

University of Missouri South African Education Program

Proposal Application Form

Name/Title: Use of South African Native Plant Extracts to Alleviate Heat Stress

Department/Campus: Animal Science

Campus Address: 159 Animal Science Research Center

Telephone: (573) 882-8234

Fax: (573) 884-7827

E-mail: lambersonw@missouri.edu

I am applying for:

☒ I. The UM/UWC Linkage Program

☐ II. The South African Partnerships

Program Proposal Abstract (Include a brief statement of the problem or need being addressed, the intended outcomes/objectives of the project, the project methodology, and the project timeline.):

Heat stress is of critical importance in human health as well as in economic losses to livestock producers. Our previous research has shown that providing a tea of *Artemisia afra*, an herb reported by indigenous South Africans to ameliorate hyperthermia, is effective in preventing detrimental effects of short-term (24 hr) heat stress on fertility of male mice. Our hypothesis in the current study is that phenotypic effects of longer term heat stress in mice and pigs may be alleviated by treatment with extracts of *Artemisia afra* and other South African native herbs reported to ameliorate hyperthermia. While in Africa conducting the first phase of the studies described in the present proposal, I will work with Drs. David Fisher and Jeremy Klassen to identify other plants that may have similar activity. In addition, we will attempt to identify other methods of delivery that have been used by indigenous peoples, as well as modern methods of extraction of active compounds (Dr. Klassen's expertise). Two animal studies will subsequently be conducted in the proposed project. First, we will expand our research on potentially important effects of *Artemisia* on activity during heat stress by exposing male mice to a longer period of heat stress. Mice in this study (n = 24) will again be implanted with core body temperature transmitters, and they will have access to activity wheels equipped with counters that quantify voluntary activity during five days of heat stress. As a second thrust, to initiate the next phase in the translational research process toward potential livestock and human applications, we will initiate studies with boars. Six mature boars will be assigned in a crossover design to be exposed to high environmental temperatures while receiving 1) no treatment/drinking water only; 2) *Artemisia* as a tea instead of drinking water; or 3) *Artemisia* as a feed supplement (30 g twice per day with feed). The boars will have semen serially collected twice per week for microscopic semen quality evaluation. Based on our previous work, we expect to identify methods of *Artemisia* treatment to mitigate heat stress effects on livestock reproduction, as well as identifying the potential for *Artemisia* to increase activity

during heat stress of mice. The latter may have applications for humans required to work under high temperature.

Project Timeline

October, 2009 – Preparation Phase: Procure young boars from the MU Swine Research Complex.

February, 2010 – Identification Phase: Travel to UWC to identify herbs from the collection of native plants as potential effective agents against heat stress effects

May – July, 2010 – Experimentation Phase: Experiments will be conducted on mice and boars.

August - December 2010 – Dissemination Phase: Analysis and manuscript preparation will be completed.

Proposed budget, including matching funding (use attached budget form): Amount

requested: \$ 10,000 Matching: \$ 20,918 TOTAL: \$ 30,918

Attach a detailed narrative proposal (maximum of 10 pp.) and a detailed budget.

Signature of Applicant: William J. Hunter

Date: July 29, 2009

Signature of department chair and/or dean: Rodney Hunt

Date: 7-30-09

Please attach:

- 1) your curriculum vitae,
- 2) a letter of endorsement from your chair and/or dean, and
- 3) a letter of commitment from your South African collaborator.

Budget Summary

Item†	Amount Requested from UMSAEP	Amount funded by other sources‡	Total
1. Air fare	\$ 2,000	\$	\$ 2,000
2. Room/board	\$ 1,400	\$	\$ 1,400
3. Ground transportation including car rental	\$ 1,200	\$	\$ 1,200
4. Personnel	\$	\$ 18,150	\$ 18,150
5. Materials/printing	\$ 400	\$ 400	\$ 800
6. Communications	\$	\$	\$
7. Other expenses	\$ 5,000	\$ 2,368	\$ 7,368
Total	\$ 10,000	\$ 20,918	\$ 30,918

Use of South African Native Plant Extracts to Alleviate Heat Stress

Collaborators: Dr. David Fisher, Faculty of Science, Univ. Western Cape
Dr. William Lamberson, Division of Animal Sciences, Univ. Missouri

Issues and Needs:

Heat stress is of critical importance in human health as well as in economic losses to livestock producers. In the past 10 years there have been 25 deaths attributed to heat stroke among football players in the U.S. More than 5,000 U.S. Army personnel were hospitalized, and 37 died due to heat illness between 1980 and 2002¹. An estimated 371 elderly die of heat-related illness annually in the U.S., with more than 1400 deaths attributed to heat-related illness among the elderly in the New York City metropolitan area alone during the 1990's². Heat stress also has large impacts on agricultural income. Economic losses in the U.S. from heat stress causing decreased reproduction in livestock average \$543 million, \$40 million, and \$51 million annually for dairy, beef, and swine, respectively. Missouri producers lose approximately \$17 million, \$2 million, and \$5 million in dairy, beef, and swine production, respectively, due to heat-related infertility.

Specific allelic variants influenced resistance to heat stress in studies from our laboratory³. We have also shown that the heritability of fertility after heat stress is low (0.14 in mice)⁴. The low heritability indicates that nongenetic factors which may respond to therapeutic agents control the majority of the variation in fertility after heat stress. Previous studies in our laboratory have revealed beneficial effects of providing "tea" made with *Artemisia afra* and the related plant, *Artemisia absinthium*, for preventing heat stress induced infertility in male mice. Mice presented with the *Artemisia* teas were also more active than mice drinking only tap water during periods of heat stress.

These results suggest that *Artemisia afra* and possibly other plants have potential for use in improving fertility of heat-stressed livestock. There may also be potential for it to ameliorate the effect of stress on humans engaged in physical activity during high temperatures.

Overall and Specific Objectives:

Artemisia afra is an herb reported by indigenous South Africans to ameliorate hyperthermia. Our previous research has shown that providing a tea of *Artemisia afra* is effective in preventing

detrimental effects of short-term (24 hr) heat stress on fertility of male mice. Our hypothesis is that phenotypic effects of longer term heat stress (and associated patterns of gene expression) in mice and pigs may be alleviated by treatment with extracts of *Artemisia afra* and other herbs anecdotally reported to ameliorate hyperthermia among indigenous South Africans. The specific objectives are to: 1) identify additional herbs associated ameliorating effects of hyperthermia from collections at the University of Western Cape, identify possible alternative methods of delivery, and possible methods of extraction of active compounds; 2) conduct animal experiments at the University of Missouri to determine if treatment with herbal extracts reduce the phenotypic effects of heat stress on activity of mice measured over a five day period; and 3) determine whether treatment with herbal extracts improves semen quality of boars exposed to high environmental temperatures in chambers in the Brody Environmental Center in the Animal Sciences Research Center at the University of Missouri.

Project Methodology:

Summary: In previous research we screened botanical collections in the Western Cape for plants thought to have therapeutic value in alleviating the effects of hyperthermia. We identified four species, *Sutherlandia frutescens* (S), *Tulbaghia violacea* (T), *Helichrysum* (H) and *Artemisia afra*. *Artemisia afra*, when presented as a 1% tea, was found to be largely effective in alleviating short term heat stress-induced infertility in male mice. An earlier animal study conducted in our laboratory animal study showed that *Artemisia afra* was not palatable to mice when presented as a ground plant mixed with ground mouse chow (it is quite bitter). It was readily accepted as a tea prepared 1% weight to volume, boiled for five minutes and then filtered. In fact, consumption of the tea was greater than consumption of tap water prior to and during heat stress in the second 24 hour experiment. Unfortunately, due to constraints resulting from biosecurity of swine units as well as difficulty in delivery through automatic watering systems, it is unlikely that teas would be a practical delivery method for pigs under commercial conditions. A dry feed additive is likely to be preferred. While in Africa working on the studies described in the present proposal, I will work with Drs. David Fisher and Jeremy Klassen to identify other plants that may have similar activity. In addition, we will attempt to identify other methods of delivery both that have been used by indigenous South Africans, as well as modern methods of extraction of active compounds Dr. Klassen's expertise.

In a second 24 hr. study conducted earlier this year, the effect of *Artemisia afra* and the related plant *Artemisia absinthium* on improving fertility of heat stressed males was confirmed. Mice in the second study were implanted with core body temperature transmitters that also tracked activity. Activity was measured by a qualitative recording of any change in signal strength from the transmitter which indicated that the transmitter has moved. Although core body temperatures were not different between heat stressed mice receiving *Artemisia afra* or the related plant *Artemisia absinthium* and those receiving tap water, activity of mice receiving either of the *Artemisias* was greater during heat stress than control mice.

Two animal studies will be conducted in the present project. First, we will follow up on potentially important effects of *Artemisia* on activity during heat stress by exposing male ICR strain mice to a longer period of heat stress. Mice in this study (n = 24) will again be implanted with core body temperature transmitters, and they will have access to activity wheels equipped with counters which quantify voluntary activity. A baseline measurement of activity will be made under thermoneutral conditions for five days (21°C), then mice will be given 1% tea made from *Artemisia afra* or *Artemisia absinthium* (or an alternative herb identified during screenings of botanical collections in South Africa in February, 2010) or be given tap water for five days before initiation of and during heat treatment (35°C; n = 8 per group). All mice will then be returned to thermoneutral conditions for ten days before switching mice among treatments and repeating the heat regimen. Dependent variables will include core body temperature, feed consumption, water consumption, time on the activity wheel and total activity (revolutions of the activity wheel).

As a second thrust, we will initiate studies with boars. There has been great interest from livestock producers in our preliminary results due to high economic losses they suffer as a result of reduced reproductive success of swine and dairy cattle during periods of hot summer temperatures. In this preliminary study, we will make use of environmental chambers in the Brody Environmental Center in the Animal Sciences Research Center at the University of Missouri. Six mature boars will be assigned in a crossover design to be exposed to high environmental temperatures while receiving no treatment, *Artemisia* as a tea in place of drinking water, or *Artemisia* as a feed supplement (30 g twice per day with feed). The boars will have semen serially collected twice per week for visual semen quality evaluation. Measurements will include an evaluation of progressive motility and 100 cells will be classified as having normal

morphology, a cytoplasmic droplet, or a noncytoplasmic droplet related abnormality. Rectal temperature will be measured with a thermistor thermometer, respiration rate will be calculated by counting breaths per minute (bpm), and skin temperature on the ear, rump and testes will be taken with an infrared temperature gun.

Project Timeline

October, 2009 - Procure young boars from the MU Swine Research Complex.

February, 2010 - Travel to UWC to identify herbs from the collection of native plants that are candidates for alleviation of the effects of heat stress.

May – July, 2010 - Animal experiments will be conducted.

August - December 2010 - Manuscripts will be prepared.

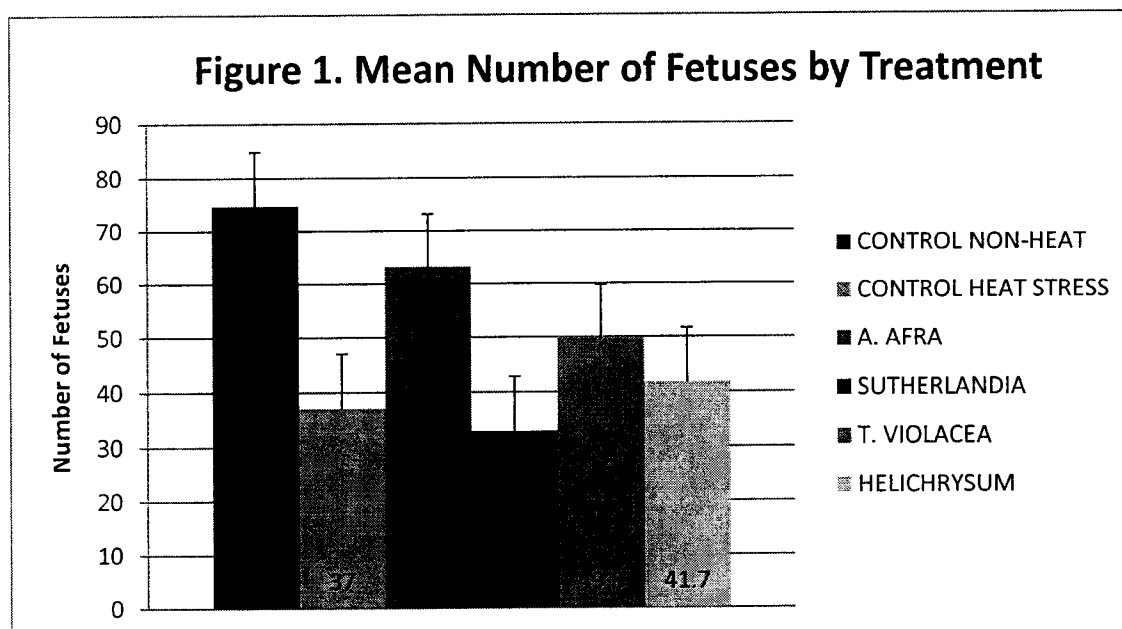
Background and rationale:

Reproductive performance is reduced in bulls, rams and boars exposed to high ambient temperatures through compromised spermatogenesis^{5,6,7} and semen quality^{5,6,7,8,9,10,11}, including decreased sperm output and motility^{12,13} and increased percentage of abnormal spermatozoa^{13,14}. Similar disruptions in spermatogenesis have also been observed in rats¹⁵ and mice¹⁶. Losses in fertility do not occur immediately following heat exposure (0 – 5d), implying that the effect of the stress is not directly detrimental to spermatozoa¹⁶, but impacts the developing spermatozoa. Subfertility of heat-stressed male mice occurs approximately d 18 to 28 post-stress, coinciding with the spermatid and spermatocyte stages of sperm development^{16,17}. Histological examination of testes has revealed tubule degradation and apoptotic cells mostly from spermatocyte origin¹⁶. Additionally, seminiferous tubule damage and vacuolation of spermatids has been exhibited in heat stressed bulls, thus identifying spermatids as being the most severely affected stage of spermatogenesis⁷.

Even minimal exposure to increased ambient temperatures can have a damaging effect on male fertility. Bulls exposed to a 40°C environment for twelve hours and male mice maintained at 32°C for 24 hours have shown disruptions of spermatogenesis and decreases in semen quality^{7,17}. While excessive increases in temperature may result in complete infertility, subfertility of males can be produced by less extreme hyperthermia. Male mice exposed to

32.7°C and 36.1°C exhibited fertility rates of ~57% and 0%, respectively, as compared to the 100% fertility of control males maintained at 21°C¹⁸.

Two studies have conducted in our laboratory to determine the effect of treatment of mice with *Artemisia afra* or other herbs on preventing heat stress induced hyperthermia in mice. The figure below shows the number of fetuses produced by males given one of four herbs for five days prior to and the day of a 24 hour period of heat stress. *Artemisia afra* largely alleviated the decrease in fertility associated with heat treatment.



In a subsequent, study prenatal survival was increased by treatment with teas of *Artemisia afra* (AF) or *Artemisia absinthium* (AB) compared to control mice that were exposed to heat, but given only tap water to drink (table 1).

Table 1: Mean percentage prenatal survival by treatment

	Estimate	Percentage
AB	1.0399 ± 0.2180	73.9%
AF	1.2263 ± 0.3518	77.3%
HSC	0.2720 ± 0.2722	56.8%
C	1.4884 ± 0.1689	81.6%

Activity was also measured during the 24 hour period of heat stress in mice in the second study. Unlike heat-stressed controls, mice given AF or AB maintained activity throughout the period of heat stress while activity of heat-stressed control mice dropped precipitously after about 10 hours and remained low for the remainder of the heat stress period.

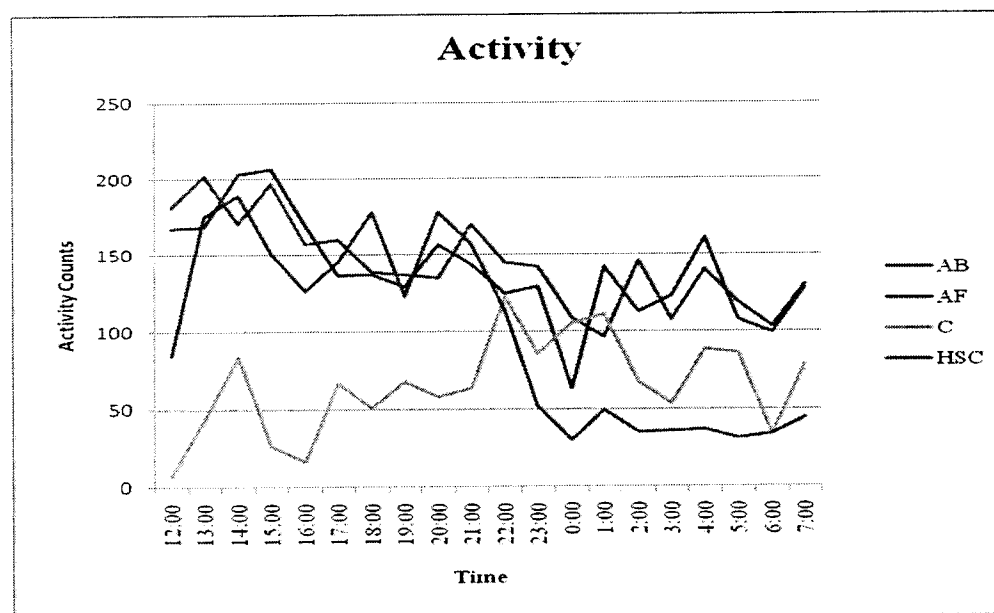


Figure 2. Activity count is a qualitative measurement of any change in signal strength which is used as an indicator that the transmitter has moved. Early in the heat stress period activity was similar among AB, AF, and HSC groups but significantly higher than C group. As time progressed HSC mice became lethargic and the two tea groups (AB and AF) were more active than controls.

Interestingly, these positive effects on activity and fertility were not associated with reductions in core body temperature (table 2). We are currently evaluating gene expression of heat-shock proteins in these mice.

Table 2: Core Body Temperature of Mice During Heat Stress

	N	AB	AF	HSC	C	P value
Body Temp. (°C)	222	39.32 ± .16 ^b	39.58 ± .16 ^b	39.32 ± .20 ^b	36.52 ± .15 ^a	<0.0001

These preliminary results suggest that *Artemisia afra* and possibly other plants have potential to improve fertility of heat-stressed livestock. There may also be potential for it to ameliorate the effect of stress on humans engaged in physical activity during high temperatures.

Project design and procedures:

Dr. Lamberson will initially travel to the University of Western Cape for two weeks to work with Dr. David Fisher and Dr. Jeremy Klassen and study botanical collections to identify additional plants that may have the effect of relieving symptoms of heat stress. An earlier animal study conducted in our laboratory animal study showed that *Artemisia afra* was not palatable to mice when presented as a ground plant mixed with ground mouse chow (it is quite bitter). It was readily accepted as a decoction prepared 1% weight to volume, boiled for five minutes and then filtered. In fact, consumption of the decoction was greater than consumption of tap water prior to and during heat stress in the second 24 hour experiment. Unfortunately, due to constraints resulting from biosecurity of swine units as well as difficulty in delivery through automatic watering systems, it is unlikely that decoctions would be a practical delivery method to pigs in commercial boar studs. A dry feed additive is likely to be preferred. While in Africa working on the studies described in the present proposal, Dr. Lamberson will work with Drs. David Fisher and Jeremy Klassen to identify other plants that may have similar activity. In addition, they will attempt to identify other methods of delivery both that have been used by indigenous South Africans, as well as modern methods of extraction of active compounds Dr. Klassen's expertise.

Upon his return animal experiments will be initiated as follows: Four sets of six ten-week-old male mice (ICR; outbred) will be used for the experiments. Mice in this study will be implanted in the abdominal cavity with a MiniMitter core body temperature transmitter, and they will be given two weeks to recover from surgery before initiation of experiments. A baseline measurement of activity will be made under thermoneutral conditions for five days (21°C), then mice will be given 1% tea made from *Artemisia afra* or *Artemisia absinthium* (or an alternative herb identified during screenings of botanical collections in South Africa in February, 2010) or be given tap water for five days before initiation of and during heat treatment (35°C; n = 8 per group). All mice will then be returned to thermoneutral conditions for ten days before switching mice among treatments and repeating the heat regimen (see timeline below). Dependent

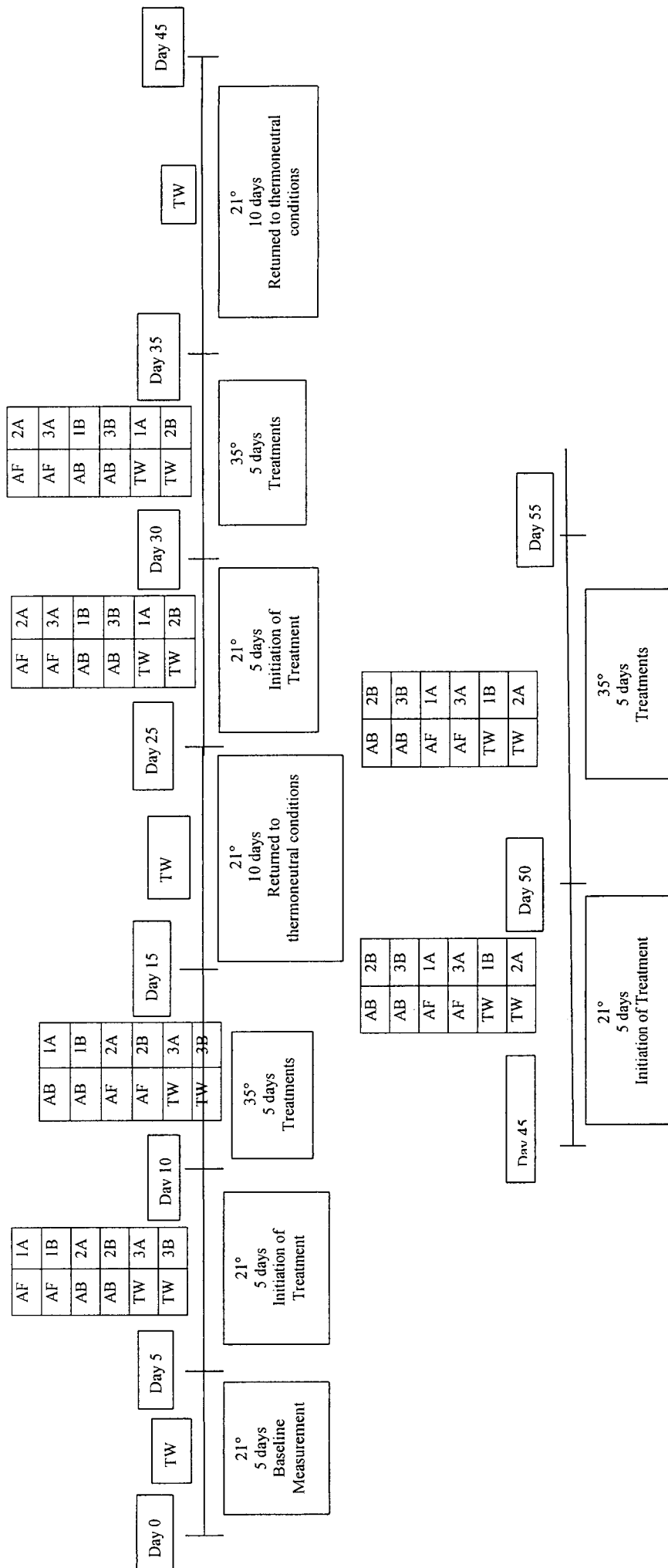
variables will include core body temperature, feed consumption, water consumption, time on the activity wheel and total activity (revolutions of the activity wheel).

Table 3. Experimental timeline

Study Day	1 to 10	10 to 15	16 to 20	21 to 40	41 to 60
Event	acclimatization	herb treatment	herb treatment	switch treatments	switch treatments
Temperature	21	21	35	and repeat	and repeat

Activity measurements will be made as follows: Mice will individually housed in a large mouse cage. Affixed to the top of each cage, but extending down into the cage, is a running wheel which measures 4.5 inches in diameter. The wheel is ~1 inch from the floor of the cage permitting easy access on and off. Attached to the running wheel is a magnet which transmits each wheel revolution to the bicycle computer attached to the roof of the cage. Revolutions are converted to distance traveled as the known wheel perimeter is calibrated into the computer. The distance each mouse runs per 24 hours, the total time spent running, average running speed, and cumulative running distance will be noted each morning between 8 and 9 am. Following notation of running data, the computer will be zeroed to permit another 24 hour collection of data. It takes between 7 and 10 days for mice to reach a consistent daily running distance. Thus, data collection will not begin until mice have acclimated to the wheel for at least 10 days. Running data and core body temperature will be collected daily throughout the trial.

In study using boars, we will use the environmental chambers in the Brody Environmental Center in the Animal Sciences Research Center at the University of Missouri. Six mature boars will be assigned in a crossover design to be exposed to high environmental temperatures while receiving no treatment, *Artemisia* as a tea in place of drinking water, or *Artemisia* as a feed supplement (30 g twice per day with feed). Thermal measurements will be taken following the procedures of Williams¹³. Thermal response measurements will be taken four times each day at 0800, 1200, 1600 and 2000 h. Rectal temperature was measured with a Model 8110-20 thermistor thermometer (Cole-Parmer Instrument Company, Vernon Hills, IL, USA), respiration rate will be measured by counting breaths per minute (bpm), and skin temperature will be taken with an infrared temperature gun (Raytek, Everett, WA, USA). Skin temperature measurements will be taken at three locations (ear, shoulder and testes) where the skin will be marked with a permanent marker and shaved.



The boars will have semen serially collected twice per week for visual semen quality evaluation following the procedures of Lovercamp, et al.²⁰. Measurements will include an evaluation of progressive motility and 100 cells will be classified as having normal morphology, a cytoplasmic droplet, or a noncytoplasmic droplet related abnormality. Boars will be collected into 500 mL styrofoam cups through a 230 mm non-woven filter by the double-glove method. The ejaculates will be extended to include approximately three billion viable cells. One-hundred spermatozoa will be randomly evaluated within 12 h of collection and classified as follows: normal (NORM), possessing a cytoplasmic droplet (CD) in the proximal, distal, or distal midpiece reflex position (PCD, DCD, or DMR, respectively) or any other non-CD related aberration (ABE). Spermatozoa classified as PCD possess a CD located on the anterior half of the sperm tail midpiece proximal to the sperm head. Spermatozoa classified as DCD possess a CD located on the posterior half of the sperm midpiece at the midpiece/principal piece junction of the sperm tail. Spermatozoa classified as ABE include spermatozoa demonstrating atypical shape and size of the head, midpiece and tail. Two additional cumulative variables to be derived from the morphological analysis include a total attached CD group ($TACD = PCD + DCD + DMR$) and a total abnormality group ($TABN = TACD + ABE$). Analyses will be performed by using differential interference contrast optics and an infinity corrected lens (60_ primary magnification and 10_ ocular magnification) of a Nikon Eclipse E800 microscope (Nikon Inc., Melville, NY, USA) equipped with a CoolSnap HQ CCD camera (Roper Scientific, Tucson, AZ, USA) operated by MetaMorph software (Universal Imaging Corp., Downingtown, PA, USA).

Resources and environment: This research will be conducted in the Animal Science Research Center (ASRC) which includes animal and laboratory facilities. Mice will be housed in the small animal facility in the ASRC (four small animal environmental chambers, 26 small animal housing rooms, and two isolation rooms). Boars will be obtained from MU's Swine Research Complex on South Farm and experiments will take place in the ASRC Unit D swine housing facility and Unit C Brody Environmental Center. Rectal temperatures will be measured with a Cole-Parmer Model 8110-20 thermistor thermometer and skin temperatures with a Raytek infrared temperature gun. Twenty-four running wheels to be used to measure mice voluntary activity are available in the ASRC Unit B in collaboration with Dr. M. Brown. Should it be determined that additional measurements of metabolic rate, hormone concentrations or gene

expression need to be made, general laboratory equipment in the ASRC available to the PI includes: indirect calorimeters (for mice), autoclaves, -80°C freezers, centrifuges, incubators, water baths, shakers, speed vac system, Nikon Eclipse 800 Microscope with Cool Snap CCD camera for immunofluorescence evaluations, thermocyclers, and counters. Real time quantitative PCR is currently performed on an Applied Biosystem ABI PRISM® 7700 Sequence Detection Systems available either at the University of Missouri Center for Phytonutrient and Phytochemical Studies or in the Department of Veterinary Pathophysiology, each within 0.5 miles of the ASRC.

Bibliography:

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Budget:

Item	Cost
Personnel	
Graduate student (12 mo)	15,000
Undergraduate student (30 wk x 15 hr x \$7.00)	3,150
Supplies	
Animals (24 mice x \$3)	72
Animal care, males (one male per cage, 24 cages x 60 d x \$1)	1440
Animals (8 boars x \$300)	2,400
ASRC Unit D swine housing (eight boars x 140 d x \$1.50)	1,680
Environmental chambers (15 d x \$35)	525
Mouse surgery supplies and transmitters	675
Consumables for semen collection and evaluation (96 collections x \$6)	576
Travel (travel to UWC and lodging for 2 wk)	
Air Fare	2000
Lodging	1000
Meals	400
Car rental	1200
Publication (one manuscript)	<u>800</u>
Total	\$30,918

We request \$10,000 for travel, lodging, car rental and supplies from the program, the graduate student stipend will be paid from Division of Animal Science funds and remaining research expenses will be paid from salary saving funds from Dr. Lamberson.

Budget Justification:

Dr. Lamberson will devote 15% of his time to the project. His responsibilities will be overall design and oversight of the project. He will be directly involved with animal experiments, data analysis, interpretation of results and manuscript preparation. Dr. Fisher will be primarily involved in assisting with logistics in South Africa and identifying appropriate plant materials as potential alleviators of heat stress. He will also be involved with interpretation of results and preparation of manuscripts. Dr. Brown will supervise mouse activity measurements. Dr. Lamberson will manage the day to day operation of the project, travel to South Africa to identify and collect plant materials. The graduate student will conduct the animal experiments and laboratory procedures, as well as have involvement data analyses, interpretation of results and manuscript preparation. Cost of mice is \$3 per mouse and cost of animal housing and care is \$1 per cage per day. Males are housed individually yielding 1440 cage days. Temperature transmitters will be surgically implanted. Cost of surgery supplies is expected to be about \$75. There is a 25% replacement rate on transmitters which cost \$100 each. Eight pigs from the Swine Research Complex will be raised as boars at a cost of \$300 each. Two extra boars will be boars will developed so that we are assured of having six trained for semen collection. Cost of housing pigs in the ASRC is \$1.50 per day. Cost of using the Unit C environmental chambers is \$35 per day. Cost of consumables for semen collection and evaluation is \$6 per collection.