYYYMMDD-MMDD) and SD card (e.g. 3a)

  - *Photo number* – The file name of the particular photograph in question. You only need to write the number (e.g. if file name is I_00013, write 13).

  - *Questions or Comments* – Write what you are uncertain of, and what specifically you would like Shauna to verify.

Logging on to computer/server

To sign in to the computer, click the Novell Logon icon. Then click the "Computer Logon" option. Enter username (ppm) and password (ppm1). At ZENworks prompt, click cancel.

To log in to the server where the photos are stored (folder…, enter the username (ppm) and password (ppm1).

All folders are stored on the drive Heteromyid (\\aae-storage)(H:). The folders are organized by the dates the camera was in a particular location (i.e. YYYYMMDD-MMDD), the camera number and SD card letter (e.g. 2a), and the type of file (i.e. 100CUDDY are photographs, and 300CUDDY are videos). All photographs will be tagged prior to working on the videos.

Using Adobe Bridge

1. To open Bridge, click on the icon on the toolbar along the bottom.

2. Make sure you are working with the correct Keyword list.

   On the left hand side there is a pane labelled Keywords. You want to make sure you are working with the list labelled ZZ_PPM. By clicking the arrow on the left hand side of the lists, you can collapse and open a list or category. Please collapse all other lists, and make sure all categories under ZZ_PPM are open.

3. Select the folder you will be working on.

   On the left hand side of the screen there is a pane that has two tabs: Favourites and Folders. Navigate to the folder you are going to work on (e.g. Heteromyid (\\aae-storage)(H:)→ 20160615-0622 → 11b → 100CUDDY). The photos will then show up in the bottom pane.

4. Click on the first image (or the image you are starting on if a folder is partially complete). A single click will bring the image up in the center pane. A double click will open the photograph in another window.

You are now ready to start tagging the photograph (see Keyword List).

When you are finished for the day, simply close Bridge. Your work is automatically saved.

Keyword list

The Keyword list is divided into categories (i.e. ear tags, image quality, interesting event, number of animals present, questions, and species). Below are descriptions of the tags under each category.

Ear tags – Select "Yes" if you see an ear tag in either ear or both. If you are unsure, check yes and QC Shauna. If there are not any ear tags present, then leave unchecked. Only Dulzura kangaroo rats (DKR), and the two *Peromyscus species* are tagged; however, not all of those species are tagged.

Image Quality:

   Bad Image – The quality of the photo is poor (e.g. camera angle is off and you cannot see the ground, image is washed out (Figure 1), condensation on the camera, etc.). You can mark a photo/video as bad even if you have tagged something in it (this will indicate a low level of confidence in the identification). To mark multiple photos/videos as "bad" select all the images that apply in the film strip pane (middle bottom pane of the Bridge screen), and then click "Bad Image" in the keyword pane (right side of the Bridge Screen).

Figure 1. An example of a bad image, and unknown species. The photo is washed out, and the camera was placed too close to the target.

> **Best of** – The photo is extraordinary (i.e. could be used in reports, presentations, posters, etc.; Fig. 2 and 3), and either shows the animal or behaviour (e.g. emerging from a burrow, using a supplemental feeding station) really well. "Good Image" can be marked for any photo (not just ones of PPM). Fill out a line on the Best of Photographs 2016 data sheet for each photographed labelled "Best of."

Interesting event:

> **Emerging from or entering a tube (Figure 2)** – After the 7 day acclimation period, we removed the top portion of the cage, and left the bottom portion in the ground. All materials are biodegradable. There are "burrows" (small mailing tubes the lead to the underground chamber).

Figure 2. A Pacific pocket mouse entering an artificial burrow.

Using supplemental feeding station (Figure 3) - We have placed 50 supplemental feeding stations throughout the reintroduction site. The stations are composed of two PVC pieces put together to make a "T." Please select if you see any animal (not just the mammals, but birds, snakes, lizards etc...) in, on, or investigating a station.

Figure 3. A Pacific pocket mouse using a supplemental feeding station.

> **Circling/Copulation** – These are reproductive behaviors, and I will show you a video of examples.

> **Predation** – I currently do not have an example of a predation, but please select if there is an image of any animal being killed by another/others.

> **Chasing –** I currently do not have an example of chasing, but please select if there are two animals in the image, and one appears to be fleeing, and the other is pursuing.

Number of animals Present: Select how many animals present in the photograph (i.e. 1, 2, or more than 3).

QC Shauna – QC stands for quality control. Tag any photo you are uncertain of. It is better to mark a photo and be wrong than to not mark a photo and miss an event. Then fill out the "Quality Control (QC) Shauna 2016" data sheet in the binder.

Species:

> **Arachnid** – We are specifically interested in noting scorpions and tarantula; however, if there is an arachnid in a photo interacting with a mouse, please tag with other or unknown.

> **Avian** – Any bird species. The most common birds you will see are Mourning doves and California towhees

> **Mammalian**

> Identifying the small mammals can be challenging; however, the more you go through the photographs, the more comfortable you will become. Please remember it is better to

tag a photograph and be wrong, than to not tag a photograph. If uncertain, you can always tag the photograph with "QC Shauna."

The main features which will assist in identifying the small mammals are size, tail length, presence of a tuft on the tail, ear shape, and locomotion (Table 1).

Table 1. A coarse comparison of the main features of the small mammals you will likely see: size [ranked largest (1) to smallest (4), and an approximate average weight), tail length (in reference to body length, presence of a tuft on the tail (yes or no), ear shape (size is relative to its body, and shape), and locomotion (bipedal or quadrupedal).

Species	Size (weight)	Tail length	Tuft on tail	Ear Shape	Locomotion
CHCA	3 (18 g)	Very long	Yes	Oblong	Quadrupedal
DKR	2 (70 g)	Very long	Yes	Round	Bipedal (long hind feet)
PPM	4 (6 g)	More or less equal	No	Small and round	Quadrupedal
Peromyscus sp.	3 (18 g)	Shorter or slightly longer	No	Large and oblong	Quadrupedal
Thomomys sp.	1 (250 g)	Shorter	No	Small and round	Quadrupedal

When using size to help identify the small mammal keep in mind that the mice will appear larger when closer to the camera, and smaller when further from the camera.

California Pocket Mouse (CHCA; Figure 4)

- Smaller than a DKR, bigger than a PPM, and around the same size as a PEER/PEMA.
- Smaller ears than PEER/PEMA, but larger than PPM
- Ears are more oblong than round
- Long tail, but not as long as a DKR, with a tuft at the end
- Fur, particularly on the rump, sticks out like spines

Figure 4. CHCA by a supplemental feeding station. Notice the long tail with a tuft on the end, the posture, and the ears.

Dulzura Kangaroo Rat (DKR; Figure 5)

- the largest of the mice
- a large, flat space between the eyes
- have a long tail with a tuft at the end
- long-hind feet
- large round ears (may have an ear tag in one or both ears)
- predominantly bipedal

Figure 5. An ear tagged DKR. Notice the long tail with a tuft at the end, a black "mustache" on the muzzle, round ears, and a wide space between the eyes.

Deer mice (Peromyscus sp.: PEER and PEMA; Fig. 6)

- There are two species of Peromyscus present at the site: cactus mouse (PEER), and deer mouse (PEMA)**.** There are subtle differences between the two that are difficult to see in these images. We have tagged deer mice in the right ear, and cactus mice in the left ear; however, not all mice are tagged (e.g. born after the tagging event, or was not trapped)**.** Therefore, cannot rely on ear tags to differentiate between the species, so we have grouped them together.
- Smaller than a DKR, but larger than a PPM
- Large ears, that are more oblong than round
- Face is longer and slightly more pointed than the other species
- Quadrupedal
- Tail length is slightly shorter or longer than the body length (depending on species)
- Dark fur line above muzzle (PPM too)

Figure 6. A *Peromyscus*. Notice the large oblong ears, and the pointy nose. The mouse is in the same spot as the DKR in figure 5.

Pacific pocket mouse (PPM; Fig. 7)

- The smallest mouse in North America
- Tiny round ears
- Dark fur line above muzzle (DKR too)
- Quadrupedal

Figure 7. A PPM. Notice size, body posture, small ears, and a "mustache" on the muzzle. The PPM is in the same spot as the DKR in figure 5, and *Peromyscus sp*. In figure 6.

Pocket gophers (Thomomys sp.; Fig. 8)

- Largest rodent we have seen on the camera
- Small eyes and ears
- Short tail
- Quadrupedal

Figure 8. A pocket gopher. Notice the size, and the small eyes, and ears.

Other –Any other mammal (e.g. coyote, or rabbit) that is not described above.

Reptilian- We are specifically interested in noting snakes and lizards.

Other/Unknown – Select if you see an animal that does not fit into one of the above categories, if you are unsure if there is an animal present, or the animal cannot be identified (Figure 1). It is better to tag a photo than not to because we will not be looking at the untagged photos, and we could potentially lose that data.