

Sample Grant Proposals

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Established Investigator

I. Title: Towards predicting plague epizootics: Understanding free-living amoeba as an inter-epizootic host for sylvatic plague

Rationale: *Yersinia pestis*, the causative agent of bubonic plague, is transmitted by flea bite. The plague is difficult to eradicate because it is widespread in wild rodents and their associated fleas, and has a continuing foci of endemicity during quiescence. Since its introduction into North America at the turn of the 20th century, plague has caused the precipitous decline of wild rodent species e.g. prairie dogs and brought their predator, the black-footed ferret, to the brink of extinction. Natural sylvatic cycles of plague are composed of two phases: i) a prolonged quiescent phase during which *Y. pestis* persists undetected in an unknown reservoir, and ii) unpredictable re-emergence of an epizootic phase characterized by rampant death of susceptible hosts (prairie dogs, the endangered black-footed ferret) and amplified levels of disease that heighten risk of transmission to other animals (e.g. mountain lions, domestic cats and dogs). Understanding how plague persists during inter-epizootic periods and then re-emerges into epizootics is paramount to improving approaches to understand risk and predict and prevent epizootic events and large scale animal death.

Hypothesis: We hypothesize that bacterivorous free-living soil amoeba (FLA) serve as an alternate host and soil reservoir for *Y. pestis* during quiescent inter-epizootic plague cycles that supports reactivation of plague epizootics.

Study Design: In this study we propose to make an experimental investigation into whether FLA that are isolated from an active plague foci can support long term survival and /or replication of *Y. pestis* in controlled laboratory culture. This will be followed by determining if such an interaction is supported in a rodent burrow soil simulating environment under conditions that are consistent with seasonal epizootic and inter-epizootic plague cycles.

Preliminary Data: Our preliminary studies provide the first evidence for replication and prolonged survival of *Y. pestis* within a laboratory strain of the FLA, *Acanthamoeba castellanii*. We have subsequently isolated and identified FLA species, including *Acanthamoeba* spp, from black-tailed prairie dog burrow soils of a plague foci in the Cathy Fromme prairie in Colorado. This prairie dog community has recently experienced a die-off from plague. These data support that colocalization of *Y. pestis* and FLA in prairie dog burrows is probable, and warrants further investigation of this interaction.

Expected Results: We anticipate developing essential method and expertise to detect the *Y. pestis*-amoeba interaction in endemic plague foci. As well, this study should advance understanding of how amoeba act as a reservoir host for *Y. pestis* during quiescence. This work has the potential to initiate pioneering efforts into detecting the *Y. pestis*-amoeba interaction in plague endemic areas, and developing strategies to predict plague recrudescence and epizootics.

Budget and Timeline: During Year One the ability of amoeba from plague endemic areas to support long term survival and and/or replication as outlined in Aim 1, will be performed. Concurrently, optimization of experiments in Aim 2 will be undertaken. In Year Two final investigation into the dynamics of bacterial survival in amoeba in a burrow soil simulating environment and manuscript preparation and submission will be accomplished. Funding [REDACTED] per year over two years is sought.

Potential Impact for Animal Health: This research aims to discover the mechanisms that allow *Y. pestis* inter-epizootic persistence and drive its re-emergence. This is especially significant as current bio-surveillance methods are focused on serology testing in wild rodents or bacterial culturing from fleas that often prove unsuccessful at detecting plague even immediately prior to epizootic events. Knowledge of *Y. pestis* survival in plague foci endemic FLA will allow specific detection of *Y. pestis* in plague foci. The approach of predicting a plague outbreak, will allow preemptive measures to be implemented (e.g insecticide spraying to kill fleas in rodent burrows prior to reactivation of an epizootic; insecticide use is usually only undertaken when rodent deaths are observed as indication of the presence of disease). Pre-emptive measures can prevent large scale prairie dog and back-footed ferret die-off and death in the first place. As such the research is consistent with the Morris Animal Foundation funding criteria for relevant research.

III. Name, Institution and email address

Principal Investigator:

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Technician:

To be determined

IV Study proposal

1. Specific, testable Hypothesis and Objectives

Yersinia pestis, the causative agent of sylvatic plague, is transmitted by flea bite. Natural sylvatic cycles of the plague are composed of two phases: 1] a prolonged quiescent phase during which *Y. pestis* persists undetected in an unknown reservoir, and 2] unpredictable re-emergence of an epizootic phase characterized by rampant death of susceptible animal hosts and heightened risk of transmission to other animal species including domestic cats and dogs. Understanding how plague persists during inter-epizootic periods and then re-emerges into epizootics is needed to improve approaches to understand risk and reduce epizootic transmission. Identification of the reservoir that supports long-term quiescent persistence of *Y. pestis* however remains enigmatic. Epidemiologists/ecologists have postulated that bacterivorous soil FLA phagocytose *Y. pestis* and serve as a soil reservoir for this pathogen, similar to what has been demonstrated for other bacterial pathogens, e.g. *Legionella pneumophila* (1-3). This model posits that, in the first step, *Y. pestis* enters and survives, and remains protected within the vegetative active trophozoite amoeba forms in the soil. In the next step which leads to recrudescence of the epizootic cycle, *Y. pestis* infected amoeba can act as a vehicle for re-infection of a foraging rodent or larval flea in the soil (Fig 1). As proof-of-concept our preliminary studies provide the first evidence for replication and prolonged survival of *Y. pestis* as residents of vacuoles in amoeba trophozoite forms of the soil FLA, *Acanthamoeba castellanii*. The next logical steps in defining if FLA can serve as natural reservoir hosts of quiescent *Y. pestis*, is to understand the natural biological context of the *Y. pestis*-amoeba interaction.

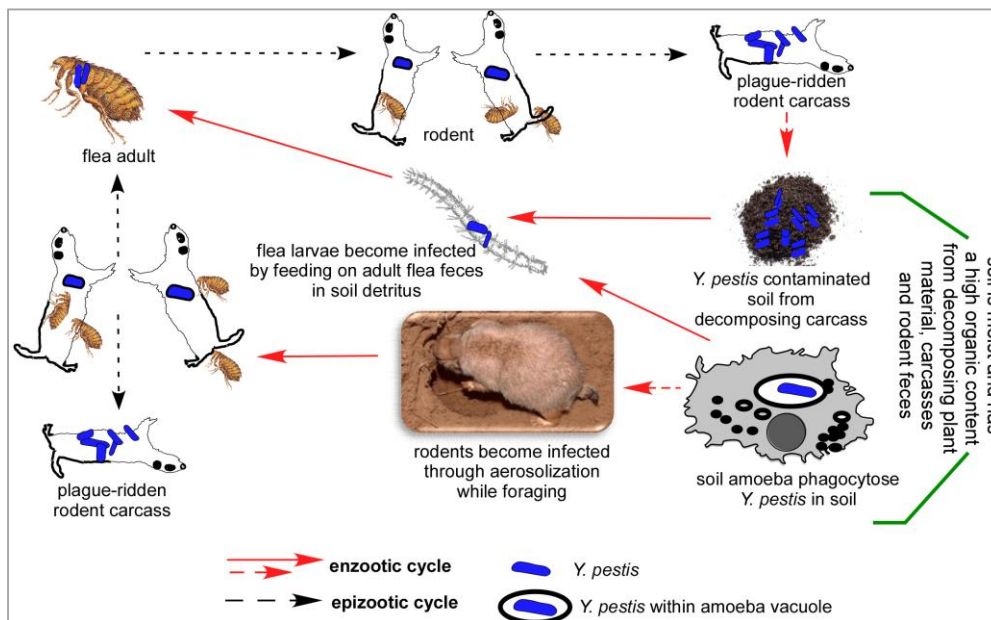


Fig. 1. Model for how amoeba serve as an alternate host reservoir of *Y. pestis* during enzootic plague periods

survival and replication of *Y. pestis*. Abundant and diverse FLA that can serve as reservoirs for bacterial pathogens have been documented in areas endemic for their associated diseases e.g. *L. pneumophila* (2, 3) and *Mycobacterium ulcerans* (4). To this end, we have cultivated viable FLA, including *Acanthamoeba* spp. from soil collected from burrows of prairie dogs that have recently experienced a die-off from plague. A quantitative determination of long-term survival and replication of *Y. pestis* in trophozoites will be achieved for the different FLA species. Defining the level of *Y. pestis* surviving in trophozoites can be used to establish the threshold for detection of infected amoeba in rodent burrow soils of endemic plague foci

Specific Aim 2: Determine if the ability of *Y. pestis* to survive long-term and/or replicate in amoeba can be recapitulated in a simulated burrow soil environment. This simulated environment should mimic the environmental conditions during epizootics or the inter-epizootic stage. Thus far we have shown experimentally that under favorable axenic laboratory medium culture conditions, *Y. pestis* has

Our proposal aims to address this knowledge gap by **determining if FLA present in burrows of black-tailed prairie dogs that have recently experienced plague epizootics, are capable of supporting long term survival and replication of *Y. pestis*.**

Specific Aim 1:

Determine if FLA present in burrows of black-tailed prairie dogs that have recently experienced plague epizootics are capable of supporting long term

the ability to survive and replicate in amoeba (Fig 2). This is the first demonstration that *Y. pestis* is able to evade digestion and survive for a prolonged period of time in bacterivorous amoeba. However it is not known if such an interaction is supported in a natural soil environment.

2. Justification , Significance and Literature Review

Impact of plague on wild rodent species and the endangered black-footed ferret. Since the introduction of plague to North America at the turn of the 20th century, *Y. pestis* has established itself in endemic wild rodent populations (5). This has led to the precipitous decline of particularly susceptible endemic rodent species e.g. prairie dogs (*Cynomys* spp.). The prairie dog predator, and only endemic US ferret population, the black-footed ferret (*Mustela nigripes*), has also been brought to the brink of extinction due to plague. To preserve the endangered black-footed ferret, a captive-breeding program aimed at latter reintroduction of this species into viable prairie dog habitat has been ongoing (6). A successful full recovery of the species however has yet to be achieved, and continues to be a challenge due to continued plague outbreaks in these areas. Amoeba as an inter-epizootic reservoir host for *Y. pestis*: *Y. pestis*, the etiological agent of plague has arisen as a clonal variant of *Yersinia pseudotuberculosis*, which can survive for long periods in soil (7), while *Y. pestis* cannot. Comparative genomics between *Y. pestis* and *Y. pseudotuberculosis* (7) has revealed that the *Y. pestis* genome has undergone genomic decay that has resulted in degradation of several metabolic pathways. This is believed to account for its host (flea or rodent) requirement for propagation. Beyond these two host environments however, there has been minimal evidence for *Y. pestis* to transmit or persist in other life stages. This is consistent with the long-standing inter-epizootic host persistence theory for *Y. pestis*, which postulates that plague is maintained naturally in infected fleas feeding on a heterogeneous

population of susceptible and relatively resistant hosts. This model does not however address the following conundrums that collectively suggest an additional environmental maintenance reservoir for *Y. pestis*. These are that: (i) bacteremia levels of relatively resistant hosts are low and insufficient for flea acquisition of *Y. pestis* from the bloodmeal to perpetuate the flea–rodent transmission cycle (8), (ii) seldom are infected fleas or seropositive rodents recovered after surveillance of endemic plague foci (9), (iii) re-emergence and epizootics are infrequent and can sometimes occur >50 years or more, beyond the lifespan of fleas and hosts, (iv) ecological modeling studies show that susceptible burrow rodent metapopulations have low probability of supporting plague quiescence and re-emergence to produce epizootics at regular intervals (10). Alternately, a soil reservoir for maintenance of *Y. pestis* during inter-epizootic periods has also been hypothesized, and under experimental conditions that assume *Y. pestis* is free-living and metabolically

active, persistence of *Y. pestis* in soil has been demonstrated, but only over short periods of time (9, 11-13). This fails to explain limited detection of the organism in soil during inter-epizootic periods at plague foci. Why amoeba? Nevertheless, the survival of *Y. pestis* inside an alternate host, like bacterivorous soil

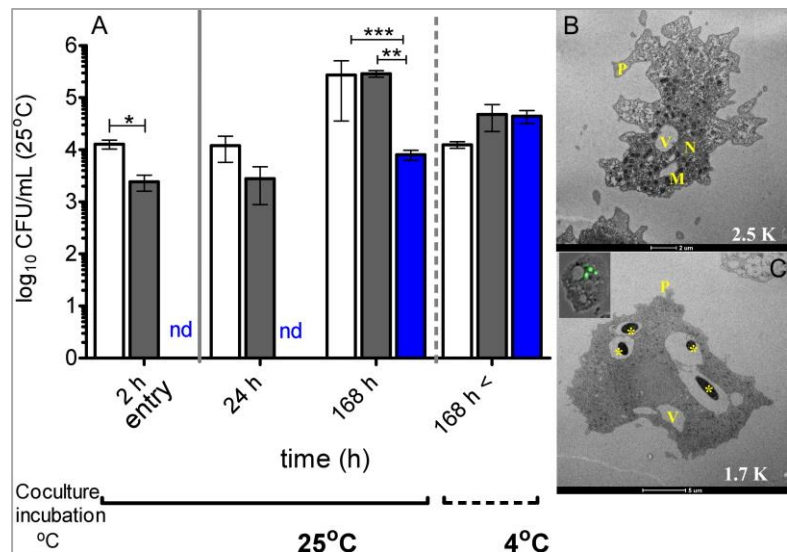


Fig. 2. Survival and replication of *Y. pestis* strains in trophozoites of *A. castellanii* at epizootic temperature of 25°C (2h, 24h and 1 week) and inter-epizootic temperature of 4°C (1 week). Error bars represent mean±SD of at least 3 independent biological replicates. Statistical significance from One Way ANOVA analysis followed by a post Tukey's test is represented by *P<0.05, **P<0.01, ***P<0.001. *Y. pestis* strains are white bars: KIM5; grey bars: KIM6+; blue bars: *phoP* mutant. nd= not detected. **Electron micrograph images** of uninfected amoeba trophozoites (B) and *Y. pestis* KIM5 infected *A. castellanii* trophozoites. (C) *Y. pestis* KIM5 infected amoeba. (*) *Y. pestis* KIM5, inset image in C represents intra-amoebal GFP-*Y. pestis* KIM5. V=vacuole, N=nucleus, P=pseudopodia, M= mitochondria

FLA, could simultaneously explain the existence of a soil reservoir, and satisfy a host requirement for *Y. pestis* and the ability to persist and maintain plague cycles. Survival of several bacterial pathogens, including *Legionella pneumophila* (1), *Francisella tularensis* (14), and *Mycobacterium avium* (15), has been mechanistically characterized within both the metabolically active trophozoite and dormant cyst forms of the host-like soil FLA *Acanthamoeba* spp. These bacteria are able to survive and are protected from the environment within amoeba (16, 17). To evade being digested by amoeba, similar mechanisms as those used to resist digestion in mammalian host immune cells (e.g. macrophages) are often used by pathogenic bacteria (18). One example, is that of *Legionella pneumophila*, the bacterial agent of a pneumonic infection called Legionnaire's disease that persists in aquatic systems in environmental FLA (18). These bacteria reside in phagosomes and evade phagocytic destruction by deploying bacterial effectors secreted from the Dot/Icm secretion system in both amoeba and macrophages (18). *L. pneumophila* demonstrates enhanced virulence following intracellular persistence in amoeba (1, 19), and intranasal infection of mice with *L. pneumophila*-containing amoeba leads to development of Legionnaire's disease (2, 3).

The prairie burrow environment is conducive to an amoeba-*Y. pestis* interaction. Black-tailed prairie dogs, *Cynomys ludovicianus*, are ground dwelling rodents that live in large social communities connected by extensive tunnels and burrows (called towns) in the short grass prairie plains in Northern Colorado. These rodents epitomize the sporadic nature of plague because they experience extinctions from plague epizootics frequently during warmer and wetter climatic conditions as opposed to absence of plague during drought conditions (20, 21). During these epizootics the microclimate maintained in the prairie dog burrow is conducive to maintaining amoeba in their trophozoite form e.g. moist soil (22, 23), high organic nutritional content maintained by plant material that rodents feed on, rodent feces, and decaying carcasses of rodents that have succumbed to plague, high bacterial content as a nutritional source for bacterivorous amoeba (21, 23). Consistent with this, *Acanthamoeba polyphaga* FLA species is commonly present in soils of the Central experimental station short grass prairie where black-tailed prairie dog

populations have often become extinct from plague (15, 24, 25). *A. polyphaga* acts as an environmental host to various pathogens (26, 27). Based on these factors a model for the survival and recrudescence of *Y. pestis* from an amoeba host has been envisaged. In this model *Y. pestis* enters and survives in vegetative active trophozoite amoeba forms during cooler quiescent periods. In the second step, during favorable warmer and wetter environmental conditions infected trophozoites will be acquired by flea or rodents resulting in reactivation of the epizootic disease cycle (Fig 1).

3. Preliminary data

To address this model we quantified the intracellular survival of bacteria after allowing bacteria to be phagocytosed for an hour and then killing of extracellular/unphagocytosed bacteria with gentamycin. Bacterial survival in trophozoites is then assessed in a nutritive axenic culture medium, PYG, which supports continued metabolic activity in trophozoites. Our preliminary studies consequently provide the first quantitative evidence for entry, prolonged survival and replication of *Y. pestis* within trophozoites (Fig. 2) of *A. castellanii*. We used a strain that contained a mutation in PhoP which is a factor that is required for *Y. pestis* to enter and survive in macrophages (Fig 2) (28, 29). Similar to its role in macrophages, PhoP appears to be required for entry in amoeba. This data is in keeping with the prevailing paradigm that bacteria use conserved mechanisms to survive both in amoeba and macrophages

(18, 30). Consistent with our quantitative data, electron microscopy (EM) shows that intact *Y. pestis* occurs in phagosomes of *A. castellanii* (Fig 2C). A previous study has observed *Y. pestis* within the FLA, *Hartmanella rhysodes* (31). These findings support our model and identifies FLA as an alternate host (besides rodents and fleas) that can support long-term survival and replication of *Y. pestis*.

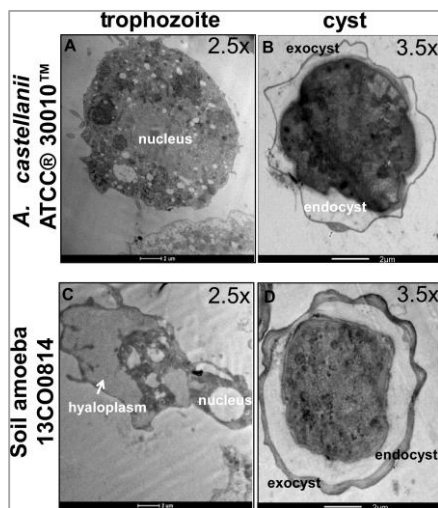


Fig. 3. Electron micrograph images of trophozoites (A, C) and double walled mature cyst forms (B, D) of lab *A. castellanii* and burrow *Acanthamoeba* spp. 13CO0814 respectively. Burrow *Acanthamoeba* has a pronounced hyaloplasm that supports it faster locomotion than *A. castellanii* in lab culture medium.

Most approaches in plague research are aimed towards development of vaccine strategies to prevent plague. Only a few studies focus on plague surveillance and predicting re-emergence as a means to preventing the disease. Plague surveillance in endemic areas is challenging based on the difficulty of detecting the pathogen in the environment during inter-epizootic cycles (32). Indeed a lack of detection of *Y. pestis* in the soil in endemic areas during inter-epizootic periods may be related to the bacteria surviving within an amoeba host at low levels, rather than freely in the soil. If *Y. pestis* can be detected during this quiescent cycle, the ability to predict its re-emergence will be practical. This could lead to novel strategies being implemented that prevent disease prior to epizootics. As the biological relevance of *Y. pestis* being harbored by amoeba has not been established, this proposal aims to establish biological relevance of *Y. pestis* in FLA that we have identified from burrows of black-tailed prairie dogs that have recently experienced plague epizootics. Therefore in a first approach to determine if *Y. pestis* can be harbored in FLA species, soil from burrows in the Cathy Fromme short grass prairie in Northern Colorado where plague epizootics resulting in black-tailed prairie dogs die-offs have very recently been recorded, was investigated for the presence of FLA. We received soil sample collections from our collaborators (Drs Eisen and Montenieri) of the Plague Group at the CDC in Fort Collins, CO. Following standard isolation and enrichment culturing methods, and characterization based on morphology and 18S rRNA sequencing, we have been able to culture a few FLA species including *Hartmanella* and *Acanthamoeba* spp., and have achieved culturing a pure isolate of the *Acanthamoeba* spp. (Fig. 3).

4. Experimental methods and design

The *Y. pestis* KIM5 (33) and isogenic KIM5 *phoP* mutant (28) avirulent strains will be used for the studies outlined below. The *Y. pestis* KIM5 strain contains the T3SS and is genetically more representative of a fully virulent *Y. pestis* strain. Because the *Y. pestis phoP* mutant strain (34) is defective in its ability to productively infect *A. castellanii* (Fig. 2) it will be used as a negative control in quantitative intracellular survival studies. The trophozoite life stage is predicted to support infection and re-emergence.

Specific Aim 1: Determine if ecologically relevant burrow *Acanthamoeba* spp. can support intracellular survival and replication of *Y. pestis*

Rationale: *Y. pestis* is expected to enter the soil in rodent burrows in a plague endemic area, from a decomposing carcass of a rodent that has succumbed to plague, or from flea feces (Fig 1). Within soil numerous FLA species are present that likely phagocytose *Y. pestis*. Within amoeba *Y. pestis* is protected from the harsh soil environment (Fig 1). *Acanthamoeba* spp trophozoites have a growing range of 4-27°C (35, 36). This is consistent with extreme lower and upper temperature limits expected in subterranean rodent burrows during seasonal inter-epizootic and epizootic periods (23, 37). These temperature limits will be used to test long term intracellular survival of *Y. pestis*. Similar to *A. castellanii* we have observed that at 4°C temperature, amoeba remain as trophozoites for over a month at least, and do not multiply as rapidly as at ambient temperature, consistent with published findings.

Methodology: Gentamycin protection assays designed to evaluate *Y. pestis* survival and replication will be used to quantify *Y. pestis* survival and replication in naturally occurring FLA. Bacteria will be grown at 25°C consistent with the burrow temperature during epizootics prior to co-culture. Co-cultures will be incubated at both ambient temperature of 25°C and 4°C to investigate the intracellular survival and/or replication at expected epizootic and inter-epizootic periods, respectively. The assay is a modification of our published methods used to determine that *Y. pseudotuberculosis*, and not *Escherichia coli* DH5α survives and replicates in *A. castellanii* trophozoites and cysts (38). *A. castellanii* will be used as a positive control for its ability to phagocytose *Y. pestis*. *Klebsiella pneumoniae* will be used as a negative control as it is utilized as a food source by burrow FLA. At least 3 independent biological replicates of interaction assays will be undertaken and a Student's T-test will be undertaken to determine significant differences in entry/uptake and efficiency of survival and replication in natural burrow amoeba *Acanthamoeba* for the two *Y. pestis* strains to be tested. **Analysis and alternatives:** *Y. pestis* is expected to survive and replicate in burrow amoeba at ambient co-culture temperatures, and survive but not replicate at 4°C, similarly to *Y. pestis*-*A. castellanii* coculture assays (Fig 2). This will also be similar to the ability of *L. pneumophila* (39, 40) and other bacterial pathogens (4, 41, 42) to survive in environmental FLA. If the burrow *Acanthamoeba* is unable to support survival and replication of *Y. pestis*, we will test the burrow *Hartmanella* FLA species. **Technical alternatives:** Flow cytometry could be used to assess survival and

replication of *Y. pestis* in trophozoites as has been achieved in other studies defining interactions between bacterial pathogens and FLA (4, 27). Overall we expect that this aim will provide biological evidence that *Y. pestis* can colonize environmentally relevant amoeba species.

Specific Aim 2 Determine if the ability of *Y. pestis* to survive long term and/or replicate in amoeba can be recapitulated in a simulated burrow soil environment.

Rationale: During sterile and highly controlled laboratory culture *Y. pestis* is able to survive and replicate in amoeba. However it is not known if a natural soil environment can support prolonged intra-amoebal survival of *Y. pestis*.

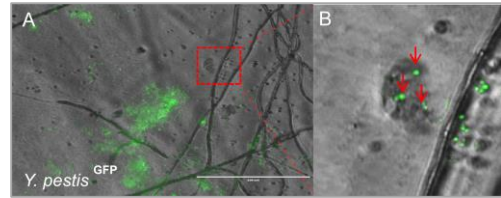


Fig. 4. GFP-*Y. pestis* within *A. castellanii* seeded on agar plates. Image B is a magnification of the red block in image C.

Experimental design: The soil collected from rodent burrows (described in preliminary studies) will be used. Temperature (°C) and relative humidity (%RH) will be maintained within the range observed in rodent burrows during epizootic (~25°C; 68%RH) and quiescent plague cycles (4-10°C, 80%RH). To allow fluorescent microscopic detection of *Y. pestis* a green fluorescent protein tagged *Y. pestis* (GFP-*Y. pestis*; shown in Figs 3 and 4) will be grown and seeded in mouse blood at a concentration of that present in a bacteremic mouse (10^8 - 10^9 cfu/mL). One mL of infected blood will be added to 1g soil. Amoeba will be added to one replicate of soil at $\sim 10^{11}$ trophozoites/g soil to produce a multiplicity of infection of 100, similar to our axenic coculture experiments (Fig 2); the other replicate will serve as a control for bacterial survival in soil lacking FLA. Similarly a soil sample, containing amoeba only, will serve as a control for changes in amoeba numbers in the absence of bacteria. Over a period of two months in which the experimental soil environment will first be incubated at epizootic conditions for two weeks, followed by inter-epizootic conditions for 1 month and then epizootic conditions again for two weeks, soil samples will be collected and processed every 3-4 days to detect changes in bacteria and amoeba numbers as follows: (i) total genomic DNA will be isolated using Mobio Powersoil PowerLyzer® Kits and quantitative real-time PCR of unique target genes encoded on their chromosomes will be undertaken to determine changes in copy numbers of bacteria and amoeba; (iii) as a qualitative determination and for detection of viable bacteria, wetted soil samples will be added to agar wells and *Y. pestis* infected amoeba allowed to proliferate. Fluorescent microscopic analysis of the agar plate has the ability to identify if GFP-*Y. pestis* are present within amoeba. Burrow amoeba was originally isolated from soil using agar plates. Our test experiment using *A. castellanii* and GFP-*Y. pestis* grown axenically, and then seeded on agar plates proves feasibility of identifying intra-amoebal fluorescing *Y. pestis* on agar plates. (Fig 4). This method can be advantageous in detecting low numbers of infected amoeba as these can subsequently be amplified in culture and processed as in Aim 1. Experiments will initially be optimized using *A. castellanii* and tested using burrow amoeba. **Analysis and alternatives:** We expect to identify increases or no alteration in bacterial numbers under epizootic conditions ideal for amoeba proliferation. Under inter-epizootic conditions it's expected that there will be decreases or no alteration in bacterial numbers as conditions should be less favorable for proliferation. However, our hypothesis that amoeba serve as a *Y. pestis* reservoir will still be accepted as long as viable intra-amoebal bacteria are demonstrated at the end of 3 months, irrespective of whether bacterial numbers are altered in either condition.

Overall outcomes: These experiments will provide the basis for specific targeting of amoeba in rodent burrows and for quantitatively defining levels of intra-amoebal bacteria expected in burrows during plague cycles. This is of paramount importance for detecting the *Y. pestis*-amoeba interaction in prairie plague foci in order to predict plague re-emergence in such areas. The ability to predict a plague epizootic could inform which prairie areas are viable for recovery of black-footed ferrets, or implementation of early interventions that prevent disease spread and extinction of prairie dog colonies. This will have the overall impact of reducing risk of disease spread to other susceptible wildlife species e.g. mountain lions or to domestic animals e.g. cats and dogs.

5. Timeline: During Year One, Aim 1 will be performed and accomplished. Concurrently, optimization of experiments in Aim 2 will be undertaken. In Year Two final investigation into the dynamics of bacterial survival in amoeba in a burrow soil simulating environment and manuscript preparation and submission will be accomplished

Morris Animal Foundation

Animal Involvement Justification

(From the proposal guidelines, single-spaced, no page limit)

Morris Animal Foundation (MAF) is dedicated to funding scientifically sound, relevant and humane studies that specifically address the health and well-being of animals. All studies receiving funding must follow MAF's Health Study Policy for Animals Involved in Research (adopted October 18, 2008), which was written to ensure that each and every animal involved in a MAF funded health study receives excellent, compassionate care throughout the study. MAF shall not fund health studies which require euthanasia as an endpoint or the induction of disease or injury, unless the nature of the disease or condition to be studied is of such significance for improving animal health that such means are justified, and that meaningful information can be obtained in no other way. Furthermore, MAF will not fund any study that induces or allows pain or distress unless such pain or distress can be controlled by appropriate anesthetic, analgesic, tranquilizing drugs, or nursing care. [Click here](#) for the full Health Study Policy.

- A. If this study does not involve live animals please indicate here by N/A: N/A
- B. Does this study involve biological samples, tissues, etc.? **N/A, Mouse blood will be acquired from commercial vendor (Bioreclamation, New York)**

If yes, describe in detail what samples will be used and where & how they will be (or were) acquired. Note: Morris Animal Foundation reserves the right to request a copy of the Institutional Animal Care and Use Committee (IACUC) application/approval and other relevant applications/approvals (e.g., wildlife permit) covering the original collection of samples, including archived samples. MAF reserves the right to request IACUC (or equivalent) review and approval for any Foundation study ***regardless of the Institution's requirements***. This would include the use of archived samples as well as clinical trials.

- C. If this study involves live animals, succinctly address the following: (please restate the questions and directives).
1. What species will be studied?
 2. State the status of your IACUC application/approval. All recipients of MAF funding will be required to submit the entire IACUC protocol and document. A copy of the IACUC approval should not be included with the application, but it is required before funding can be awarded.
 3. List the USDA category for pain and distress (B, C, D, E): _____

Note: Any study beyond category C will require review by MAF's Animal Welfare Advisory Board (AWAB). In general MAF does not fund studies beyond category C (category D studies will only be considered if they conform with MAF's Health Study Policy, category E studies will not be considered).

4. Does this proposal involve client-owned animals? _____
If yes, the protocol for client-owned animals must be approved by the appropriate peer review committee before the project is funded. *If this proposal involves client-owned animals, an informed client consent form must be submitted with this proposal. For a suggested list of items to be considered in an informed client consent form, [click here](#).*
5. Explain how animals will be acquired (e.g., client-owned, USDA licensed breeder, institutional "herds" or "colonies") and verify that the animals are suitable for the study (e.g., have no physiologic, physical or pharmacologic issues that would interfere with results)
6. How many animals will be used? _____
 - a. Summarize numerical justification
7. Does this study induce disease, injury, pain or distress in animals? Note: any study requiring the induction of disease, injury, pain, or distress will have an additional evaluation by MAF's AWAB.

If yes,

- a. Defend the necessity of experimental design
 - b. Explain how pain and/or distress will be controlled
 - c. Justify that no alternative, including clinical studies, can be used to accomplish study objectives and the disease/condition to be studied is of such significance for improving the health of the species.
8. Explain the environment and housing conditions (quality of life) in which the animals will live (address species-appropriate exercise, enrichment, socialization, veterinary care, etc.)
9. What will happen to the animals upon completion of the study?
- If adoption, explain the adoption process. Provide assurance that whenever possible and when in the animal's best interest, investigators shall make companion animals available for adoption at the end of the study or return the animals to the owner/responsible agency in an environment that promotes animal welfare and excellent quality of life.
10. If euthanasia, provide the following additional information (note: any study requiring euthanasia as an endpoint will have an additional evaluation by a MAF's AWAB).
- i. Total number that will be euthanized and justification for numbers
 - ii. Method of euthanasia
 - iii. Justification that no alternatives can be used to accomplish study goal(s) and that the disease/condition to be studied is of such significance for improving the health of the species that a terminal endpoint is deemed necessary.
 - iv. Reason for euthanasia in lay language (this wording may be shared with staff, donors and media)
 - v. Provide objective criteria for determining when euthanasia is appropriate or necessary (note: Morris Animal Foundation wants assurance that an animal will not be allowed to suffer and that monitoring for pain and suffering is adequate)

Note: Morris Animal Foundation does not consider the use of CO2 alone to be an appropriate method of euthanasia

Please note:

1. If an animal is used in an invasive study, MAF may require that a guarantee is provided, through principal investigator and institutional signatures that the animal will not participate in any future invasive study or procedure
2. MAF does not allow inclusion of ancillary data in MAF funded research that includes animal use protocols not in agreement with our Health Study Policy, even if it is obtained using other funding sources.
3. Morris Animal Foundation considers euthanasia acceptable when an animal develops unanticipated illness or injury that results in pain and suffering that cannot be alleviated with standard veterinary interventions.

VI. Recombinant DNA/Biohazards: Recombinant DNA will not be used in this study. The *Y. pestis* strains used in this study are designated avirulent and will be used at Biosafety Level 2 (BSL2). The BSL2 practices using avirulent *Y. pestis* have been approved by the Washington State University Institutional Biosafety committees [REDACTED]

VII. Facilities and equipment:

Paul G. Allen School for Global Animal Health, College of Veterinary Medicine, WSU

The Paul G. Allen Center for Global Animal Health, opened in 2012, is a 62,000-square-foot state-of-the-art infectious disease research facility designed and equipped to meet today's standards for investigating pathogens and emerging diseases. The facility includes administrative offices and a CDC and USDA certified Biosafety Level 3 (BSL3) laboratory on the first floor, and two floors of BSL2 laboratories, meeting rooms, office space, and open areas to encourage direct interactions among the students, faculty and staff. In addition to individual faculty laboratories, there are common equipment rooms and shared facilities including licensed work sites for bioinformatics.

Laboratory facilities: Dr. Vadyvaloo has an assigned laboratory in the Allen Center. Additional resources and laboratories are available to Dr. Vadyvaloo throughout the College of Veterinary Medicine (CVM) complex. The Allen School and other CVM laboratories are fully equipped for contemporary research in molecular biology, infectious diseases, and immunology. The BSL2 laboratories have been approved by the Institutional Biosafety Committee for pathogen use, recombinant DNA experiments and radioisotope use.

Equipment: The allocated laboratory space is equipped with four 4-ft biological safety cabinets, four CO₂ incubators, various bacterial incubators and shakers for recombinant work in *Escherichia coli* and *Yersinia pestis*, a Thermolyne Locator Jr Plus nitrogen freezer for cryostorage of amoeba cell lines, Thermo Revco -86°C freezers for long term storage of strains and samples, standard DNA, RNA and protein electrophoresis equipment, 2 BioRad C1000Touch PCR cyclers and a CFX96/384 Real Time PCR System, various Eppendorf centrifuges, biospectrophotometer, Agilent Bioanalyzer (RNA/DNA quality analysis) and a Biorad GenePulser Xcell for electroporation, Nanodrop (DNA/RNA quantification), pipettes (single and multi-channel), EVOS fluorescence microscope, spectrophotometer, and heating blocks. Gamma counters, HPLC and FPLC are available nearby.

Additional equipment is available for use in shared facilities in the Allen Center including a Leica DM4000 epifluorescence upright microscope with 4 laser lines: (1) Excitation 360/40, emission 470/40 (for DAPI and Hoechst); (2) Excitation 480/40, emission 527/30 (for Alexa 488, FITC, GFP); (3) Excitation 545/40, emission 610/75 (for Alexa 546, Alexa 568, mCherry, DsRed); (4) Excitation 640/30, emission 690/50 (for Cy5, Alexa 647). Also available are TECAN Infinite M1000 fluorescence/luminescence microplate reader, a Millipore guava easyCyte™ HT flow cytometer, a BioRad ChemiDoc™ MP gel imaging system, Sorvall WX Ultra 100 and MTX 150 ultracentrifuges, Sorvall high speed centrifuges, an Illumina MiSeq personal sequencer, Zeiss Primovert microscope for tissue culture, and Lonza 4D-Nucleofector.

Office: The PI has a private 150 sq. ft. office space located next to her research laboratory. Shared office space is also available for technicians, postdocs, and graduate students working in the laboratory

Computer: Dr. Vadyvaloo has networked computers in her laboratory and office including multiple specialized software packages, and wireless connectivity is available throughout the building.

Additional resources available on the WSU campus

A Leica TCS SP8 X point scanning confocal microscope with 7 laser lines is available in nearby BSL2 lab space in the CVM *Department of Integrative Physiology and Neuroscience*.

Microscopy and Imaging: The Franchesci Center maintains the full complement of microscopy services and instrumentation. This includes confocal microscopes (BioRad MRC 1024; Zeiss LSM 510), 2 transmission electron microscopes (FEI TEM T20, Philips CM-200), 2 scanning electron microscopes (Hitachi S-570, FEI Quanta 220F) electron microscopes, Fluorescence microscope (Leica) and laser dissection microscope (PALM MicroBeam Laser Dissection Core). All are fitted with imaging instrumentation. The full-set of tissue processing for these microscopy needs are available at the Center. All necessary ancillary equipment, computers for image processing and analysis are maintained for student and faculty use. The center provides project consultation and has a skilled staff to assist students and faculty in a wide range of research projects

Laboratories in the neighboring *Animal Diseases and Biotechnology Facility* are fully equipped and approved for research in molecular biology, infectious diseases, and radioisotope use, as described above.

VIII. REFERENCES

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infection of the amoebae *Acanthamoeba castellanii*, *Hartmannella vermiformis*, and *Naegleria lovaniensis*. Infect Immun **81**:1399-1410.

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IX. PROPOSAL BUDGET

Category	Year 1	Year 2	Total	
<u>Personnel:</u>				
1. Principal investigator (Viveka Vadyvaloo, PhD)*	■	■	■	
2. Co-investigator #1 (Javier Benavides-Montano, DVM)	■	■	■	
3. Technician ■				
Salary (40%)	■	■	■	
Fringe benefits (33.2%)	■	■	■	
Total Salaries & Wages	■	■	■	
<u>Supplies & Expenses:</u>				
1. Consumables (plastic ware, tips, tubes, tissue culture flasks, etc.)	■	■	■	
2. Chemicals and media components (glucose, magnesium chloride, brain-heart infusion broth, sodium chloride, etc.)	■	■	■	
3. Molecular reagents (Mobio Powersoil Kit, qPCR reagents, etc.)	■	■	■	
4. Publication/Page charges		■	■	
5. Annual Usage Fee for the Microscopy facility	■	■	■	
6. Microscopy reagents	■	■	■	
Provide justification in the designated section.				
Total Supplies & Expenses:				
<u>Animal Use & Care:</u>				
Animal Purchase:				
Animal Per diem:				
Total Animal Care:				
Subtotal of All Categories:	■	■	■	
Maximum of 8% - Indirect Costs:**	■	■	■	
Grand Total Requested from MAF:	■	■	■	

X. Itemized Budget Justification

Salaries and benefits:

Principal Investigator (Viveka Vadyvaloo, PhD)

Dr. Vadyvaloo has >9 years' experience working on *Y. pestis*. As PI, she will coordinate, supervise and participate in generating experimental data for the proposed work. She will oversee and advise all technical aspects of the research outlined in the specific aims. Dr. Vadyvaloo will be responsible for data analysis and interpretation, as well as writing and submitting reports and manuscripts for publication. Dr. [REDACTED] No salary or benefits are requested.

Co-Investigator (Javier Benavides-Montaña, DVM)

Mr. Benavides-Montaña has 2 years' experience with *Y. pestis* in amoeba and is responsible for generating the preliminary data presented in this proposal. Under the supervision of Dr. Vadyvaloo, he will assist in conducting the experiments described in both aims, will participate in the data analysis and interpretation, and manuscript preparation. No salary or benefits are requested.

Laboratory technician (To be determined)

Under the direct supervision of Dr. Vadyvaloo the technician will assist in conducting the experiments outlined in Aims 1 and 2, and will provide support to Mr. Benavides-Montaña on all experimental aspects of the project. The technician will also maintain amoeba and bacterial culture stocks, prepare media, and oversee general laboratory BSL-2 protocol and maintenance. Salary [REDACTED] and [REDACTED] benefits are requested to cover the cost of the technician at 40% effort, for a total cost of [REDACTED]

Supplies and expenses:

A total [REDACTED] is requested to cover the supplies and expenses associated with the completion of the specific aims in this proposal.

1. Consumables and plastic-ware: tissue culture flasks, tubes, pipets, pipet tips, petri plates etc. [REDACTED] is requested over two years for laboratory consumables and plastic-ware expenses.
2. Chemicals and reagents: chemicals to make media (NaCl, MgCl, glucose, vitamins, minerals etc.), brain heart infusion, etc. [REDACTED] is requested to cover the costs of chemicals and reagents over the two years.
3. Molecular biology reagents: Mobio Powerlyzer® Powersoil® soil DNA extraction kit, primers and probes for qRT-PCR, qRT-PCR reagents, DNA stain, DNA loading buffer. [REDACTED] is requested to purchase the necessary molecular biology reagents.
4. Publication and page charges: [REDACTED] is requested in year 2 to defray publication expenses.
5. Use of the microscopy (electron) facility: [REDACTED] per year is requested to pay the annual usage fee of the Franceschi Microscopy & Imaging Center.
6. Microscopy reagents: (uranyl acetate, electron grids, cacodylate buffer, etc.) [REDACTED] is requested to cover the cost of microscopy reagents over the two year period.

XI. Other Support

Current support:

Project number: [REDACTED]

Source: College of Veterinary Medicine Washington State University Intramural Grant program

[REDACTED]

[REDACTED]

[REDACTED]

[REDACTED]

[REDACTED]

[REDACTED]

[REDACTED]

Overlap: The above funded project has conceptual and scientific overlap with the currently proposed study. In terms of % effort and resources there is no overlap because the latter is focused on understanding survival and replication of *Y. pestis* in ecologically relevant amoeba that have been identified by studies that have been accomplished from the currently funded project.

Pending support:

Project number: [REDACTED]

[REDACTED]

[REDACTED]

[REDACTED]

[REDACTED]

[REDACTED]

[REDACTED]

[REDACTED]

Overlap: No overlap exists between the above study and the currently proposed study.

XII. Prior MAF Support during the last three years

None

BIOGRAPHICAL DATA

NAME: Viveka Vadyvaloo

POSITION/ROLE ON PROJECT: Principal Investigator

CURRENT POSITION: Assistant Professor, Paul G. Allen School for Global Animal Health
Washington State University, PO Box 647090, Pullman, WA 99164-7090

EDUCATION/TRAINING

INSTITUTION AND LOCATION	DEGREE (if applicable)	Completion Date MM/YYYY	FIELD OF STUDY
University of Durban-Westville, Durban, South Africa	BS	12/1995	Microbiology, Human Physiology
University of Kwazulu-Natal, Pietermaritzburg, South Africa	MS	12/1999	Microbial genetics
University of Stellenbosch, Stellenbosch, South Africa	PhD	06/2003	Biochemistry
Howard Hughes Medical Institute at UCLA, Los Angeles, California	Postdoctoral Fellow	08/2004	Biochemistry
NIH/NIAID/ Rocky Mountain Laboratories, Hamilton, Montana	Postdoctoral Fellow	09/2005	Bacterial pathogenesis
NIH/NIAID/Rocky Mountain Laboratories, Hamilton, Montana	Postdoctoral Fellow	09/2008	Vector-borne disease; plague

Positions and Honors

Positions and Employment

2009 - 2010 Research Assistant Professor, Paul G. Allen School for Global Animal Health,
Washington State University, Pullman, WA

2010 - present Assistant Professor, Paul G. Allen School for Global Animal Health, Washington
State University, Pullman, WA

Other Experience and Professional Memberships

2003 - Member, American Society of Microbiology

2010 - Member, North West Regional Center of Excellence

2011 - Member, Entomological Society of America

Honors

1997 AECI MS Fellowship, (AECI/CSIR – South Africa)

2000 Prestigious Equity PhD Fellowship, National Research Foundation of South Africa

2014 NSF ADVANCE at WSU External Mentor Program Award, NSF/WSU

2014 International Travel Award , Washington State University

Selected Peer-Reviewed Publications

- Martínez-Chavarría LC, **Vadyvaloo V**. 2015. *Yersinia pestis* and *Yersinia pseudotuberculosis* infection: a regulatory RNA perspective. *Front Microbiol.* 6:956. doi: 10.3389/fmicb.2015.00956. eCollection 2015. Review. PubMed PMID: 26441890; PubMed Central PMCID: PMC4585118.
- Vadyvaloo V**, Hinz AK. 2015. A LysR-Type Transcriptional Regulator, RovM, Senses Nutritional Cues Suggesting that It Is Involved in Metabolic Adaptation of *Yersinia pestis* to the Flea Gut. *PLoS One.* 10(9):e0137508. doi: 10.1371/journal.pone.0137508. eCollection 2015. PubMed PMID: 26348850; PubMed Central PMCID: PMC4562620.
- Santos-Montañez J, Benavides-Montañón JA, Hinz AK, **Vadyvaloo V**. 2015. *Yersinia pseudotuberculosis* IP32953 survives and replicates in trophozoites and persists in cysts of *Acanthamoeba castellanii*. *FEMS Microbiol Lett.* 362(13):fnv091. doi: 10.1093/femsle/fnv091. Epub 2015 May 29. PubMed PMID: 26025069.
- Vadyvaloo V**, Viall AK, Jarrett CO, Hinz AK, Sturdevant DE, Joseph Hinnebusch B. 2015. Role of the PhoP-PhoQ gene regulatory system in adaptation of *Yersinia pestis* to environmental stress in the flea digestive tract. *Microbiology.* 161(6):1198-210. doi: 10.1099/mic.0.000082. Epub 2015 Mar 24. PubMed PMID: 25804213.
- Bobrov AG, Kirillina O, **Vadyvaloo V**, Koestler BJ, Hinz AK, Mack D, Waters CM, Perry RD. 2015. The *Yersinia pestis* HmsCDE regulatory system is essential for blockage of the oriental rat flea (*Xenopsylla cheopis*), a classic plague vector. *Environ Microbiol.* 17(4):947-59. doi: 10.1111/1462-2920.12419. Epub 2014 Mar 11. PubMed PMID: 25586342; PubMed Central PMCID: PMC4295937.
- Martínez LC, **Vadyvaloo V**. 2014. Mechanisms of post-transcriptional gene regulation in bacterial biofilms. *Front Cell Infect Microbiol.* 4:38. doi: 10.3389/fcimb.2014.00038. eCollection 2014. Review. PubMed PMID: 24724055; PubMed Central PMCID: PMC3971182.
- Rempe KA, Hinz AK, **Vadyvaloo V**. 2012. Hfq regulates biofilm gut blockage that facilitates flea-borne transmission of *Yersinia pestis*. *J Bacteriol.* 194(8):2036-40. doi: 10.1128/JB.06568-11. Epub 2012 Feb 10. PubMed PMID: 22328669; PubMed Central PMCID: PMC3318476.
- Vadyvaloo V**, Jarrett C, Sturdevant DE, Sebbane F, Hinnebusch BJ. 2010. Transit through the flea vector induces a pretransmission innate immunity resistance phenotype in *Yersinia pestis*. *PLoS Pathog.* 6(2):e1000783. doi: 10.1371/journal.ppat.1000783. PubMed PMID: 20195507; PubMed Central PMCID: PMC2829055.
- Vadyvaloo V**, Jarrett C, Sturdevant D, Sebbane F, Hinnebusch BJ. 2007. Analysis of *Yersinia pestis* gene expression in the flea vector. *Adv Exp Med Biol.*;603:192-200. Review. PubMed PMID: 17966415.
- Erickson DL, Waterfield NR, **Vadyvaloo V**, Long D, Fischer ER, Ffrench-Constant R, Hinnebusch BJ. 2007. Acute oral toxicity of *Yersinia pseudotuberculosis* to fleas: implications for the evolution of vector-borne transmission of plague. *Cell Microbiol.* 9(11):2658-66. Epub 2007 Jun 24. PubMed PMID: 17587333.

BIOGRAPHICAL DATA

NAME: Javier Antonio Benavides Montaña

POSITION/ROLE ON PROJECT: Co-Investigator

CURRENT POSITION: PhD Student, Paul G. Allen School for Global Animal Health
Washington State University, PO Box 647090, Pullman, WA 99164-7090

EDUCATION/TRAINING

INSTITUTION AND LOCATION	DEGREE (if applicable)	Year of Graduation	FIELD OF STUDY
Universidad de Caldas, Manizales, Colombia	BS	1999	Veterinary Medicine and Animal Production Science
University of Manizales, Caldas, Colombia	M.Ed	2005	Education and University Teaching
Federal University of Viçosa – Brazil	MSc	2006	Veterinary Medicine

Positions and Honors

Positions and Employment

2000-2004 Diego Villegas Toro. Clinical hospital, Universidad de Caldas. Residency in small animal medicine; Clinical Parasitology; Teaching Assistant.

2007-present Award Academy Excellence I – 07, Faculty of Agriculture Science (Palmira), National University of Colombia.

2011-present Fulbright Scholar, Washington State University, Pullman, WA, USA

Honors

2010 Best Research Award. XXXVII Congreso Sociedad Colombiana de Entomologia (SOCOLEN), Bogota, July 2010.

Selected Peer-Reviewed Publications (English language)

Santos-Montañez J, **Benavides-Montaña JA**, Hinz AK, Vadyvaloo V. 2015. *Yersinia pseudotuberculosis* IP32953 survives and replicates in trophozoites and persists in cysts of *Acanthamoeba castellanii*. FEMS Microbiol Lett. 362(13):fnv091. doi: 10.1093/femsle/fnv091. PubMed PMID: 26025069.

Lu X, Liu Q, **Benavides-Montaña JA**, Nicola AV, Aston DE, Rasco BA, Aguilar HC. 2013. Detection of receptor-induced glycoprotein conformational changes on enveloped virions by using confocal micro-Raman spectroscopy. Journal of Virology. 87(6):3130 – 3142. doi: 10.1128/JVI.03220-12. PMCID: PMC3592171

Liu Q, Stone JA, Bradel-Tretheway B, Dabundo J, **Benavides-Montaña JA**, Santos-Montañez J, Biering SB, Nicola AV, Iorio RM, Lu X, Aguilar HC. 2013. Unraveling a three-step spatiotemporal mechanism of triggering of receptor-induced Nipah virus fusion and cell entry. Plos Pathogens. 9(11):e1003770. Doi: 101371/journal.ppat.1003770. PMCID: PMC3837712

Benavides-Montaña JA, Rocha HGR, Campos VEB, Ferreira APBR, Souza e Silva CH, Carvalho GD, et al. 2009. Humoral immune responses to the synthetic immunogens SBbo23290 (*Babesia bovis*) and SBm7462 (*Rhipicephalus (Boophilus) microplus*). *Veterinary Immunology and Immunopathology*. 128(1-3):284-285. doi:10.1016/j.vetimm.2008.10.160

Benavides-Montaña JA, Rocha HGR, Nogueira da Gama M, Campos VEB, Ferreira APBR, Carvalho GD, et al. 2009. Biological parameters of ticks collected from animals immunized with peptides SBbo23290 and SBm7462. *Veterinary Immunology and Immunopathology*. 128(1-3):285-286. doi:10.1016/j.vetimm.2008.10.161

Fellowship Training

I. Title and Abstract:

Title: Integrated metabolomic and genomic approach to metabolic variation across horse breeds

Rationale: Equine Metabolic Syndrome (EMS) is defined by a clustering of clinical signs, namely hyperinsulinemia, insulin resistance and adiposity, which predispose horses to the development of laminitis. Certain breeds appear to be more susceptible to EMS, while other breeds seem to be at lower risk. Genetic selection in horses has resulted in metabolic and athletic phenotypes that allowed horses to efficiently perform different types of work. These underlying metabolically efficient, or “thrifty” alleles, in particular those that regulate energy intake, storage and use, coupled with changes in equine husbandry practices in recent times, including dense high caloric feed and limited exercise, may explain much of the increasing prevalence of equine metabolic disease phenotypes in modern environments.

Hypothesis/Objectives: We *hypothesize* that breed differences in key metabolic phenotypes are due to high frequencies of alleles that modify metabolic traits. Our *objectives* are to **1)** further dissect the metabolic differences between breeds at the molecular level using total serum metabolite profiling; and **2)** use these breed-specific molecular metabolic profiles to identify candidate genes underlying breed metabolic differences.

Study Design: In **Objective 1** serum metabolite profiles will be analyzed before and after an oral sugar test in 274 horses from 5 breeds with distinct metabolic phenotypes. These data will be used to **1)** identify the metabolites and metabolic pathways that are significantly different between breeds using functional annotation and mapping of metabolites to known pathways, pathway and metabolite set enrichment analysis, network analysis, and pathway activity profiling; and **2)** to correlate the metabolite/pathway differences with the previously identified breed differences in key hormonal and biochemical measurements. In **Objective 2** candidate genes responsible for metabolic differences between breeds will be identified by using high-density SNP genotype data to locate genomic regions and specific haplotypes that are highly differentiated between breeds. Metabolites and metabolic pathways that are significantly different between breeds will then be used to provide context for narrowing the focus to specific candidate genes within these genomic regions.

Preliminary Data: Our data demonstrate significant differences among Morgans, Arabians, Welsh ponies, Tennessee Walking Horses and Quarter Horses in EMS-defining metabolic traits (e.g. insulin dynamics, lipid metabolism, adipokines) that mirror EMS risk. We have also demonstrated the use of SNP genotype data to identify regions of breed differentiation/selection across a wide breed panel, and across Quarter Horses and Welsh Ponies, and provided examples in the *GYS1* and *MSTN* genes that selection for certain performance traits results in near-fixation of alleles that alter energy metabolism. Finally, we have demonstrated the potential for serum metabolomic data to lead to insight into the metabolic differences between horses and clinical phenotypes.

Expected Results: We expect to detect major breed differences in metabolites/metabolic pathways that are correlated to previously identified biochemical and hormonal differences. We also expect to identify and prioritize candidate genes within genomic regions of interest that influence a spectrum of metabolic traits, particularly the susceptibility to metabolic syndrome.

Budget and Timeline: [REDACTED] Approximately one year is required to achieve each objective.

Potential Impact for Animal Health: Elucidating the evolution of the genetic basis of metabolic efficiency and metabolic syndrome is a novel, unexploited approach to the study of the genetic basis of obesity, energy dysregulation and EMS. This project will provide novel insights into disease biology, allowing the identification of new therapeutic targets, and increasing our understanding of the pathophysiology of EMS and its associated clinical features. Moreover, the identification of genes underlying the EMS phenotype will also directly impact equine health by allowing for the development of genetic tests to identify horses at risk for the development of obesity and laminitis prior to the onset of clinical disease.

July 15th, 2015

To the Scientific Advisory Board – Fellowship Training Proposal

I am a Postdoctoral Research Associate in the Equine Genetics and Genomics Laboratory at the University of Minnesota under the supervision of Drs. Molly McCue and James Mickelson. I have been working in this laboratory since the Fall semester of 2014, with the intent of taking further steps towards a successful career in animal research. My goal is to obtain a tenure-track position at a major institution where I can establish a laboratory to conduct research in the field of large animal genetics and genomics, as well as be involved in teaching. My professional vision is to be part of a strong and collaborative program that provides me the opportunity to perform cutting-edge research in animal health, disease and performance and to learn constantly from my peers. I also have a passion for teaching, and would like to continue working with students both in the classroom and in the laboratory, throughout my career.

I obtained my Bachelor's degree in Biological Sciences from the University of Brasilia (Brazil) in 2005, and my Ph.D. in Biomedical Sciences from Texas A&M University in 2014. I understood the importance of research early on in my career, so while I was an undergraduate in Brazil, I was an intern at the Laboratory of Animal Molecular Genetics at EMBRAPA, Brazil's Federal Agricultural Research Institute. During that period, I worked on the genetics of bovine and sheep reproduction, and this experience not only consolidated my passion for animal research, but also provided me with knowledge that laid the foundation for my subsequent endeavors. After that, I did another internship at the Laboratory of Uterine Biology and Pregnancy at Texas A&M University, where I continued working on the genetics of sheep reproduction. Then, I decided to come back to the United States to pursue a graduate degree in animal genetics.

My doctoral studies, conducted in the Molecular Cytogenetics and Genomics Laboratory at Texas A&M University under the supervision of Dr. Terje Raudsepp, included generating a whole genome integrated map for the alpaca (*Lama pacos*). This research project, funded by Morris Animal Foundation [REDACTED] allowed us to develop a genome-wide set of molecular markers that successfully integrated the alpaca genome sequence assembly with the physical chromosomal maps for this species. As part of this research project, and also with funds obtained from a *Veterinary Student Scholars Program Grant* from Morris Animal Foundation awarded to me in 2011, we successfully mapped candidate genes involved in deafness associated with depigmentation in alpacas. Therefore, it is safe to say that Morris Animal Foundation played an instrumental role in the successful completion of my doctorate, and for that I am very grateful.

During my undergraduate and graduate careers, I was either the author or co-author of a total of 24 publications in national and international conference proceedings, as well as 12 articles in international peer-reviewed journals. Moreover, I was fortunate to win several honors and awards including *Outstanding Graduate Student* and *High Impact Achievement* awards from the Texas A&M College of Veterinary Medicine, and the *U.S. Senator Phil Gramm* fellowship for excellence in research, teaching and as a scholar-mentor, among others.

Up until this point, my research background has been very diverse, ranging from the annotation of transposable elements in a nematode species to my current research in the genetic basis of EMS, including bovine, sheep and camelid studies. I believe that my experience has provided me with a skillset that will allow me to pursue excellence in all aspects of my postdoctoral career, including research, grant and manuscript writing, and communicating my findings to fellow researchers and to the public.

My postdoctoral research project aims at identifying candidate genes associated with Equine Metabolic Syndrome, or EMS. The opportunity to work with equine genetics provided me with invaluable knowledge on the potentially harmful outcomes of genetic diseases in horses. Moreover, I was able to understand the importance of preventative approaches to genetic diseases, especially in the case of EMS, which can lead to fatal consequences in affected horses. The identification of candidate genes associated with this syndrome is an important first step towards early diagnostics and preventative measures that can be taken prior to the development of clinical signs. I

believe it also lays the foundation for the discovery of the pathophysiology of the disease, as well as of targets for novel therapies that can greatly improve the health and welfare of potentially affected individuals.

Therefore, the research study presented in this Fellowship Proposal aims at identifying candidate genes associated with the EMS phenotype and breed-specific metabolic profiles. In order to accomplish that, we will identify genome-wide signatures of positive selection in four breeds with distinct metabolic profiles (Arabian, Morgan, Quarter Horse and Welsh Pony) using genotyping data for 2 million SNP markers. Then, we will use a comprehensive set of phenotypic, environmental and epidemiologic measurements, as well as breed-specific global metabolic profiles derived from serum metabolomics analysis, to refine and prioritize the genes and pathways associated with EMS phenotypes and distinct serum metabolic profiles in horses and ponies.

We anticipate that these findings will be critical to gain further knowledge on the genetic basis of this syndrome, and for our long-term goal of developing genetic tests for early EMS diagnosis. I am confident that our laboratory, which is well-known for its solid research history and use of cutting-edge technologies, will provide me with the ideal facilities and human resources to conduct the proposed research study. My mentor, Dr. Molly McCue, is a very successful DVM/PhD scientist who has dedicated years to studying the genetics of equine metabolic diseases, having published her numerous findings in high impact journals. My co-mentor, Dr. James Mickelson, is a well-established researcher who helped establish the field of equine genetics and genomics, having played a significant role in developing and using the first ever genetic analysis tools in the horse.

With the help of previous Morris Animal Foundation funding, my interest and passion for animal research have been translated into scientific knowledge and experience that allowed me to develop my career up until this point. Continued support from Morris Animal Foundation will allow me to pursue my newfound interest in the genetic basis of EMS, as well as support me in building my skills in equine genetics and taking further steps towards developing my career as an independent investigator. Thank you in advance for your consideration of my proposal.

Sincerely,

Felipe Avila, Ph.D.
Equine Genetics and Genomics Laboratory
College of Veterinary Medicine
University of Minnesota

Dear members of the Large Animal Scientific Advisory Board,

It is my distinct pleasure to write this mentor letter for Dr. Felipe Avila in support of his Morris Animal Foundation Postdoctoral Fellowship proposal. I had the opportunity to recruit Felipe to our research group in the fall of 2014. Since that time I've had the opportunity to become familiar with Felipe's strengths and have watched him step in and become an integral part of our research team.

Felipe came to our group with some background in reproductive genetics and cytogenetics but with genuine interest in learning how to take advantage of high dimensional data sets and 'omic technologies. The work done in our lab and the focus of Felipe's research thus far has required Felipe to acquire an entirely new set of skills in the last ten months. His motivation and dedication to this task has been inspiring. It is not necessarily an easy transition for a bench top scientist that has been heavily invested in molecular work to step away from pipettes and into the world of computational biology. This transition has required Felipe to learn core computing skills including some basic programming. While this transition results in a steep learning curve with many daily opportunities for frustration, Felipe has maintained a remarkably positive attitude and has been tenacious in his efforts.

Felipe has taken two large projects previously overseen by a senior postdoc in the laboratory and he has moved both of these projects forward substantially, presenting at the International Plant and Animal Genome meeting barely 4 months into his post-doc and having an abstract accepted for the Havemeyer Equine Genomics meeting in July 2015. These projects, which focus on the identification of genomic regions highly differentiated between breeds or undergoing positive selection, form the basis of the genomic preliminary data in Felipe's post-doc proposal. As part of this work, Felipe has learned how to manipulate and analyze single nucleotide polymorphism (SNP) genotyping data and the analysis of whole genome sequence data, and has developed the genomic analysis pipeline outlined in objective 2a. The work outlined in this proposal builds on the skills he has developed thus far, and his preliminary data, by adding a functional high dimensional data set in the form of non-targeted serum metabolomic data. This functional data will give Felipe the opportunity to explore new statistical analyses and to tie molecular phenotypes to his genomic regions of interest; which will round out Felipe's post-doctoral training.

At the completion of his postdoctoral training, Felipe will truly have a diverse research portfolio, with experiences ranging from genome annotation and FISH mapping to high dimensional data analysis. Further, Felipe will have had the opportunity to perform genetic research in a range of large animal species which fits with his long-term goal of establishing a research lab focusing on large animal genetics and genomics. In addition to his diverse training, Felipe has several other strengths which make him well-suited to become an independent investigator. As mentioned above he is hard-working and tenacious, which is demonstrated not only by his work in my laboratory, but also by his stellar academic record which includes primary or co-authorship on 12 articles in international peer-reviewed journals. He also has excellent people skills, and has shown a talent for teaching/mentoring while working with visiting/rotating PhD students and veterinary students in the lab.

As the primary mentor for Felipe's postdoctoral fellowship I am committed to mentoring Felipe not only on his research projects but also in his professional development. This includes supporting Felipe as he develops his technical writing skills through mentored manuscript and grant writing experiences (such as this one). Further I will support Felipe and his passion for teaching by identifying opportunities for him to become involved in teaching during his postdoctoral training. Finally, as Felipe has mentioned in his training plan, I am committed to helping him network in the professional community through opportunities to speak and professional contacts at meetings, as well as through a number of visiting scientists that have visited/will visit the lab during his tenure here. Our laboratory group meets almost weekly for 1 to 2 hours to discuss current literature, new data analyses, or data from a specific student's project. In addition, I meet with each student in my laboratory including Felipe weekly in a one-on-one meeting to discuss data and/or troubleshoot ongoing projects.

My previous mentoring experience includes being a graduate faculty member in 3 graduate programs at the University of Minnesota, and serve as a faculty mentor for both T32 and T35 awards, and I am the co-director of our post-doctoral T32 in Comparative Medicine and Pathology. I have served/am serving as the primary mentor for 6 PhD students and 3 MS students, 3 post-doctoral trainees, and as the primary clinical mentor for 5 internal medicine residents. I am also currently the co-advisor for 2 PhD students and have served on an additional 10 graduate committees (5 PhD and 5 MS).

Student	Degree	Role	Dates	Current position
[REDACTED]	[REDACTED]	[REDACTED]	[REDACTED]	
[REDACTED]	[REDACTED]	[REDACTED]	[REDACTED]	
[REDACTED]	[REDACTED]	[REDACTED]	[REDACTED]	
[REDACTED]	[REDACTED]	[REDACTED]	[REDACTED]	
[REDACTED]	[REDACTED]	[REDACTED]	[REDACTED]	
[REDACTED]	[REDACTED]	[REDACTED]	[REDACTED]	
[REDACTED]	[REDACTED]	[REDACTED]	[REDACTED]	
[REDACTED]	[REDACTED]	[REDACTED]	[REDACTED]	[REDACTED]
[REDACTED]	[REDACTED]	[REDACTED]	[REDACTED]	[REDACTED]
[REDACTED]	[REDACTED]	[REDACTED]	[REDACTED]	
[REDACTED]	[REDACTED]	[REDACTED]	[REDACTED]	[REDACTED]
[REDACTED]	[REDACTED]	[REDACTED]	[REDACTED]	[REDACTED]
[REDACTED]	[REDACTED]	[REDACTED]	[REDACTED]	[REDACTED]
[REDACTED]	[REDACTED]	[REDACTED]	[REDACTED]	[REDACTED]
[REDACTED]	[REDACTED]	[REDACTED]	[REDACTED]	[REDACTED]

This proposal is the result of Felipe's intellectual contribution, with input from Dr. Mickelson and myself in formulating the research objectives. Felipe produced the initial completed draft of the proposal and we worked together to edit content for clarity and to fit with in the 5 allotted pages—which is a challenge for this novel and sophisticated idea! As the primary mentor I am committed to funding the research portion of this project. All samples have been collected and the money is in-hand to generate metabolomics data from more than half the number of outlined horses. I have pending grant proposals to cover the remaining data generation, and in the event that these pending proposals are not funded, I will commit internal dollars to ensure that Felipe has data to complete this project.

In summary, I would like to reiterate that Felipe is a bright, hard-working and capable scientist who has a bright future ahead of him. He is very deserving of this award and will take full advantage of the opportunities that this fellowship would provide.

Sincerely,

Molly McCue DVM, MS, PhD, Diplomate, American College of Veterinary Internal Medicine
Associate Professor, University of Minnesota College of Veterinary Medicine

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July 6, 2015

Dear Review Committee members:

It is my great pleasure to provide you with this letter in support of Dr Felipe Avila's postdoctoral fellowship application. I have known Felipe for approximately 9 months as a co-mentor of his outstanding work using dense genotyping data from multiple breeds and populations to locate genomic loci of interest in a number of different phenotypes in the horse. I have organized this letter according to your wishes.

Candidate accomplishments, perceived strengths, motivation, academic abilities and potential. Felipe came to us having been trained by Terje Raudsepp who you know as one of the world's most accomplished animal geneticists from her work on horse and alpaca genomics and the genetics of reproduction and infertility. During his time with Terje Felipe made advances in the alpaca genome and solved a problem in their karyotype that puzzled cytogeneticists for decades. Felipe has authorship on 12 publications, with three of them as lead author from his PhD, and I will leave it to you to count the many abstracts and presentations. It all adds up to a beautiful portfolio presenting the first phase of his training towards becoming an independent scientist.

Felipe easily stepped into our group and made an immediate impact. He is very motivated to succeed and learns new technologies very rapidly, enabling him to quickly go from a primarily laboratory based scientist at TAMU to a computational biologist at Minnesota with an entirely different set of tools and an entirely different type of data. He now has a handle on all the necessary approaches to working with dense genotyping data, analyzing it to localize regions of reduced variation, scan these regions for underlying genes and develop databases to catalog gene functions. And, I have no concerns about his ability to work with the metabolomic data that will be generated in his fellowship proposal.

His current work is coming together nicely now in the areas of identifying loci of interest for metabolic traits which in some ways is moving Felipe into a new area that relates more to animal health. We are thrilled that he has already been invited to give platform presentations of his preliminary work at the Plant and Animal Genome conference (Jan 2015) and the Havemeyer Horse Genomics Workshop (July 2015). I wish I had space to tell you about more of the other types of loci he is identifying, such as regions of selection in different sub-types of Quarter Horses that have different performance traits, loci under selection in Thoroughbreds and Standardbreds, and potential size loci in drafts, ponies and miniature horses.

Lastly, here, Felipe is incredibly kind, calm, good-natured, generous, and polite. He is extremely well-organized, logical and well-spoken. All of these traits are apparent in his one on one meetings, his oral presentations to our group meetings, and his talk at the PAG meeting. They also come through in his day to day work where to make progress in the very complex projects he is undertaking requires on organized dedicated workflow with multiple stages going on at once and incredible patience to deal with time consuming computations on computer networks.

To conclude Felipe has all the tools necessary to become an outstanding independent researcher, able to utilize all types of "omics" data, and I have no doubts whatsoever that he will achieve this goal.

Mentoring relationship. I am a co-mentor to Felipe with Molly McCue. Together Molly and I maintain a dynamic, productive and internationally-recognized group of 6 – 8 grad students/postdocs/faculty/techs/vet students in basic and applied equine genetics and genomics. Molly is Felipe's primary mentor with clear strengths in current methods of genome analysis and understanding of equine pathobiology. I consider my role as

providing independent opinions on genomic results and hypotheses in discussions and adding context to results from my earlier expertise in genetics with the basic biochemistry and biology behind many of the phenotypes we study. Our (usually weekly) group meetings mainly consist of directed study on major papers in current topics of genome analysis. In addition Molly and I have weekly joint one on one 30 – 60 min sessions with each student or fellow. I have a similar role in a canine genomics group which can be beneficial as both groups use similar approaches and encounter similar problems and can benefit from each other's work.

[illegible]

Roles in the preparation of this fellowship application and status of research project funding. As with all Fellowship applications this document is a joint product of the trainee and the advisors. Felipe assembled the first draft of the proposal from his readings of the literature, other proposals we have submitted, and his own drafting of his preliminary work. The advisors worked on the first draft (mainly to shorten it!) and ensure clarity and flow. Current funding has generated all data necessary for Objective 2. As indicated in the Support pages, multiple proposals are submitted to complete funding for Objective 1.

Training Plan

I have several training objectives that will support my career development and provide me with the necessary skills for success in this proposed study, as well as in my future in animal research. They include development of technical research skills relative to metabolomics and genomics, and non-technical aspects such as writing, networking, developing critical thinking skills, and academic competences.

Research training: based on previous training and experience, my skillset reflects that of a trained molecular cytogeneticist and geneticist. Therefore, I seek to broaden my knowledge by incorporating different ‘omics’ tools to my scientific repertoire. This fellowship will allow me to further develop my skills on computational biology applied to genomics and dense genotyping data, which I have been learning since I started working in this laboratory, in the Fall of 2014. Moreover, I am thrilled to gain a more in depth understanding of Equine Metabolic Syndrome and its clinical phenotypes, as well as the use of metabolomics to address the pathophysiology of this disease. I plan to learn and successfully apply the techniques listed in this research study, such as: the use of bioinformatics tools to identify genomic signatures of selection; collection and analysis of various metabolic measurements in horses; statistical analyses applied to ‘omics’ data, among others. For that, I plan to attend workshops at the University of Minnesota’s Supercomputing Institute on handling and analyzing big genotyping data and at the Center for Mass Spectroscopy and Proteomics on analysis of metabolomic data.

Technical writing: during this fellowship I will have to opportunity to write scientific reports, abstracts and manuscripts stemming from our findings, with the guidance and input of my mentors, whom have extensive experience with scientific writing. In addition to mentored writing of abstracts, manuscripts, and reports, I plan to receive formal training in grant and manuscript writing through short courses offered at the University of Minnesota, including the "Getting Started" and "Write Winning Grants" workshops, and the "Scientific Writing Series" sponsored by the Center for Translational Science Institute.

Networking and development of critical thinking skills: I believe that the Equine Genetics and Genomics Laboratory will provide me with the ideal environment to establish networks with other scientists, as well as critical thinking skills. Drs. McCue and Mickelson mentor a large group of undergraduate and graduate students, and laboratory technicians from various backgrounds including DVMs and veterinary medicine students. We hold weekly meetings in which we discuss research articles, as well as the progress of our research projects. Being part of a talented, multidisciplinary group is critical to learn various aspects of my project from veterinarians and researchers alike, and to develop critical thinking skills by discussing outcomes and pitfalls, and by incorporating inputs and/or suggestions to my work. Moreover, my mentors have an extensive array of collaborators, with whom we exchange ideas, knowledge, and technologies. I also plan to attend and present my findings at national and international scientific meetings and workshops (e.g. Plant and Animal Genome, Havemeyer Equine Genome Workshop). My mentors will serve as networking facilitators at such meetings, helping me develop independent collaborations which will be useful in my career as a researcher. Finally, it is worth to mention that I will have the opportunity to work with Dr. Susan Van Riper at the University of Minnesota Informatics Institute, a nationally recognized expert on statistical analysis of metabolomic data.

Mentoring plan: Dr. McCue D.V.M., M.S., Ph.D., a board-certified large animal internal medicine specialist (DACVIM), will serve as my primary mentor. Dr. McCue is internationally recognized as a leader in equine genetics and genomics. Her primary research focus is the genetics of neuromuscular and metabolic diseases affecting the horse. Dr. McCue will provide guidance for questions related to the clinical phenotypes and various metabolic measurements, analysis of the large scale datasets (SNP and metabolomic data), statistics, and biologic interpretation of results. She will also provide guidance and critical evaluation for reports, abstracts, and publications related to this work. Dr. Mickelson M.S., Ph.D., as my co-mentor, will provide independent opinions on data acquisition and analysis, adding context to results from his extensive expertise in genetics, biochemistry, and biology. It is worth to mention that the technology used to genotype the animals in this study - the 2 million equine SNP array - was developed by Drs. McCue and Mickelson.

Institutional environment: apart from the human resources available from the talented, multidisciplinary group of mentors and fellow students which I am part of, the laboratory is equipped with all the necessary, cutting edge resources for the completion of this research study. Computing resources available in the lab include 7 Windows computers with a dual installed Linux virtual machine, containing all the software needed for the proposed analyses. Additionally, the laboratory maintains an account with the University of Minnesota Supercomputing Institute (MSI) which includes access to the Computational Genetics and Basic Sciences computing laboratories. Finally, all metabolomics analytical work will be performed at the University of Minnesota Center for Mass Spectroscopy and Proteomics.

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July 10, 2015

To the Large Animal Scientific Review Board,

It is my distinct pleasure to provide you with this letter in support of Dr. Felipe Avila's postdoctoral fellowship application.

Felipe proposes to continue his postdoctoral training with Drs. McCue and Mickelson, who comprise an established and very successful research team with a history of mentorship of both graduate and postdoctoral students. The team they have formed is a leader in the field of applying state-of-the-art high-throughput genomics approaches to identify and understand genes and mutations that underlie important health, disease and performance traits in horses. Their team is integrated, highly productive, and scientifically rigorous. This team invests heavily in preparing students for technical proficiency, basic sciences comprehension, and grantsmanship. Dr. Avila could not have selected a better program in which to continue his training in a career leading him to a position as an independent scientist in animal health and disease.

Our College's research program emphasizes three Signature Research programs in Comparative Medicine, Emerging and Zoonotic Disease and Population Systems. Animal genomics research (both large and small animal) occupies a key position in the Comparative Medicine program. I would also like to note that the University of Minnesota as a whole has invested heavily in support of genomics, proteomics, metabolomics and computational biology work through its core facilities and infrastructure that has enabled groups such as the equine genomics group to have established themselves and propose higher order and complex projects such as Dr. Avila's. Further, Dr. McCue, the primary mentor for this proposal, is uniquely positioned to help Dr. Avila access these University resources with her intimate involvement with the newly formed University of Minnesota Informatics Institute (UMII) as both a member of the UMII Scientific Advisory Board and as a UMII Transdisciplinary Research Faculty Fellow.

As the chair of Dr. McCue's home department I will do everything possible to ensure that Dr. Avila's training is well-structured and he has the physical resources necessary to achieve his training goals.

To conclude, the college is strongly supportive of this training fellowship, which brings together a strong candidate and a solid mentoring team to work on an important disease in the horse. Please do not hesitate to contact me with any questions.

Sincerely,

Thomas Molitor, PhD
Distinguished Teacher
Professor and Chair,
Veterinary Population Medicine
College of Veterinary Medicine

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July 10, 2015

RE: Salary Verification for Morris Animal Foundation Fellowship Proposal

To Whom It May Concern:

Dr. Felipe Avila is a Postdoctoral Research Associate who joined the Equine Genetics and Genomics Laboratory, Department of Veterinary Population Medicine, in August of 2014. We are requesting an annual salary of [REDACTED] indirect costs for his position.

The requested salary rate is in line with salaries paid to postdocs in the College of Veterinary Medicine [REDACTED] This salary rate is also below NIH guidelines for a fellow with his equivalent experience.

Respectfully,

Natalie L. Dillon
Administrative Director
University of Minnesota
Veterinary Population Medicine

III. Resubmission Summary

N/A

IV. Name, Institution and Email Address

Fellow:

Felipe Fagundes de Avila, PhD
Postdoctoral Research Associate
Department of Veterinary Population Medicine
College of Veterinary Medicine
University of Minnesota
[REDACTED]

Mentor:

Molly McCue, DVM, MS, PhD, Diplomate of ACVIM
Associate Professor
Department of Veterinary Population Medicine
College of Veterinary Medicine
University of Minnesota
[REDACTED]

Co-Mentor:

James Mickelson, MS, PhD
Professor
Department of Veterinary Biosciences
College of Veterinary Medicine
University of Minnesota
[REDACTED]

1. Hypothesis and Objectives. Equine Metabolic Syndrome (EMS) is defined by a clustering of clinical signs, namely hyperinsulinemia, insulin resistance and adiposity, which predispose horses to the development of laminitis^{1,2}. Certain breeds (Morgan, Arabian, Welsh Pony, Tennessee Walking Horse) appear to be more susceptible to EMS, while other breeds (Quarter Horse) seem to be at lower risk³. Our preliminary data demonstrate significant breed differences in EMS-defining metabolic traits (e.g. insulin dynamics, lipid metabolism, adipokine concentrations) that mirror EMS risk. We *hypothesize* that breed differences in these key metabolic phenotypes are due to high frequencies of alleles that modify metabolic traits within breeds. The *goals of this proposal* are to **1)** further dissect the metabolic differences between breeds at the molecular level using total serum metabolite profiling; and **2)** use these breed-specific molecular metabolic profiles to identify candidate genes underlying breed metabolic differences.

Objective 1: Determine the molecular basis of breed metabolic variation by connecting metabolic pathways to the hormonal and biochemical differences between breeds. Global serum metabolite profiles will be analyzed before and after an oral sugar test in 274 horses from 5 breeds with distinct metabolic phenotypes. These data will be used to **1)** identify the metabolites and metabolic pathways that are significantly different between breeds using functional annotation and mapping of metabolites to known pathways, pathway and metabolite set enrichment analysis, network analysis and pathway activity profiling; and **2)** correlate the metabolite/pathway differences with the previously identified breed differences in key hormonal and biochemical measurements.

Objective 2: Identify candidate genes responsible for metabolic differences between breeds. In *objective 2a*, genomic regions and specific haplotypes that are highly differentiated between breeds will be identified using high-density SNP genotype data from the breeds in objective 1. In *objective 2b*, metabolites and metabolite pathways that are significantly different between breeds will be used to provide functional context for narrowing the focus to specific candidate genes within these genomic regions.

Combined, this project will expand our knowledge of the metabolic and genomic bases for across-breed differences in metabolic profiles and how they relate to EMS physiological and clinical phenotypes, and as a result greatly increase our understanding of the complex biology of EMS.

2. Justification, Significance and Literature Review. EMS refers to a cluster of clinical abnormalities minimally represented by three criteria: documented or suspected insulin resistance (IR), obesity and/or increased regional adiposity, and a predisposition to laminitis^{1,2}. Other reported abnormalities include hypertriglyceridemia, dyslipidemia, increased low-density lipoprotein concentrations, hyperleptinemia, arterial hypertension, and increased systemic inflammatory markers^{4,7}, although there is disagreement between studies⁸. Using morphometric, biochemical and hormonal phenotypes combined with epidemiologic and environmental data from 610 horses/ponies, we have demonstrated that variability in metabolic phenotypes is due to both environmental and individual factors, including genetics (preliminary data). In addition, these data highlight the fact that while breeds can share key metabolic features of EMS, they can also differ in the magnitude of these responses or of other features, such as fasting insulin, triglyceride, non-esterified fatty acid and adipokine concentrations. We *hypothesize* that the differences in the severity and secondary features of the EMS phenotype between breeds are the result of the variable frequencies of genetic risk alleles within breeds. Thus, the heterogeneity of the EMS phenotype across individuals and breeds is a result of the combination of underlying genetic alleles, the interactions between these alleles and the environment, and the resulting molecular pathophysiology. In this proposal, we build upon our ongoing work on the genetics of individual metabolic variation to focus on the molecular and genetic differences underlying breed-specific metabolic profiles.

Intense selective breeding in domestic animals leads to the fixation of phenotypic traits within breeds. Since domestication, selection in horses has included metabolic and athletic phenotypes that allowed horses to efficiently perform different types of work. Thus, many modern breeds likely have alleles segregating at high frequency or approaching fixation (i.e., 100% frequency) that modulate energy utilization by mechanisms such as enhanced anaerobic and/or aerobic metabolism, increased muscle mass and strength, increased cardiovascular and respiratory fitness, localized accumulation of adipose tissue, and improved economy of locomotion, among others⁹. These metabolically efficient or “thrifty” alleles, in particular those that regulate energy intake, storage and use, coupled with changes in equine husbandry practices in recent times including dense high caloric feed and limited exercise, may help explain the increasing prevalence of equine metabolic disease phenotypes in modern environments. Our proposal is based on the hypothesis that genetic predisposition to the development of

metabolic syndrome phenotypes in certain breeds is due to modern environments interacting with genotypes selected for under different environmental pressures. Studies demonstrating the overlap between genetic loci under selection in the human genome and loci conferring susceptibility to metabolic traits including obesity and type 2 diabetes lend validity to this hypothesis^{10,11}. Similarly, in the horse, we have identified selection for a mutation in the *GYS1* gene in Belgian draft horses, resulting in excess skeletal muscle glycogen associated with Polysaccharide Storage Myopathy Type 1 in modern environments¹³ and selection at the myostatin (*MSTN*) gene locus in Quarter Horses, which alters muscle fiber type proportions and which we hypothesize also alters many aspects of muscle and adipose tissue energy metabolism (**preliminary data**). Elucidating the evolution of the genetic basis of metabolic efficiency and metabolic syndrome across horse breeds is a novel, unexploited approach to the study of the genetic basis of obesity, energy dysregulation and EMS.

The current lack of information regarding the genetic basis of and variation in EMS within and across breeds restricts understanding of the pathophysiology, as well as limits the ability to predict disease risk and to identify individuals who can benefit from management changes and/or therapeutic intervention prior to the onset of disease/laminitis. To further untangle this complex phenotype, we propose collection of global serum metabolomics data both before and after an oral glucose challenge in our large across-breed cohort of horses and ponies. Non-targeted studies such as this provide unbiased data on the presence/absence and proportions of metabolites contained within a sample, and will provide a snapshot into the metabolic state of these horses both before and after a dynamic challenge (i.e., OST). Biologic differences between breeds will be explored by **1)** identification of breed differences in metabolite concentrations; **2)** functional annotation, mapping and visualization of metabolites within known predefined metabolic pathways; **3)** pathway overrepresentation analysis of significant metabolite lists; **4)** metabolite set enrichment analysis; **5)** the construction of metabolite networks and network topology analysis; and **6)** pathway activity profiling. Identified metabolic differences between breeds will be linked to putative candidate genes through a set of analyses designed to detect regions of low genetic diversity and positive selection in breeds of varying metabolic and EMS phenotypes.

We expect the major contribution of the proposed research to be the detection of breed differences in metabolites/metabolic pathways that are correlated to previously identified biochemical and hormonal differences, and the identification and prioritization of candidate genes that influence a spectrum of metabolic traits, particularly the susceptibility to metabolic syndrome. These findings will provide novel insights into disease biology, allowing the identification of new therapeutic targets, and increasing our understanding of the pathophysiology of EMS and its associated clinical features. Moreover, the identification of genes underlying the EMS phenotype will also directly impact equine health by allowing for the development of genetic tests to identify horses at risk for the development of obesity and laminitis prior to the onset of clinical disease.

3. Preliminary Data.

Clinical and breed variation in metabolic phenotype. We have collected 11 phenotypes, epidemiologic and environmental data from a total of 610 horses/ponies from 166 farms. These data include individuals from 5 breeds with distinct metabolic phenotypes (Arabians, Morgans, Quarter Horses, Tennessee Walking Horses and Welsh Ponies). Using these data, we have been able to rigorously assess several key aspects of variation in metabolic phenotypes and EMS, including: **1)** how metabolic measures differ in horses when parsed by clinical status (i.e., non-obese, non-laminitic [NO-NL], non-obese, laminitic [NO-L], obese, non-laminitic [O-NL], and obese, laminitic [O-L] and **2)** how *individual* (i.e., age, **breed**, gender, etc) and *environmental* (i.e., dietary adaptation, exercise, etc) variables impact trait measures (**Table 1**).

Our data indicate that fasting insulin, triglycerides, and insulin 75 minutes post-oral sugar challenge (OST) are consistently elevated, whereas adiponectin is consistently decreased in individuals with a history of laminitis, regardless of obesity status, age, gender or breed (**Table 1**). In addition to differences between clinical groups, we have also identified significant differences in the relevant biochemical measurements between breeds regardless of clinical phenotype (**Table 2**). For example, Quarter Horses have significantly lower fasting insulin and insulin OST, and lower leptin in relation to other breeds, whereas Welsh Ponies tend to have higher insulin OST. Moreover, Welsh Ponies have significantly higher fasting serum triglyceride and NEFA concentrations than horses as a whole (data not shown), even after correction for obesity and laminitis status. Heritability estimates from whole genome SNP genotype data in Morgans indicate many of these traits are highly heritable (**Table 1**).

Table 1. Key findings and estimated heritability for phenotypes in 610 horses and ponies.

Response variables	Predicted mean trait values by clinical group ^ε					Predictor (explanatory) variables significantly associated with outcome measures (p < 0.001)	Estimated heritability (Morgans)
	NO-NL	NO-L	O-NL	O-L	Overall p-value		
N:H RATIO	0.64 ^c	0.66 ^a	0.68 ^{ab}	0.70 ^b	4.60x10⁻¹⁹	breed, gender, obesity, laminitis	21.24%
G:H RATIO	1.19 ^a	1.19 ^a	1.24 ^b	1.24 ^b	2.10 x10⁻¹⁷	gender, obesity	10.48%
GLU (mg/dl)	77.5 ^a	79.8 ^a	78.2 ^a	78.6 ^a	0.44	season	NA*
INS (uIU/ml)	5.1 ^c	9.5 ^{ab}	7.8 ^a	13.3 ^b	1.40 x10⁻¹⁹	breed, age, obesity, laminitis	32.19%
GLU OST (mg/dl)	98.3 ^a	100.7 ^a	101.9 ^a	102.8 ^a	0.15	dietary starch, hours in stall	NA*
INS OST (uIU/ml)	19.5 ^b	35.2 ^a	32.6 ^a	42.8 ^a	2.90 x10⁻¹¹	breed, gender, age, obesity, laminitis	34.39%
TG (mg/dl)	23.8 ^b	33.7 ^a	26.8 ^b	34.4 ^a	6.40 x10⁻⁰⁸	laminitis, season, thyroxine	NA*
NEFA (mmol/L)	0.20 ^a	0.21 ^a	0.20 ^a	0.21 ^a	0.96	hours grazing, hours in stall, season	NA*
LEP (ng/ml)	4.2 ^a	4.0 ^a	6.1 ^b	6.0 ^b	2.32 x10⁻¹¹	breed, age, latitude, season	54.50%
APN (ng/ml)	4240 ^b	2495 ^a	3752 ^b	2418 ^a	1.15 x10⁻⁰⁸	breed, gender, obesity, season	13.58%
ACTH (pg/ml)	26.9 ^a	29.3 ^a	28.1 ^a	27.7 ^a	0.48	gender, laminitis, season	55.65%

^εValues are adjusted for breed, sex, age, month, latitude, diet, exercise, and L-thyroxine supplementation. Different letters within a row indicate a significant pair-wise difference (p < 0.05, Holm adjustment for multiple comparison). *NA indicates heritability estimates <2%. Abbreviations: neck- [NH] and girth- [GH] to height ratio, fasting glucose (GLU), insulin (INS), adrenocorticotrophic hormone (ACTH), triglyceride (TG), non-essential fatty acid (NEFA), leptin (LEP) and adiponectin (APN) concentrations; and insulin (INS OST) and glucose (GLU OST) post oral sugar challenge.

Breed-specific genomic regions. Our use of low-density SNP genotype data to identify regions of breed differentiation/selection across a wide breed panel has been demonstrated earlier¹²⁻¹⁵. Here we propose a similar approach in the relevant 5 breeds using ~1.8 million SNP markers, with genomic signatures of selection identified by calculating d_i statistics in non-overlapping 10 kb windows across the genome^{15,16} (**Figure 1**). The highest d_i values for the Quarter Horse (QH) are located within a 3-Mb window on chromosome 18 around the myostatin gene (*MSTN*), which we have previously reported as a signature of selection in this breed¹⁵. We have recently demonstrated that a SINE insertion in the *MSTN* promoter region results in altered skeletal muscle fiber type proportions¹⁷ and that the SINE alters metabolic phenotype in QH. Specifically, QHs with the SINE allele have less apparent adiposity (lower G:H RATIO), lower fasting and OST insulin, and leptin concentrations. In contrast, serum adiponectin concentrations are positively associated with the SINE (*manuscript in preparation*). In the Welsh Pony (WP), the highest d_i values were found to be on ECA6 around the interleukin-1 receptor-associated kinase 3 (*IRAK3*) gene, which is down-regulated in association with obesity and metabolic syndrome in humans¹⁸. These data demonstrate the feasibility of identifying breed-specific genomic regions associated with metabolic phenotypes in our cohort.

Characterization of serum metabolites and metabolic pathways. Metabolomic analysis, completed by a commercial laboratory (Metabolon®) using a combination of GC-MS and LC-MS, was performed on serum samples obtained from 20 Welsh ponies before and at 75 min during an OST. Ponies were classified as healthy [CON] (n = 10, insulin < 30 mU/L) or having insulin dysregulation [ID] (n = 10, insulin > 60 mU/L) at 75 min post OST. The serum

Table 2. Breed differences in metabolic trait measures. Values are adjusted for clinical status, sex, age, month, latitude, diet, exercise, and L-thyroxine supplementation.

	Morgan	Arab	Pony	QH	TW	Overall p-
N:H	0.69 ^b	0.65 ^a	0.69 ^b	0.68 ^{ab}	0.68 ^{ab}	3.10x10⁻⁰⁶
INS	9.6 ^b	5.9 ^{ab}	11.7 ^c	5.5 ^a	10 ^{bc}	4.00x10⁻⁰⁶
INS OST	34.3 ^a	25 ^{ab}	41.8 ^b	16.1 ^a	45.6 ^b	1.50x10⁻⁰⁶
TG	29.9 ^{ab}	26.8 ^{ab}	32.3 ^{ab}	24.1 ^b	34.5 ^{ab}	2.60x10⁻⁰³
LEP	6.2 ^a	4.5 ^{ab}	5.1 ^a	3.2 ^b	5.8 ^a	1.79x10⁻⁰⁶
ACTH	26.4 ^b	30.1 ^{ab}	35.9 ^a	23.8 ^b	22.6 ^b	6.60x10⁻⁰⁵

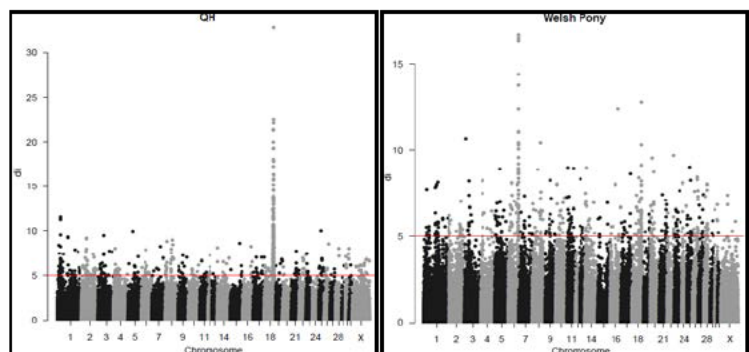


Figure 1. Genome-wide d_i values for QH and WP. Regions of putative positive selection (d_i values above the red line) were those in the top 99% of each empirical distribution

metabolomic profiles comprised 646 biochemical compounds, of which 506 were of known identity. Statistically significant differences between ID and CON, or between baseline and post OST, were identified for > 130 biochemicals, including hexoses (glucose, mannose, fructose), metabolites involved in the tricarboxylic acid cycle (citrate, fumarate, malate), fatty acid metabolism (palmitoleate, eicosapentanoate, palmitocarnitine, laurycarnitine), and branched-chain amino acid oxidation (4-methyl-2-oxopentanoate, 3-methyl-2-oxoalate and isovalerylglutamate). These data demonstrate the potential for serum metabolomic data to lead to insight into the metabolic differences between horses and clinical phenotypes.

4. Experimental Methods and Design.

Objective 1: Determine the molecular basis of breed metabolic variation by connecting metabolic pathways to the hormonal and biochemical differences between breeds.

Rationale. Global serum metabolomics data collected both before and after an oral glucose challenge in an across-breed cohort of horses and ponies will provide a *comprehensive* and *unbiased* approach to understanding the molecular physiology/pathophysiology of breed phenotypic variation/EMS by providing data on the proportions of all measureable metabolites. Further, annotation and assembly of metabolite data into pathways and networks and correlation of these pathways/networks to hormonal and biochemical differences between breeds can potentially pinpoint the molecular alterations leading to, or resulting from, hormonal/biochemical differences (e.g., the effects of hyperinsulinemia, etc). Metabolites, metabolite ratios, and networks/pathways will also provide a more precise foundation for investigation of the genetic component of metabolic trait variation in **objective 2**.

Experimental Methods and Design. Fasting and 75 min post oral glucose challenge (OST) serum samples from 274 previously-phenotyped individuals from 5 breeds (39 Arabians, 75 Morgans, 46 Quarter Horses, 39 Tennessee Walking Horses and 75 Welsh Ponies) will be used. Based on power calculations performed in Metaboanalyst for multivariate models using preliminary metabolomic data, to determine group (i.e. breed) differences with a specificity of 0.95 and a sensitivity of 0.85, the number of needed individuals per group is 38-75 depending on the desired confidence interval (i.e., 90% vs 95%)¹⁹⁻²¹

All analytical work will be performed at the U of M Center for Mass Spectroscopy and Proteomics. Samples will be deproteinized and aliquots lyophilized²². An initial QC analysis to assay technical, sample preparation and biologic variability will be used to set acceptable QC limits. Then, raw data will be pre-processed to provide structured data and appropriate format for subsequent analysis using Progenesis QI²³ (for UHPLC-MS) or XCMS²⁴ (for GC-MS) software. Background subtraction will be performed as indicated by initial QC investigation. Data will be auto-scaled (i.e., mean centered and divided by the sample standard deviation) to avoid skewing due to dynamic range differences, and log-transformed to ensure normality, if necessary (i.e., for regression-based analyses). Sample and feature outliers will be removed and missing values will be imputed²². Unidentified metabolites of statistical interest will be subjected to secondary fragmentation and resulting patterns searched against existing spectral libraries containing annotated fragmentation patterns.

Statistical differences between breeds and correlations between metabolic features and continuous measured outcome variables (i.e., INS, INS OST, TG, GHR, APN, etc)²⁶ at each time point (baseline and post-OST) will be performed in MetaboAnalyst¹⁹⁻²¹. Comparison of temporal profiles (i.e. fasting and post-OST) will be evaluated using the MetATT tool using multivariate empirical Bayes time series analysis (MEBA)²⁷. These analyses will identify differences between breeds, time points, and the interactions between breed and time point.

Biological patterns, functions and metabolic pathways differences will be further explored by functional annotation, mapping and visualization of metabolites within known predefined metabolic pathways using MBRole²⁸ and Interactive Pathways Explorer (iPath)²⁹, with data from KEGG³⁰, HMDB³¹, PubChem (pubchem.ncbi.nlm.nih.gov), and ChEBI³² databases. Pathway overrepresentation analysis and metabolite set enrichment analysis will be performed using MESA. The construction of metabolite networks and network topology analysis and pathway activity profiling will be performed with MetPA and PAPI, respectively. The differences in metabolites, pathways, networks and pathway activity estimates between breeds will highlight key metabolic functions that are differentiated by breed.

Expected outcomes and potential pitfalls. We expect to identify up to several thousand metabolites, as observed in the human serum metabolome project²⁶. Several dozen to hundreds of metabolites may differ significantly by

breed or temporal sampling, and several metabolites and metabolic pathways are likely to be correlated to phenotypic measurements such as INS, INS OST, APN, LEP, TG, etc.

Objective 2. Identify candidate genes responsible for metabolic differences between breeds.

Rationale. Domestic animal breeds originate through artificial selection in which breeders work to fix desirable phenotypic traits. In the horse, there is evidence that this selection process has focused on metabolic and athletic traits that have resulted in several breeds that are metabolically efficient, and by extension at high risk for the development of EMS. When the alleles underlying these traits reach high frequency or become fixed within a breed, genetic association studies lose their power to identify these loci. To identify high allele frequency loci harboring alleles responsible for breed differences in metabolic traits, we first identify genomic regions within each breed where there is evidence of decreased genetic diversity and haplotype differentiation relative to other breeds, which together suggest that the region harbors genes/alleles responsible for breed phenotypic differentiation. Then, we identify the genes present in these genomic regions of interest (ROIs) (**objective 2a**). In **objective 2b**, we capitalize on the breed differences in metabolic phenotypes identified in objective 1 to prioritize genes within these ROIs. Gene lists from ROIs will be prioritized for further association investigation using the metabolic pathways that are different between breeds and correlated with key hormonal measures. Identification of putative metabolic pathways and candidate genes that result in breed phenotypic differences constitutes a key step in linking certain phenotypes to causal genotypes and is critical to understanding the complex biology underlying EMS.

Experimental Methods and Design - Objective 2a. Genomic ROIs will be identified in all breeds using an F_{st} -based statistic (d_i) calculated in non-overlapping 10 kb regions across the genome, with regions in the top 1% of the empirical data picked as putative regions of genomic differentiation (**preliminary data**)^{15,16}. Haplotype sharing within and specific haplotypes within ROIs will be delineated by the hapQTL method of quantifying local haplotype sharing³³ for which we will choose significant SNP markers with $-\log(\text{Bayes Factor}) > 5$ with at least 2 significant markers overlapping the above regions. Finally, extended haplotype homozygosity analysis will be conducted to identify core haplotypes containing putative causative alleles^{34,13}. The BioMart software suite (www.biomart.org) will be used to query the Ensembl database and create lists of genes located within the haplotype boundaries identified above. The equine genome (EquCab2) and syntenic regions of the human genome sequence (GRCh37) will be queried to ensure candidate genes are not missed due to inaccurate annotation of EquCab2. Gene ids, gene structure information including start and end positions and exonic regions will be retrieved. All genes will be annotated with gene ontology (GO) terms to facilitate identification of genes with relevant biologic function.

Experimental Methods and Design - Objective 2b. Mapping and annotation of metabolites to known metabolic pathways in objective 1 using MBRole and iPath allow for changes in metabolite concentrations to be linked to the gene products (and subsequently genes) that are involved in metabolic pathways. Genes in ROIs for a given breed will be prioritized as candidate genes if the gene's product plays a role in the formation of the metabolites or in pathways that are different within the breed. Metabolite correlation networks (MetPA) can also be used to prioritize genes when metabolites do not map to canonical pathways. In this instance, breed-specific sub-networks containing genes identified within ROIs will be extracted from the metabolite networks. The biological coherency of the subnetwork will be assessed by calculating the average metabolite interaction weight between all possible interactions²¹, and used to reconstruct biochemical pathways related to breed metabolic differences.

Expected results and potential pitfalls. As shown in preliminary results, there may be many dozens of loci showing differentiation between the 5 breeds which contain compelling genes in pathways highlighted by metabolomic analysis. We anticipate that the myostatin locus on ECA18 will be highlighted as a major driver of the metabolic differences in QHs as compared to other breeds, and as such should serve as a 'proof of principle' positive control and that the ECA6 locus in Welsh ponies may provide another major target. It is possible that we do not identify any genes within genomic ROIs that are strongly correlated with breed-specific networks. In this circumstance, we will simply use differential network analysis to identify candidate genes for further investigation, regardless of ROIs.

Timeline. **Objective 1:** Sample preparation and submission; months 1-6. Serum metabolite profile analysis; months 6-12. **Objective 2a:** Identification of signatures of selection; months 12-14. **Objective 2b:** Candidate gene prioritization; months 14-20. Manuscript(s) preparation: months 20-24.

Morris Animal Foundation

Animal Involvement Justification

(From the proposal guidelines, single-spaced, no page limit)

Morris Animal Foundation (MAF) is dedicated to funding scientifically sound, relevant and humane studies that specifically address the health and well-being of animals. All studies receiving funding must follow MAF's Health Study Policy for Animals Involved in Research (adopted October 18, 2008), which was written to ensure that each and every animal involved in a MAF funded health study receives excellent, compassionate care throughout the study. MAF shall not fund health studies which require euthanasia as an endpoint or the induction of disease or injury, unless the nature of the disease or condition to be studied is of such significance for improving animal health that such means are justified, and that meaningful information can be obtained in no other way. Furthermore, MAF will not fund any study that induces or allows pain or distress unless such pain or distress can be controlled by appropriate anesthetic, analgesic, tranquilizing drugs, or nursing care. [Click here](#) for the full Health Study Policy.

- A. If this study does not involve live animals please indicate here by N/A: NA
- B. Does this study involve biological samples, tissues, etc.? YES

If yes, describe in detail what samples will be used and where & how they will be (or were) acquired. Note: Morris Animal Foundation reserves the right to request a copy of the Institutional Animal Care and Use Committee (IACUC) application/approval and other relevant applications/approvals (e.g., wildlife permit) covering the original collection of samples, including archived samples. MAF reserves the right to request IACUC (or equivalent) review and approval for any Foundation study *regardless of the Institution's requirements*. This would include the use of archived samples as well as clinical trials.

ALL SAMPLES HAVE BEEN COLLECTED AS PART OF PRIOR STUDIES. Information regarding prior collection is provided below. Blood samples were collected before and 75 minutes after an oral sugar test from the jugular vein.

- C. If this study involves live animals, succinctly address the following: (please restate the questions and directives).
1. What species will be studied? **EQUINE**
 2. State the status of your IACUC application/approval. All recipients of MAF funding will be required to submit the entire IACUC protocol and document. A copy of the IACUC approval should not be included with the application, but it is required before funding can be awarded.

Samples were collected under the University of Minnesota IACUC protocol # 1109B04448 approved 10/16/11 (M McCue PI)

3. List the USDA category for pain and distress (B, C, D, E): C

Note: Any study beyond category C will require review by MAF's Animal Welfare Advisory Board (AWAB). In general MAF does not fund studies beyond category C (category D studies will only be considered if they conform with MAF's Health Study Policy, category E studies will not be considered).

4. Does this proposal involve client-owned animals? YES

If yes, the protocol for client-owned animals must be approved by the appropriate peer review committee before the project is funded. *If this proposal involves client-owned animals, an informed client consent form must be submitted with this proposal. For a suggested list of items to be considered in an informed client consent form, [click here](#).*

Informed client consent attached.

5. Explain how animals will be acquired (e.g., client-owned, USDA licensed breeder, institutional "herds" or "colonies") and verify that the animals are suitable for the study (e.g., have no physiologic, physical or

pharmacologic issues that would interfere with results)

6. How many animals will be used? 274 (for this study)

a. Summarize numerical justification **Based on power calculation –see experimental design**

7. Does this study induce disease, injury, pain or distress in animals? Note: any study requiring the induction of disease, injury, pain, or distress will have an additional evaluation by MAF's AWAB.

If yes,

a. Defend the necessity of experimental design

Collection of blood samples is necessary for accurate phenotyping of horses and ponies for metabolic traits. Samples have been previously collected (under the UMN IACUC referenced above as well as under IACUC protocol #0804A30547, Approved 4/20/2010, renewed 4/21/2011).

b. Explain how pain and/or distress will be controlled

Jugular venipuncture was performed by licensed veterinarians, and caused minimal distress.

c. Justify that no alternative, including clinical studies, can be used to accomplish study objectives and the disease/condition to be studied is of such significance for improving the health of the species.

An alternative method to characterize the serum metabolites in horses of different breeds/metabolic phenotype without the collection of blood samples is unavailable.

8. Explain the environment and housing conditions (quality of life) in which the animals will live (address species-appropriate exercise, enrichment, socialization, veterinary care, etc.)

Animals are client owned and living in their normal environment.

9. What will happen to the animals upon completion of the study? **N/A, animals are client owned**

If adoption, explain the adoption process. Provide assurance that whenever possible and when in the animal's best interest, investigators shall make companion animals available for adoption at the end of the study or return the animals to the owner/responsible agency in an environment that promotes animal welfare and excellent quality of life.

10. If euthanasia, provide the following additional information (note: any study requiring euthanasia as an endpoint will have an additional evaluation by a MAF's AWAB.

i. Total number that will be euthanized and justification for numbers

ii. Method of euthanasia

iii. Justification that no alternatives can be used to accomplish study goal(s) and that the disease/condition to be studied is of such significance for improving the health of the species that a terminal endpoint is deemed necessary.

iv. Reason for euthanasia in lay language (this wording may be shared with staff, donors and media)

v. Provide objective criteria for determining when euthanasia is appropriate or necessary (note: Morris Animal Foundation wants assurance that an animal will not be allowed to suffer and that monitoring for pain and suffering is adequate)

Note: Morris Animal Foundation does not consider the use of CO2 alone to be an appropriate method of euthanasia

Please note:

1. If an animal is used in an invasive study, MAF may require that a guarantee is provided, through principal investigator and institutional signatures that the animal will not participate in any future invasive study or procedure
2. MAF does not allow inclusion of ancillary data in MAF funded research that includes animal use

protocols not in agreement with our Health Study Policy, even if it is obtained using other funding sources.

3. Morris Animal Foundation considers euthanasia acceptable when an animal develops unanticipated illness or injury that results in pain and suffering that cannot be alleviated with standard veterinary interventions.

Informed Client Consent Equine Metabolic Syndrome (EMS) Study

We are delighted that you have agreed to allow us to examine and obtain blood samples from your horses/ponies, in addition to obtaining hay/pasture/feed samples, in order to determine if your horse(s) have a group of risk factors for laminitis that have been termed Equine Metabolic Syndrome (EMS). We will use the results of this study to determine early predictors for laminitis in horses and ponies. We will also save some of your horse's genetic material (DNA) so we can identify the underlying genetic causes that predispose horses to EMS. The results will be made available to you. Identification of risk factors in your horses will allow you to identify high risk horses and change their management, and hopefully prevent future episodes of laminitis. The results of our study will be published in scientific journals **without** the identity of the farms involved or horses involved being disclosed. Records will be kept confidential indefinitely.

The examination will consist of a physical examination of your horse, the determination of a body condition score (BCS), measurements of height, circumference of the neck and girth and weight (estimated by weight tape). We will also evaluate your horse for any signs of lameness/laminitis and will collect historical data regarding prior episodes of laminitis. Digital photographs will be taken to allow assessment of BSC by a second researcher. We will collect blood samples before and after an oral sugar test for measurement of glucose, triglyceride, free fatty acid, insulin, GLP-1, C-peptide, and ACTH concentrations. The oral sugar test will be performed by administering 50 g/kg dextrose as syrup by mouth using a dose syringe. All blood samples will be collected after a short fasting period (approximately 6 hours). A portion of the blood samples will be used to extract DNA for future genetic analysis. Serum and/or plasma will also be frozen for future studies on EMS phenotyping.

All of these procedures are routine and non-invasive. We do not anticipate any complications. Horses may experience a slight discomfort from the needle when blood is sampled. If you notice any swelling at this site, please contact us, and we will arrange for medical care if necessary and cover any costs incurred. Dextrose is a non-structural carbohydrate and insulin resistant horses are more sensitive to non-structural carbohydrates (NSC) and at higher risk for laminitis development when excess levels of NSC are consumed in the diet over a period of time. However, *the oral glucose tolerance test will only involve a one-time administration of dextrose in an amount that is small enough to not place an insulin resistant horse at a higher risk of developing laminitis.* The amount of dextrose being administered is approximately equivalent to the amount of NSC in a typical grain meal. We have performed this procedure in more than 500 horses without any complications.

Once you are enrolled in the study, the examination and blood collection will take approximately 90 minutes. We may contact you in the future to follow-up with you regarding any bouts of laminitis your horse has experienced after our visit.

The contacts for this study are Dr. Molly McCue (612) 624-9320 or Dr. Ray Geor (517) 355-9593. Either the researchers or the owner of the farms involved in this study have the right to withdraw at any time. The cost of the examinations, blood measurements, and hay/pasture/feed analysis will be covered entirely by the research team.

Informed consent: I, _____ understand that there may be unforeseen risks involved in any research activity. If I have any concerns about the performance of this study I can contact the Department Chairman at The University of Minnesota Dr. Tom Molitor at (612) 625-7755 or the Institution animal care and use committee (IACUC).

Owner signature and date

Investigator signature and date

VII. Recombinant DNA/Biohazards:

NA

VIII. Cited References

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PROPOSAL BUDGET

Note: First Award – complete year 1, year 2 and total only. **Pilot Study** – complete year 1 only. **Fellowship Training** – complete salary, fringe benefits, indirect costs and total for year 1 and year 2 only. For Established Investigator, First Award and Pilot Study; Fellowship Training lines can be removed.

Category	Year 1	Year 2	Year 3	Total
<u>Personnel:</u>				
1. Principal investigator (name)*				
2. Co-investigator #1 (name)				
3. Co-investigator #2 (name)				
4. Technician Salary (X%) Fringe benefits (Y%)				
5. Student Assistant Salary (X%) Fringe benefits (Y%)				
Total Salaries & Wages				
Fellowship Training Only: Salary (90.5%) Fringe benefits (9.5%)				
<u>Supplies & Expenses:</u>				
1.				
2.				
3.				
4.				
Provide justification in the designated section.				
Total Supplies & Expenses:				
<u>Animal Use & Care:</u>				
Animal Purchase:				
Animal Per diem:				
Total Animal Care:				
Subtotal of All Categories:				
Maximum of 8% - Indirect Costs: ** (8%)				
Grand Total Requested from MAF:				

* Salary requests for principal investigators must be clearly defined and justified in the following budget justification section. You may request salary for technicians, residents, graduate students, and postdoctoral fellows, based on their percentage of time involved in the project

** Indirect costs may be claimed only if you are charged for indirect costs by your institution for work carried out in this proposal. **You must make this calculation yourself.** If your institution charges less than 8%, claim only that amount and indicate the percentage.

X. Itemized Budget Justification (one-page limit): Salaries, supplies and animal care costs not justified may be deleted from the budget of an approved/funded proposal. The role of each investigator should be clearly defined. Investigator salary requests will be thoroughly scrutinized. Indicate and justify a percent effort on this grant for all individuals, including technicians, graduate students, etc., for whom MAF salary funds are requested.

First Award

I. Title and Abstract (1pp)

Title: Biomechanical and Genetic Risk Factors for Osteochondrosis in Standardbred Pacers and Trotters

Rationale: Osteochondrosis (OC) is a common manifestation of developmental orthopedic disease in young horses that is influenced by both genetic and environmental risk factors. Some OC lesions may heal spontaneously, but those present after 8 months of age are typically permanent and nearly always require surgical intervention. While OC can affect individuals of any breed, Standardbreds have a particularly high prevalence of tarsal lesions (10-26%). Differences in tarsal OC lesion prevalence and distribution between Standardbred pacers and trotters have been reported, but it is unknown if these are due to differences in genetic risk, biomechanical forces related to gait, or a combination of the two.

Hypothesis/Objectives: We hypothesize that Standardbred pacers and trotters share genetic risk factors for OC, but that biomechanical differences in their natural gait patterns influence which early lesions heal and which become permanent. In **Objective 1** we will *prospectively follow the development of tarsal OC lesions in a cohort of Standardbred pacer and trotter foals* to determine if gait preference has an impact on lesion healing/persistence. In **Objective 2** we will *determine if previously validated genetic risk factors (Preliminary Data) are shared between pacers and trotters and determine if these risk alleles are associated with development and/or persistence of OC lesions* in our experimental cohort.

Study Design: 86 sire-matched foals (n = 43 pacer; n = 43 trotter) will be enrolled in this study between birth and 60 days of age. **Objective 1:** Four standard radiographic views of both tarsi will be taken using a portable digital x-ray unit at 60 day intervals until the foal is sold or reaches 12 months of age. Radiographs will be assembled serially for each foal and evaluated blindly for the presence/absence, as well as spontaneous healing or persistence, of OC lesions. Foals will also be observed in their normal paddock/pasture turnout and their activity will be recorded (amount of time spent moving and at which gait). Differences in presence, location, and progression of OC lesions, based on gait preference (pace vs trot) and activity will be determined. **Objective 2:** DNA from all foals will be submitted for single nucleotide polymorphism (SNP) genotyping on a custom Sequenom genotyping assay (**Preliminary Data**). Mixed model association analysis will be performed to identify risk alleles significantly associated with 1) development of any OC lesion during the course of the study; 2) development of a permanent OC lesion.

Preliminary Data: A genome-wide association study (GWAS) in 182 Standardbreds revealed regions associated with tarsal OC on equine (ECA) chromosome 14. Follow-up genotyping of 240 putative risk alleles using a Sequenom assay in the GWAS population and an independent population of Standardbreds (n = 139) resulted in validation of the two risk loci on ECA14 as well as risk loci on ECA10 and 21.

Expected Results: We expect that $\geq 70\%$ of the foals will have evidence of OC at one or more time points, with $\sim 20\%$ developing permanent lesions. Further, the amount of time spent pacing/trotting will correlate with the location of permanent lesions (i.e. distal intermediate ridge of the tibia in pacers vs. medial malleolus in trotters). Finally, we expect that one or more putative risk alleles within the validated risk loci on ECA14, 10, or 21 will be associated with disease in our cohort, but that there will not be differences between pacers and trotters.

Budget and Timeline: 2 years, total budget [REDACTED], most of which is devoted to radiographic exams [REDACTED] and genotyping [REDACTED]. **Months 1-21:** sample collection and radiographs (Year 1 n = 60 foals; Year 2 n = 26 foals). **Months 17-19:** genotyping. **Months 21-24:** final data analysis and compilation of results.

Potential Impact for Animal Health: Prospective evaluation of differences in OC lesion formation between pacer and trotter foals will provide insight into the role that biomechanical forces related to gait may play in the development of disease. Further, this approach will allow us to determine if there are differences in genetic risk factors not just between pacers and trotters, but between horses who spontaneously heal their OC lesions and those who go on to develop permanent lesions. Defining the roles of these putative risk factors will both improve our understanding of the pathophysiology of OC and facilitate early interventions for at-risk horses to help reduce risk of clinical disease.

II. Letters

Candidate Letter of Intent

UNIVERSITY OF ILLINOIS
AT URBANA-CHAMPAIGN

Department of Veterinary Clinical Medicine
College of Veterinary Medicine
1008 West Hazelwood Drive
Urbana, IL 61802



25 June 2015

To the Selection Committee:

As a veterinarian board-certified in large animal surgery, I have an ongoing interest in musculoskeletal disease in horses as this is a major reason for presentation of patients for clinical evaluation, with complaints ranging from poor performance to impaired quality of life. As a researcher, I am especially interested in the role that genetics plays in developmental and degenerative joint disease. This encompasses predisposing genetic factors, changes in gene expression during the course of disease, and genetic therapies for treatment. I believe that clinician-scientists play a vital role in bridging the gap between benchtop research and clinical applications, and I hope to fulfill such a role as I advance in my career. I recently accepted a tenure-track Assistant Professor position in academic veterinary medicine and am working to establish an independent research program while maintaining involvement in clinical practice, the didactic and clinical teaching of veterinary students, and the training of interns and residents. The work outlined in this First Award proposal is an outgrowth of the projects I completed during my PhD training, and is the natural next step to support my growth into an independent researcher.

My previous research training has been diverse, ranging from cognitive psychology to molecular genetics, from very basic to purely clinical. I deliberately sought out these different experiences so that I would have a clear idea of where I wanted to focus my own research career. I discovered that while I enjoyed both basic and clinical research, I was most interested in being able to bridge the gap between them as a clinician-scientist. My PhD mentors supported my efforts to combine my clinical interest in orthopedics with my basic research interest in genetics, which resulted in a thesis project primarily focused on the genetic risk factors for osteochondrosis (OC; a developmental orthopedic disease), and secondarily focused on genetic determinants of gait and performance in horses. Specific approaches used in the course of this work included single nucleotide polymorphism (SNP) genotyping, genome-wide association (GWA) analysis, traditional Sanger sequencing of candidate genes, and whole-genome sequencing analysis. I wrote 14 grant proposals related to this research, three of which were funded. To date, however, I have not received funding as a principal investigator for more than \$20,000 for any single, extramural award. I have presented the results of my research at several national and international meetings since 2011 (e.g. Plant and Animal Genome XVIII Conference, 2011; Dorothy Russell Havemeyer Foundation 10th International Equine Genome Mapping Workshop, 2013; Plant and Animal Genome XXII Conference, 2014), and have abstracts accepted at two upcoming meetings (Dorothy Russell Havemeyer Foundation 11th International Equine Genome Mapping Workshop July 2015; American College of Veterinary Surgeons Surgical Summit October 2015). Additionally, my PhD research has generated four peer-reviewed publications to date (*Osteoarthritis Cart* 21:1638-1647, 2013; *J Hered* 105:163-172, 2014; *Anim Genet* 45:153, 2014; *Equine Vet J* 47:438-444, 2015), with a fifth manuscript submitted for review (*BMC Genomics*).

One of these publications described the effect of hock OC on race performance in a cohort of Standardbred pacers and trotters (*Equine Vet J* 47:438-44, 2015). An unexpected novel finding of this report was that there were gait-related differences for both OC lesion distribution and the overall risk for developing disease in our study population. These differences could have been due to biomechanical factors, genetic factors, or a combination of the two, but my existing data set did not allow further exploration of these possibilities. In collaboration with my new colleagues at the University of Illinois, including Dr. Kevin Kline (co-investigator on this award), I am now ideally positioned to follow up on this finding in this First Award by prospectively evaluating foals radiographically for the development and progression (healing/permanence) of OC lesions and correlating these findings with observed activity and natural gait preferences. Additionally, I will be able to evaluate specific risk alleles for OC in this group of foals based on recent work done in collaboration with my mentor, Dr. Molly McCue, in which we validated several genetic risk loci for hock OC by demonstrating association with disease in two independent populations of Standardbreds (work supported by a Morris Animal Foundation Pilot Award; manuscript submitted for review). Not only will I be able to compare these risk alleles between pacers and trotters, but also between foals that spontaneously heal their OC lesions and those that go on to require treatment for permanent lesions. This type of comparison has never been reported, and has the potential to open up an entirely new line of inquiry for me to pursue moving forward.

The work outlined in this First Award represents the next step towards a major goal of my planned independent research program, namely development and testing of a risk model for development of OC in individual horses based upon genetic variants of putative functional effect and environmental risk factors. The independent collaborations I have established at the University of Illinois will open up many future research opportunities by giving me access to a large local cohort of mares and foals. At the same time, the First Award supports my efforts to meet my training and career development goals under the ongoing guidance of my mentor, Dr. McCue, who is both an internationally recognized expert in my chosen field of equine genetics/genomics and a singularly successful researcher who has established a highly productive lab in her relatively short career.

In my eyes, my roles as a clinician and as a researcher are inherently intertwined, with each enhancing the other. My clinical training is the lens through which I frame my scientific inquiries, providing crucial context and perspective. As equine genetics research advances with the aid of next-generation technologies, allowing us to ask and answer more complex questions, the results will continue to be translated into tangible benefits for our patients. I certainly plan to be a part of this process as my career develops, and this First Award will help me take the next step towards my goal of becoming an independent clinician-scientist.

Sincerely,

Annette M. McCoy, DVM, MS, PhD
Diplomate, American College of Veterinary Surgeons
Assistant Professor, Equine Surgery
Department of Veterinary Clinical Medicine
College of Veterinary Medicine
University of Illinois

Mentor Letter

UNIVERSITY OF MINNESOTA

Twin Cities Campus

*Veterinary Population Medicine
College of Veterinary Medicine*

*225 Veterinary Medical Center
1365 Gortner Avenue
St. Paul, MN 55108
Office: 612-625-7755*

To the Review Committee:
June 20, 2015

It is my pleasure to write a letter indicating my strongest support for Dr. Annette McCoy's first award application. I have had the pleasure of working closely with Dr. McCoy since she began her PhD in my laboratory in 2010, and I have continued to collaborate with Dr. McCoy since she began her faculty position at the beginning of 2015. The objectives outlined in this first award are the follow-up to the project Annette worked on in my laboratory as a PhD student. As part of her thesis work, Annette phenotyped a population of Standardbred horses for osteochondrosis (OC), characterizing the impact of OC and early surgical intervention on racing performance and identifying genetic risk loci using genome-wide association (GWAS) analysis. During Annette's study on the impact of early surgical intervention on racing performance, she identified differences in tarsal OC prevalence and site predilection in Standardbred pacers and trotters, a finding that had not been previously reported. Further, Annette's GWAS study identified two chromosomal loci associated with tarsal OC, and Annette successfully obtained funding from Morris Animal Foundation (MAF) in 2014 to validate these findings in a second independent population of European Standardbred trotters. Annette has now successfully completed this MAF funded work and for the first time, validated a genetic locus identified for OC in a second independent horse population. The hypothesis in Annette's First Award comes directly from her findings in both her performance and genetic studies, and the work outlined in this proposal are the natural next steps to these findings.

The goals of the First Award are to support new investigators that are proposing the highest quality science. I can state that without a doubt that Dr. McCoy's proposal represents both. The proposed research in this proposal builds on the genetics/genomics and computational skills Annette acquired during her PhD, however, this work represents Annette's own intellectual pursuit and is feasible due to the new collaborations she has established since her arrival at the University of Illinois. The project plan is Annette's own design and represents an opportunity for Annette to establish her own independent research program. The objectives in this proposal fit perfectly into her long-term goal of developing a risk model of OC that incorporates both genetic and environmental (including biomechanical) risk factors. Further, the genetic risk factors identified will likely be informative across breeds and predilection sites, thus the impact of this work will extend beyond predicting risk for tarsal OC in the Standardbred. Finally, the prospective nature of the study will allow for an estimation of the number of OC lesions in foals that spontaneously heal and has the potential to identify the genetic factors that are responsible for spontaneous healing.

Funding of this proposal will also have an impact on the career of a truly outstanding scientist. I cannot overstate Annette's potential as an independent research scientist; she is highly intelligent, extremely organized, dedicated to her career, and an incredibly hard worker. During her PhD, Annette not only studied OC, but also capitalized on the population of Standardbred horses she used for her thesis to map two additional traits by genome wide association (GWAS), one for racing performance and another for gait. She has also worked with a collaborator to gather samples from > 100 Thoroughbred horses to eventually study the genetic basis of a juvenile osteoarthritis, and laid the groundwork for the functional genomics and proteomics evaluation of an experimental model of post-traumatic osteoarthritis in the horse. In addition to generating research data and research samples, during her tenure in my laboratory, Annette co-authored 14 grant proposals (3 funded), published 5 manuscripts and authored a book chapter. Annette has also mentored undergraduate students and veterinary summer scholars, and in 2012 the college awarded her the Vaughn Larson Award in recognition of her leadership. Annette has taken an active role in her professional

development outside of research, participating in the University's Preparing Future Faculty Program and in teaching in the didactic veterinary curriculum. Needless to say she has been highly productive, setting the bar for her fellow graduate students, and for those who will follow in her footsteps.

As Dr. McCoy's primary mentor I will continue to be involved in her research training and on-going project development. As Dr. McCoy has been establishing her own laboratory and ongoing projects at the University of Illinois, we have continued to be in contact 2-3 times per month for Skype meetings. Annette has also contributed to the on-going training of students in my laboratory by being involved in our weekly data analysis meetings (via Skype) and has often volunteered to help with feedback for upcoming research presentations, both via reviewing slides and by providing input during presentation "practice sessions". Moving forward (at least until Annette's own lab becomes busy enough that we can no longer impose on her time), Annette will continue to be involved in some of the on-going activities in my laboratory. I view this as a win-win situation; these interactions provide Annette the opportunity to help mentor graduate students and also give my students an opportunity to get additional feedback and/or a different perspective. In addition to nearly weekly discussions and her on-going involvement with my laboratory group, Annette and I have identified opportunities to develop clinical research projects, hone her skills in grant and manuscript writing, network with investigators both inside and outside the University of Minnesota and the University of Illinois, and to further develop her teaching skills. As stated above, the research project outlined in this proposal stems from the work on OC that she initiated during her PhD, and also from other collaborations she has independently developed. To support these and other on-going projects, I have worked with Dr. McCoy to identify potential funding sources and continue to mentor her in the grant writing process. Additionally, I will work with Dr. McCoy to ensure that she publishes a minimum of 2-3 (or more) high quality manuscripts each year to ensure that she stay on track in her career development. However, in large part Dr. McCoy's independence will be facilitated by her taking sole ownership of the OC and OA projects she has initiated in my laboratory. Both OC and OA are disease processes that are in alignment with Dr. McCoy's clinical training; whereas my clinical training and interests are in internal medicine with the main projects in the my lab being focused on muscle, metabolic, and neurologic diseases/biology.

My previous mentoring experience includes being a graduate faculty member in 3 graduate programs at the University of Minnesota. I serve as a faculty mentor for both T32 and T35 awards, and I am the co-director of our post-doctoral T32 in Comparative Medicine and Pathology. I have served/am serving as the primary mentor for 6 PhD students and 3 MS students, and 3 post-doctoral trainees, and as the primary clinical mentor for 5 internal medicine residents. Two of my PhD students have completed their degrees and a third will defend in August 2015. One of three MS students has completed her masters and gone on to a PhD degree at the University of Minnesota. Two of three post-docs have completed their training. Of the PhD and post-doctoral students who have completed their training, three have become faculty members (2 tenure track) and the forth went into industry. I am also currently the co-advisor for 2 PhD students and have served on an additional 10 graduate committees (5 PhD and 5 MS). I currently informally mentor another tenure track faculty member at the University of Minnesota, including serving on a K01 award mentoring committee, mentoring on the balance between research and clinical work and serving as a co-investigator on a canine genomics grants.

In summary, Annette is a very bright, talented, and motivated individual. Her academic career and productivity to date has been stellar; she is most deserving of a First Award and will take full advantage of the opportunity. I have had the pleasure of watching her grow as a scientist during her PhD and would be honored to continue to mentor her. I appreciate your consideration of her application.

Sincerely,

Molly McCue DVM, MS, PhD, Diplomate, American College of Veterinary Internal Medicine
Associate Professor, University of Minnesota College of Veterinary Medicine

Letter of Support from Department Head

UNIVERSITY OF ILLINOIS AT URBANA-CHAMPAIGN

Department of Veterinary Clinical Medicine
College of Veterinary Medicine
1008 West Hazelwood Drive
Urbana, IL 61802



June 23, 2015

To: Morris Animal Foundation

I am writing this letter to indicate my support for Dr. Annette McCoy's proposal, "Biomechanical and Genetic Risk Factors for Osteochondrosis in Standardbred Pacers and Trotters." We were pleased to have Dr. McCoy join our department in January, 2015 as an Assistant Professor in our Equine Medicine and Surgery section.

The Department of Veterinary Clinical Medicine is strongly committed to Dr. McCoy's research work, and have provided her a furnished office and an assigned research laboratory. She will receive ample startup funding to be used for her research projects. In addition she will have access to shared equipment and space in the department and college. Dr. McCoy's clinical and teaching activities will be restricted to 25% to allow her to focus on her research activity. Her research appointment will be increased if the level of external funding supporting scholarship is obtained.

The Department and College are committed to the development, advancement and retention of its faculty members. As Department Head, I am personally committed to these goals and will attempt to provide all necessary resources for Dr. McCoy to be successful.

Sincerely,

Karen L. Campbell, DVM, MS
Diplomate, ACVIM & ACVD
Professor and Department Head

III. Resubmission Summary: N/A

IV. Name, Institution, and E-mail Address of PI and all Co-Investigators (1pp)

Annette M. McCoy, DVM, MS, PhD, DACVS; University of Illinois; [REDACTED]

Molly E. McCue, DVM, MS, PhD, DACVIM; University of Minnesota; [REDACTED]

Kevin H. Kline, PhD; University of Illinois [REDACTED]

V. Study Proposal (5pp limit)

Specific, Testable Hypothesis and Objectives

Our long-term goal is to develop a predictive model that will allow for estimation of an individual horse's risk for the development of osteochondrosis (OC). A crucial component of this is improved understanding of the interplay between the genetic and environmental factors that underlie disease risk. We have recently reported differences in tarsal OC lesion prevalence and distribution between Standardbred pacers and trotters (see **Literature Review**), however it is unknown if these are due to differences in genetic risk, biomechanical forces related to gait, or a combination of the two. We have identified genetic risk loci for tarsal OC that are shared between Standardbred pacers and trotters (see **Preliminary Data**), therefore, *we hypothesize that Standardbred pacers and trotters share genetic risk factors for OC, but that biomechanical differences in their natural gait patterns influence which early lesions heal and which become permanent*. To address this hypothesis, in this proposal we will:

Objective 1: Prospectively follow the development of tarsal OC lesions in a cohort of Standardbred pacer and trotter foals to determine if gait preference has an impact on lesion healing/persistence.

Objective 2: Determine if previously validated genetic risk factors are shared between pacers and trotters, and determine if these risk alleles are associated with development and/or persistence of OC lesions in our experimental cohort.

Justification, Significance, and Literature Review

Osteochondrosis (OC) is a commonly diagnosed developmental orthopedic disease in the horse, as well as other domestic animal species (particularly the dog and pig), which is characterized by abnormal cartilage within a joint that occurs secondary to focal failure of endochondral ossification (the process by which a cartilage template becomes bone in the limbs of a growing animal). It is a complex disease, with interactions of genetics and environment (e.g. diet, exercise) determining expression and severity of lesions. Manifestations of disease can vary from mild to severe, and there is evidence that many lesions heal spontaneously (van Weeren and Barneveld, 1999; Dik et al., 1999). However, “permanent” OC lesions (present after 8 months of age [Dik et al., 1999]) nearly always require surgical intervention to prevent ongoing joint damage. If left untreated, OC can potentially lead to severe degenerative joint disease and can be career- or even life-threatening. Prevention of OC is an as-yet unattained goal of the equine industry, and thus this disease represents an animal health and welfare issue requiring ongoing research efforts.

OC is recognized across many breeds, with reported overall prevalence ranging from 6.25% in feral horses in the Western United States (Valentino et al, 1999) to greater than 40-50% in European Warmblood and Coldblood breeds (Lepeule et al, 2009; Wittwer et al, 2006). In addition to differences in overall prevalence, it is recognized that lesions are more common at one predilection site (i.e. fetlock, tarsus, or stifle) than another in different breeds. In Warmbloods, for example, average reported prevalence of OC lesions in the fetlock is 22.3%, while average prevalence in the tarsus and stifle are reported to be 11.5% and 7.0%, respectively (e.g. Stock et al., 2005; van Grevenhof et al., 2009). By comparison, in Standardbreds, OC of the tarsus is most common, with an average reported prevalence of 14.7% as compared to 3.3% and 6.3% in the fetlock and stifle, respectively (e.g. Grondahl and Dolvik, 1993; Lykkjen et al., 2012; Ricard et al., 2013). These differences in predilection sites and prevalence between breeds may be due to population differences in environmental and/or genetic risk factors, but this is not yet completely understood.

It has been postulated that OC could be caused either by abnormal forces on normal cartilage or by normal forces on abnormal cartilage (Pool, 1986). The evidence for the role of exercise in the development of OC is mixed, with one large study showing that the amount of exercise during the first 5 months of life affected the distribution but not the total number of lesions (van Weeren and Barneveld, 1999), and another reporting that reduced or irregular activity in the first weeks of life increased severity of lesions (Lepeule et al., 2013). However, to date there have been no studies examining the role that differences in natural gait preferences may play on the development of OC. Recently, we reported differences in tarsal OC lesion prevalence and distribution between Standardbred pacers and trotters (McCoy et al., 2015). Trotters were significantly

more likely to be affected with OC than were pacers. Further, among OC-affected individuals, the odds of a trotter having a medial malleolus (MM) lesion were 5 times higher than a pacer, while the odds of a pacer having a lesion at the distal intermediate ridge of the tibia (DIRT) were 3.7 times higher than a trotter. Since the natural pacing gait is demonstrated in young pacing-bred foals before any training occurs (United States Trotting Association, 2013), it is possible that gait-specific biomechanical forces may have an effect on lesion development and/or distribution. At least three differences between the biomechanics of the trot and the pace have been reported that may have biological significance (Drevemo et al., 1980; Wilson et al., 1988a; Wilson et al., 1988b; Robilliard et al., 2007). In objective 1, prospective radiographic evaluation of foals that preferentially pace and those that preferentially trot will be used to identify differences in the development and persistence of OC lesions between these two groups. This will determine if the gait-based differences are related to overall OC incidence, differences in the site(s) of primary lesion development and/or differences in primary lesion healing.

Pacing and trotting lines are carefully maintained and are as genetically distinct as any two separate breeds (Cothran et al., 1987). Thus, a second possibility is that genetic risk factors for OC may vary between pacers and trotters, and that differences in genetic risk factors are responsible for differences in prevalence and lesion localization. We recently performed a genome-wide association study (GWAS) in a cohort of Standardbreds with tarsal OC that was specifically selected to minimize the effect of environmental confounders on disease association (see **Preliminary Data**). Two distinct loci on equine (ECA) chromosome 14 were found to be associated with OC in this population. Putative risk alleles from within our GWAS regions of interest, as well as from chromosomal regions previously reported to be associated with equine OC, were evaluated in both our GWAS population and an independent population of Standardbreds (see **Preliminary Data**). This work resulted in validation of the two risk loci on ECA14, as well as risk loci on ECA 10 and 21. These data support our hypothesis that genetic risk factors for OC are in fact shared between pacers and trotters, although it is possible that modifying population-specific risk factors also play a role in disease manifestation.

Prospective evaluation of differences in OC lesion formation between pacer and trotter foals will provide insight into the role that biomechanical forces related to gait may play in the development of disease. Further, this approach will allow us to determine if there are differences in modifying genetic risk factors not just between pacers and trotters, but between horses who spontaneously heal their OC lesions and those who go on to develop permanent lesions. Defining the roles of these putative risk factors will both improve our understanding of the pathophysiology of OC and is an important step in developing a predictive model for OC that can facilitate management changes and early disease intervention in at-risk individuals.

Preliminary Data

GWAS in discovery population: The discovery cohort was comprised of 182 similarly bred Standardbred yearlings born on a single breeding farm in the eastern United States between 2007 and 2012 and raised under similar management conditions (70 affected with tarsal OC, 112 unaffected). Horses were genotyped on either the Illumina Equine SNP50 or SNP70 beadchips. Markers were imputed to a set of 74,595 single nucleotide polymorphism (SNP) markers for analysis (McCoy and McCue, 2014). Mixed model association analysis with gender and gait covariates was performed utilizing GEMMA software (Zhou and Stephens, 2012), which incorporates a relationship matrix to account for population structure. Five SNPs located within two distinct regions on ECA14 were most highly associated with OC, and were nearly genome-wide significant after a Bonferroni correction was applied based on the number of effective independent markers ($p < 5.1 \times 10^{-5}$; corrected genome-wide significance level 1.86×10^{-6} [Li et al., 2012]). Repeat GWAS after imputation to nearly 250,000 markers identified the same regions of association on ECA14, defined by 30 SNPs at $p < 4.76 \times 10^{-5}$.

Validation of risk loci: Approximately 1.5 million alleles, discovered via whole-genome sequencing of 18 horses from the discovery population (9 affected with OC, 9 unaffected) were evaluated from a total of 32 regions that were either identified as regions of interest in our GWAS or were chromosomal regions

previously reported to be associated with hock OC. 240 putative risk alleles from loci on 10 chromosomes were prioritized for follow-up according to predicted functional effect and segregation with OC status in the sequenced horses. These alleles were genotyped in both the GWAS cohort (above) and an independent validation cohort consisting of 139 Norwegian Standardbreds (60 affected with tarsal OC, 79 unaffected; described in Lykkjen et al., 2010) using a custom high-throughput Sequenom assay. Mixed model association analysis was performed in GEMMA with sex and gait included as covariates. Variants from within the two GWAS loci on ECA14 were most highly associated with disease status in both populations ($p=0.0004-0.022$ in the GWAS cohort; $p=0.014-0.049$ in the validation cohort). Variants with $p < 0.05$ from chromosomal regions on ECA10 and 21 were also shared between the two populations. These additional regions were identified from the GWAS, although they were less significantly associated with OC than the regions on ECA14. The association of putative risk alleles from within the same regions with disease status in two independent populations of Standardbreds suggest that these are true risk loci in this breed. Work is ongoing to evaluate specific risk alleles within these loci.

Experimental Methods and Design

Objective 1: Prospectively follow the development of tarsal OC lesions in a cohort of Standardbred pacer and trotter foals to determine if gait preference has an impact on lesion healing/persistence.

Rationale and working hypothesis: We recently reported differences in OC prevalence and lesion distribution between Standardbred pacers and trotters (McCoy et al., 2015), but the retrospective nature of this study did not allow further investigation of this finding. Prospective evaluation of the development and persistence of OC lesions, combined with field observation of foals to assess their activity and preferred gait will provide insight into the role of gait-related biomechanical forces in the development of disease. We hypothesize that pacer and trotter foals develop similar early OC lesions, but that biomechanical differences in their natural gait patterns influence which lesions heal and which become permanent.

Study cohort: 86 sire-matched Standardbred foals ($n = 43$ pacer; $n = 43$ trotter) will be enrolled in this study before 60 days of age (see below for power calculation). Foals will be raised under similar management conditions on one of three breeding farms in the central United States, located within 75 miles of each other. Approximately 80 foals are raised each year among the participating farms, and most of the foals are sold at breed-recognized yearling sales each fall. We expect to enroll the majority of foals needed during the first foaling season ($n = 60$) and the remainder during the second foaling season ($n = 26$).

Power calculation for sample size: The overall prevalence of tarsal OC in our cohort, based on historical expectations from the participating breeding farms and reported breed prevalence, is expected to be approximately 20%. Lesions of the distal intermediate ridge of the tibia (DIRT) and medial malleolus (MM) are the most commonly reported across all studies of tarsal OC. We previously reported that DIRT lesions were present in 70% of affected individuals (expected overall prevalence 14%) with a relative risk (RR) of 3.7 for pacers compared to trotters, and that MM lesions were present in 36% of affected individuals (expected overall prevalence 7.2%) with a RR of 5.01 for trotters compared to pacers. Based on expected prevalence and RR, power calculations to determine sample size was performed using the formula:

$$N = 4 / \text{prevalence} * (\sqrt{RR} - 1)^2$$

This results in a required sample size 34 horses per group for DIRT lesions, and 37 horses per group for MM lesions. We selected the larger of these, and added 15% to account for potential loss to follow-up, resulting in a final sample size of 43 pacers and 43 trotters.

Serial radiographs: Four standard radiographic views – lateral, cranio-caudal, dorsolateral-palmaromedial oblique, and dorsomedial-palmarolateral oblique – of both tarsi will be taken using a portable digital x-ray unit beginning within one week of turning 60 days of age, and then at 60 day intervals until the foal is sold or reaches 12 months of age (i.e. 60 ± 7 , 120 ± 7 , 180 ± 7 , 240 ± 7 , 300 ± 7 , 360 ± 7 days). Foals will be excluded from data analysis if they are lost to follow-up before 8 months of age. Routine physical exams will be performed on each foal on each day that radiographs are collected. Radiographs will be taken using

routine physical restraint (halter/leadrope) whenever possible; for foals that are intractable to physical restraint alone, a single dose of sedation (xylazine 0.2mg/kg IV) will be administered. Best practices for radiographic safety (ALARA principles) will be followed at all times. Based on pilot work we have recently performed in foals ranging from 2 weeks to 3 months of age, with our projected personnel and equipment resources, we expect to be able to radiograph 10-20 foals in a day.

Field observation: Foals will be video monitored in their normal paddock/pasture turnout at least 1 day (8-12 hours) per week. The video will subsequently be evaluated for the following activities: lying down, standing/nursing, walking, trotting and/or pacing, and moving at a faster gait (canter and/or gallop). A standardized activity log will be maintained for each foal to record the number of minutes (rounded to 30 second intervals) spent on each activity for a minimum of two consecutive hours each week.

Data analysis: Radiographs will be randomized and blindly evaluated for the presence or absence of OC lesions. They will then be assembled serially for each foal to determine where lesions developed and whether they healed spontaneously or became permanent. An OC lesion will be considered permanent if it is seen on radiographs of a foal ≥ 8 months of age. Differences in presence (yes/no), location (DIRT, MM, lateral trochlear ridge of the talus [LTR], medial trochlear ridge of the talus [MTR]) and progression (healed/permanent) of OC lesions between pacers and trotters will be determined using Chi-squared analysis. Survival analysis (logrank statistic) will be used to determine if there is a difference in lesion development and healing between pacers and trotters over time.

Overall activity will be assessed as percent time spent moving (combined time at the walk, trot/pace and canter/gallop divided by total observation time) during all observation periods prior to each set of radiographs (i.e. each 60 day period will be considered separately). Pacing will be evaluated in all foals as a categorical variable (observed/not observed). For those foals observed to pace, it will further be evaluated as a percentage of their total activity. Logistic regression models will be constructed to test the relationship between overall activity (in each 60 day period) and pacing with categorical outcome variables: 1) presence/absence of any OC lesion; 2) presence/absence of specific OC lesions (DIRT, MM, LTR, MTR); and 3) whether an observed OC lesion healed or became permanent. Sex and sire will also be included as predictor variables. For example:

$$OC_{R3} = \mu + \text{activity}_{T1} + \text{activity}_{T2} + \text{pace} + \text{sex} + \text{sire} + \epsilon$$

where the outcome is the presence of an OC lesion (yes/no) on the third set of radiographs (R3), pace is a categorical predictor, and activity levels during each of the two 60-day periods prior to the third set of radiographs are considered separately (T1, T2).

Expected Outcomes, Potential Pitfalls, and Alternative Approaches: Based on previously published studies (e.g. Dik et al., 1999), we expect $\geq 70\%$ of the foals will have evidence of tarsal OC at one or more time points, with $\sim 20\%$ developing permanent lesions. Further, differences in preferred gait will affect the location of permanent lesions (i.e. DIRT in pacers, MM in trotters). The biggest potential pitfall for this objective is that we may not have as many foals affected with OC as we expect, resulting in insufficient power to detect differences between groups. Should this appear to be the case based on preliminary analysis of findings from year 1, we will plan to enroll additional foals during the second year of the study. There are sufficient numbers of foals raised on the three participating farms that this could be easily accomplished without having to recruit additional participants. Should this become necessary, internal funds will be committed to accomplish the additional radiographs.

Objective 2: Determine if previously validated genetic risk factors are shared between pacers and trotters, and determine if these risk alleles are associated with development and/or persistence of OC lesions in our experimental cohort.

Rationale and working hypothesis: The presence of OC across domestic horse populations, including a feral horse population (Valentino et al., 1999), as well as shared major predilection sites and lesion morphology suggests a unified underlying pathophysiology and shared genetic risk across breeds. Our recent validation

of shared risk loci for tarsal OC in two independent populations of Standardbreds (see **Preliminary Data**) supports this idea. Thus, we hypothesize that Standardbred pacers and trotters share genetic risk factors for OC, and further, that alleles from within validated risk loci on ECA14, 10, and 21 will be associated with disease in our study cohort.

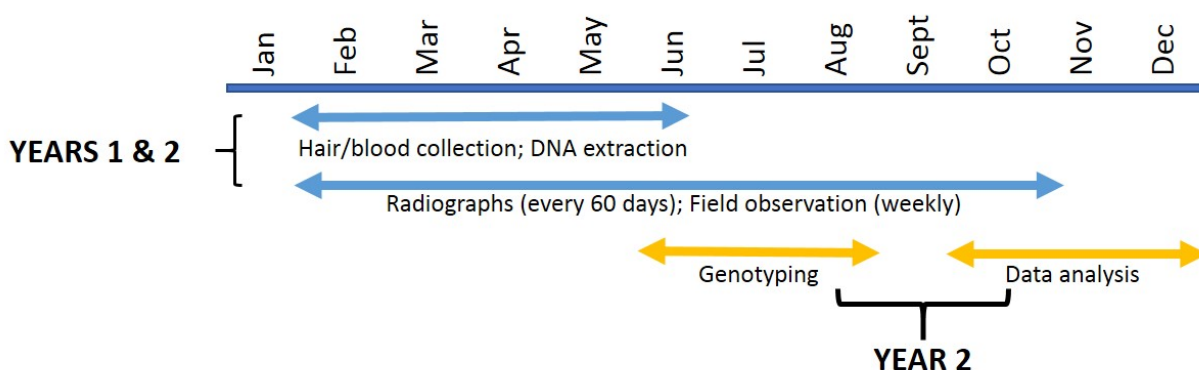
Study cohort and sample collection: The study cohort is described under **Objective 1**. Hair roots and/or whole blood will be collected from all foals at the time of study enrollment (<60 days of age). DNA will be isolated from collected samples using the Gentra® Puregene® Blood Kit (Qiagen, Valencia, CA) per manufacturer recommendations. Quantity and purity of extracted DNA will be assessed using spectrophotometric readings at 260 and 280nm (NanoDrop 2000, Thermo Scientific, Wilmington, DE).

Genotyping: Genotyping will be completed by Neogen GeneSeek (Lincoln, NE). The Sequenom assay has been previously designed and all SNPs validated in our GWAS cohort (see **Preliminary Data**). This assay includes 338 SNPs multiplexed into wells of 48; 98 of these SNPs are ancestry informative markers (AIMs) included to help control for population structure, while 240 are SNPs selected from chromosomal regions of interest associated with OC. Evaluation of additional risk alleles (not included in the Sequenom assay) within validated risk loci on ECA14, 10, and 21 is ongoing. We will genotype these risk alleles in our study cohort via restriction fragment length polymorphism (RFLP) or Sanger sequencing, as appropriate.

Data analysis: Genotyping data will be analyzed using mixed model association analysis as implemented in the GEMMA software (Zhou and Stephens, 2012). Outcomes for which models will be created are 1) development of any OC lesion during the course of the study; and 2) development of a permanent OC lesion. Association testing will be performed using the options to create a centered relatedness matrix (-gk2) and perform all three possible frequentist tests: Wald, likelihood ratio, and score (-fa4). The relatedness matrix will be constructed using the AIMs. Gender and gait will be included in the model as covariates. SNPs will be pruned prior to analysis using the default GEMMA parameters of minor allele frequency (MAF) < 1% and missingness < 95%.

Expected Outcomes, Potential Pitfalls, and Alternative Approaches: Our study cohort includes individuals of the same breed and with OC lesions in the same location (tarsus) as our GWAS and validation cohorts (see **Preliminary Data**). Further, our GWAS cohort was comprised of both pacers and trotters. Thus, we expect that putative risk alleles found within validated loci on ECA14, 10, and 21 will be associated with disease in our entire study cohort and that there will not be difference between pacers and trotters. However, it is possible that there are true differences in modifying alleles conferring disease risk between these two groups. We are addressing this potential limitation by using a Sequenom assay that contains SNPs from multiple chromosomal regions of interest associated with OC in other populations of horses. If SNPs from other loci are associated with disease in our study cohort, this will need to be investigated further and would be the subject of future studies.

Timeline



VI. Animal Involvement Justification

Morris Animal Foundation

Animal Involvement Justification

(From the proposal guidelines, single-spaced, no page limit)

Morris Animal Foundation (MAF) is dedicated to funding scientifically sound, relevant and humane studies that specifically address the health and well-being of animals. All studies receiving funding must follow MAF's Health Study Policy for Animals Involved in Research (adopted October 18, 2008), which was written to ensure that each and every animal involved in a MAF funded health study receives excellent, compassionate care throughout the study. MAF shall not fund health studies which require euthanasia as an endpoint or the induction of disease or injury, unless the nature of the disease or condition to be studied is of such significance for improving animal health that such means are justified, and that meaningful information can be obtained in no other way. Furthermore, MAF will not fund any study that induces or allows pain or distress unless such pain or distress can be controlled by appropriate anesthetic, analgesic, tranquilizing drugs, or nursing care. [Click here](#) for the full Health Study Policy.

A. If this study does not involve live animals please indicate here by N/A: _____

B. Does this study involve biological samples, tissues, etc.? yes

If yes, describe in detail what samples will be used and where & how they will be (or were) acquired. Note: Morris Animal Foundation reserves the right to request a copy of the Institutional Animal Care and Use Committee (IACUC) application/approval and other relevant applications/approvals (e.g., wildlife permit) covering the original collection of samples, including archived samples. MAF reserves the right to request IACUC (or equivalent) review and approval for any Foundation study *regardless of the Institution's requirements*. This would include the use of archived samples as well as clinical trials.

- Hair root samples and/or whole blood will be collected from each individual included in the study for the purpose of DNA extraction. Samples will be acquired from the animals located on their home farm. Routine restraint (i.e. halter and lead rope) should be sufficient to allow collection of these samples; sedation will not be required.
- Foals will be enrolled in this study between birth and 60 days of age. Standard radiographic views (lateral, cranio-caudal, dorsolateral-plantarolateral oblique, and dorsomedial-plantarolateral oblique) of both tarsi (hocks) will be taken using a portable digital x-ray unit starting at 60 days of age (± 7 days) and then at 60 day intervals until the foal is sold at a yearling sale or reaches 12 months of age. Radiographs will be taken using routine physical restraint whenever possible (i.e. halter and leadrope); if a foal is intractable to physical restraint alone, a single dose of sedation (xylazine 0.2mg/kg IV) will be administered. In the case of unweaned foals, the mare will be routinely restrained (halter/leadrope) within sight of the foal while radiographs are taken. Best practices for radiographic safety (ALARA principles) will be followed at all times, including the use of personal protection equipment (lead aprons, thyroid shields, and gloves). Total time for each radiographic exam (based on pilot work in foals 2 weeks-3 months of age) is expected to be 15-20 minutes.
- Foals will also be video monitored in their normal paddock/pasture turnout at least one day a week. The video will be reviewed for at least 2 consecutive hours of observation per foal,

and activity will be recorded (amount of time spent moving, and at which gait). Observation may also be done in person.

C. If this study involves live animals, succinctly address the following: (please restate the questions and directives).

1. What species will be studied? **Equine (horse)**
2. State the status of your IACUC application/approval. All recipients of MAF funding will be required to submit the entire IACUC protocol and document. A copy of the IACUC approval should not be included with the application, but it is required before funding can be awarded.
 - **The protocol for hair root/blood collection for the purpose of DNA isolation has been reviewed and approved by the University of Illinois IACUC (protocol #15031 2/20/2015).**
 - **The protocol for serial radiographs and field observation has been reviewed and approved by the University of Illinois IACUC (protocol #15086 5/18/2015)**
3. List the USDA category for pain and distress (B, C, D, E): C

Note: Any study beyond category C will require review by MAF's Animal Welfare Advisory Board (AWAB). In general MAF does not fund studies beyond category C (category D studies will only be considered if they conform with MAF's Health Study Policy, category E studies will not be considered).

4. Does this proposal involve client-owned animals? yes

If yes, the protocol for client-owned animals must be approved by the appropriate peer review committee before the project is funded. *If this proposal involves client-owned animals, an informed client consent form must be submitted with this proposal. For a suggested list of items to be considered in an informed client consent form, [click here](#).*

- **A copy of the client consent forms is included for review. These forms have been approved by the University of Illinois Veterinary Teaching Hospital and have also been reviewed as part of the IACUC approval process.**
5. Explain how animals will be acquired (e.g., client-owned, USDA licensed breeder, institutional "herds" or "colonies") and verify that the animals are suitable for the study (e.g., have no physiologic, physical or pharmacologic issues that would interfere with results)
 - **Individuals included in the study will be University- or client-owned foals that are housed at their home farm. There is no physiologic, physical, or pharmacological issue that would be anticipated to interfere with the study results. If a foal sustains an unrelated illness or injury during the course of the study, they will be excluded from the final analysis.**
 6. How many animals will be used? 86
 - a. Summarize numerical justification
 - **The overall prevalence of hock osteochondrosis (OC) in Standardbreds is expected to be approximately 20%, based on numerous radiographic surveys in the literature. Lesions of the distal intermediate ridge of the tibia (DIRT) and medial malleolus (MM) are the most commonly reported across all studies of OC in the hock.**
 - **Previous work we have done looking at OC in Standardbred pacers and trotters (McCoy et al. *Equine Vet J* 47:438-444, 2015 DOI:10.1111/evj.12297) suggests the following expectations for prevalence and relative risk (RR) of specific lesions:**
 - **DIRT – prevalence ~14% (70% of affected individuals), RR 3.7 for pacers compared to trotters**

- MM – prevalence ~7.2% (36% of affected individuals), RR 5.01 for trotters compared to pacers
 - Power calculations based on the expected prevalence of lesions was completed using the formula: $N = 4 / \text{prevalence} * (\text{sqrt}(\text{RR}) - 1)^2$
 - RR 3.7, prevalence 14%: N = 34 in each group
 - RR 5, prevalence 7.2%: N = 37 in each group
 - Based on these calculations, and accounting for 15% loss to follow-up, we plan to enroll 43 pacers and 43 trotters in the study, for a total of 86 horses
7. Does this study induce disease, injury, pain or distress in animals? Note: any study requiring the induction of disease, injury, pain, or distress will have an additional evaluation by MAF's AWAB.
- **No, this study does not induce disease, injury, pain (beyond momentary discomfort from a hair root collection or venipuncture), or distress in animals. All of these foals are regularly handled as part of their normal routine.**

If yes,

- a. Defend the necessity of experimental design
 - b. Explain how pain and/or distress will be controlled
 - c. Justify that no alternative, including clinical studies, can be used to accomplish study objectives and the disease/condition to be studied is of such significance for improving the health of the species.
8. Explain the environment and housing conditions (quality of life) in which the animals will live (address species-appropriate exercise, enrichment, socialization, veterinary care, etc.)
- **The horses in this study will be housed at their home farms. Management of these horses will proceed per standard protocol for these locations and will not be affected by participation in the study.**
9. What will happen to the animals upon completion of the study?
- If adoption, explain the adoption process. Provide assurance that whenever possible and when in the animal's best interest, investigators shall make companion animals available for adoption at the end of the study or return the animals to the owner/responsible agency in an environment that promotes animal welfare and excellent quality of life.
- **After completion of the study, the horses will return to their regular management protocol. Their disposition will not be affected by participation in the study.**
10. If euthanasia, provide the following additional information (note: any study requiring euthanasia as an endpoint will have an additional evaluation by a MAF's AWAB).
- **Euthanasia is not an endpoint for this study.**
 - i. Total number that will be euthanized and justification for numbers
 - ii. Method of euthanasia
 - iii. Justification that no alternatives can be used to accomplish study goal(s) and that the disease/condition to be studied is of such significance for improving the health of the species that a terminal endpoint is deemed necessary.
 - iv. Reason for euthanasia in lay language (this wording may be shared with staff, donors and media)

- v. Provide objective criteria for determining when euthanasia is appropriate or necessary (note: Morris Animal Foundation wants assurance that an animal will not be allowed to suffer and that monitoring for pain and suffering is adequate)

Note: Morris Animal Foundation does not consider the use of CO2 alone to be an appropriate method of euthanasia

Please note:

1. If an animal is used in an invasive study, MAF may require that a guarantee is provided, through principal investigator and institutional signatures that the animal will not participate in any future invasive study or procedure
2. MAF does not allow inclusion of ancillary data in MAF funded research that includes animal use protocols not in agreement with our Health Study Policy, even if it is obtained using other funding sources.
3. Morris Animal Foundation considers euthanasia acceptable when an animal develops unanticipated illness or injury that results in pain and suffering that cannot be alleviated with standard veterinary interventions.

VI. Animal Involvement Justification: Informed Client Consent Forms

OWNER CONSENT FORM	Owner or Agent Name _____
	Horse Name or Tattoo _____
	(Check here <input type="checkbox"/> if additional animals are listed on page 2)

Biomechanical and Genetic Risk Factors for Osteochondrosis in Standardbred Pacers and Trotters Development of Osteochondrosis in Standardbred Pacers and Trotters

Clinical Investigators: Annette McCoy, DVM, MS, PhD, DACVS. Veterinary Teaching Hospital, University of Illinois, Urbana, IL

Purpose of Study: Prospectively evaluate differences between pacer and trotter foals in the development of osteochondrosis lesions in the hock.

Eligibility: Standardbred foals (pacer- and trotter-bred) less than 60 days of age are eligible for enrollment in the study. Study participation will continue until the foals are sold at yearling sales and/or reach 12 months of age.

Procedures: Standard radiographic views (lateral, cranio-caudal, dorsolateral-plantaromedial oblique, and dorsomedial-plantarolateral oblique) of both tarsi (hocks) will be taken using a portable digital x-ray unit at 30-60 day intervals from the time of enrollment until the foal is sold at a yearling sale or reaches 12 months of age. If needed, a single dose of intravenous sedation will be administered to obtain radiographs. Foals will also be observed in their normal pasture/paddock turnout at least weekly, for a minimum of 2 hours, and their activity will be recorded (amount of time spent moving, and at which gait). Observation may be done in person or by video monitoring.

Possible Benefits / Owner Incentives: Radiographs will be made available to the owners at no charge, if desired. While this study does not provide any additional immediate financial benefits to you, in the future, information obtained will help us to better understand osteochondrosis, a disease that has enormous economic impact in the equine industry.

Associated and Unforeseen Risks: This study involves routine restraint for serial radiographs +/- intravenous sedation. None of these procedures should pose a significant risk to any participant. Rarely, horses may have an adverse reaction to sedative drugs. All reasonable efforts will be made to minimize known or potential risks associated with all procedures, and therefore there will not be compensation in case of problems arising from participation in this study. There will be no cost to you for participation in this study.

Confidentiality: Neither you nor your horse(s) will be mentioned by name in any report arising from this study. Results pertaining specifically to your horse(s) may be released to you or your designated representative upon request after completion of the study; otherwise all records will remain entirely confidential

Compensation and Financial Obligations: There will be no compensation or financial obligation associated with participation in this study.

Questions about this project may be directed to: Dr. Annette McCoy, Department of Veterinary Clinical Medicine, University of Illinois, at [REDACTED]

Acknowledgements:

Please note: Before being accepted into any clinical trial, all animals must be evaluated and officially enrolled by the study principal investigator. All owners must sign an official study consent form before their pet will be accepted into a clinical trial. The cost of pre-evaluations may be the responsibility of the owner. Please be sure to contact the study investigator if you have any questions or concerns.

I understand that my horse's participation in this study is entirely voluntary. Refusal to participate or to continue participation carries no medical penalty, and I am free to withdraw my horse from this study at any time without medical penalty or prejudice. I understand that my voluntary removal will constitute disqualification from further participation in this study.

I also understand that my horse may be required to withdraw from the study for violation of eligibility requirements, or noncompliance with restrictions and/or procedures during the study. This also constitutes disqualification. I may also be required to withdraw from the study to protect my horse's health (such as with the occurrence of significant injury, adverse reactions, or illness whether or not a consequence of the study), or if the study is terminated early.

I have not withheld information regarding my horse's medical history. I acknowledge that I have read and understand this consent form and all my questions have been answered to my satisfaction. I have been given a copy of this consent form if I have requested a copy. I am aware that this research has been reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Illinois (UI).

As a volunteer, I give my informed consent to the Board of Trustees of the UI and the Veterinary Teaching Hospital (VTH) to enroll my horse in this study, according to the explanations and conditions presented in this document. I agree to hold harmless the Board of Trustees of the UI, the VTH, and its officers, employees, agents and assigns from any and all liability, claims and actions that may arise from participation in this study.

IACUC Approval #: 15086

_____	_____	____/____/____
Owner's Printed Name (or authorized agent)	Signature of Owner (or authorized agent)	20____ Date

_____	_____	____/____/____
Witness' Printed Name	Signature of Witness	20____ Date

Laura Garrett

VTH Director/Chief of Staff	Signature of Director/Chief of Staff	Date: May 20, 2015
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My additional horses that I approve to be included in the above study/studies		
Horse Name or Tattoo	Horse Name or Tattoo	
Owner's Printed Name (or authorized agent)	Signature of Owner (or authorized agent)	<div> <div> <div></div> <div>20</div> </div> <div> <div></div> <div></div> </div> </div> Date

(Original to medical records)

OWNER CONSENT FORM	Owner or Agent Name _____
	Horse Name or Tattoo _____
	(Check here <input type="checkbox"/> if additional animals are listed on page 2)

Biomechanical and Genetic Risk Factors for Osteochondrosis in Standardbred Pacers and Trotters

Genetic Factors Underlying Disease and Performance Traits in the Horse

Clinical Investigators: Annette McCoy, DVM, MS, PhD, DACVS. Veterinary Teaching Hospital, University of Illinois, Urbana, IL

Purpose of Study: Identify genes and alleles in the horse that underlie 1) genetic risk for the development of osteochondrosis, and 2) the ability to perform alternative gaits.

Eligibility: Known phenotype for OC (affected or unaffected) based on radiographs and/or surgical records, and/or known phenotype for alternative gait (gaited or non-gaited individual) based on history or official race records.

Procedures: A single 10ml blood sample and hair root sample will be collected for the purpose of DNA extraction. The DNA samples will be examined using known markers from the horse genome to identify differences between horses.

Possible Benefits / Owner Incentives: While this study does not provide any immediate financial benefits to you, in the future, information obtained will help us to better understand diseases and performance traits that have enormous economic impact in the equine industry.

Associated and Unforeseen Risks: This study involves routine restraint for a single blood draw from the jugular vein and collection of hair root samples. None of these procedures should pose a significant risk to any participant. Rarely, horses may develop a clot or infection at the site of blood collection that in isolated cases may lead to long-term health problems. All reasonable efforts will be made to minimize known or potential risks associated with all procedures, and therefore there will not be compensation in case of problems arising from participation in this study. If heat or swelling is noted over the jugular vein, please contact your local veterinarian for immediate care. There will be no cost to you for participation in this study.

Confidentiality: Neither you nor your horse(s) will be mentioned by name in any report arising from this study. Results pertaining specifically to your horse(s) may be released to you or your designated representative upon request after completion of the study; otherwise all records will remain entirely confidential

Compensation and Financial Obligations: There will be no compensation or financial obligation associated with participation in this study.

Questions about this project may be directed to: Dr. Annette McCoy, Department of Veterinary Clinical Medicine, University of Illinois, at [REDACTED].

Acknowledgements:

Please note: Before being accepted into any clinical trial, all animals must be evaluated and officially enrolled by the study principal investigator. All owners must sign an official study consent form before their pet will be accepted into a clinical trial. The cost of pre-evaluations may be the responsibility of the owner. Please be sure to contact the study investigator if you have any questions or concerns.

I understand that my horse's participation in this study is entirely voluntary. Refusal to participate or to continue participation carries no medical penalty, and I am free to withdraw my horse from this study at any time without medical penalty or prejudice. I understand that my voluntary removal will constitute disqualification from further participation in this study.

I also understand that my horse may be required to withdraw from the study for violation of eligibility requirements, or noncompliance with restrictions and/or procedures during the study. This also constitutes disqualification. I may also be required to withdraw from the study to protect my horse's health (such as with the occurrence of significant injury, adverse reactions, or illness whether or not a consequence of the study), or if the study is terminated early.

I have not withheld information regarding my horse's medical history. I acknowledge that I have read and understand this consent form and all my questions have been answered to my satisfaction. I have been given a copy of this consent form if I have requested a copy. I am aware that this research has been reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Illinois (UI).

As a volunteer, I give my informed consent to the Board of Trustees of the UI and the Veterinary Teaching Hospital (VTH) to enroll my horse in this study, according to the explanations and conditions presented in this document. I agree to hold harmless the Board of Trustees of the UI, the VTH, and its officers, employees, agents and assigns from any and all liability, claims and actions that may arise from participation in this study.

I approve the participation of my horse(s) in the following studies:

- ☐ Investigation of genetic risk factors underlying **OC** (willing to share with us if your horse ever had OC/OCD)
- ☐ Investigation of genetic risk factors affecting **gait**

IACUC Approval #: 15031

_____	_____	____/____/____
Owner's Printed Name (or authorized agent)	Signature of Owner (or authorized agent)	20____ Date

_____	_____	____/____/____
Witness' Printed Name	Signature of Witness	20____ Date

Brendan McKiernan		Feb. 24, 2015
VTH Director/Chief of Staff	Signature of Director/Chief of Staff	Date

My additional horses that I approve to be included in the above study/studies
--

Horse Name or Tattoo		Horse Name or Tattoo
Owner's Printed Name (or authorized agent)	Signature of Owner (or authorized agent)	<div> <div></div> <div>20</div> <div></div> </div> <div> <div></div> <div></div> <div></div> </div> <div>Date</div>

(Original to medical records)

VII. Recombinant DNA/Biohazards: N/A

VIII. Facilities and Equipment

Foals will be recruited for this study from the University of Illinois (UIUC) Horse Farm and from 2 nearby private breeding farms that routinely collaborate with Dr. Kline (Co-investigator). Approximately 80 foals are raised each year among the participating farms, and most of the foals are sold at breed-recognized yearling sales each fall. Management is similar among the facilities, including exercise regimens: foals are kept with their dams in a large box stall or small run for the first ~2 weeks of life, and then are turned out in a large paddock or pasture with the other foals/mares. After weaning, the foals are turned out together. At each facility, personnel experienced in handling foals and mares will be available to assist with restraint during exams. Large box stalls will be available for all exams. Radiographs will be taken with a MinXray HF8020 portable high frequency veterinary x-ray unit with a wireless plate and laptop running Metron software for digital image capture. Radiographs will be uploaded as DICOM images to the UIUC College of Veterinary Medicine PACS system for long-term secure storage. Video observation will be performed using a GoPro HERO3+ camera with waterproof housing and mounting hardware allowing it to be attached to a fence. Digital video will be uploaded to Dr. McCoy's (PI) lab server space for storage and analysis.

All necessary lab work will be performed in Dr. McCoy's laboratory. Dr. McCoy has approximately 400 sq ft of laboratory space. Laboratory equipment available in the McCoy lab includes: 2 water baths, a dry bath, 8 pipetters, 2 multi-channel pipetters, 1 each repeater pipette and serological pipette, 2 BioRad horizontal gel electrophoresis with 26 well combs and power supplies, 1 BioRad mini-horizontal gel electrophoresis unit, 1 Bio-Rad T100 96 well plate format thermal cycler, a refrigerated microcentrifuge, Thermo Scientific Sorvall ST16R 15,200 rpm refrigerated table-top centrifuge, Nanodrop 2000 spectrophotometer, fume hood with UV light, Spectrolinker XL-1500 UV crosslinker, one 15 cu. ft -20 C freezer, and one 20 cu. ft. -80 C freezer. Equipment in the neighboring colleague's lab that will be available includes additional pipetters, thermal cyclers, and water baths, as well as a fluorescent plate reader and gel imaging system, biopulverizers, and a Spex mill. Shared larger equipment available within the college includes a Millipore water purification system and 4 ABI Real-Time PCR systems.

Available computing resources in the McCoy lab include 2 windows computers, both Dell dual quad (8) processor workstations, with a dual installed Linux virtual machine, and maintain (Linux and/or Windows) versions of PLINK, R (SNP Matrix, Pedigreemm, HClust, etc), PHASE, fastPHASE, Sequencher, and others. The computers are also be fully equipped with word processing, spreadsheet, and database software. Additionally, Dr. McCoy has access to the UIUC High-Performance Biological Computing center (HPCBio). This includes access to HPCBio's computational infrastructure (including two Campus Clusters with 512 computing nodes each, and the Blue Waters supercomputer with 1.5 petabytes of memory and 25 petabytes of disk storage) and a broad suite of genomics software. Large data sets are stored both locally and remotely at HPCBio. Remote access to these resources is available.

Sequenom assay genotyping will be performed by Neogen GeneSeek (Lincoln, NE). Sanger sequencing will be performed by the the University of Illinois Roy J Carver Biotechnology Center's High-Throughput Sequencing and Genotyping Unit. The facility maintains ABI 3730xl DNA Analyzers for fragment analysis and Sanger sequencing and offers custom oligonucleotide preparation.

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X. Budget**PROPOSAL BUDGET**

Note: First Award – complete year 1, year 2 and total only. **Pilot Study** – complete year 1 only.
Fellowship Training – complete salary, fringe benefits, indirect costs and total for year 1 and year 2 only.

Category	Year 1	Year 2	Year 3	Total
<u>Personnel:</u>				
1. Principal investigator (Annette McCoy)*				
2. Co-investigator #1 (Kevin Kline)				
3. Co-investigator #2 (Molly McCue)				
4. Technician (Sulalita Chaki) Salary (10% effort * \$31,751) Fringe benefits (39.14%)				
5. Student Assistant (TBD) Salary (200 hours @ \$10/hr) Fringe benefits (0.15%)				
Total Salaries & Wages				
<u>Supplies & Expenses:</u>				
1. Radiographs (\$100/foal/session)				
2. Sequenom genotyping (\$40/sample)				
3. Consumable supplies				
4. Sequencing and RFLP genotyping				
5. Statistical consulting				
6. Conference travel				
Provide justification in the designated section.				
Total Supplies & Expenses:				
<u>Animal Use & Care:</u>				
Animal Purchase: N/A				
Animal Per diem: N/A				
Total Animal Care: N/A				
Subtotal of All Categories:				
Maximum of 8% - Indirect Costs:**				
Grand Total Requested from MAF:				

* Salary requests for principal investigators must be clearly defined and justified in the following budget justification section. You may request salary for technicians, residents, graduate students, and postdoctoral fellows, based on their percentage of time involved in the project

** Indirect costs may be claimed only if you are charged for indirect costs by your institution for work carried out in this proposal. **You must make this calculation yourself.** If your institution charges less than 8%, claim only that amount and indicate the percentage.

XI. Itemized Budget Justification

Personnel

Dr. Annette McCoy (PI): Dr. McCoy is a veterinarian board-certified in Large Animal Surgery with research experience in equine genetics and genomics and clinical expertise in musculoskeletal disease in the horse. Dr. McCoy generated the preliminary data for this proposal. She will coordinate and oversee the radiographs and field observation for Aim 1 of this proposal. She will also be primarily responsible for radiograph interpretation and will verify scoring of activity levels from video observations. For Aim 2, Dr. McCoy will oversee sample preparation and will perform all data analysis. Dr. McCoy will be responsible for primary drafts of manuscripts for publication.

Dr. Molly McCue (Mentor/Co-I): Dr. McCue is a veterinarian board-certified in internal medicine with extensive research experience in equine genetics and genomics, particularly in the area of statistical genetics. Dr. McCue is the Mentor for this First Award and will provide guidance and subject expertise during data analysis and interpretation and will collaborate on production of manuscripts for publication. Dr. McCue and Dr. McCoy will have weekly meetings via Skype to discuss the progress of the project.

Dr. Kevin Kline (Co-I): Dr. Kline is a professor in the Animal Science department with a shared research and extension appointment, specializing in horse production and management. He runs the University of Illinois horse farm and has extensive connections in the Standardbred community. Dr. Kline will oversee recruitment of foals into the study and will collaborate on production of manuscripts for publication.

Sulalita Chaki (Technician): Ms. Chaki is a technician in Dr. McCoy's laboratory experienced in numerous molecular biology techniques. She will perform DNA isolation, PCR, and genotyping by sequencing and RFLP. We are requesting 10% salary support (plus fringe) for Ms. Chaki.

Student Assistant: We are requesting salary support for 200 hours (plus fringe) for a student assistant for this project. The student will have primary responsibility for field monitoring (setting up cameras for video observation, reviewing and logging videos) and will assist with taking radiographs as needed.

Supplies & Expenses

Radiographs: Radiographs will be taken at 60, 120, 180, 240, 300, and 360 days (+/- 7 days) of age. Images will be obtained with a digital x-ray unit and stored on the hospital server. The cost of 1 full set of hock films (including use of equipment and personnel, plus data storage) is [REDACTED] we anticipate 6 sets of films per foal. 86 foals x [REDACTED]. Because films will be taken over 2 foaling seasons, this breaks down to [REDACTED] in Year 1 (n = 60 foals) and [REDACTED] in Year 2 (n = 26 foals).

Sequenom genotyping: Primers and multiplex design for the Sequenom assay were completed during the preliminary work for this proposal. Genotyping will cost [REDACTED] per horse. [REDACTED]

Consumable supplies: We are requesting [REDACTED] in each year of the award for consumable supplies that will be used during sample preparation as well as genotyping by sequencing and RFLP. This includes the cost of disposable plastic ware, PCR primers, agarose gels, polymerases, restriction enzymes, etc.

Sequencing and RFLP genotyping: Additional risk alleles (not included in the Sequenom assay) will be genotyped in our study cohort via restriction fragment length polymorphism (RFLP) or Sanger sequencing. The cost for sequencing is [REDACTED]/sample. We are requesting [REDACTED] in the second year of the study which will allow us to genotype approximately 15 variants per horse in our entire cohort.

Statistical Consulting: We will work with experts at ATLAS Statistics (University of Illinois College of Liberal Arts and Sciences) during our final data analysis. We are budgeting for 15 hours of consultation time in the second year of the study. [REDACTED]

Conference Travel: We are requesting [REDACTED] in the final year of the study to fund PI travel to two conferences – one focused on clinical medicine/orthopedics, and one on genetics. This is based on projected expenses related to conference registration, airfare, and accommodations.

XIII. Prior MAF Support during last three years for McCoy (PI)

MAF ID # D15EQ-813

Validation of Putative Genetic Risk Alleles for Osteochondrosis in Standardbreds

Funding period: January 2015- Dec 2015

Total Amount: [REDACTED]

Brief summary of the objectives: Osteochondrosis (OC) is a widely recognized manifestation of developmental orthopedic disease that affects weanling and yearling horses across breeds. Manifestations of OC can vary from mild to severe, but nearly always require surgical intervention to prevent ongoing joint damage. While OC is known to be influenced by environmental risk factors, heritability estimates suggest that as much as 50% of disease risk is due to genetics. It is likely that genetic risk results from a combination of alleles in several different genes. We have identified a number of putative genetic risk alleles for OC in a cohort of Standardbred yearlings, and in this proposal we seek to validate these findings in an independent population. Identification and validation of specific risk alleles is crucial to developing a genetic risk model for OC that could be applied to individual horses. *We hypothesize that one or more alleles underlying OC risk are present within the genomic regions identified in our discovery population.* The **objective** of this proposal is to confirm that specific putative risk alleles also associate with disease in an independent population of Standardbreds and can thus be considered to be true risk alleles. Deep resequencing of the regions surrounding validated alleles will be completed to confirm that the associated allele is the true functional allele. Horses included in this study are Norwegian Standardbreds (n = 162; 80 affected with hock OC, 82 unaffected). DNA will be submitted for genotyping on a previously designed Sequenom genotyping assay. Mixed model analysis will be performed to identify alleles significantly associated with OC in the validation cohort. Those alleles which are associated with OC in both the discovery and validation populations will be considered to be true risk alleles. Random forest analysis will be used to determine the relative contribution of individual variants to OC and to illuminate potential interactions between variants. Deep Sanger resequencing will be performed around each of the alleles confirmed to be associated with OC in the validation cohort to make sure that functional alleles are not missed.

Summary of results to date: A GWA analysis was performed in 182 Standardbred horses from a single breeding farm in the United States (70 affected with tarsal OC, 112 unaffected). The GWA was performed using GEMMA (Genome-wide Efficient Mixed Model Analysis) software, incorporating a relatedness matrix constructed from a LD-pruned marker set. Two distinct loci on ECA 14 were most highly associated with OC status in this cohort ($p=1.8 \times 10^{-4}$ to 8×10^{-6}). Eight additional regions of interest were identified on 7 other chromosomes, including ECA10 ($p=3.9 \times 10^{-4}$ to 8.3×10^{-5}) and 21 ($p=4.3 \times 10^{-4}$ to 1.5×10^{-4}). Variant discovery was subsequently performed via whole-genome sequencing in 18 horses (9 OC cases and 9 controls). 215,712 variants were identified within the 10 regions of interest identified in the GWA, and 1,271,635 variants were identified from 22 previously reported regions of association for tarsal OC. These 1,487,347 variants were prioritized based on predicted functional effect and segregation with OC status. 240 variants from regions on 10 chromosomes were selected for follow-up genotyping using a Sequenom genotyping assay. The 240 variants, along with 98 ancestry informative markers (AIMs), were genotyped in both the 182 horses in the GWA cohort as well as an independent validation cohort of 139 Norwegian Standardbreds (60 affected with tarsal OC, 79 unaffected).

Within the GWA cohort, variants from ECA10 (n=2, $p=0.0076$ -0.015), 14 (region 1, n=3, $p=0.019$ -0.049; region 2, n=4, $p=0.0008$ -0.018), and 21 (n=5, $p=0.008$ -0.043) were most highly associated with OC status after GEMMA mixed model analysis. Within the validation cohort, the variant most highly associated with OC status ($p=0.0014$), as well as two additional highly associated SNPs ($p=0.0089$ -0.0058), were from “region 1” on ECA14, the top GWA region of association in the discovery population. Three additional genotyped variants highly associated with OC status in the Norwegian horses were from chromosomal regions of interest previously reported in a GWA in this same population (ECA1 and ECA3).

We have successfully demonstrated shared risk loci for OC in two independent populations of Standardbreds. This is the first successful validation of findings from a genome-wide association study for OC in horses. The genotyping results from this study are included in a manuscript that will be submitted before the end of July 2015.

List of Publications: McCoy, AM, Beeson, SK, Lykkjen, S, Ralston, SL, **McCue, ME**. Identification and Validation of Risk Loci for Osteochondrosis in Standardbreds. *BMC Genomics*

List of Presentations: None to date

List of patents resulting from MAF awards: none at this time.

XIII. Prior MAF support during last three years for McCue (Mentor)

MAF ID # D15EQ-029

Role of endocrine disrupting chemicals in equine metabolic syndrome.

Funding period: January 2015- Dec 2015

Total Amount: [REDACTED]

Brief summary of the objectives: Our prior work has demonstrated that equine metabolic syndrome (EMS) and its metabolic components, including hyperinsulinemia, adiposity and key biochemical and hormonal measures, are influenced by both environmental and individual animal factors. We have collected 11 morphometric, biochemical and hormonal phenotypes along with epidemiologic and environmental data from 610 horses/ponies from 166 farms. Multi-level regression modeling demonstrated that 23-49% of the variability in phenotype was due to shared environment (farm). Individual factors (ie. age, breed, sex, laminitis status, obesity) accounted for 3-16%, and environmental factors (ie diet, exercise, season) accounted for 4-18% of the phenotypic variation in the data. However, a large portion of the phenotypic variability in this cohort was not explained by our 16 predictors. Thus, a significant amount of metabolic variation in EMS remains unexplained, which we believe is due in large part to as yet unmeasured environmental risk factors and undetermined specific genetic risk alleles. Epidemiologic studies have linked environmental exposure to synthetic and naturally occurring chemicals that disturb endogenous endocrine signaling pathways ('endocrine disrupting chemicals' [EDCs]) with underlying components and long term consequences of metabolic syndrome (MetS) in humans; and our preliminary data links potential EDC exposure to laminitis and abnormal insulin responses in horses. Specifically, we have examined proximity of our cohort of horses to US EPA 'Superfund sites' containing EDCs as a possible contributor to environmental risk. Horses from farms < 30 miles of a Superfund site were significantly more likely to have a history of laminitis ($p=0.002$) and have higher insulin responses post oral sugar challenge (OST INS, $p=0.00005$) when compared to horses on farms > 30 miles from a Superfund site, suggesting that exposure to POP/EDCs may play a role in the phenotypic variation seen in our cohort. Concurrently, our genetic studies on EMS have identified 3 non-synonymous SNPs in the trans-activation domain of the equine aryl hydrocarbon receptor (*AHR*) gene. The aryl hydrocarbon receptor (*Ahr*) is a ligand-dependent transcription factor that mediates a wide range of cellular effects resulting from exposure to synthetic and naturally occurring chemicals, including EDCs. One of the *AHR* SNPs that we identified (*Val556Met*) was associated with OST INS values ($p=0.002$) and two haplotypes across 7 SNPs in the *AHR* trans-activation domain were strongly associated with OST INS ($p=0.0007$ and 0.0009 ; overall $p=0.0002$). Taken together, these data suggest that further investigation of the relationship between these *AHR* variants and metabolic traits, and a potential interaction between *AHR* genotype and environmental EDCs, is needed.

We hypothesized that EDCs are an important environmental factor for the development of EMS and that genotypic variation in the *AHR* may mediate the effects of EDCs in horses. Our **objectives** in this proposal are to: **1) Determine the role of endocrine disrupting chemicals in metabolic variation in horses, and 2) to identify interactions between EDCs and *AHR* genotype.** We propose to quantify EDCs in banked serum samples from 139 Welsh Ponies from 14 farms and 161 Morgan horses from 18 farms (total $n=300$ from 32 farms) using cell-based reporter bioassays that measure dioxin and organochlorine compound levels based on their ability to interact with the *Ahr* and estrogen receptors (ER), respectively. Toxic equivalency factors (TEFs) will be calculated from the *Ahr* assay and β -estradiol equivalents will be calculated from the ER assay. TEFs and β -estradiol equivalents will be correlated to EMS phenotypic responses using multi-level, multivariate, multiple regression modeling. The roles of *AHR* genotype and genotype-by-environment (ie EDC level) interaction, will be determined by inclusion of *AHR* genotype in the statistical models.

Progress to date: Frozen heparinized serum samples from all 300 horses were to BD Systems in the Netherlands in the second week of March. BDS has generated data on the first 50 samples for both the DR-CALUX and the ER α -CALUX. DR-CALUX results are as anticipated. ER α -CALUX assays are lower than

expected, which BDS believes is due to the fact that estrogens in horse serum are mainly present as sulfate conjugates. BDS is currently evaluating de-conjugation procedures before moving forward with additional ER α -CALUX assays.

List of Publications: We anticipate a minimum of one publication for each objective upon completion.

List of Presentations: None to date

List of patents resulting from MAF awards: none at this time.

MAF ID# D15EQ-031

Gene Loci for Recurrent Exertional Rhabdomyolysis in Thoroughbreds and Standardbreds

Funding period: January 2015- Dec 2015

Total Amount: [REDACTED]

Brief summary of the objectives: Approximately 5 - 10% of Thoroughbred (TB) and Standardbred (STB) racehorses suffer from recurrent exertional rhabdomyolysis (RER), in which they exhibit sporadic bouts of painful cramping and muscle cell damage following mild to moderate exercise. This condition also affects TB and TB crossbreds used for performance events such as three day eventing, hunter jumper, and barrel racing competitions; the same or a closely related form of exertional rhabdomyolysis is present in many other breeds including the Quarter Horse. Losses due to RER come from the cost of veterinary care, lost training time, and less frequent participation in competition. Previous research is consistent with an underlying genetic basis to RER susceptibility, with gender, temperament, diet, age, and activity being contributing factors. We hypothesize that genes of moderate to major effect underlie RER susceptibility in both TB and STB horses, and the goals of this proposal are to identify these gene loci and their underlying functional mutations associated with RER risk in both breeds. This will be achieved through three objectives: **1)** an enhanced genome-wide association analysis (GWAS) with SNP markers and high-density haplotype analysis to precisely locate RER susceptibility loci; **2)** identifying the sequence variants in these regions through whole genome sequencing of multiple cases and controls; and, **3)** genotyping high-priority variants, based on their allele frequencies in the sequenced horses and their predicted SNP effects, in the entire case and control population. We will first impute our existing GWAS data to 670K genome-wide SNPs to increase the genetic mapping power, and confirm the chromosomal regions and underlying haplotypes associated with RER risk in each breed. We will then use whole genome sequencing (10 cases and 10 controls in each breed) to identify the sequence variants in the RER-associated regions; these variants will be prioritized based on their predicted effects on gene or encoded protein function and estimated allele frequencies in cases vs controls. The highest-priority variants will then be genotyped on the entire cohort to identify the most likely RER functional mutations.

Summary of results to date: Using genotype data from an equine haplotype map resource being developed in our laboratory, we have now imputed the whole genome SNP data to greater than 1,450,000 SNPs in the Thoroughbreds and 540,000 in the Standardbreds with >90% imputation accuracy and minor allele frequency of greater than 5%. Using these data we have now repeated the GWAS analysis in each population independently and across both breeds. This has led us to concentrate our GWAS to 2 loci (ECA16 and ECA30) in the Thoroughbreds and 1 locus (ECA14) in the Standardbreds, with one locus (ECA11) shared by both breeds. We have also selected and submitted the 10 Standardbreds (5 RER and 5 controls) for whole genome sequencing based on haplotypes at the ECA14 and ECA11 loci, and selected the 10 Thoroughbreds to be sequenced based on haplotypes at the ECA11, ECA16, and ECA30 loci.

List of Publications: We anticipate a minimum of one publication for each objective upon completion.

List of Presentations: We will also be reporting on the results of this project at the Havemeyer Equine Genome Mapping Workshop in Hannover Germany in July 2015.

List of patents resulting from MAF awards: none at this time.

XIII. Prior MAF support during last three years for Kline (Co-investigator)

None

XIV. Biographical Data

NAME Annette Marie McCoy	POSITION TITLE Assistant Professor, Dep't of Veterinary Clinical Medicine University of Illinois College of Veterinary Medicine 1008 W Hazelwood Dr., Urbana, IL 61802		
EDUCATION/TRAINING <i>(Begin with baccalaureate or other initial professional education, such as nursing, include postdoctoral training and residency training if applicable.)</i>			
INSTITUTION AND LOCATION	DEGREE <i>(if applicable)</i>	MM/YY	FIELD OF STUDY
Michigan State University, East Lansing, MI	B.S.	05/2002	Animal Science (GPA 3.98/4.0)
Michigan State University, East Lansing, MI	D.V.M.	05/2006	Veterinary Medicine (GPA 3.93/4.0)
University of Minnesota, St. Paul, MN	Internship	06/2006-07/2007	Large Animal Medicine and Surgery
Colorado State University, Ft. Collins, CO	M.S.	05/2010	Clinical Sciences (GPA 4.0/4.0)
Colorado State University, Ft. Collins, CO	Residency	07/2007-07/2010	Equine Surgery and Lameness
University of Minnesota, St. Paul, MN	Ph.D.	09/2010-5/2014	Comparative and Molecular Biosciences (GPA 4.0/4.0)
University of Minnesota, St. Paul, MN	Post-doctoral	5/2014-10/2014	Computational Genetics

A. Role: Principal Investigator

B. Positions and Honors

Positions

Professorial Assistant, Michigan State University 1999-2001
Research Assistant, Michigan State University 2001-2002
Veterinary Research Scholar, Kansas State University 5/2004-8/2004
Internship, Large Animal Medicine and Surgery, University of Minnesota 2006-2007
Residency, Equine Surgery and Lameness, Colorado State University 2007-2010
Postdoctoral Fellow/PhD Candidate, University of Minnesota 2010-2014
Postdoctoral Associate, University of Minnesota 5/2014-10/2014
Assistant Professor, University of Illinois 2015-present

Honors

Board Certification: Large Animal Surgery (American College of Veterinary Surgeons) 2011
Stephen J. O'Brian Award (American Genetic Association) 2015
Doctoral Dissertation Fellowship (Univ of Minnesota) 2013-2014
NIH T32 Training Grant in Comparative Medicine and Pathology 2010-2013
Council of Graduate Students Travel Award (Univ of Minnesota) 2013
Vaughn Larson Scholarship Award (Univ of Minnesota) 2012
AAEP Foundation Past Presidents' Research Fellow 2011
ACVS Resident's Forum 2010, 2nd place, Large Animal Clinical Research Presentation
Mitzy H. Yount Memorial Scholarship (Colorado State Univ) 2009-2010
Doctor of Veterinary Medicine, *With Highest Honors*
J.P. Hutton-W.F. Riley Equine Award (Michigan State Univ) 2006
Phi Zeta Award (Michigan State Univ) 2006

CVM Alumni Council Student Leadership Award (Michigan State Univ) 2006
Theriogenology Award (Michigan State Univ) 2006
Phi Zeta Research Day Award for Best Oral Presentation by a Veterinary Student (Michigan State Univ) 2004
Bachelor of Science in Animal Science, *With Highest Honors*
Richard Lee Featherstone Society Prize (Michigan State Univ) 2002
American Society of Animal Science Scholarship Award (Michigan State Univ) 2002
Alumni Distinguished Scholarship (Michigan State Univ) 1999-2003

Professional Organizations

American College of Veterinary Surgeons
American Veterinary Medical Association
American Association of Equine Practitioners
The Society of Phi Zeta, Zeta Chapter
Orthopaedic Research Society
Veterinary Orthopedic Society

C. Peer-reviewed Publications

1. **McCoy AM**. 2015. Animal models of osteoarthritis: comparisons and key considerations. *Vet Path* 2015 June 10 [Epub ahead of print] DOI:10.1177/0300985815588611.
1. **McCoy AM**, Ralston SL, McCue ME. 2015. Short- and long-term performance of Standardbred pacers and trotters after early surgical intervention for tarsal osteochondrosis. *Equine Vet J* 47:438-444 DOI: 10.1111/evj.12297.
2. **McCoy AM**, McCue ME. 2014. Validation of imputation between equine genotyping arrays. *Anim Genet* 45:153 DOI: 10.1111/age.12093.
3. **McCoy AM**, Schaefer R, Petersen JL, Morrell PL, Slamka MA, Mickelson JR, Valberg SJ, McCue ME. 2014. Evidence of positive selection for a glycogen synthase (*GYS1*) mutation in domestic horse populations. *J Hered* 105:163-172. DOI: 10.1093/jhered/est075.
 - selected for the 2015 Stephen J. O'Brian Award from the American Genetic Association
4. **McCoy AM**, Toth F, Dolvik NI, Ekman S, Ellemann J, Olstad K, Ytrehus B, Carlson CS. 2013. Articular osteochondrosis: a comparison of naturally-occurring human and animal disease. *Osteoarthritis Cart* 21:1638-1647. DOI: 10.1016/j.joca.2013.08.011. PMCID:3815567.
5. **McCoy, AM**, LR Goodrich. 2012. Use of a radiofrequency probe for tenoscopic-guided annular ligament desmotomy. *Equine Vet J* 44:412-415. DOI: 10.1111/j.2042-3306.2011.00454.x.
6. **McCoy AM**, ES Hackett, AE Wagner, KR Mama, DA Hendrickson. 2011. Pulmonary gas exchange and plasma lactate in horses with gastrointestinal disease undergoing emergency exploratory laparotomy: a comparison with an elective surgery patient population. *Vet Surg* 40:601-609. doi: 10.1111/j.1532-950X.2011.00840.x [Epub 2011 May 3].
7. **McCoy AM**, ES Hackett, RJ Callan, BE Powers. 2009. Alimentary-associated carcinomas in Vietnamese pot-bellied pigs: 5 cases (2000-2008). *J Am Vet Med Assoc* 235:1336-1341.
8. Beilock S, BI Bertenthal, **AM McCoy**, TH Carr. 2004. Haste does not always make waste: expertise, direction of attention, and speed versus accuracy in performing sensorimotor skills. *Psychonomic Bulletin and Review* 11:373-379.

NAME Molly Elizabeth McCue		POSITION TITLE Associate Professor; Veterinary Population Med Department College of Veterinary Medicine, University of Minnesota 225 VMC, 1365 Gortner Ave, St. Paul, MN 55108	
EDUCATION/TRAINING <i>(Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)</i>			
INSTITUTION AND LOCATION	DEGREE <i>(if applicable)</i>	YEAR(s)	FIELD OF STUDY
Kansas State University, Manhattan Kansas	BS	1998	Animal Science
Kansas State University, Manhattan Kansas	DVM	2000	Veterinary Medicine
University of Georgia, Athens Georgia	Internship	2001	Large Animal Medicine and Surgery
Kansas State University, Manhattan Kansas	MS	2004	Clinical Sciences (Epidemiology)
Kansas State University, Manhattan Kansas	Residency	2004	Equine Internal Medicine
University of Minnesota, St. Paul Minnesota	PhD	2007	Comparative and Molecular Biosciences
University of Minnesota, St. Paul Minnesota	Post-doctoral	2008	Genetic Epidemiology

A. Role: Mentor/Co-investigator

B. Positions and Honors

2008-2013 Assistant Professor, University of Minnesota
2009-present Faculty, Paul and Sheila Wellstone Muscular Dystrophy Center, Uof Minnesota
2013-present Faculty, Microbial and Plant Genomics Institute, University of Minnesota
2013-present Associate Professor, University of Minnesota
2014-present Informatics Institute Transdisciplinary Faculty Fellow, University of Minnesota
Board Certification: Diplomate American College of Veterinary Internal Medicine (Large Animal)
2014 Inventor Recognition Award University of Minnesota
2013-present NSRP8 Equine Genome Co-coordinator
2011 NRSP8 Equine Genome Workshop Chair
2010 NRSP8 Equine Genome Workshop co-Chair
2008 Inventor Recognition Award University of Minnesota
2007-2008 Morris Animal Foundation Fellow
2007-2008 University of Minnesota Doctoral Dissertation Fellow
2007-2008 University of Minnesota Women's Leadership Institute
2007 Best Graduate student research Award University of Minnesota
2004-2007 NIH Comparative Medicine and Pathology Post-Doctoral Fellowship
Doctor of Veterinary Medicine *Summa Cum Laude*
Bachelor of Science, Animal Science and Veterinary Medicine *Magna Cum Laude*

C. Selected Peer-Reviewed Papers (last 2 years, out of 48 total)

* indicates manuscript by a graduate or ^ post-doctoral student in Dr. McCue's laboratory

1. ^Petersen JL, Mickelson JR, Valberg SJ, **McCue ME**. Genome-wide SNP data shows little differentiation between the Appaloosa and other American stock horse breeds. *Animal Genetics* Article first published online: 22 MAY 2015 DOI: 10.1111/age.12301.
2. **McCue ME**, Geor RJ, Shultz NE. Equine Metabolic Syndrome: a complex disease influenced by genetics and the environment. *Journal of Equine Veterinary Science* 35 (5) (2015): 367-375.
3. ^Petersen JL, Valberg SJ, Mickelson JR, **McCue ME**. Haplotype diversity in the equine myostatin gene with focus on variants associated with race distance propensity and muscle fiber type proportions *Animal Genetics* published online: 26 August 2014 DOI: 10.1111/age.12205. PMID:25160752.

4. *McCoy AM, Ralston SL, **McCue ME**. Short- and long-term racing performance of Standardbred pacers and trotters after early surgical intervention for tarsal osteochondrosis. *Equine Veterinary Journal* 47:438-444, 2015 DOI: 10.1111/evj.12297. PMID:24819047.
5. Schubert M, Ermini L, Der Sarkissian C, Jónsson H, Ginolhac A, Schaefer R, Martin MD, Fernández R, Kircher M, **McCue ME**, Willerslev E, Orlando L. Characterization of ancient and modern genomes by SNP detection and phylogenomic and metagenomic analysis using PALEOMIX. *Nature Protocols* 9:1056-1082, 2014. PMID:24722405.
6. *McCoy AM, Schaefer R, Petersen JL, Slamka MA, Morrell PL, Mickelson JR, Valberg SJ, **McCue ME**. Evidence of Positive Selection for the *GYS1* mutation in Equine Polysaccharide Storage Myopathy. *Journal of Heredity* 105:163-172, 2014. PMCID: PMC3920812.
7. Martinson K, Coleman B, Rendahl AK, **McCue ME**. Estimation of body weight and development of a body weight score for adult equines using morphometric measurements. *Journal of Animal Science* 92:2230-2238, 2014. PMID:24663191.
8. ^Fritz KL, Hendrickson JA, Kaese HJ, Rendahl AK, Dynes KM, Lucio MA, Cuomo FM, Brinkmeyer-Langford CL, Skow LC, Valberg SJ, Mickelson JR, Rutherford MS, **McCue ME**. Genetic risk factors for insidious Equine Recurrent Uveitis in Appaloosa horses. *Animal Genetics* 45:392-399, 2014. PMID:24467435.
9. ^Petersen JL, Cleary K, Mickelson JR, **McCue ME**. The American Quarter Horse: Population Structure and relationship to the Thoroughbred. *Journal of Heredity* 105:148-162, 2014. PMCID: PMC3920813.
10. *McCoy AM, **McCue ME**. Validation of imputation between equine genotyping arrays. *Animal Genetics* 45:153, 2014. PMCID: PMC4000747.
11. *Teixeira, R, Rendahl AK, Anderson, S, Sigler, D, Buchanan, B, Coleman, B, Mickelson, JR, **McCue ME**. Coat color genotypes and risk and severity of melanoma in gray Quarter Horses. *Journal of Veterinary Internal Medicine* 27:1201-8, 2013. PMID:23875712.
12. Lykkjen, S, Dolvik, NI, **McCue ME**, Rendahl, AK, Mickelson, JR, Roed, KH. Equine developmental orthopaedic diseases – a genome-wide association study of first phalanx plantar osteochondral fragments in Standardbred trotters. *Animal Genetics*. 44:766-9, 2013. PMID:23742657.
13. ^Petersen JL, Mickelson JR, Cothran EG, Rendahl AK, Andersson LS, Axelsson J, Bailey E, Bannasch D, Binns MM, Borges AS, Brama P, de Câmara Machado A, Distl O, Felicetti M, Fox-Clipsham L, Graves KT, Guérin G, Haase B, Hasegawa T, Hemmann K, Hill EW, Leeb T, Lindgren G, Lohi H, Lopes MS, McGivney BA, Mikko S, Orr N, Penedo MCT, Piercy RJ, Raekallio M, Rieder S, Røed KH, Silvestrelli M, Swinburne J, Tozaki T, Vaudin M, Wade CM, **McCue ME**. Genetic diversity in the modern horse illustrated from genome-wide SNP data. *PLoS One* 2013; 8(1): e54997 DOI:10.1371/journal.pone.0054997.
14. ^Petersen JL, Mickelson JR, Rendahl AK, Valberg SJ, Andersson LS, Axelsson J, Bailey E, Bannasch D, Binns MM, Borges AS, Brama P, de Câmara Machado A, Capomaccio S, Cappelli K, Cothran EG, Distl O, Fox-Clipsham L, Graves KT, Guérin G, Haase B, Hasegawa T, Hemmann K, Hill EW, Leeb T, Lindgren G, Lohi H, Lopes MS, McGivney BA, Mikko S, Orr N, Penedo MCT, Piercy RJ, Raekallio M, Rieder S, Røed KH, Swinburne J, Tozaki T, Vaudin M, Wade CM, **McCue ME**. Genome-wide analysis reveals selection for important traits in domestic horse breeds. *PLoS Genetics* 2013; 9(1): e1003211, DOI:10.1371/journal.pgen.1003211.

NAME Kevin Halden Kline	POSITION TITLE Professor of Animal Sciences and Extension Specialist, Horses College of ACES, University of Illinois 388 Animal Sciences Lab, 1207 W Gregory Dr., Urbana, IL 61801		
EDUCATION/TRAINING <i>(Begin with baccalaureate or other initial professional education, such as nursing, include postdoctoral training and residency training if applicable.)</i>			
INSTITUTION AND LOCATION	DEGREE <i>(if applicable)</i>	MM/YY	FIELD OF STUDY
University of Illinois, Urbana, IL	BS	05/1978	Animal Sciences
University of Illinois, Urbana, IL	MS	05/1980	Animal Sciences
University of Illinois, Urbana, IL	PhD	05/1987	Animal Sciences

A. Role: Co-investigator

B. Positions and Honors

8/1981 – 1/1988 Extension Specialist – Academic Professional; University of Illinois, Urbana, IL
1/1988 – 6/1994 Assistant Professor of Animal Sciences; University of Illinois, Urbana, IL
6/1994 – 6/2001 Associate Professor of Animal Sciences and Extension Specialist, Horse;
University of Illinois, Urbana, IL
6/2001 – present Professor of Animal Sciences and Extension Specialist, Horse; University of
Illinois, Urbana, IL

Honors

2013 College of ACES Spitler Teaching Award (University of Illinois)
2008 College of ACES Senior Faculty Award for Excellence in Extension (University of Illinois)

Other Positions

Director, Horsemen's Council of Illinois (HCI)
Chair, Illinois Equine Foundation
Consultant, State Racing Commission

C. Selected Peer-reviewed Publications

- Schliewert E, Lascola K, O'Brien R, Clark-Price S, Wilkins P, Foreman J, Mitchell M, **Kline K**. Comparison of radiographic and computed tomography images of the lung in healthy neonatal foals. *Am J Vet Res* 2015; 76:42–52.
- Heffron B, Benoit M, Bishop J, Costello S, Hurt L, Simpson L, Taddei L, **Kline K**, Negrusz A., Equine total carbon dioxide testing in Illinois in 2012. *J Anal. Toxicol.* 2014 Oct; 38(8):536-40.
- Warren S, Gonzalez P, Kline K. Feed form affects growth and stomach ulcers in yearling horses. *Journal of Agricultural Science and Technology A* (2014) 449-453.
- Flores, R.S., C.R. Byron and **K.H. Kline**. 2011. Effect of Feed Processing Method on Average Daily Gain and Gastric Ulcer Development in Weanling Horses. *Equine Vet J.* 31: 124-128.
- Butudom, P, J. H. Foreman, **K. H. Kline** and E. L. Whittem. 2010. Validation and comparison of two methods of measuring lactate in equine plasma. *Equine Vet. J.* 42 (suppl.38): 155-160.
- Bonoma, T.A., A.C. Brogren, **K.H. Kline** and K.M. Doyle. 2008. Effects of feeding distillers dried grains with solubles on growth and feed efficiency of weanling horses. *J. Equine Vet. Sci.* 28: 725-727.

7. Markey, A.D. and **K.H. Kline**. 2006. Effects of Dietary Fat and Yeast Culture Supplementation on Total Tract Digestibility by Horses. *The Professional Animal Scientist*. 22: 261-266.
8. **Kline, K.H.**, D. Fitzpatrick, L.Taddei, and A. Sukta. 2006. Effect of Dose of Furosemide on Plasma TCO₂ Changes in Standardbred Horses. *J. Equine Vet. Sci.* 26: 317 – 321.
9. Andrew, JE, **K.H. Kline** and J.L. Smith. 2006. Effects of Feed Form on Growth and Blood Glucose in Weanling Horses. *J. Equine Vet. Sci.* 26: 349-355.
10. **Kline, K.H.**, L.P. Frey, J.H. Foreman and J.T. Lyman. 2005. Effects of intravenous sodium bicarbonate and sodium acetate on equine acid-base status. *J. Equine Vet. Sci.* 25: 349 – 354.
11. **Kline, K.H.** Milkshakes for the Horse. 2004. *J. Equine Vet. Sci.* 24: 374 – 378.
12. Bush, J, Freeman D, **Kline K**, Merchen N, Fahey G. 2001. Dietary fat supplementation effects on in vitro nutrient disappearance and in vivo nutrient intake and total tract digestibility by horses. *J. Anim. Sci.* 79: 232 – 239
13. Frey, L. P., **K. H. Kline**, J. H. Foreman and J. T. Lyman. 2001. Using calcium carbonate as an osmolar control treatment for acid-base studies in horses. *J. Anim. Sci.* 79: 1858 – 1862.
14. Vischer, C. M., J. H. Foreman, P. D. Costable, G. J. Benson, **K. H. Kline**, D. E. Freeman, K. L. Campbell and T. C. Grubb. 1999. Hemodynamic effects of thyroidectomy in sedentary horses. *Am. J. Vet. Res.* 60: 14-21.
15. Frey, L. P. and **K. H. Kline**. 1995. Effects of pre-race exercise, frusemide, sex and ambient temperature on blood sodium, bicarbonate and pH values in Standardbred horses. *Equine Vet. J.* 27, 170-174.

XV. Letters of Support: N/A

Pilot Study

I-Title and abstract: The First Phylogenetic and Spatial Analysis of Parvovirus Strains Circulating in the Serengeti Maasai Mara Ecosystem

Rationale: Despite being widely distributed among domestic and wild carnivores, little is known about the impact that canine/feline parvovirus (CPV/FPLV) has on free-living carnivore species. Parvovirus has been reported to cause mortality in the young of wild carnivores and is suspected to have triggered declines in naïve wolf populations worldwide. African wild carnivores in captivity are also susceptible to parvovirus disease, however no epidemiological research has been conducted in free-living populations.

Furthermore, the epidemiological relationship that links parvovirus infection in domestic animals and wildlife is poorly understood. Mass domestic dog vaccination campaigns (MDV) targeting parvovirus are ongoing in communities living in the periphery of the Serengeti-Maasai Mara ecosystem, but the impact that these activities are having on wild populations is not studied yet. This information is required to optimize control measures to prevent disease transmission between domestic and wild carnivores in this protected area.

Hypothesis/Objectives: The hypothesis of this study is that domestic and wild carnivores play a significant and linked role in the epidemiology of parvovirus in the Serengeti-Maasai Mara ecosystem. The objectives are: 1) to estimate the prevalence of infection in our samples and the susceptibility of different species to be infected by canine and feline parvovirus. 2) to determine which strains of parvovirus are circulating in wild and domestic carnivores living in and around this ecosystem; 3) to carry out phylogenetic analyses of these strains to assess the likelihood of cross-species transmission and the possible introduction of new strains across national borders; and 4) to evaluate the influence that proximity to human communities has on the prevalence of parvovirus infection in wild carnivores. The data from this study will lead to a broader research project that will investigate the influence that mass dog vaccination has on the ecology of parvovirus in wild and domestic carnivores.

Study design: This study is divided in four activities: 1) The detection of parvovirus infection by real-time PCR (polymerase chain reaction), in archived blood and organ samples of wild and domestic carnivore species; 2) Sequencing of the VP2 protein DNA, and phylogenetic analyses that will enable circulating parvovirus strains to be characterised; 3) Spatial analysis of the results to enable the distribution of circulating strains within the ecosystem to be mapped.

Preliminary data: In 2014 a molecular study was carried out in Spain and newly adapted PCR techniques for sequencing the whole VP2 protein DNA developed. Spatial analysis data confirmed proximity to urban areas to be a risk factor for parvovirus infection of Iberian wolves. These results support the hypothesis that parvovirus spills over into wild populations from domestic carnivores. Furthermore, research conducted in Tanzania confirmed the presence of CPV-2a and CPV-2b in wild and domestic animals, but no data is available about Feline panleukopenia virus and other strains of CPV-2.

Expected results: This study will increase our understanding of the epidemiology of parvovirus in the Serengeti-Maasai Mara ecosystem by 1) determining which parvovirus strains are currently circulating, 2) assessing the susceptibility of different carnivore species to these strains, and 3) determining the influence that proximity to human settlements has on the prevalence and distribution of different parvovirus strains among wild populations.

Budget and timeline: This study has duration of one year with a total budget of 10.800 dollars.

Potential impact for animal health: Having evaluated the epidemiology of parvovirus in the Tanzanian environment we will be better able to refine intervention measures to reduce transmission from domestic to wild carnivores. Due to the difficulties of assessing the health of litters, the impact of parvovirus on African carnivore populations is unknown. However, in captive environments large carnivores have been shown to be susceptible to parvovirus-induced disease. The introduction of new, and potentially more pathogenic, strains into the ecosystem is a potential health risk for the young of wild carnivores. Our results will enable the appropriate strain of vaccine to be selected for MDV efforts targeting communities living around the Serengeti Maasai Mara ecosystem. Additionally, subsequent research projects will build upon the data generated in this project to test the efficacy of this intervention strategy. These outputs will help to minimize the impact that human settlements, and associated domestic animals, have on the health of threatened populations of African wild carnivores.

II. Resubmission Summary:

This proposal was sent in 2014 to The Morris Foundation and we are very grateful for the response we obtained. Due to the fairly positive and encouraging comments -"This project is highly relevant in terms of wildlife health, and epidemiology of viral pathogens that can pass between domestic and wild animal hosts"- we decided to resubmit the application since our project is mandatory to assess the health and control measures currently conducted on the Serengeti Maasai Mara Ecosystem. We have introduced some changes and new explanations in the proposal, and a specific response to the comments can be found below.

Proposal Review

Brief summary of proposed research (1-2 sentences):

This molecular epidemiology study will determine the host range of parvoviruses in domestic animals (dogs specially) relative to wild carnivores in Tanzania. Parvovirus spillover-spillback has huge implications for the evolution of this virus, as other work has shown the ability of parvoviruses to evolve in novel hosts and change their host range. This has implications for vaccination strategies of domestic dogs, and the effects on wildlife.

Critique of scientific merit and proposed approach:

Stool and blood samples of domestic dogs and wildlife are available between 2002 and 2009 from the districts surrounding Serengeti National Park. First, samples will be tested for parvovirus using real-time PCR, the positive samples will have their entire VP2 gene sequenced for phylogenetic analyses. Spatial analyses will be conducted on the samples, in addition to determining their genetic similarity and likely patterns of evolutionary divergence. The goal is to measure cross-species epidemiology, spillover to domestic cats and dogs, and potential spillback to wildlife populations.

Comments on relevance of topic to Morris Animal Foundation:

This project is highly relevant in terms of wildlife health, and epidemiology of viral pathogens that can pass between domestic and wild animal hosts.

Comments on potential impact of project on identified field:

The impact will be large for understanding how diversity in parvoviruses evolves, and potentially the evolution of pathogenicity, as the virus is transmitted among hosts.

For Fellowship Training and First Awards, comment on candidate, mentor and training environment:

N/A

Overall assessment of scientific merit, relevance of topic, and potential impact on identified field:

Describe any animal involvement in this project including live animals, client-owned animals, laboratory animals, samples, etc.

Samples are of convenience, from vaccination campaigns of domestic dogs and cats, and other wildlife studies in and around Serengeti National Park. No additional samples will be obtained for this study.

Potential for inventions:

None

Additional pertinent comments or information:

Are any of the samples linked to clinical cases of parvovirus infection? It was suggested in panel discussion that the condition of many samples in this collection may be degraded, and that the quality of DNA from samples should be determined after extraction and before RT-PCR procedures are applied to amplify and sequence them. Further, providing additional information about how much variation is expected in the VP2 gene within each strain (e.g. how many variable sites, SNPs), to give an idea of how well the virus phylogeny may be resolved.

Reply

Are any of the samples linked to clinical cases of parvovirus infection?

We will randomly select the samples and no link to clinical cases of parvovirus infection will be favored, in order to be able to establish the prevalence of infection.

In the case of dog samples (*Canis lupus familiaris*) and cats (*Felis catus*), we will choose the available samples with spatiotemporal relationships with those from wild carnivores. However, all information available about these individuals, such as age or sex, will be collected for statistics purposes.

Wild carnivores samples were collected opportunistically from chemically immobilized wild animals and postmortem examinations. In this case, all data from clinical reports and postmortem examinations will be examined to detect clinical cases of parvovirus infection and infer statistics associations.

Prevalence of parvovirus infection is high in wild and domestic asymptomatic carnivores (Allison et al., 2013; Castanheira et al., 2014). Our preliminary studies conducted in Tanzania followed the same sampling method, and they found a percentage of positives of 10,4% in dogs and 9,1% in free-living carnivore. In this study only CPV-2a and CPV-2b were searched; consequently, we expect higher rates since we are adding CPV-2c and FPLV to the research.

It was suggested in panel discussion that the condition of many samples in this collection may be degraded, and that the quality of DNA from samples should be determined after extraction and before RT-PCR procedures are applied to amplify and sequence them.

In order to assess the quality of the purified DNA from samples, before performing real-time PCR, housekeeping gene amplification will be performed by real-time PCR. This will be carried out using the universal primer set that amplifies a fragment of 248 bp of the b-actin gene previously described (Bellis et al., 2003).

All samples to be used in this current study will be obtained from the sample bank collected and stored by the Carnivore Disease Project (CDP). Many of these samples have been used in previous genetic studies and, for example, the preliminary study mentioned above has obtained good results using these samples. Furthermore, the real-time PCR technique we have adapted in our laboratory in Spain (INIA-CISA) in the preliminary study, has successfully confirmed the presence of DNA coding for VP2 in 39 out of 213 of the samples of free-living carnivores. Moreover, our newly adapted nested PCR successfully amplified 19 sequences of 1746 bp, which were later sequenced, confirming the success of the procedure. Some of these samples

were extracted from carcasses in decomposition, in the hot and humid climate conditions of Balear Islands, which have an average of around 80% humidity and 16°C temperature (Instituto Geográfico Nacional). For these reasons we expect similar success rates in this new study conducted in Tanzania.

Further, providing additional information about how much variation is expected in the VP2 gene within each strain (e.g. how many variable sites, SNPs), to give an idea of how well the virus phylogeny may be resolved.

In this resubmission we have added the requested information.

For differencing FPLV from CPV-2 groups the residues 80, 93, 103, 232, 323, 564 and 568 are very useful. A mutation Met87Leu is needed for differencing FPLV/CPV-2 from CPV-2a/-2b/-2c. Another important residue for the molecular epidemiology of the virus is the position 426. The classification based on residue 426 of the VP2 protein is currently accepted for differencing the FPLV/CPV-2/CPV-2a (Asn), CPV-2b (Asp) and CPV-2c (Glu). Despite this classification could be controversial, this is epidemiologically useful since they represent the chronological evolution of different epidemic strains (Buonavoglia et al., 2001; Nakamura et al., 2004). In order to establish recent events of spillover and spillback between domestic dogs and free living carnivores, we believe that this classification would be useful.

Allison et al. (2015) found that the residue 300 of VP2 is highly polymorphic and mutations are necessary for host adaptation. This result is not observed in our preliminary study on free-living carnivores from Spain (Calatayud et al., manuscript in revision). We have found Glycine in all our CPV-2 sequences isolated from wolves (*Canis lupus signatus*), foxes (*Vulpes vulpes*), genets (*Genetta genetta*) and badgers (*Meles meles*); as well as in all CPV-2 selected from the Genbank, which includes species as *Canis* spp –wild and domestic–, bobcat (*Lynx rufus*), stone marten (*Martes foina*) and puma (*Puma concolor*); and Alanine in all FPLV.

We believe that the differences found between Allison et al. (2015) and our study (as well as other sequences found in the Genbank), is because we detect subtypes CPV-2b and CPV-2c, and Allison et al. worked with CPV-2a. We think that subtypes CPV-2b and CPV-2c are less host specific than CPV-2 and CPV-2a and thus they don't need mutations in residue 300. This would be addressed in the present study, in which we expect to find similar results for subtypes CPV-2b and CPV-2c.

III. Name, Institution, and email address of Principal Investigator and all co-investigators.

Principal investigator: Principal investigator: Dr. Felix Lankester,
Paul G. Allen School for Global Animal Health, Washington State University,

[REDACTED]
[REDACTED]

Co-investigator: Olga Calatayud DVM, MSc Wild Animal Health.

[REDACTED]

IV. Study Proposal:

1. Specific, Testable Hypothesis and Objectives:

The hypothesis of this study is that domestic dogs and cats play a determinant role in the epidemiology of parvovirus in wild carnivores in the Serengeti-Maasai Mara ecosystem. Domestic animals maintain the virus in the ecosystem and contribute new strains to the ecology of the virus. Thus, a close phylogenetic relationship among virus strains may be found, and wild carnivores analyzed near human settlements may show a higher prevalence of the virus.

The final objective of this study is improving measures for conservation of the African wild carnivores. These measures should be chosen in accordance to the current scenario found in Tanzania, what is our subject of study. We have marked to understand the molecular epidemiology of parvovirus in this environment, through four objectives:

- 1) Estimate the prevalence of infection in our samples and the susceptibility of different species to be infected by canine and feline parvovirus (FPLV).
- 2) Characterize and classify the strains circulating among carnivores in Tanzania to improve the current vaccination programs in domestic dogs.
- 3) Perform a phylogenetic comparison of the strains infecting wild and domestic carnivores and assess the cross-species transmission. Also compare these strains with the strains found in the Genbank to know if the global evolution of parvovirus is influencing the Tanzanian epidemiology by the introduction of exotic strains.
- 4) Evaluate the proximity to humanized areas as an influent factor of infection in wild populations.

This study aims to understand the role of domestic animals in the epidemiology of parvovirus, by investigating routes of transmission and species acting as reservoir of infection to know if parvovirus is self-sustaining in the Serengeti Maasai Mara wildlife. This knowledge may be useful to assess preventive measures such as parvovirus vaccination of domestic dogs, and the control of animal translocations across national borders. Furthermore, data obtained in this study will lead to a broader research project that will investigate the influence that mass dog vaccination has on the ecology of parvovirus in wild and domestic carnivores.

2. Justification, Significance and Literature Review:

The Serengeti Maasai Mara ecosystem is a UNESCO Biosphere Reserve due to its natural heritage and biodiversity, and it has an important touristic potential for Kenya and Tanzania development. Growing evidence points to the importance of conserving carnivores because they have a pivotal role in maintaining ecosystem dynamics through trophic cascades (Ripple and Beschta, 2004). In addition to this, some of the carnivore species inhabiting this ecosystem, such as the wild dog (*Lycaon pictus*), are classified as threatened by the IUCN (Woodroffe & Sillero-Zubiri, 2012).

The introduction of non-native fauna, such as domestic animals, has different consequences in ecosystems, and the transmission of infectious diseases to naive wild populations is an important side effect of this kind of human activity. There are published several cases where introduced infectious diseases have been involved in the decline and local extinction of native species, such as the previous rabies and distemper outbreaks in the Serengeti Maasai Mara ecosystem. The impact may be more severe when an introduced host population maintains a generalist pathogen and it spillover into less abundant native populations (Cleaveland et al., 2002). This could be the case of parvovirus in Tanzania, where domestic dogs occupied the same landscape than threatened carnivore populations (Woodroffe and Donnelly, 2011).

According to Mech et al. (2008) parvoviruses may undermine certain wild population growth. Moreover, some species among the Tanzanian carnivores, such as the cheetah (*Acinonyx jubatus*), has low genetic diversity levels (Durant et al., 2008) and could be more vulnerable to infectious agents, as has been previously reported (Acevedo-Whitehouse et al., 2003).

Parvoviruses are non-enveloped, icosahedral viruses with a small capsid and a single-stranded DNA consisting of approximately 5000 base pairs (bp). VP2 is the most abundant structural protein and it determines the raised region of the capsid, known as the 'threefold spike'. This region is highly antigenic and serves as a target for neutralizing antibodies. It determines adaptations to hosts and also characterises the emergent strains (Shackelton et al., 2005).

CPV and FPLV viruses belong to the feline subgroup of parvoviruses and are grouped together with other viruses such as mink enteritis virus, racoon parvovirus and blue fox parvovirus. CPV and FPLV have a genome homology of 98% and most differences are accumulated in the VP2 domain (Truyen et al., 1998). CPV-2 was first identified by Polymerase Chain Reaction (PCR) in a domestic dog in USA in 1978 (Appel et al., 1979), following the emergence of a previously unrecognized disease in dogs, that rapidly became pandemic. However, FPLV has been known since the 1920s (Verge & Cristoforoni, 1928), and evolutionary studies have traditionally considered that FPV is the ancestor of CPV-2. According to Truyen (1998), CPV emerged from a FPV-like virus that was most likely present in a wild carnivore species -possibly the red fox (*Vulpes vulpes*)-, thereby underlining the importance of wildlife in the epidemiology of these viruses. On the other hand, recent phylogenetic studies suggest that both viruses were derived separately from common ancestors, and evolved independently before the first description of CPV in dogs (Allison et al., 2013).

CPV and FPLV follow different epidemiologic patterns. Since the emergence of CPV-2 in the 1970s, antigenic drift has been constantly changing this parvovirus. The original 1978 virus strain was replaced worldwide by CPV-2a in 1979–1980. The 2b variant was detected in the United States in 1984 and 2c in Germany in 1996 (Decaro et al. 2008). The initial CPV-2, that was unable to infect cats, regained this ability to infect these domestic animals and other carnivores with the new antigenic subtype CPV-2a (Truyen et al. 1998). It is assumed that CPV follows an epidemic pattern since its growth rate and host range have increased. Moreover, new antigenic variants have arisen over the past three decades due to a high substitution rate of about 10^{-4} substitutions per site per year. By contrast, the FPLV follows endemic patterns in cats since it has maintained a constant population size, and varies at slower rates (Shackelton et al., 2005). These viruses are currently widespread but no molecular studies have been done in East Africa. The distribution of CPV-2 strains varies depending on the continent. While the most predominant strain in Asia is 2a (Ohshima et al., 2008), in America is 2b and 2c (Hong et al., 2007), and few studies are done in Africa. In Tunisia, for example, the three strains have been found (Touihri et al., 2009).

Despite their wide host range, little is known about either the distribution of the CPV and FPV virus clades, or the susceptibility of free-ranging animals to infection. Molecular detection in hosts other than domestic cats and dogs are usually found in artificial settings such as zoos or wildlife centres (Barlow et al., 2012). However, the few genetic studies that have studied wild carnivores suggest that parvoviruses are widely distributed in these species, and that a high level of strain variation exists (Truyen et al., 1998).

Symptoms of parvovirus infection are similar in wild and domestic carnivores and primary effect of CPV is mortality in young pups (Mech et al., 2008). Parvoviruses cannot induce mitosis and can only replicate in actively dividing cells during the S phase of the cell cycle, for this reason, cubs are more vulnerable to disease (Shackelton et al., 2005). Lymphopenia or leukopenia and hemorrhagic enteritis are the most pronounced clinical symptoms and these manifestation can vary from severe to subclinical (Hoelzer and Parrish, 2010). Maternal acquired immunity is crucial for protection during the early stages of life, and pups without this protection become vulnerable when they emerge from the den (Mech et al., 2008). However, recent studies have demonstrated that the new strain CPV-2c prompts more severe pathogenicity with higher mortality rates and even vaccinated adult dogs can develop the disease (Decaro et al., 2008). CPV-2c seems to be more infectious and to induce a greater frequency of disease than previous strains (Nakamura et al., 2001).

Transmission without direct contact may be mainly thanks to the faecal-oral route. Parvoviruses may be shed in faeces for more than six months and they are highly resistant to changes in pH, temperature, solvents and desiccation. It can persist for over seven months in the environment and can be transmitted long distances by fomites (Kerr et al., 2005; Mech et al., 2008). These characteristics enable them to spread rapidly and the emergence of new strains could represent a risk for naïve populations. An example of this is CPV-2, that has been reported to cause mortality in young wild carnivores and is suspected to have triggered declines in naïve wolf populations worldwide (Mech et al., 2008).

For these reasons, it is important to understand the molecular epidemiology of parvoviruses,

since it would allow us to detect the threatened populations at risk. Furthermore, the study of the host-parasite systems is important for the understanding of host range alteration and the evolution of virulence, which will determine the emergence of new diseases (Ikeda et al., 2002). This information is needed to determine the optimum control measures to minimize the impact of domestic animals in the ecosystem, and establish which techniques will be most effective and sustainable for protecting endangered carnivores from disease. This is one of the gaps in knowledge established by the IUCN (International Union for Conservation of Nature), Red List of Threatened Species, for the conservation of some African carnivores (Woodroffe & Sillero-Zubiri, 2012).

3. Preliminary Data:

Two preliminary studies have been carried. First, we conducted a study in Tanzania on 11 wild carnivores species and asymptomatic domestic dogs (Mwalongo et al., 2014). Blood and tissues samples were collected from 2002 in Serengeti National Park and they come from the same sample bank that we are using in this current study. We tested for CPV-2a and CPV-2b by deploying convectional PCR and both strains were detected, with hosts being jackals (*Canis aureus*), African wild dogs, lions (*Panthera leo*), dwarf mongoose (*Helogale parvula*) and domestic dog (Table 1).

	Total prevalence of CPV-2a and CPV-2b (95% CI)	Positives to CPV-2a	Positives to CPV-2b
Wild carnivores	9.1 (4.6-13.6)	2/154	12/154
Domestic dogs	10.4 (3.58-17.22)	5/77	3/77

Table 1: Results from the preliminary study in Tanzania

This preliminary study reveals the presence of CPV-2a and CPV-2b in domestic dogs and wild carnivores from Tanzania; however, further investigations are needed. These results suggest that cross species could happen but sequencing the VP2 protein and comparing the strains found in different species would allow us to investigate the evolution and transmission patterns of the virus. Furthermore, a spatial analysis would confirm the spillover from domestic to native carnivores. This information would help us to determine the importance of the contact between species and therefore, the relevance of adopting control measures. For economic limitations, this previous study did not search the CPV-2c and FPLV strains and therefore there is a gap in the knowledge about the parvovirus strains circulating in the ecosystem, that need to be studied in order to assess the correct vaccination programs.

The second preliminary study has been conducted in the Animal Health Research Centre in Spain (INIA-CISA) (Calatayud et al., manuscript in revision). It is an epidemiological research of parvovirus infection in free-ranging carnivores in Spain. We conducted real-time PCR on spleen samples from 213 wild carnivores belonging to five families. Some of these samples were extracted from carcasses in decomposition. The real-time PCR technique we have adapted in our laboratory in Spain (INIA-CISA) has successfully confirmed the presence of DNA coding for VP2 in 39 of the total samples. The newly adapted nested PCR successfully amplified the sequences of 1746 bp from 19 of these positive samples, and we expect similar success rates in this current study.

The phylogenetic study revealed a high diversity of sequences, finding 15 different nucleotide sequence types (ntST). All positive Iberian wolves and one wildcat (*Felis silvestris*) were infected by CPV-2c, while the stone martens were infected by FPLV and CPV-2b. This research supports the hypothesis that cross-species transmission of parvovirus between carnivores takes place and that parvovirus host specificity is less strict than previously believed. The prevalence of infection of parvovirus in Iberian wolves was 67.6% (C.I.=50.2–82%) and a spatial analysis revealed that the proximity to urban areas is a risk factor for wolves to get infected (Millán et al., in press). The virus would not seem to persist in wild carnivore populations without the anthropogenic influence in the Spanish scenario.

Regarding the phylogenetic analysis, several aminoacid residues of the VP2 protein have been proven to be essential for molecular epidemiology and taxonomy. In first place, for differencing FPLV from CPV-2 groups the residues 80, 93, 103, 232, 323, 564 and 568 are very useful

(Allison et al., 2015). A mutation Met87Leu is needed for differencing FPLV/CPV-2 from CPV-2a/-2b/-2c. Another important residue for the molecular epidemiology of the virus is the position 426. The classification based on residue 426 of the VP2 is currently accepted for differencing the FPLV/CPV-2/2a (Asn), CPV-2b (Asp) and CPV-2c (Glu). Despite this classification could be controversial, this is epidemiologically useful since they represent the chronological evolution of different epidemic strains (Buonavoglia et al., 2001; Nakamura et al., 2004). In order to establish recent events of spillover introduction from domestic dogs to wild carnivores we believe that this classification would be useful.

Allison et al. (2015) found that the residue 300 of VP2 is highly polymorphic and mutations are necessary for host adaptation. This result is not observed in our preliminary study on free-living carnivores from Spain (Calatayud et al., manuscript in revision). We have found Glycine in all our CPV-2 sequences isolated from wolves, foxes, genets and badgers; as well as in all CPV-2 selected from the Genbank, which includes species as *Canis* spp –wild and domestic–, bobcat, stone marten and puma; and Alanine in all FPLV.

We believe that the differences found between Allison et al. (2015) and our study (as well as other sequences found in the Genbank), are because we detect subtypes CPV-2b and CPV-2c, and Allison et al. worked with CPV-2a. We think that subtypes CPV-2b and CPV-2c are less host-specific than CPV-2 and CPV-2a and thus they don't need mutations in residue 300. This question would be addressed in the present study, where we expect to find similar results for subtypes CPV-2b and CPV-2c.

4. Experimental Methods and Design:

To achieve our main goal we will conduct 1) investigation of the presence of CPV-2 and FPLV in free-living and domestic carnivores in Tanzania by real-time PCR; 2) characterization of the parvovirus strains by sequencing the complete VP2 gene and further inferring the phylogenetic associations of the obtained isolates; 3) Spatial analysis of the prevalence.

Main outcomes will be represented as:

- 1) Prevalence of subgroup of parvoviruses in both wild and domestic carnivores, to know the susceptibility of these animals.
- 2) Classification of the subgroups of parvoviruses (FPLV, CPV-2, 2a, 2b, 2c) infecting different carnivore species and the construction of a phylogenetic tree.
- 3) Distribution map to visualize the distribution of the isolates and their relationship among humanized areas.

4.1. Investigation of the presence of CPV-2 and FPLV

4.1.1. Stool samples of free-living carnivores.

Wild carnivores samples used in this study were collected opportunistically from chemically immobilized wild animals and postmortem examinations performed in the Serengeti National Park between 2002 and 2009. The samples were collected by the Carnivore Disease Project (CDP) under the Tanzanian Wildlife Research Institute (TAWIRI) permission.

We will select 150 samples available from the CDP belonging the following species: lions, jackals, African wild dogs, dwarf mongooses, cheetah, hyenas (*Crocuta crocuta*), leopards (*Panthera pardus*), civets (*Civettictis civetta*), aard wolves (*Proteles cristata*), bat-eared foxes (*Otocyon megalotis*), and servals (*Leptailurus serval*). These samples will be selected randomly to be able to establish the prevalence of infection. However, data from clinical reports and postmortem examinations will be examined to detect clinical cases of parvovirus infection and infer statistic associations.

4.1.2 Stool samples of domestic dogs.

Since 2003 the Serengeti Health Initiative (SHI) has been carrying out mass dog vaccination campaigns in districts surrounding the Serengeti National Park. During these campaigns domestic dogs and cats are brought to the central village point, where they receive a health check and a sub-cutaneous inoculation against rabies, distemper and parvovirus. In addition, a 10 ml blood sample is collected from the cephalic vein of all dogs that appear to be under one year of age. These samples were frozen and are available for the current study. We will choose 150 of these available samples with spatiotemporal relationships with those from wild carnivores. They will be randomly selected in each area to infer the prevalence of infection, and

no link to clinical cases of parvovirus infection will be favored. All information available about these individuals, such as age or sex, will be collected for statistic purposes.

4.1.3. Real-time PCR detection of CPV-2 and FPLV in all samples

This step will be done in the Nelson Mandela African Institution of Science and Technology laboratory in Arusha, Tanzania. Total DNA of the samples will be isolated by the method of spin columns, which it is routinely used in our lab. In order to assess the quality of the purified DNA, housekeeping gene amplification will be performed by real time PCR. This will be carried out using the universal primer set that amplifies a fragment of 248 bp of the b-actin gene previously described (Bellis et al., 2003).

Once validated the quality of the DNA, a real time PCR based on Taqman probes will be performed in all the samples (Decaro et al., 2007). This real time PCR is capable to detect with a high sensibility and specificity all positives to CPV-2, 2a, 2b and 2c, plus all positives to FPLV. A vaccine will be used as positive control (Nobivac® puppy).

4.2. Characterization and sequencing of the strains

This step will be done in the INIA-CISA (Animal Health Research Institute, Madrid, Spain), following the same steps than in the preliminary study conducted in Spanish wild carnivores:

4.2.1. Characterization.

We will characterize the VP2 coding region of all positive samples. This region will be amplified by two nested PCR using two sets of primers as described previously, and we will use a vaccine as positive control (Nobivac® puppy).

4.2.2. Sequencing.

We will sequence the amplicons obtained from the PCRs described above, using seven internal primers designed in previous studies. Due to the fact that we are trying to amplify long fragments of naturally infected carnivores without clinical signs -and probably with low copies of virus in the target tissues-, we expect that some positive samples could be negative or weak positive. In the last case, in order to obtain clear sequences, we will clone these amplicons in *Escherichia coli* vectors using a commercial kit.

4.2.3. Phylogenetic analysis

This analysis comprises the following steps:

4.2.3.1. Alignment of the sequences with MEGA 6 software to obtain a consensus sequence for each positive sample, plus translation of the nucleotide sequences into amino acid.

4.2.3.2. Blast search in the Genbank of the consensus sequences. All sequences of the VP2 genes available in the database will be included in the study.

4.2.3.3. Phylogenetic analysis with MEGA 6 Software using different algorithms (Neighbor-Joining and Maximum Likelihood), based on p-distances (nucleotide and amino acid).

The main outcome of this step will be to describe for each obtained sequence: a) the identification of the parvovirus (FPLV/CPV-2) and CPV-2 subtype, b) the phylogenetic relationships within the different subtypes.

4.3. Spatial analysis of the prevalences

The main outcome will be to explore the statistic relationship among the strains and other variables, such as host species (domestic vs. wild animals), geographical distribution, age, gender, etc. We will use ArcGIS Software for spatial analysis.

5. Timeline:

The project is expected to last one year, from June 2016 to July 2017. Each column represents approximately a trimester.

Tasks	2016		2017	
Sample selection and preparation of laboratorial works				
DNA extraction and real time PCR screening of 300 samples (wild carnivores and domestic dogs).				
Amplification of VP2 gene in positive samples				
Sequencing VP2/sequence assembly/phylogenetic study				
Epidemiological study				
Final report				

V. Animal Involvement Justification:

Morris Animal Foundation Animal Involvement Justification

Morris Animal Foundation (MAF) is dedicated to funding scientifically sound, relevant and humane studies that specifically address the health and well-being of animals. All studies receiving funding must follow MAF's Health Study Policy for Animals Involved in Research (adopted October 18, 2008), which was written to ensure that each and every animal involved in a MAF funded health study receives excellent, compassionate care throughout the study. MAF shall not fund health studies which require euthanasia as an endpoint or the induction of disease or injury, unless the nature of the disease or condition to be studied is of such significance for improving animal health that such means are justified, and that meaningful information can be obtained in no other way. Furthermore, MAF will not fund any study that induces or allows pain or distress unless such pain or distress can be controlled by appropriate anesthetic, analgesic, tranquilizing drugs, or nursing care. [Click here](#) for the full Health Study Policy.

A. If this study does not involve live animals please indicate here by N/A: N/A

B. Does this study involve biological samples, tissues, etc.? Yes

If yes, describe in detail what samples will be used and where & how they will be (or were) acquired. Note: Morris Animal Foundation reserves the right to request a copy of the Institutional Animal Care and Use Committee (IACUC) application/approval and other relevant applications/approvals (e.g., wildlife permit) covering the original collection of samples, including archived samples. MAF reserves the right to request IACUC (or equivalent) review and approval for any Foundation study *regardless of the Institution's requirements*. This would include the use of archived samples as well as clinical trials.

This study involves blood and spleen samples, which have been previously collected. We distinguish samples collected from free-living and domestic animals below:

Stool samples of free-living carnivores.

Wild carnivores samples used in this study were collected opportunistically from chemically immobilized wild animals (blood samples) and postmortem examinations (spleen samples) performed in the Serengeti National Park between 2002 and 2009. The samples were collected by the Carnivore Disease Project (CDP) under the Tanzanian Wildlife Research Institute (TAWIRI) permission.

We will select 150 samples available from the CDP belonging the following species: lions (*Panthera leo*),

Jackals (*Canis aureus*), African wild dogs (*Lycaon pictus*), dwarf mongooses (*Helogale parvula*), cheetah (*Acinonyx jubatus*), hyenas (*Crocuta crocuta*), leopards (*Panthera pardus*), civets (*Civettictis civetta*), aard wolves (*Proteles cristata*), bat-eared foxes (*Otocyon megalotis*), and servals (*Leptailurus serval*). These samples will be selected randomly to be able to establish the prevalence of infection.

Stool samples of domestic dogs.

Since 2003 the Serengeti Health Initiative (SHI) has been carrying out mass dog vaccination campaigns in districts surrounding the Serengeti National Park. During these campaigns domestic dogs and cats are brought to the central village point, where they receive a health check and a sub-cutaneous inoculation against rabies, distemper and parvovirus. In addition, a 10 ml blood sample is collected from the cephalic vein of all dogs that appear to be under one year of age. These samples were frozen and are available for the current study. We will choose 150 of these available samples with spatiotemporal relationships with those from wild carnivores. They will be randomly selected in each area to infer the prevalence of infection, and no link to clinical cases of parvovirus infection will be favored.

C. If this study involves live animals, succinctly address the following: (please restate the questions and directives). N/A

1. What species will be studied? N/A
2. State the status of your IACUC application/approval. All recipients of MAF funding will be required to submit the entire IACUC protocol and document. A copy of the IACUC approval should not be included with the application, but it is required before funding can be awarded.
3. List the USDA category for pain and distress (B, C, D, E): N/A
 Note: Any study beyond category C will require review by MAF's Animal Welfare Advisory Board (AWAB). In general MAF does not fund studies beyond category C (category D studies will only be considered if they conform with MAF's Health Study Policy, category E studies will not be considered).
4. Does this proposal involve client-owned animals? N/A
 If yes, the protocol for client-owned animals must be approved by the appropriate peer review committee before the project is funded. *If this proposal involves client-owned animals, an informed client consent form must be submitted with this proposal. For a suggested list of items to be considered in an informed client consent form, [click here](#).*
5. Explain how animals will be acquired (e.g., client-owned, USDA licensed breeder, institutional "herds" or "colonies") and verify that the animals are suitable for the study (e.g., have no physiologic, physical or pharmacologic issues that would interfere with results) N/A
6. How many animals will be used? N/A
 - a. Summarize numerical justification
7. Does this study induce disease, injury, pain or distress in animals? Note: any study requiring the induction of disease, injury, pain, or distress will have an additional evaluation by MAF's AWAB. No
 If yes,
 - a. Defend the necessity of experimental design
 - b. Explain how pain and/or distress will be controlled
 - c. Justify that no alternative, including clinical studies, can be used to accomplish study objectives and the disease/condition to be studied is of such significance for improving the health of the species.
8. Explain the environment and housing conditions (quality of life) in which the animals will live (address species-appropriate exercise, enrichment, socialization, veterinary care, etc.) N/A
9. What will happen to the animals upon completion of the study? N/A
 If adoption, explain the adoption process. Provide assurance that whenever possible and when in the animal's best interest, investigators shall make companion animals available for adoption at the end of the study or return the animals to the owner/responsible agency in an environment that promotes animal welfare and excellent quality of life.
10. If euthanasia, provide the following additional information (note: any study requiring euthanasia as an endpoint will have an additional evaluation by a MAF's AWAB. N/A
 - i. Total number that will be euthanized and justification for numbers
 - ii. Method of euthanasia
 - iii. Justification that no alternatives can be used to accomplish study goal(s) and that the disease/condition to be studied is of such significance for improving the health of the species that a terminal endpoint is deemed necessary.
 - iv. Reason for euthanasia in lay language (this wording may be shared with staff,

donors and media)

- v. Provide objective criteria for determining when euthanasia is appropriate or necessary (note: Morris Animal Foundation wants assurance that an animal will not be allowed to suffer and that monitoring for pain and suffering is adequate)

Note: Morris Animal Foundation does not consider the use of CO₂ alone to be an appropriate method of euthanasia

Please note:

1. If an animal is used in an invasive study, MAF may require that a guarantee is provided, through principal investigator and institutional signatures that the animal will not participate in any future invasive study or procedure
2. MAF does not allow inclusion of ancillary data in MAF funded research that includes animal use protocols not in agreement with our Health Study Policy, even if it is obtained using other funding sources.
3. Morris Animal Foundation considers euthanasia acceptable when an animal develops unanticipated illness or injury that results in pain and suffering that cannot be alleviated with standard veterinary interventions.

VI. Recombinant DNA/Biohazards: N/A

VII. Facilities and Equipment.

Facilities at the Nelson Mandela African Institution of Science and Technology laboratory in Arusha, Tanzania.

The laboratory has the full equipment for real time PCR detection: real time thermocycler, DNA extraction materials, cabins, centrifuges...

Facilities at the Animal Health Research Centre (INIA-CISA).

The laboratory has the full equipment for PCR detection, sequencing and clonning: thermocycler, cabins, centrifugues, automatic sequencer, microbiological lab...

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IX. Budget

Category	
<u>Personnel:</u>	
1. Co-investigator (Olga Calatayud)	██████
Total Salaries	██████
<u>Supplies & Expenses:</u>	
1. PCR reagents and plastics for real time detection	██████
2. Service of molecular diagnostics	██████
3. Transport, accommodation and meals in Tanzania.	██████
4. Courier and permits for sample transport.	██████
Total Supplies & Expenses:	██████
Subtotal of All Categories:	\$ 10000
Maximum of 8% - Indirect Costs:**	\$ 800
Grand Total Requested from MAF:	\$ 10800

X. Itemized Budget Justification

Salary Co-Investigator (Olga Calatayud): This will afford the salary of two months of work, one of them at the Nelson Mandela African Institution of Science and Technology laboratory in Arusha, Tanzania; and the other month at the Animal Health Research Centre, in Madrid, Spain.

Service of molecular characterization: The samples will be analyzed in the Animal Health Research Center (INIA-CISA), as an external service. The responsible of the analysis is Dr. Fernando Esperón, which participates in this proposal as resource personnel.

Transport, accommodation and meals in Tanzania: These costs will be used for a stay of a month of the Co-Investigator Olga Calatayud at the Nelson Mandela African Institution of Science and Technology laboratory in Arusha, Tanzania. During this stay she will collaborate in DNA extraction and perform the real-time PCR for parvovirus detection. She has full expertise on parvovirus detection and characterization, since her MSc research project was entitled: "Molecular characterization of parvovirus infection in free ranging carnivores in Spain".

Courier and permits for sample transport: Necessary to transport the samples from the Nelson Mandela African Institution of Science and Technology (Tanzania) laboratory to the Animal Health Research Centre (Spain).

XI. Other Support: N/A.

XII. Prior MAF Support during the last three years: N/A.

XIII. Biographical Data (two-page limit for each individual):

Full name:	Dr. Felix Lankester
Present position:	Clinical Assistant Professor – Paul G. Allen School for Global Animal Health, Washington State University
	Post-Graduate Supervisor – Nelson Mandela African Institute of Science and Technology (NM-AIST)
	Regional Representative – Global Animal Health Tanzania
	Director, Serengeti Health Initiative
	Veterinary Director for Pandrillus Foundation

Education/Training:

2010 – present. PhD candidate - University of Glasgow, UK. Title: Malignant catarrhal fever: a field-based vaccine trial and an economic impact assessment of the disease and its control on pastoralist livelihoods in Tanzania.

2002 – 2003. Master of Science in Wild Animal Health - Institute of Zoology & Royal College of Veterinary Surgeons, London University, UK; graduated with merit

1990 – 1995. Bachelor of Veterinary Science - Liverpool University, UK

Previous positions:

2009 - 2012	Director of Tanzanian Programs – Lincoln Park Zoo
2006 - 2009	Country Director of Pandrillus Foundation – Cameroon
2004 - 2009	Project Director and Head Veterinarian of the Limbe Wildlife Centre (LWC)
2003 - 2004	Consultant to the Mediae Production Company, Nairobi, Kenya
2004 -2004	Associate Producer for ‘Born to Run’ (Sky Sports)
2002 - 2003	Two phases of volunteering as a veterinary surgeon for The Orang-utan Foundation, Kalimantan, Borneo
1997 - 2001	Assistant Producer for Granada Wild (Partridge Films), Bristol, UK
1995 - 2002	Veterinary surgeon of mixed and small animal practices in SW England

Honours/distinctions/membership of societies, institutions, committees:

2012-present	International Society for Veterinary Epidemiology and Economics Bursary Award
2010-present	Inspector of the Pan African Sanctuaries Alliance
2009-present	Member of Tanzania Veterinary Association
1995-present	Member of Royal College of Veterinary Surgeons

Selected Peer Reviewed Publications (four last years):

- Mwalongo, O., Shahada, F., Bigambo, M., Gwakisa, P. & **Lankester, F.** (2014) Prevalence of canine parvovirus in domestic dogs around Serengeti National Park (Tanzania). *International Journal of Science and Research*. 3 (10), 1864 - 1868
- Cleaveland, S., Hampson, K., Lembo, T., Townsend, S. and **Lankester, F.** (2014). Role of dog sterilisation and vaccination in rabies control programs. *Veterinary Record*. 2014 175: 409-410. doi: 10.1136/vr.g6352
- **Lankester, F.**, Hampson, K., Lembo, T., Taylor, L., Palmer, G. & Cleaveland, S. (2014). Implementing Pasteur’s vision for rabies elimination. *Science* 345 (6204), 1562 – 1564.
- Cleaveland, S., **Lankester, F.**, Townsend, S., Lembo, T. and Hampson, K. (2014). Rabies control and elimination: a test case for One Health. *Veterinary Record*. One Health Feature, 188-193. doi: 10.1136/vr.g4996
- Bartley, K., Deane, D., Percival, A., Dry, I., Grant, D., Inglis, N., Mclean, K., Manson, E., Imrie, L., Haig, D., **Lankester, F.** and Russell, G. (2014). Identification of immuno-reactive capsid proteins of malignant catarrhal fever viruses. *Journal of Veterinary Microbiology*. 173 (1-2), 17-26
- Cleaveland, S., Beyer, H., Hampson, K., Haydon, D., **Lankester, F.**, Lembo, T. *et al.*, (2014), ‘The changing landscape of rabies epidemiology and control’, *Onderstepoort Journal of Veterinary Research* 81(2), Art. #731, 8 pages. <http://dx.doi.org/10.4102/ojvr.v81i2.731>

- Cleaveland, S., Beyer, H., Hampson, K., Haydon, D., **Lankester, F.**, Lembo, T., Meslin, F., Morders, M., Mtema, Z., Sambo, M., Townsend, S. (In press). The changing landscape of rabies epidemiology and control, Onderstepoort Journal of Veterinary Research
- Prado-Martinez, J... **Lankester, F.J.**...and Marques-Bonet, T. (2013) Great ape genetic diversity and population history. *Nature*. 499, 471–475
- Lyons S, Sharp C, LeBreton M, Djoko CF, Kiyang JA, **Lankester F.**, Bibila, T.G., Fair J., Wolfe N. and Simmonds P. (2012) Species Association of Hepatitis B Virus (HBV) in Non-Human Apes; Evidence for Recombination between Gorilla and Chimpanzee Variants. *PLoS ONE* 7(3): e33430. doi:10.1371/journal.pone.0033430
- Cleaveland S., Keyyu J., Auty H., Lembo T., Hampson K., **Lankester F.J.** and Kazwala R. (2012). Domestic animal health programs to protect wildlife. *Animal Health and Biodiversity – Preparing for the Future*. OIE Booklet on the OIE Global Wildlife Conference, Paris
- Köndgen S, Leider M, **Lankester F**, Bethe A, Lübke-Becker A, Leendertz, F.H. and Ewers, C. (2011). *Pasteurella Multocida* Involved in Respiratory Disease of Wild Chimpanzees. *PLoS ONE* 6(9): e24236. doi:10.1371/journal.pone.0024236
- Wevers D, Metzger S, Babweteera F, Bieberbach M, Boesch C, Cameron K, Couacy-Hymann E, Cranfield M, Gray M, Harris LA, Head J, Jeffery K, Knauf S, **Lankester F**, Leendertz SA, Lonsdorf E, Mugisha L, Nitsche A, Reed P, Robbins M, Travis DA, Zommers Z, Leendertz FH, Ehlers B. (2011) Novel adenoviruses in wild primates: a high level of genetic diversity and evidence of zoonotic transmissions. *J Virol*. 85 (20): 10774-84. Epub 2011. PMID: 21835802
- Gonder, M.K., Locatelli, S., Ghobrial, L., Mitchell, M., Kujawski, J.T., **Lankester' F.J.**, and Tishkoff, S. (2010) Evidence from Cameroon reveals differences in the genetic structure and histories of chimpanzee populations. In: *PNAS*, 108 (12) 4766-4771
- **Lankester, F.**, Anyam Kiyang, J., Bailey, W and Unwin, S.J. (2010) *Dientaomeba fragilis*: initial evidence of pathogenicity in the western-lowland gorilla (*Gorilla gorilla gorilla*). In: *Journal of Zoo and Wildlife Medicine*. 41(2): 350-352.
- Ghobrial, L., **Lankester, F.J.**, Kiyang, A.J., Akih, E.A., de Vries, S., Fotso, R., Gadsby, E.L., Jenkins, P.D. and Gonder, M. K. (2010) Tracing the origins of rescued chimpanzees reveals widespread chimpanzee hunting in Cameroon. In: *BMC Ecology* 10:2
- Leendertz, F., Deckers, M., Schempp, W., **Lankester, F.**, Boesch, C., Mugisha, L., Dolan, A., Gatherer, D., McGeoch, D. and Ehlers, B. (2009) Novel cytomegaloviruses in free-ranging and captive great apes: phylogenetic evidence for bidirectional horizontal transmission. In: *J Gen Virol* 90, 2386-2394.
- **Lankester, F.**, Matz-Rensing, K., Kiyang, J., Jensen, S., Weiss, S. And Leendertz, F. (2008) Fatal ulcerative colitis in a western-lowland gorilla (*Gorilla gorilla gorilla*). In: *J. Med. Primatol* 37: 297-302
- Thalmann, O., Fischer, A., **Lankester, F.**, Paabo, S. and Vigilant, L. (2007) The complex evolutionary history of gorillas: insights from genomic data. In: *Mol. Biol. Evol.* 24(1):146-158
- ***Lankester, F.**, ***Leendertz, F.** and Guislain, P. (2006) Anthrax in Western and Central African great apes. In: *American Journal of Primatology*. 68:928-933 (*authors contributed equally)
- **Lankester, F.** and Divers, S. (2005) Phallic prolapse and subsequent amputation in a Nile crocodile (*Crocodylus niloticus*). In: *Journal of Zoo and Wildlife Medicine*.
- **Lankester, F.** (2004) A probable case of fatal hyper-infection syndrome associated with nematodiasis in a wild orang-utan (*Pongo pygmaeus*). In: *World Association of Wildlife Veterinarians*. 14 (55): 3–6.

Full name:	Olga calatayud
Present position:	PhD candidate
Present work address:	N/A
Role on project	Resource personnel / molecular analysis

Education/Training:

2007. Bachelor of Veterinary Medicine (University Cardenal Herrera-CEU, Valencia, Spain).

2013-2014. Master degree in "Wild Animal Health" at the Royal Veterinary College/ Zoological Society of London, UK. MSc Research Project entitled: "Molecular characterization of parvovirus infection in free ranging carnivores in Spain".

Previous positions:

2015	Veterinarian in the small animal clinic Drs. Bustillo, Valencia, Spain
2014	Researcher in the Animal Health Research Center CISA-INIA, Madrid, Spain.
2013	Veterinarian in the small animal clinic <i>Albert</i> , Picassent, Spain.
2012	Veterinarian at Bylakuppe Rabies and Disease Control Programme, (<i>Vets Beyond Borders Australia NGO</i>). Bylakuppe, India.
2011	Veterinarian in The Thai Society for the Conservation of Wild Animals, Thailand.
2011	Veterinarian in charge of The Society for the Prevention of Cruelty to Animals of Paterna, Valencia, Spain.
2009-2010	Veterinarian in <i>Sanaga-Yong Chimpanzee Rescue Center</i> , Cameroon.
2008-2009	Veterinarian in the Small Animals Hospital, <i>Hospital Veterinario Benicarló</i> , Castellón, Spain.
2008	Animal keeper at the Zoo of primates "La Vallée des Singes", Poitiers, France.
2006-2007	Practices at Valencia Zoo, Spain.
2005-2006	Practices at the Wildlife Recovery Center <i>La Granja</i> , Valencia, Spain.
2005	Practices at the Zoo <i>Selwo-Aventura</i> , Málaga, Spain.

Membership of societies:

2008-present: Member of College of Veterinary Surgeons of Valencia, Spain.

Full name:	Fernando Esperón Fajardo
Present position:	Responsible of the Unit of Diagnostic of Wildlife Diseases, Epidemiology and Environmental Group (EySA), (CISA-INIA)
Present work address:	Animal Health Research Centre (CISA-INIA), Ctra. Algete a El Casar s/n, 28130 Valdeolmos, Madrid, Spain.
Role on project	Resource personnel/ molecular analysis

Education/Training:

2005. PhD degree. Department of Animal Health, Faculty of Veterinary Medicine (University Complutense of Madrid, Spain). Thesis entitled: “Environmental pollutants in stranded odontocetes from Canary Archipelago. Health implications”.

2003. Degree of Master on Research. Department of Animal Health, Faculty of Veterinary Medicine (University Complutense of Madrid, Spain).

1999. Bachelor of Veterinary Medicine (University Complutense of Madrid, Spain).

Previous positions:

2008 – present	Responsible of the Unit of Diagnostic of Wildlife Diseases, Epidemiology and Environmental Group (EySA), (CISA-INIA)
2006 - 2008	University graduate position (TRAGSA), Animal Health Research Centre (CISA-INIA).
2005-2006	University graduate position, Department of Animal Health, Faculty of Veterinary Medicine (University Complutense of Madrid)
2001-2005	Predoctoral Fellowship, Animal Health Research Centre (CISA-INIA).
2001-2003	Chief Executive Officer and founding partner, Sil-Ex, SLL (Private laboratory, diagnostics exotic and wildlife).
1999-2001	Small Companion Animals, DVM.

Honours/distinctions/membership of societies, institutions, committees:

2014-present	Member of Wildlife Diseases Association (WDA) and regional sections: European Wildlife Diseases Association (EWDA) and Latin American Wildlife Diseases Association (LAWDA).
2005-present	Teaching contributor on practices classes, Department of Pharmacology, Faculty of Veterinary Medicine (University Complutense of Madrid).
2002-2003	Honorific Contributor, Department of Compared Anatomy and Morphology, Faculty of Veterinary Medicine (University Complutense of Madrid).
1997-2000.	Cofounder and President of the Veterinary Association for the Assistance of Exotic Pets and Wildlife (AVAFES-Madrid).

Selected Peer Reviewed Publications (four last years):

- 2015. Javier Millán, José Vicente López-Bao, Emilio J. García, Álvaro Oleaga, Luis Llaneza, Vicente Palacios, Alejandro Rodríguez, Ana de la Torre, Edward J. Dubovi, Fernando Esperón. Patterns of exposure of Iberian wolves (*Canis lupus*) to canine viruses in human-dominated landscapes. Ecohealth. In press. ECH-15-0042.
- 2015. Millán J., López-Roig, M., Delicado, V., Serra-Cobo, J., Esperón F. 2014. Widespread infection with hemotropic mycoplasmas in bats in Spain, including a hemoplasma highly related to *Candidatus Mycoplasma hemohominis*. Comp. Immunol. Microbiol. Infect. Dis.; 39:9-12.
- 2014. Esperón F., Vázquez, B., Sánchez, A., Fernández-Pinero, J., Yuste, M., Neves, E., Nogal, V., and Maria Jesus Muñoz. 2014. *Seroprevalence of paramyxoviruses in synanthropic and semi-free range birds*. Avian Diseases 58(2):306-8.

- 2014. Sacristán C, Esperón F, Herrera-León S, Iglesias I, Neves E, Nogal V, Muñoz MJ, de la Torre A. *Virulence genes, antibiotic resistance and integrons in Escherichia coli strains isolated from synanthropic birds from Spain*. Avian Pathol. 2014;43(2):172-5.
- 2013. Cabello J, **Esperón F**, Napolitano C, Hidalgo E, Dávila JA, Millan J. (2013). *Molecular identification of a novel gammaherpesvirus in the endangered Darwin's fox (Lycalopex fulvipes)*. J Gen Virol. 94, 2745–2749.
- 2013. **Esperón F**, Martín MP, Lopes F, Orejas P, Carrero L, Muñoz MJ, Alonso R. 2013. *Gongylonema sp. infection in the scops owl (Otus scops)*. Parasitology International, 62:502-504.
- 2013. Rubio-Guerri, C.; Melero, M.; **Esperón, F.**; Bellière, E.N.; Arbelo, M; Crespo, J.L.; Sierra, E.; García-Párraga, D.; Sánchez-Vizcaíno, J.M. 2013. *Unusual striped dolphin mass mortality episode related to cetacean morbillivirus in the Spanish Mediterranean sea*. BMC Veterinary Research 2013, 9:106.
- 2013. Rubio-Guerri, C.; Melero, M.; Rivera-Arroyo, B.; Bellière, E.N.; Crespo, J.L.; García-Párraga, D.; **Esperón, F.**; Sánchez-Vizcaíno, J.M. *Simultaneous diagnosis of Cetacean morbillivirus infection in dolphins stranded in the Spanish Mediterranean sea in 2011 using a novel Universal Probe Library (UPL) RT-PCR assay*. Veterinary Microbiology, 165: 109-114.
- 2013. Carballo, M. , **Esperón, F.** , Sacristán, C. , González, M. , Vázquez, B. , Aguayo, S. and Torre, A. *Occurrence of tetracycline residues and antimicrobial resistance in gram negative bacteria isolates from cattle farms in Spain*. Advances in Bioscience and Biotechnology, 4: 295-303.
- 2012. Arbelo, M.; Bellière, E.N.; Sierra, E.; Sacchinni, S.; **Esperón, F.**; Andrada, M.; Rivero, M.; Diaz-Delgado, J.; Fernández, A. *Herpes virus infection associated with interstitial nephritis in a beaked whale (Mesoplodon densirostris)*. BMC Veterinary Research 13;8:243.
- 2012. **Esperón, F.**; García-Párraga, D.; Bellière, E.N.; Sánchez-Vizcaíno, J.M. *Molecular diagnosis of lobomycosis-like disease in a bottlenose dolphin in captivity*. Medical Mycology, 50(1):106-9.
- 2011. Bellière, E.N.; **Esperón, F.**; Sánchez-Vizcaíno, J.M. *Genetic comparison among dolphin morbillivirus in the 1990-1992 and 2006-2008 Mediterranean outbreaks*. Infection and Genetics Evolution, 11(8):1913-20.
- 2011. Bellière, E.N.; **Esperón, F.**; Fernández, A.; Arbelo, M.; Muñoz, M.J.; Sánchez-Vizcaíno J.M. *Phylogenetic analysis of a new Cetacean morbillivirus from a short-finned pilot whale stranded in the Canary Islands*. Research in Veterinary Science, 90: 324-328

Other relevant facts.

- Edwige Nina Bellière. “Advances on cetacean virology: molecular characterization of new sequences of Morbilliviruses and Herpesviruses”. PhD thesis. Directors: J.M. Sánchez-Vizcaíno and F. Esperón. October, 2012.
- Esperón F, Bellière E, Sánchez-Vizcaíno J (2010) Chapter 4: New perspectives on the Cetacean morbillivirus epidemiology and taxonomy. In: Pearce AG, Correa LM (eds) Dolphins: Anatomy, Behavior and Threats (Animal Science, Issues and Professions). Nova Science Publishers, Inc., p 107-128.



Tanzania Wildlife Research Institute

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TWIRI/RS-171/VOL.IV/90/52

Our Ref:

Your Ref:

To the Morris Animal Foundation,

28th November, 2014

Date:

RE: LETTER OF SUPPORT FOR THE PROJECT A FIRST PHYLOGENETIC AND SPATIAL ANALYSIS OF PARVOVIRUS STRAINS CIRCULATING IN THE SERENGETI MAASAI MARA ECOSYSTEM.

The Tanzanian Wildlife Research Institute (TAWIRI) is aware of the project entitled, "*A first phylogenetic and spatial analysis of parvovirus strains circulating in the Serengeti Maasai Mara ecosystem*".

The Serengeti Maasai Mara ecosystem is a protected area of international importance within which African carnivores play a pivotal role. As a consequence their conservation is of great value. However the influence of human settlements and the transmission of infectious diseases from domestic to wild species are principal threats for wild carnivore populations and the mitigation of these impacts will be key to their conservation.

Current mass dog vaccination programmes are being undertaken in this environment with parvovirus one of the vaccines used. However, there have been no studies in the Serengeti Maasai Mara ecosystem about the epidemiology of this pathogen, resulting in a knowledge gap regarding the strains of the virus that are circulating, the susceptibility of carnivore species to infection, and the broader impact that vaccination of domestic dogs against parvovirus has on wild carnivores.

Given these issues, TAWIRI supports this project's application to the Morris Animal Foundation Grants in 2014 to seek funding to support this important work.

Yours sincerely,

TANZANIA WILDLIFE RESEARCH INSTITUTE

Dr. Angela Mwakatobe
FOR: DIRECTOR GENERAL

TAWIRI is responsible for the co-ordination of all wildlife research in Tanzania

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(NM-AIST)**

School of Life Sciences and Bioengineering

P.O. Box 447, Arusha, Tanzania

E-mail: Joram.buza@nm-aist.ac.tz



20th November, 2014

The Morris Animal Foundation,

Dear Sir/Madam,

Re: Letter of support for the project A first phylogenetic and spatial analysis of parvovirus strains circulating in the Serengeti Maasai Mara ecosystem

The Nelson Mandela African Institution of Science and Technology (NMAIST) is aware of the project entitled A first phylogenetic and spatial analysis of parvovirus strains circulating in the Serengeti Maasai Mara ecosystem.

The Serengeti Maasai Mara ecosystem is a protected area of international importance within which African carnivores play a pivotal role. As a consequence, their conservation is of great value. However the influence of human settlements and the transmission of infectious diseases from domestic to wild species are principal threats for wild carnivore populations and the mitigation of these impacts will be key to their conservation.

Current mass dog vaccination programmes are being undertaken in this environment with parvovirus one of the vaccines used. However, there have been no studies in the Serengeti Maasai Mara ecosystem about the epidemiology of this pathogen, resulting in a knowledge gap regarding the strains of the virus that are circulating, the susceptibility of carnivore species to infection, and the broader impact that vaccination of domestic dogs against parvovirus has on wild carnivores.

Given these issues, NMAIST *REQUESTS* to the Morris Animal Foundation Grants in 2014 to seek funding to support this important work. If the funding application is successful, and the study proceeds, we expect that NMAIST students will be able to collaborate on, and benefit from, the project.

Yours sincerely,

Prof. Joram Buza, Dean of the School of Life Sciences



18th November 2014

Project Title:

A first phylogenetic and spatial analysis of parvovirus strains circulating in the Serengeti Maasai Mara ecosystem

To Whom It May Concern:

This is a letter of support for the above project. Over the past 20 years research work that colleagues and I have conducted in the Serengeti ecosystem has resulted in a functional research network being established and many biological samples, currently stored in a frozen archive in Tanzania, being collected.

We are happy to offer this project the support of our Tanzanian infrastructure to collect new samples and to grant the PI access to our archive as we feel that the project's objectives will be of value to disease surveillance efforts in the ecosystem. Moreover the samples within our bank are there to be used and this project provides an excellent opportunity to answer some fundamental questions about the ecology of parvovirus. We therefore give our full support to the project and hope that the funding application is successful.

Yours truly,

Prof. Sarah Cleaveland

Nelson Mandela African Institution of Science & Technology
Arusha
Tanzania

And

Boyd Orr Centre for Population and Ecosystem Health
Institute of Biodiversity
Animal Health & Comparative Medicine
University of Glasgow
Glasgow G12 8QQ
UK
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Veterinary Student Scholar

Assessing admixture between historically isolated mitochondrial DNA lineages in Bornean and Sumatran orang-utans (*Pongo* spp.) in North American zoos

Alyssa Karklus

University of Wisconsin School of Veterinary Medicine

Class of 2020

with

Dr. Graham L. Banes, Assistant Professor, Department of Surgical Sciences, SVM
Director, Molecular Ecology and Evolution Laboratory at Henry Vilas Zoo

and

Dr. Kurt Sladky, Clinical Associate Professor, Department of Surgical Sciences, SVM

Background and Significance

Orang-utans include two species found on the islands of Borneo (*Pongo pygmaeus*) and Sumatra (*P. abelii*), which diverged around 400,000 years ago (Locke *et al.*, 2011). The Bornean orang-utan is further divided into multiple geographically and reproductively isolated subpopulations, with three subspecies currently recognized as having shared a common ancestor around 176,000 years ago. Fewer than 15,000 orang-utans are thought to survive on Sumatra (Wich *et al.*, 2016), while the population of Bornean orang-utans, currently numbering 105,000 individuals, is projected to decline by more than 57,000 before the year 2025 (Ancrenaz *et al.*, 2016).

In the face of declining wild populations, the need to conserve healthy and genetically viable populations of orang-utans in zoos is becoming increasingly important. Many zoos across North America participate in captive breeding programs with the aims to maintain healthy captive populations and to contribute to the conservation of wild populations. Typically, such *ex-situ* conservation involves interbreeding the least related individuals, thus reducing the risk of “inbreeding depression” and augmenting genetic diversity (Ballou and Lacy, 1995). However, the opposite phenomenon, “outbreeding depression,” may have potentially negative effects on a population's overall fitness. Hybridization of distinct populations or subspecies, particularly if they occupy different habitat types or have been isolated for more than 500 years, has been linked to equal or greater detriments than inbreeding depression, including developmental, genetic, and other abnormalities (Banes *et al.*, 2016).

Orang-utans are at particular risk of outbreeding depression, both in the wild and in zoos. In an effort to support dwindling wild populations, sanctuaries of orphaned and displaced orang-utans have been established in Borneo and Sumatra, with the ultimate goal of reintroducing individual orang-utans to the wild. Genetic analyses have determined that certain reintroductions from these sanctuaries have resulted in hybridized and introgressed offspring. Notably, some of the hybridized and introgressed individuals have exhibited poor reproductive success and overall ill health; however, the full effects of the admixture remain unclear (Banes *et al.*, 2016). Orang-utans in captivity may represent a similar situation. In the 1920s, orang-utans were collected for exhibition in zoos from across the species' natural range, and therefore may have been indiscriminately hybridized through captive breeding (Elder, 2016). However, the extent of hybridization and introgression among Bornean orang-utans in zoos is yet to be fully characterized. This information is an essential precursor to investigating the effects of such introgression on the health and viability of this critically endangered population. If interbreeding distinct orang-utan subspecies can be linked to reduced fitness or reproductive success, it may be necessary to develop new and independent breeding programs for orang-utans in zoos worldwide, in order to preserve the health of both captive and wild populations. Conversely, if no ill effects are observed as a result of hybridization, this could simplify the procedure for reintroducing more than 1,500 orphaned and displaced orang-utans housed in rehabilitation centers on Borneo and Sumatra (Banes *et al.*, 2016).

Aims and Hypothesis

I propose to determine the ancestral maternal origins of all 219 orang-utans in the North American zoo population, housed in 53 institutions in the United States, Canada, and Mexico. I will generate mitochondrial DNA sequences from each matriline and compare these with published sequences from orang-utans of known geographic origin. By inferring the subspecies or subpopulation of wild-caught founder individuals, I will assess the extent of hybridization and introgression in the captive orang-utan population over the last century. I hypothesize that orang-utans in North American zoos will derive ancestrally from all three recognized subspecies and from all recognized genetically distinct populations.

Brief Description of Approaches and Methodologies

Samples from all orang-utans in North American zoos have been collected by my faculty advisor and are housed in the Molecular Ecology and Evolution Laboratory at Henry Vilas Zoo, where I will be based for the duration of the project. I will extract genomic DNA from these samples and design a protocol to amplify the complete mitochondrial DNA control region from at least one orang-utan of each matriline, using the Polymerase Chain Reaction (PCR) (Banes and Galdikas, 2016). The results of the PCR will be visualized on agarose gels and the PCR products will be prepared for sequencing. Under the direction of my faculty advisor, I will become familiar with evaluating the quality of DNA sequences via chromatograms, and using this information, inferring and

collapsing haplotypes. Then, by aligning haplotypes and inferring Bayesian phylogenetic trees, I will assign DNA sequences to populations of known origin.

Complete kinship and pedigree data for orang-utans is available in the form of the International Orangutan Studbook (Elder, 2016) and the *Pongo* database maintained by Dr Banes. By aligning my newly generated sequences with those from orang-utans of known geographic origin, I will identify the ancestral origins of orang-utans in North American zoos. Using *Pongo* and the studbook, I will trace the extent of hybridization and introgression over the past century. This information could serve as preliminary data to influence captive breeding programs and to assess the potential for outbreeding depression within captive orang-utan populations.

Role of the Student and Qualifications of the Mentor

During the duration of this project, I anticipate developing skills in many key areas of biological research. The laboratory work will provide basic transferable research skills in molecular genetics. I will develop experience in the operation and maintenance of a genetics laboratory and become familiar with biosafety levels and working with non-human primate samples. Moreover, I will gain familiarity with the cost of laboratory research and genetic analysis and learn how to manage a research budget. I will also gain a greater understanding of research ethics and procedures, and develop skills in scientific writing. In particular, I will co-author a manuscript for submission to a peer-reviewed journal, and will assist in writing grants to expand this study in future. As my study will likely result in pilot data that can be used to prepare a larger, full-genome-wide research program, I will be involved in determining future directions for longer-term study.

My primary mentor, Dr. Graham Banes, is the director of the Molecular Ecology and Evolution Laboratory at the Henry Vilas Zoo in Madison, WI, as well as an Assistant Professor at the University of Wisconsin School of Veterinary Medicine (SVM). The Zoo is located within 2 miles of the SVM. His research focuses on the use of molecular genetics to the study and conservation of wildlife, including orang-utans. He currently leads *The Orangutan Conservation Genetics Project*, a partner program of the World Association of Zoos and Aquariums, and curates one of the largest biomaterials collections for any critically endangered mammal: more than 3,000 samples from wild, zoo-housed and ex-captive orang-utans were collected over 7 years.

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February 28, 2017

Morris Animal Foundation
Suite 17a
720 S. Colorado Blvd.
Denver, CO 80246

RE: Application of Ms. Alyssa Karklus for MAF Student Scholar Award

The Research Committee has identified Ms. Alyssa Karklus as the 2017 University of Wisconsin nominee for an MAF Student Scholar Award. This letter is to confirm that Ms. Karklus is in good academic standing in the veterinary curriculum, has identified excellent mentors in Dr. Graham L. Banes and Dr. Kurt Sladky, and that a letter of support for Ms. Karklus' application authored by Drs. Banes and Sladky is included with the application materials.

Little or no information is available regarding the genetic diversity of orang-utans, and wild populations of orang-utans are continuing to decrease in numbers. Ms. Karklus' project will take advantage of the unique availability of DNA samples from the entire population of orang-utans in North America zoos to generate novel data that will be extremely valuable in the ongoing effort to sustain orang-utans in the wild and zoos. The Henry Vilas Zoo is in close proximity to the School of Veterinary Medicine, and Dr. Banes and Dr. Sladky have an excellent collaborative relationship that will support Alyssa's training and completion of the proposed research.

If further information is needed to consider her application, please do not hesitate to contact me.

Sincerely,

Dale E. Bjorling, DVM, MS
Associate Dean for Research and Graduate Training

School of Veterinary Medicine • Office of Research & Graduate Training

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advancing animal and human health with science and compassion



February 17, 2017

Morris Animal Foundation
Veterinary Student Scholars
10200 East Girard Ave., Suite B430
Denver, CO 80231

Dear Morris Animal Foundation Veterinary Student Scholar Program,

We are writing to enthusiastically support Alyssa Karklus' application for the Morris Animal Foundation Veterinary Student Scholar Program. Alyssa is a first-year veterinary student at the University of Wisconsin School of Veterinary Medicine with an interest in pursuing a career in Zoological Medicine. She has several unique experiences working with zoo and wildlife species. In her proposed research project, Alyssa will determine the ancestral maternal origins of orang-utans in the North American zoo population. The results of her study will serve as preliminary data for future research on the potential for outbreeding depression within captive orang-utan populations, and may influence orang-utan pairings within captive breeding programs. During her project, Dr. Graham Banes will serve as Alyssa's primary mentor and Dr. Kurt Sladky will serve as her secondary mentor. For Alyssa, this project will provide excellent laboratory training in molecular genetics and experience collecting and analyzing biological data; developing manuscript preparation and grant writing skills; working with primate biological samples and gaining an appreciation for the benefits of, and ethics associated with, endangered species research; and learning to manage a research project budget.

We have read her proposal and agree to supervise the project as described. We understand that we are responsible for providing any remaining funding for the project, and we assure the Morris Animal Foundation that these funds are available. Thank you very much for your consideration, and please do not hesitate to contact us with any further questions or concerns.

Kurt K. Sladky, MS, DVM

Dr Graham L Banes, PhD

Morris Animal Foundation
Animal Involvement Justification

(From the proposal guidelines, single-spaced, no page limit)

Morris Animal Foundation (MAF) is dedicated to funding scientifically sound, relevant and humane studies that specifically address the health and well-being of animals. All studies receiving funding must follow MAF's Health Study Policy for Animals Involved in Research (adopted October 18, 2008), which was written to ensure that each and every animal involved in a MAF funded health study receives excellent, compassionate care throughout the study. MAF shall not fund health studies which require euthanasia as an endpoint or the induction of disease or injury, unless the nature of the disease or condition to be studied is of such significance for improving animal health that such means are justified, and that meaningful information can be obtained in no other way. Furthermore, MAF will not fund any study that induces or allows pain or distress unless such pain or distress can be controlled by appropriate anesthetic, analgesic, tranquilizing drugs, or nursing care. [Click here](#) for the full Health Study Policy.

A. If this study does not involve live animals please indicate here by N/A: N/A

B. Does this study involve biological samples, tissues, etc.? Yes

If yes, describe in detail what samples will be used and where & how they will be (or were) acquired. Note: Morris Animal Foundation reserves the right to request a copy of the Institutional Animal Care and Use Committee (IACUC) application/approval and other relevant applications/approvals (e.g., wildlife permit) covering the original collection of samples, including archived samples. MAF reserves the right to request IACUC (or equivalent) review and approval for any Foundation study ***regardless of the Institution's requirements***. This would include the use of archived samples as well as clinical trials.

Genetic samples, comprising blood products, feces, shed hair, and oral (saliva) swabs, were collected over a seven-year period from 53 accredited zoological institutions in the United States. All samples were collected non-invasively or during routine medical procedures with ethics approval from each institution's Animal Care and Use committees. Sample collection was also approved by letter of recommendation from the Orangutan Species Survival Plan, and conformed to the additional ethical principles of both the American Society of Primatologists and the International Primatological Society.

C. If this study involves live animals, succinctly address the following: (please restate the questions and directives). This study does not involve live animals, only biological samples.

1. What species will be studied?

2. State the status of your IACUC application/approval. All recipients of MAF funding will be required to submit the entire IACUC protocol and document. A copy of the IACUC approval should not be included with the application, but it is required before funding can be awarded.

3. List the USDA category for pain and distress (B, C, D, E):

Note: Any study beyond category C will require review by MAF's Animal Welfare Advisory Board (AWAB). In general MAF does not fund studies beyond category C (category D studies will only be considered if they conform with MAF's Health Study Policy, category E studies will not be considered).

4. Does this proposal involve client-owned animals?

If yes, the protocol for client-owned animals must be approved by the appropriate peer review committee before the project is funded. *If this proposal involves client-owned animals, an informed client consent form must be submitted with this proposal. For a suggested list of items to be considered in an informed client consent form, [click here](#).*

5. Explain how animals will be acquired (e.g., client-owned, USDA licensed breeder, institutional "herds" or "colonies") and verify that the animals are suitable for the study (e.g., have no physiologic, physical or

pharmacologic issues that would interfere with results)

6. How many animals will be used?

a. Summarize numerical justification

7. Does this study induce disease, injury, pain or distress in animals?

Note: any study requiring the induction of disease, injury, pain, or distress will have an additional evaluation by MAF's AWAB.

If yes,

a. Defend the necessity of experimental design

b. Explain how pain and/or distress will be controlled

c. Justify that no alternative, including clinical studies, can be used to accomplish study objectives and the disease/condition to be studied is of such significance for improving the health of the species.

8. Explain the environment and housing conditions (quality of life) in which the animals will live (address species-appropriate exercise, enrichment, socialization, veterinary care, etc.)

9. What will happen to the animals upon completion of the study?

If adoption, explain the adoption process. Provide assurance that whenever possible and when in the animal's best interest, investigators shall make companion animals available for adoption at the end of the study or return the animals to the owner/responsible agency in an environment that promotes animal welfare and excellent quality of life.

10. If euthanasia, provide the following additional information (note: any study requiring euthanasia as an endpoint will have an additional evaluation by a MAF's AWAB.

i. Total number that will be euthanized and justification for numbers

ii. Method of euthanasia

iii. Justification that no alternatives can be used to accomplish study goal(s) and that the disease/condition to be studied is of such significance for improving the health of the species that a terminal endpoint is deemed necessary.

iv. Reason for euthanasia in lay language (this wording may be shared with staff, donors and media)

v. Provide objective criteria for determining when euthanasia is appropriate or necessary (note: Morris Animal Foundation wants assurance that an animal will not be allowed to suffer and that monitoring for pain and suffering is adequate)

Note: Morris Animal Foundation does not consider the use of CO2 alone to be an appropriate method of euthanasia

Please note:

1. If an animal is used in an invasive study, MAF may require that a guarantee is provided, through principal investigator and institutional signatures that the animal will not participate in any future invasive study or procedure
2. MAF does not allow inclusion of ancillary data in MAF funded research that includes animal use protocols not in agreement with our Health Study Policy, even if it is obtained using other funding sources.
3. Morris Animal Foundation considers euthanasia acceptable when an animal develops unanticipated illness or injury that results in pain and suffering that cannot be alleviated with standard veterinary interventions.