Animal Care and Use Committee
003 Ed Warren Life Sciences Building | East Carolina University | Greenville NC 27834 – 4354
252-744-2436 office | 252-744-2355 fax

September 7, 2021

Kyle Summers, Ph.D.
Department of Biology, ECU

Dear Dr. Summers:

Your Animal Use Protocol entitled, " Field studies of Peruvian poison frogs " (AUP #D376) was reviewed by this institution's Animal Care and Use Committee on 09/02/2021. We understand the permit cannot be obtained in advanced, therefore please send a copy of the permit once obtained to the IACUC office.
The following action was taken by the Committee:

"Approved as submitted"

****Please contact Aaron Hinkle prior to any hazard use****

A copy of the protocols is enclosed for your laboratory files. Please be reminded that all animal procedures must be conducted as described in the approved Animal Use Protocol. Modifications of these procedures cannot be performed without prior approval of the ACUC. The Animal Welfare Act and Public Health Service Guidelines require the ACUC to suspend activities not in accordance with approved procedures and report such activities to the responsible University Official (Vice Chancellor for Health Sciences or Vice Chancellor for Academic Affairs) and appropriate federal Agencies. **Please ensure that all personnel associated with this protocol have access to this approved copy of the AUP/Amendment and are familiar with its contents.**

Sincerely yours,

Jamie DeWitt, Ph.D.
Vice-Chair, Animal Care and Use Committee

JD/GD

enclosure

EAST CAROLINA UNIVERSITY
ANIMAL USE PROTOCOL (AUP) FORM
LATEST REVISION APRIL, 2017

Project Title:

Field studies of Peruvian poison frogs

	Principal Investigator	Secondary Contact
Name	Kyle Summers	Andrew Rubio
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For IACUC Use Only

AUP #	D376		
New/Renewal	New		
Full Review/Date 8/23/2021	DR/Date		
Approval Date	9/2/2021		
Study Type	Genomic		
Pain/Distress Category	D		
Surgery	Survival	Multiple	
Prolonged Restraint			
Food/Fluid Regulation			
Other			
Hazard Approval/Dates	Rad	IBC	EHS 8/30/2021
OHP Enrollment			
Mandatory Training			
Amendments Approved			

I. Personnel

A. Principal Investigator(s):

Kyle Summers

B. Department(s):

Biology

C. List all personnel (PI's, co-investigators, technicians, students) that will be working with live animals and describe their qualifications and experience with these specific procedures. If people are to be trained, indicate by whom:

Name/Degree/Certification	Position/Role(s)/ Responsibilities in this Project	Required Online IACUC Training (Yes/No)	Relevant Animal Experience/Training (include species, procedures, number of years, etc.)
Kyle Summers, Ph. D.	*Supervisor – will supervise colony maintenance, protocol implementation and record-keeping*	Yes	30 years of experience working with amphibians in the field and lab, especially with frogs of the genus Ranitomeya (20 ears)
Andrew Rubio, M.S.	*Grad student researcher – will maintain frogs in the lab, implement protocols, maintain records, and supervise undergrads (TBD)*	Yes	5 years of experience working with amphibians in the field and lab, especially with frogs of the genus Ranitomeya (last 3 years)
Click here to enter text.	Click here to enter text.	Choose an item.	Click here to enter text.
Click here to enter text.	Click here to enter text.	Choose an item.	Click here to enter text.

II. Regulatory Compliance

A. Non-Technical Summary

Using language a non-scientist would understand, please provide a clear, concise, and sequential description of animal use. Additionally, explain the overall study objectives and benefits of proposed research or teaching activity to the advancement of knowledge, human or animal health, or good of society. (More detailed procedures are requested later in the AUP.)

Do not cut and paste the grant abstract.

Mullerian mimicry is a form of mimicry in which toxic organisms converge and mimic each other's warning signals. The genetic basis of mimicry has been heavily studied, as it provides insight into key

evolutionary processes. An organism that displays an array of color morphs is the mimic poison frog (*Ranitomeya imitator).* The bright and conspicuous colorations of these frogs are meant to alert potential predators that they are chemically defended. Mimic poison frogs have undergone a "mimetic radiation" across different geographic regions of Northern Peru. In each region, this species has evolved to display a different kind of color pattern to mimic other chemically defended frogs in that region. However, the genes underlying color and pattern variation in these poison frogs remain unknown.

This project is aimed on the genomic of color pattern and ecology of *R. imitator* as follows: 1. Collection of toeclips for genetic analyses, 2. Preparation of skin and liver tissues for genetic and genomic analyses, 3. Collection of gut contents for diet analyses, and 4. assessment of territorial behavior.

B. Ethics and Animal Use

B.1. Duplication

Does this study duplicate existing research? No

If yes, why is it necessary? (note: teaching by definition is duplicative)

Click here to enter text.

B.2. Alternatives to the Use of Live Animals

Are there less invasive procedures, other less sentient species, isolated organ preparation, cell or tissue culture, or computer simulation that can be used in place of the live vertebrate species proposed here? No

If yes, please explain why you cannot use these alternatives.

Click here to enter text.

B.3. Consideration of Alternatives to Painful/Distressful Procedures

a. Include a literature search to ensure that alternatives to all procedures that may cause more than momentary or slight pain or distress to the animals have been considered.

1. Please list all of the potentially painful or distressful procedures in the protocol:

Whole tadpoles will be subjected to euthanasia before collecting skin tissue (see below). Toe clips will be removed with surgical scissors, as described below.

2. For the procedures listed above, provide the following information (please do not submit search results but retain them for your records):

Date Search was performed:	7-26-21
Database(s) searched:	Pubmed and Scopus

Time period covered by the search (i.e. 1975-2013):	1980-2021
Search strategy (including scientifically relevant terminology):	Paired search terms strategy: Toe-clipping, genetic analysis, amphibians, collection, tissue, alternatives: used pairwise strategy
Other sources consulted:	Herpetology textbook/guides

3. In a few sentences, please provide a brief narrative indicating the results of the search(es) to determine the availability of alternatives and explain why these alternatives were not chosen. Also, please address the 3 Rs of refinement, reduction, and replacement in your response. Refinement refers to modification of husbandry or experimental procedures to enhance animal well-being and minimize or eliminate pain and distress. Replacement refers to absolute (i.e. replacing animals with an inanimate system) or relative (i.e. using less sentient species) replacement. Reduction involves strategies such as experimental design analysis, application of newer technologies, use of appropriate statistical methods, etc., to use the fewest animals or maximize information without increasing animal pain or distress.

In searching the literature, we did not identify any procedures that were superior to the ones we are currently implementing. We also were able to identify a source that recommends the form of euthanasia in this protocol (Chen & Combs 1999). The main painful/distressful procedure involved is collecting and euthanizing frogs, and taking tissue samples (toe clips) for molecular genetic and genomic analyses. The procedures proposed cause the minimum amount of pain given the requirement to collect tissue specimens. There are no alternatives that would be less invasive of painful. For example, getting blood samples from frogs this small is not practical and potentially fatal to the subject. This project involves sacrificing some individuals, and procedures for euthanasia are described below. This project involves sacrificing individuals to collect skin tissue for RNA sequencing. For these analyses, metamorphic tadpoles will be euthanized by administering an overdose of benzocaine (20%), which will be rubbed on the venter. Death will be ensured by double pithing. Before euthanasia, each tadpole will be placed on a plastic sheet and have reflectance measurements taken with a spectrometer. This involves holding light probe (a blunt probe at room temperature) a few millimeters away from the skin, to record the reflectance from that patch of skin (the location of each patch measure is determined beforehand and is the same for each tadpole). This procedure can be done quite rapidly (several minutes) and imposes minimal stress. With respect to the 3 R's, replacement is not possible because our experiments focus on specific taxa with their own unique evolutionary history. These are non-model species, so the kinds of surrogate resources (e.g. independent cell lines in the laboratory) are not available. Our experimental design reduces the number of animals to be used to a minimum by focusing on a very limited set of variables. In terms

of refinement, the pain and suffering imposed by the rapid form of anesthesia and euthanasia used should be minimal, and the animals are cared for and treated well beforehand. To refine our sampling methods, we have included the use of combined analgesics/antibiotics to reduce pain and reduce the risk of infection for toe clip collection. Chen MH, Combs CA. A alternative anesthesia for amphibians: ventral application of benzocaine. *Herpetol. Rev.* 1999: 30-34.

C. Hazardous Agents

1. Protocol related hazards (chemical, biological, or radiological): Please indicate if any of the following are used in animals and the status of review/approval by the referenced committees:

HAZARDS	Oversight Committee	Status (Approved, Pending, Submitted)/Date	AUP Appendix I Completed?
Radioisotopes	**Radiation**	Click here to enter text.	Choose an item.
Ionizing radiation	**Radiation**	Click here to enter text.	Choose an item.
Infectious agents (bacteria, viruses, rickettsia, prions, etc.)	**IBC**	Click here to enter text.	Choose an item.
Toxins of biological origins (venoms, plant toxins, etc.)	**IBC**	Click here to enter text.	Choose an item.
Transgenic, Knock In, Knock Out Animals---breeding, cross breeding or any use of live animals or tissues	**IBC**	Click here to enter text.	Choose an item.
Human tissues, cells, body fluids, cell lines	**IBC**	Click here to enter text.	Choose an item.
Viral/Plasmid Vectors/Recombinant DNA or recombinant techniques	**IBC**	Click here to enter text.	Choose an item.
Oncogenic/toxic/mutagenic chemical agents	**EH&S**	Approved 8/30/2021	No
Nanoparticles	**EH&S**	Click here to enter text.	Choose an item.
Cell lines, tissues or other biological products injected or implanted in animals	**DCM**	Click here to enter text.	Choose an item.
Other agents		Click here to enter text.	Choose an item.

2. Incidental hazards

Will personnel be exposed to any incidental zoonotic diseases or hazards during the study (field studies, primate work, etc)? If so, please identify each and explain steps taken to mitigate risk:

This research will involve frogs from the field, which have toxins in their skin (acquired from their diet). We avoid unnecessary handling of the frogs by coaxing them into plastic cups, although this is mainly for their benefit to minimize the stress of handling. Other hazards include excess heat, sunstroke, and vectorborne infectious diseases. We carry lots of water and avoid excessive exertion to minimize the risk of overheating. We wear hats, sunglasses and protective (but light) clothing to avoid sunstroke and sunburn. To avoid infectious disease-carrying insect vectors like mosquitoes, we wear long sleeved shirts and pants and apply insect repellent. We also take malaria prophylaxis. To avoid waterborne parasites (e.g. amoebae) we drink only purified water or bottled drinks.

III. Animals and Housing

A. Species and strains:

Peruvian Mimic Poison Frogs, *Ranitomeya imitator*

B. Weight, sex and/or age:

Wt = 0.5g, sex – approx.. ½ male, ½ female, 1-5 yrs old

C. Animal numbers:

1. Please complete the following table:

Total number of animals in treatment and control groups	**Additional animals (Breeders, substitute animals)**	**Total number of animals used for this project**
We will collect toes (non-fatal) from 10 individuals from 7 different sites in Peru (five sites along the Huallaga river and 2 sites near Varadero). In addition, we will collect acoustic data from these individuals (non-fatal).	+14 (2 per site)	=70+14=84
We will sacrifice and collect the skin and liver of 8 tadpoles at pre-metamorphic late stage (Gosner stage 42) from 4 different sites in Peru (Santa Rosa, Tarapoto, Pongo, and Varadero).	+8 (2 per site)	=32+8=40

We will collect gut contents from 8 adults from each of the 7 different sites in Peru (five sites along the Huallaga river and 2 sites near Varadero) by using a non-fatal stomach pumping technique.	+8 (2 per site)	= 56+8= 64
We will conduct non-fatal behavioral studies on 80 adult male frogs (20 from different sites in Peru; Santa Rosa, Tarapoto, Pongo, and Varadero).	+0	=80

2. Justify the species and number (use statistical justification when possible) of animals requested:

We plan to collect toe clips and acoustic data (both non-fatal) of 80 frogs from 7 different sites in Peru for molecular genetic analyses (PCR and sequencing). These collections do no impose excess stress on individual or populations, and should prove sufficient to enable us to determine whether divergence in population calling parameters (e.g. call frequency, call duration, etc.) is associated with changes in specific genes that have been associated with acoustic behavior in other taxa.

we plan to sacrifice and collect skin samples from 32 tadpoles at pre-metamorphic late stage (Gosner stage 42), for genomic analyses. This provides a samples size of 8 individuals per morph (Sacue, Varadero, Huallaga, and Tarapoto) which should allow sufficient sample sizes to investigate the evolutionary genomics of trait (color and pattern) expression in these frogs, using current methods of transcriptome assembly, read mapping and differential gene expression analysis (we use the program DEseq2 for this purpose), as well as various gene network analyses.

we plan to collect gut contents from 8 adults from each of the 7 different sites in Peru (five sites along the Huallaga river and 2 sites near Varadero) by using a non-fatal stomach pumping technique. This quantity should allow sufficient sample to determine whether different color morphs or populations from different geographic locations are eating different types of prey, with different types of toxins.

Finally, we will run a non-fatal behavioral study on 80 adult male frogs (20 of each morph; Tarapoto, Sauce, Varadero, Huallaga) to investigate whether territorial males respond aggressively to all male color morphs or only males of their own color and pattern.

3. Justify the number and use of any additional animals needed for this study:

We have added 14 additional toe clippings from adults and 8 skin/liver collections from tadpoles and collect additional gut contents from 8 adults.

a. For unforeseen outcomes/complications:

We put 14 extra toe clippings, 8 extra skin/liver collections, and 8 extra gut contents in case anything goes wrong during collection (e.g. accidental sample loss).

b. For refining techniques:

Click here to enter text.

c. For breeding situations, briefly justify breeding configurations and offspring expected:

Click here to enter text.

d. Indicate if following IACUC tail snip guidelines: No

(if no, describe and justify)

NA

4. Will the phenotype of mutant, transgenic or knockout animals predispose them to any health, behavioral, physical abnormalities, or cause debilitating effects in experimental manipulations? No **(if yes, describe)**

Click here to enter text.

5. Are there any deviations from standard husbandry practices?

No **If yes, then describe conditions and justify the exceptions to standard housing (temperature, light cycles, sterile cages, special feed, prolonged weaning times, wire-bottom cages, etc.):**

Click here to enter text.

6. The default housing method for social species is pair or group housing (including mice, rats, guinea pigs, rabbits, dogs, pigs, monkeys). Is it necessary for animals to be singly housed at any time during the study? Yes **(If yes, describe housing and justify the need to singly house social species):**

Juveniles are kept alone as it is easier to feed them and monitor their condition. These are not group-living animals. This study will only be carried out in the field, and retention times will be brief.

7. Are there experimental or scientific reasons why routine environmental enrichment should not be provided? No

(If yes, describe and justify the need to withhold enrichment)

Click here to enter text.

8. If wild animals will be captured or used, provide permissions (collection permit # or other required information):

Permits from the Peruvian government must be obtained during the visit to Peru (they do not give them in advance). Hence, we cannot provide permit numbers at this time.

However, we have obtained permits from the Peruvian Ministry of Natural Resources (SERFOR) for our previous research in Peru, which spans a period of over 20 years.

9. List all laboratories or locations outside the animal facility where animals will be used. Note that animals may not stay in areas outside the animal facilities for more than 12 hours without prior IACUC approval. For field studies, list location of work/study site.

NA

IV. Animal Procedures

A. Outline the Experimental Design including all treatment and control groups and the number of animals in each. Tables or flow charts are particularly useful to communicate your design. Briefly state surgical plans in this section. Surgical procedures can be described in detail in IV.S.

Acoustic data will be collected by visiting five different sites along the Huallaga River: Santa Rosa, Callanayacu, Chipesa, Ricardo Palma, Achinamisa, and two sites near Varadero (southwest of the village of Varadero, and northeast of the village of Varadero). We will record males by approaching calling males to within 5 meters, and recording the call for 15 minutes using a Sensheimer directional microphone (model ME66-K6) and a Marantz digital audio recorder (Model PMD661MKII). Temperature will be noted before all recordings so that we can control for temperature variation during analyses. After recording, each male will be captured, photographed, and have one toe removed as a tissue sample for later genetic analyses. We take toes from the hindlimbs only, as this is less likely to interfere with motility. We take the second (longest) digit (approximately 2/3 of the toe). We observed the frogs for 10 minutes to make sure iti s Ok and bleeding has stopped. Toe clips will be preserved in plastic tubes in alcohol. In addition, we will collect both snout vent length (SVL) and weight of each adult frog.

Tadpoles will be collected at pre-metamorphic late stage (stage 42) from pools in Santa Rosa, Tarapoto, Pongo, and Varadero. We will aim for a sample size of 8 tadpoles of each morph/species. Before euthanasia, each tadpole will be placed on a plastic sheet and have reflectance measurements taken with a spectrometer. This involves holding light probe (a blunt probe at room temperature) a few millimeters away from the skin, to record the reflectance from that patch of skin (the location of each patch measure is determined beforehand and is the same for each tadpole). This procedure can be done quite rapidly (several minutes) and imposes minimal stress. We will euthanize each tadpole by applying 20% benzocaine gel to their venter followed by pinching the cerebral region (destroying the brain with a metal probe). We will use forceps, a scalpel,

and surgical scissors to remove the skin and liver of tadpoles. Instruments will be cleaned with alcohol between procedures. Tadpole skins and livers will be preserved in separate 1.5 ml tubes in RNALater solution. The rest of the tadpole will also be preserved in alcohol, in a separate tube. Tubes will be kept cool in a portable cooler, and will be transferred to a freezer as quickly as possible.

We will collect gut contents from 8 adults from each of the seven populations by using a non-fatal stomach pumping technique. A gavage tube (a small metal tube with a ball shape on one end that prevent damage to the gut) is inserted into the throat. Water is passed through it into the gut, causing the frog to vomit up its current gut contents. Gut contents will be separately stored in a 1.5 microcentrifuge tube and preserved with formalin.

We will run behavioral studies on 80 adult male frogs (20 of each morph; Tarapoto, Sauce, Varadero, Huallaga) to investigate whether territorial males respond aggressively to all male color morphs or only males of their own color and pattern. We will hand-paint plastic frogs with non-toxic acrylic paint to represent the four color morphs. Plastic 3D-frogs and a portable speaker playing advertisement calls (used for close range courtship) will be placed in territories of live male frogs. We will use a GoPro to record the level of interaction/aggression between live male frogs and plastic 3D frogs with colors representing a different color morph.

In sections IV.B-IV.S below, please respond to all items relating to your proposed animal procedures. If a section does not apply to your experimental plans, please leave it blank.

Please refer to DCM and IACUC websites for relevant guidelines and SOPs.

B. <u>Anesthesia/Analgesia/Tranquilization/Pain/Distress Management For Procedures Other than Surgery:</u>

For all procedures, provision of pre-emptive (pre-procedural) analgesia is required, unless specifically exempted by DCM veterinarians. For major survival surgical procedures and extensive non-surgical procedures requiring anesthesia, post-procedural analgesia must be provided for a minimum of 3 full days following anesthetic recovery, unless specifically exempted by DCM veterinarians. Analgesic administration should be continued for at least 1 full day following recovery from minor surgical and non-surgical procedures. Please

contact DCM veterinary staff for recommendations and guidance when formulating anesthetic regimens.

Adequate records describing anesthetic monitoring and recovery must be maintained for all species. Please see Guidelines for Intra-operative and Intra-procedural Monitoring on the IACUC website.

If anesthesia/analgesia must be withheld for scientific reasons, please provide compelling scientific justification as to why this is necessary:
Anesthesia is not practical for these frogs, but we will apply a standard antibiotic/analgesic before and after toe clip removal with cleaned surgical scissors (disinfected in ethanol).

1. **Describe the pre-procedural preparation of the animals:**
   a. **Food restricted for** 0 **hours**
   b. **Food restriction is not recommended for rodents and rabbits and must be justified:**
   Click here to enter text.
   c. Water restricted for 0 **hours**
   d. Water restriction is not recommended in any species for routine pre-op prep and must be justified:
   Click here to enter text.

2. Anesthesia/Analgesia *for Procedures Other than Surgery*

	Agent	**Concentration**	**Dose (mg/kg)**	**Max Volume**	**Route**	**Frequency**	**Number of days administered**
Pre-procedure analgesic	Polysporin and pramoxine	40ug/ml	NA	0.001 ml	topical	Once	NA
Pre-anesthetic	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.
Anesthetic	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.
Post procedure analgesic	Polysporin and pramoxine	40ug/ml	NA	0.001 ml	topical	Once	NA

Other	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.

3. Reason for administering agent(s):
To prevent infection and relieve distress
4. For which procedure(s):
Toe clip removal
5. Methods for monitoring anesthetic depth:
NA
6. Methods of physiologic support during anesthesia and recovery:
NA
7. Duration of recovery:
NA
8. Frequency of recovering monitoring:
NA
9. Specifically what will be monitored?
NA
10. When will animals be returned to their home environment?
Animals will be observed for 10 minutes to make sure the bleeding has stopped.
11. Describe any behavioral or husbandry manipulations that will be used to alleviate pain, distress, and/or discomfort:
NA

C. Use of Paralytics

1. Will paralyzing drugs be used? No
2. For what purpose:
Click here to enter text.
3. Please provide scientific justification for paralytic use:
Click here to enter text.
4. Paralytic drug:
Click here to enter text.
5. Dose:
Click here to enter text.
6. Method of ensuring appropriate analgesia during paralysis:
Click here to enter text.

D. Blood or Body Fluid Collection

1. Please fill out appropriate sections of the chart below:

	Location on animal	Needle/catheter size	Volume collected	Frequency of procedure	Time interval between collections
Blood Collection	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.
Body Fluid Collection	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.
Other	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.

E. Injections, Gavage, & Other Substance Administration

1. Please fill out appropriate sections of the chart below:

	Compound	Location & Route of admin	Needle/catheter/gavage size	Max volume admin	Freq of admin (ie two times per day)	Number of days admin (ie for 5 days)	Max dosages (mg/kg)
Injection/ Infusion	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.
Gavage	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.
Other	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.

3. **Pharmaceutical grade drugs, biologics, reagents, and compounds are defined as agents approved by the Food and Drug Administration (FDA) or for which a chemical purity standard has been written/established by any recognized pharmacopeia such as USP, NF, BP, etc. These standards are used by manufacturers to help ensure that the products are of the appropriate chemical purity and quality, in the appropriate solution or compound, to ensure stability, safety, and efficacy. For all injections and infusions for CLINICAL USE, PHARMACEUTICAL GRADE compounds must be used whenever possible. Pharmaceutical grade injections and**

infusions for research test articles are preferred when available. If pharmaceutical grade compounds are not available and non-pharmaceutical grade agents must be used, then the following information is necessary:

a. **Please provide a scientific justification for the use of ALL non-pharmaceutical grade compounds. This may include pharmaceutical-grade compound(s) that are not available in the appropriate concentration or formulation, or the appropriate vehicle control is unavailable.**
b. **Indicate the method of preparation, addressing items such as purity, sterility, pH, osmolality, pyrogenicity, adverse reactions, etc. (please refer to ECU IACUC guidelines for non-pharmaceutical grade compound use), labeling (i.e. preparation and use-by dates), administration and storage of each formulation that maintains stability and quality/sterility of the compound(s).**

Click here to enter text.

F. Prolonged restraint with mechanical devices

Prolonged restraint in this context means *beyond routine care and use procedures* for rodent and rabbit restrainers, and large animal stocks. Prolonged restraint also includes *any* use of slings, tethers, metabolic crates, inhalation chambers, primate chairs and radiation exposure restraint devices.

1. **For what procedure(s):**

Click here to enter text.

2. **Explain why non-restraint alternatives cannot be utilized:**

Click here to enter text.

3. **Restraint device(s):**

Click here to enter text.

4. **Duration of restraint:**

Click here to enter text.

5. **Frequency of observations during restraint/person responsible:**

Click here to enter text.

6. **Frequency and total number of restraints:**

Click here to enter text.

7. **Conditioning procedures:**

Click here to enter text.

8. Steps to assure comfort and well-being:

Click here to enter text.

9. Describe potential adverse effects of prolonged restraint and provide humane endpoints (criteria for either humanely euthanizing or otherwise removing from study):

Click here to enter text.

G. Tumor Studies, Disease Models, Toxicity Testing, Vaccine Studies, Trauma Studies, Pain Studies, Organ or System Failure Studies, Shock Models, etc.

1. Describe methodology:

Click here to enter text.

2. Expected model and/or clinical/pathological manifestations:

Click here to enter text.

3. Signs of pain/discomfort:

Click here to enter text.

4. Frequency of observations:

Click here to enter text.

5. Describe potential adverse side effects of procedures and provide humane endpoints (criteria for either humanely euthanizing or otherwise removing from study):

Click here to enter text.

H. Treadmills/Swimming/Forced Exercise

1. Describe aversive stimulus (if used):

Click here to enter text.

2. Conditioning:

Click here to enter text.

3. Safeguards to protect animal:

Click here to enter text.

4. Duration:

Click here to enter text.

5. Frequency:

Click here to enter text.

6. Total number of sessions:

Click here to enter text.

7. Describe potential adverse effects of procedures and provide humane endpoints (criteria for either humanely euthanizing or otherwise removing from study):

Click here to enter text.

I. Projects Involving Food and Water Regulation or Dietary Manipulation

(*Routine pre-surgical fasting not relevant for this section)*

1. Food Regulation

a. Amount regulated and rationale:

Click here to enter text.

b. Frequency and duration of regulation (hours for short term/weeks or months for long term):

Click here to enter text.

c. Frequency of observation/parameters documented (i.e. recording body weight, body condition, etc.):

Click here to enter text.

d. Describe potential adverse effects of procedures and provide humane endpoints (criteria for either humanely euthanizing or otherwise removing from study):

Click here to enter text.

2. Fluid Regulation

a. Amount regulated and rationale:

Click here to enter text.

b. Frequency and duration of regulation (hours for short term/weeks or months for long term):

Click here to enter text.

c. Frequency of observation/parameters documented (body weight, hydration status, etc.):

Click here to enter text.

d. Describe potential adverse effects of procedures and provide humane endpoints (criteria for either humanely euthanizing or otherwise removing from study):

Click here to enter text.

3. Dietary Manipulations

a. **Compound supplemented/deleted and amount:**
Click here to enter text.

b. **Frequency and duration (hours for short term/week or month for long term):**
Click here to enter text.

c. **Frequency of observation/parameters documented:**
Click here to enter text.

d. **Describe potential adverse effects of procedures and provide humane endpoints (criteria for either humanely euthanizing or otherwise removing from study):**
Click here to enter text.

J. Endoscopy, Fluoroscopy, X-Ray, Ultrasound, MRI, CT, PET, Other Imaging

1. Describe animal methodology:
Click here to enter text.

2. Duration of procedure:
Click here to enter text.

3. Frequency of observations during procedure:
Click here to enter text.

4. Frequency/total number of procedures:
Click here to enter text.

5. Method of transport to/from procedure area:
Click here to enter text.

6. Describe potential adverse side effects of procedures and provide humane endpoints (criteria for either humanely euthanizing or otherwise removing from study):
Click here to enter text.

7. Please provide or attach appropriate permissions/procedures for animal use on human equipment:
Click here to enter text.

K. Polyclonal Antibody Production

1. Antigen/adjuvant used and justification for adjuvant choice:

Click here to enter text.

2. **Needle size:**
   Click here to enter text.
3. **Route of injection:**
   Click here to enter text.
4. **Site of injection:**
   Click here to enter text.
5. **Volume of injection:**
   Click here to enter text.
6. **Total number of injection sites:**
   Click here to enter text.
7. **Frequency and total number of boosts:**
   Click here to enter text.
8. **What will be done to minimize pain/distress:**
   Click here to enter text.
9. **Describe potential adverse effects of procedures and provide humane endpoints (criteria for either humanely euthanizing or otherwise removing from study):**
   Click here to enter text.

L. Monoclonal Antibody Production

1. Describe methodology:
Click here to enter text.

2. Is pristane used: Choose an item.

Volume of pristane:
Click here to enter text.

3. Will ascites be generated: Choose an item.

i. **Criteria/signs that will dictate ascites harvest:**
   Click here to enter text.
ii. **Size of needle for taps:**
   Click here to enter text.
iii. **Total number of taps:**
   Click here to enter text.
iv. **How will animals be monitored/cared for following taps:**
   Click here to enter text.

4. What will be done to minimize pain/distress:
Click here to enter text.

5. Describe potential adverse effects of procedures and provide humane endpoints (criteria for either humanely euthanizing or otherwise removing from study):
Click here to enter text.

M. Temperature/Light/Environmental Manipulations

1. Describe manipulation(s):
Click here to enter text.

2. Duration:
Click here to enter text.

3. Intensity:
Click here to enter text.

4. Frequency:
Click here to enter text.

5. Frequency of observations/parameters documented:
Click here to enter text.

6. Describe potential adverse effects of procedures and provide humane endpoints (criteria for either humanely euthanizing or otherwise removing from study):
Click here to enter text.

N. Behavioral Studies

1. Describe methodology/test(s) used:
We will place plastic models in the field, play calls from a tape recorder, and observe and record male responses.

2. Will conditioning occur? If so, describe:
No

3. If aversive stimulus used, frequency, intensity and duration:
NA

4. Length of time in test apparatus/test situation: *(i.e., each test is ~10 mins)*
Each test will be about half an hour

5. Frequency of testing and duration of study: *(i.e., 5 tests/week for 6 months)*
10 tests per week for 2 months (optimistically)

6. Frequency of observation/monitoring during test:
Continuous

7. Describe potential adverse effects of procedures and provide humane endpoints (criteria for either humanely euthanizing or otherwise removing from study):
NA

O. Capture with Mechanical Devices/Traps/Nets

1. Description of capture device/method:
Frogs are captured by scooting them into plastic cups

2. Maximum time animal will be in capture device:
Half an hour

3. Frequency of checking capture device:
Every 10 min

4. Methods to ensure well-being of animals in capture device:
Check for signs of lethargy, dehydration

5. Methods to avoid non-target species capture:
Experience with key diagnostic features (calls, color patterns)

6. Method of transport to laboratory/field station/processing site and duration of transport:
Plastic containers

7. Methods to ensure animal well-being during transport:
Check for lethargy and dehydration

8. Expected mortality rates:
zero

9. Describe potential adverse effects of procedures and provide humane endpoints (criteria for either humanely euthanizing or otherwise removing from study):
Following transport to a central area, tadpoles will be euthanized for collection of tissue/skin samples, adults will have toes removed and returned to original site for release. Animals that are in distress and not salvageable will be euthanized and preserved.

P. Manipulation of Wild-Caught Animals in the Field or Laboratory

1. Parameters to be measured/collected:
Collection of skin and toes for transcriptomic/genetic analyses.

2. Approximate time required for data collection per animal:
10 min

3. Method of restraint for data collection:
Frogs will be placed in small plastic boxes, then held by hand (with rubber gloves) during toe removal, tadpoles will be placed in plastic tubes with water, then placed on a small plastic board for euthanasia prior to preservation for genetic analyses.

4. Methods to ensure animal well-being during processing:
Frequent checking for lethargy/dehydration

5. Disposition of animals post-processing:

Preserved (tadpoles) or released at point of capture (adults)

6. Describe potential adverse effects of procedures and provide humane endpoints (criteria for either humanely euthanizing or otherwise removing from study):

Following transport to a central area, tadpoles will be euthanized for collection of tissue/skin samples, adults will have toes removed and returned to original site for release. Animals that are in distress and not salvageable will be euthanized and preserved.

Q. Wildlife Telemetry/Other Marking Methods

1. Describe methodology (including description of device):

Click here to enter text.

2. Will telemetry device/tags/etc. be removed? Choose an item. **If so, describe:**

Click here to enter text.

3. Describe potential adverse effects of procedures and provide humane endpoints (criteria for either humanely euthanizing or otherwise removing from study):

Click here to enter text.

R. Other Animal Manipulations

1. Describe methodology:

Click here to enter text.

2. Describe methods to ensure animal comfort and well-being:

Click here to enter text.

3. Describe potential adverse effects of procedures and provide humane endpoints (criteria for either humanely euthanizing or otherwise removing from study):

Click here to enter text.

S. Surgical Procedures

All survival surgical procedures must be done aseptically, regardless of species or location of surgery. Adequate records describing surgical procedures, anesthetic monitoring and postoperative care must be maintained for all

species. Please see Guidelines for Intra-operative and Intra-procedural Monitoring on the IACUC website.

1. Location of Surgery (Building & Room #):

Click here to enter text.

2. Type of Surgery (check all that are appropriate):

Click here to enter text.

☐ **Non-survival surgery (animals euthanized without regaining consciousness)**

☐ **Major survival surgery (major surgery penetrates and exposes a body cavity or produces substantial impairment of physical or physiologic function)**

☐ **Minor survival surgery**

☐ **Multiple survival surgery**

If yes, provide scientific justification for multiple survival surgical procedures:

Click here to enter text.

3. Describe the pre-op preparation of the animals:

a. **Food restricted for** Click here to enter text. **hours**
b. **Food restricted is not recommended for rodents and rabbits and must be justified:**

Click here to enter text.

c. Water restricted for Click here to enter text. **hours**

d. Water restriction is not recommended in any species for routine pre-op prep and be justified:

Click here to enter text.

4. Minimal sterile techniques will include (check all that apply):

Please refer to DCM Guidelines for Aseptic Surgery for specific information on what is required for each species and type of surgery (survival vs. non-survival).

☐ **Sterile instruments**

How will instruments be sterilized?

Click here to enter text.

If serial surgeries are done, how will instruments be sterilized between surgeries:

Click here to enter text.

- ☐ **Sterile gloves**
- ☐ **Mask**
- ☐ **Cap**
- ☐ **Sterile gown**
- ☐ **Sanitized operating area**
- ☐ **Clipping or plucking of hair or feathers**
- ☐ **Skin preparation with a sterilant such as betadine**
- ☐ **Practices to maintain sterility of instruments during surgery**
- ☐ **Non-survival (clean gloves, clean instruments, etc.)**

5. **Describe all surgical procedures:**

a. Skin incision size and site on the animal:

Click here to enter text.

b. Describe surgery in detail (include size of implant if applicable):

Click here to enter text.

c. Method of wound closure:

Click here to enter text.

i. Number of layers

Click here to enter text.

ii. Type of wound closure and suture pattern:

Click here to enter text.

iii. Suture type/size/wound clips/tissue glue:

Click here to enter text.

iv. Plan for removing of skin sutures/wound clip/etc:

Click here to enter text.

6. **Anesthetic Protocol:**

For all procedures, provision of pre-emptive (pre-procedural) analgesia is required, unless specifically exempted by DCM veterinarians. For major survival surgical procedures and extensive non-surgical procedures requiring anesthesia, post-procedural analgesia must be provided for a minimum of 3 full days following anesthetic recovery, unless specifically exempted by DCM veterinarians. Analgesic administration should be continued for at least 1 full day following recovery from minor surgical

and non-surgical procedures. Please contact DCM veterinary staff for recommendations and guidance when formulating anesthetic regimens.

a. If anesthesia/analgesia must be withheld for scientific reasons, please provide compelling scientific justification as to why this is necessary:

Click here to enter text.

b. Anesthesia/Analgesia For Surgical Procedures

	Agent	Dose (mg/kg or %)	Volume	Route	Frequency	Number of days administered
Pre-operative analgesic	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.
Pre-anesthetic	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.
Anesthetic	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.
Post-operative Analgesic	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.
Other	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.

c. Methods that will be used to monitor anesthetic depth (include extra measures employed when paralyzing agents are used):

Click here to enter text.

d. Methods of physiologic support during anesthesia and immediate post-op period (fluids, warming, etc.):

Click here to enter text.

e. List what parameters are monitored during immediate post-op period. Provide the frequency and duration:

Click here to enter text.

f. Describe any other manipulations that will be used to alleviate pain, distress, and/or discomfort during the immediate post-op period (soft bedding, long sipper tubes, food on floor, dough diet, etc.):

Click here to enter text.

g. List criteria used to determine when animals are adequately recovered from anesthesia and when the animals can be returned to their home environment:

Click here to enter text.

7. Recovery from Surgical Manipulations (after animal regains consciousness and is returned to its home environment)

Click here to enter text.

a. What parameters (behavior, appetite, mobility, wound healing, etc.) will be monitored:

Click here to enter text.

b. How frequently (times per day) will animals be monitored:

Click here to enter text.

c. How long post-operatively (days) will animals be monitored:

Click here to enter text.

8. Surgical Manipulations Affecting Animals

a. Describe any signs of pain/discomfort/functional deficits resulting from the surgical procedure:

Click here to enter text.

b. What will be done to manage any signs of pain or discomfort (include pharmacologic and non-pharmacologic interventions):

Click here to enter text.

c. Describe potential adverse effects of procedures and provide humane endpoints (criteria for either humanely euthanizing or otherwise removing from study):

Click here to enter text.

V. Euthanasia

Please refer to the AVMA Guidelines for the Euthanasia of Animals: 2013 Edition and DCM Guidelines to determine appropriate euthanasia methods.

A. Euthanasia Procedure*. All investigators, even those conducting non-terminal studies, must complete this section in case euthanasia is required for humane reasons.*

Tadpoles and adult frogs if necessary, will be euthanized by administering an overdose of benzocaine (20%), which will be rubbed on the venter.

1. Physical Method- If a physical method is used, the animal should be first sedated/anesthetized with CO_2 or other anesthetic agent. If prior sedation is not possible, a scientific justification must be provided:
Click here to enter text.

2. Inhalant Method- Choose an item.
(if other, describe the agent and delivery method)
Click here to enter text.

3. Non-Inhalant Pharmaceutical Method (injectables, MS-222, etc.)- Please provide the following:

a. Agent:
Benzocaine

b. Dose or concentration:
20% gel on venter

c. Route:
Transepithelial

B. Method of ensuring death (can be physical method, such as pneumothorax or decapitation for small species and assessment method such as auscultation for large animals):
Double pithing or cerebral crushing/transection for tadpoles

C. Describe disposition of carcass following euthanasia:
Preservation for molecular analyses (storage in RNAlater)

I acknowledge that humane care and use of animals in research, teaching and testing is of paramount importance, and agree to conduct animal studies with professionalism, using ethical principles of sound animal stewardship. I further acknowledge that I will perform only those procedures that are described in this AUP and that my use of animals must conform to the standards described in the Animal Welfare Act, the Public Health Service Policy, The Guide For the Care and Use of Laboratory Animals, the Association for the Assessment and Accreditation of Laboratory Animal Care, and East Carolina University.

Please submit the completed animal use protocol form via e-mail attachment to iacuc@ecu.edu. You must also carbon copy your Department Chair.

PI Signature: Kyle Summers **Date: 8-10-21**

Veterinarian: DocuSigned by: Karen Oppelt 5735B5A085F747B... **Date:** 9/3/2021 | 11:40 AM EDT

IACUC Vice-Chair: DocuSigned by: Jamie DeWitt A7E4042678EA4B3... **Date:** 9/7/2021 | 9:53 AM EDT

APPENDIX 1-HAZARDOUS AGENTS			
Principal Investigator:	Campus Phone:	Home Phone:	
IACUC Protocol Number:	Department:	E-Mail:	
Secondary Contact: Department:	Campus Phone:	Home Phone:	E-Mail:
Chemical Agents used:	Radioisotopes used:		
Biohazardous Agents used:	Animal Biosafety Level:	Infectious to humans?	
PERSONAL PROTECTIVE EQUIPMENT REQUIRED:			
Route of Excretion:			
Precautions for Handling Live or Dead Animals:			
Animal Disposal:			
Bedding/Waste Disposal:			
Cage Decontamination:			
Additional Precautions to Protect Personnel, Adjacent Research Projects including Animals and the Environment:			
Initial Approval Safety/Subject Matter Expert Signature & Date _____			