University of California, Los Angeles
Chancellor's Animal Research Committee (ARC)

Renewal Application

General Information	
Title:	[redacted]
Protocol #:	[redacted]
PI:	[redacted]
Status:	ARCHIVED
Approval Period:	3/18/2019-3/17/2020
Received Date:	1/31/2019
Type:	Renewal
Species:	32 Dog (Pain Category D)
Create Date:	1/2/2019 11:09:20 PM
Created By:	[redacted]
Owner:	[redacted]

Updated Sections

Animal Care
Euthanasia Medications
Experimental Design
Locat ons
Medicat ons and Experimental Drugs
Pain Category
Pain Category Assignments
Pain L terature Search
Personnel
PI Assurance
Pre-Review
Rat onale
Renewal Summary
Response to Pre-Review
Species Surgery
Surgery

Renewal

1. Please indicate whether any adverse effect or unanticipated problems have been experienced, including higher than anticipated mortality/morbidity regardless of the cause. If so, please provide an explanation of how these events/problems were resolved.

Yes

One animal had a sudden cardiac arrest in the middle of the experiment on December 18, [redacted] The death occurred after all surgery was completed and setup for neuromuscular stimulation was being prepared. The vitals were all stable right up to sudden drop in heart rate. The etiology for this is unclear. Animal was on the ventilator and chest compression was performed but the heart did not recover.

Results from necropsy are still pending. I continue to work with the DLAM vets on the best anesthetic regimen and monitoring plan.

2. To assist the ARC in documenting scientific progress arising from use of animals under this protocol, please provide ONE of the following:

- ○ Citation(s) of presentations or articles resulting from this protocol (either accepted or submitted). Please include an abstract.
- ◉ A brief (1-2 sentence) update regarding progress made toward achieving the scientific objective(s) of this protocol.
- ○ A copy of the most recent annual progress report submitted to the funding agency.

If the scientific progress documentation is in a text format, please paste (or type) it here. Otherwise, you will need to submit it to the ARC as a hard copy.

The goal of the research project is to understand how laryngeal paresis and paralysis affects voice production and the optimal ways to treat these conditions. We have only recently begun the experiments and have successfully recorded data from two recent experiments (September 26 and December 12, [redacted] We are actively analyzing the results. The proposed studies continue a series of experiments designed to evaluate and treat laryngeal paresis and paralysis.

Research Summary

Your answers to the questions on this page determine the other sections needed to be filled out.

1. What is the Title of the Project?

[redacted]

2. Check all that apply:

- ☐ Tumor Formation (spontaneous or implanted)
- ☐ Chronic Disease (diabetes, EAE, status epilepticus, etc.)
- ☑ Tissue Collection (blood and all other tissues, including those collected after euthanasia)
- ☐ Antibody/Ascites Production
- ☑ Surgical Procedures (survival, non-survival)
- ☐ Non Surgical Procedures (injection of experimental drugs, behavioral studies)
- ☑ Gas Anesthetic Agent(s) (use of isoflurane, halothane, etc)
- ☐ Hazardous Agents (carcinogens, paraformaldehyde, rDNA, vectors, etc.)
- ☐ Radioisotopes or radioactive implants
- ☐ Prolonged Physical Restraint (physical restraint of unanesthetized animals for periods longer than 15 minutes)

☐ Genetically Modified Animals

☐ Tissue Sharing (use of tissues only)

3. Will the research be conducted exclusively on tissue received from another investigator?

No

If yes, do your funding sources require an ARC approved protocol?

No

4. Check all that apply:

☐ Experiments done entirely at another institution

NOTE: For experiments conducted entirely at another institut on please subm t the most recent approval not ce and a copy of the most recently approved protocol from the other institution with your submission. Please also indicate the PHS Assurance number and AAALAC accreditation status.

☐ Experiments done entirely at VAGLAHS

☐ Program Project/Training Grant

Administrative approval only – no animals associated with this protocol.

☐ Breeding Colony: #

NOTE: If you will be breeding animals for this protocol and do not already have an approved breeding protocol on file w th the ARC, you must submit an Appl cat on to Establish and/or Maintain a Breeding Colony at this time. Check the box above but leave the "Breeding Colony Number" field above empty. The ARC Staff will update the Breeding Colony Number following the submission of a breeding colony application.

5. If you are seeking approval for a training grant, list all individual projects supported by the program project or training grant, including the principal investigators' names and their current ARC approval numbers. If no animal research is currently being supported by the overall grant, please assure the Committee that, should an investigator of a project covered by the overall grant initiate research involving animals, ARC approval will be obtained prior to the distribution of funds.

Personnel

There can be only one Principal Investigator per protocol. To edit a person's contact information or add a new person to our system, click on the People tab above.

Prior to the submission of an amendment to add personnel, please ensure that these individuals have completed all applicable animal use certification requirements and have a Medical History Questionnaire (MHQ) on file with the Occupation Health Facility (OHF). If you are only requesting the removal of personnel, please email the ARC administrative office (arc@research.ucla.edu). An amendment application is NOT required if you are only removing personnel.

Principal Investigator

[redacted] View Person Detail

Email:	[redacted]	**UID:**	[redacted]
Phone:	[redacted]	**Degree:**	[redacted]
Fax:	[redacted]	**Dept:**	[redacted]
Status:	Faculty		

What role will this person be performing in this protocol?

Principal Investigator

Which species will this person handle in this protocol?

Dog

Will this person handle animal tissue in this protocol?

Yes

Will this person be involved with Survival Surgery Procedures?

No

Will this person handle rDNA and/or infectious materials?

No

Will this person handle highly toxic chemicals and/or carcinogens?

No

Please provide a brief account of the person's qualifications and experience with the animal model(s) and procedures in this protocol. Please include a description of any experience obtained beyond the required ARC/DLAM training courses. If this individual does not have any relevant previous experience, please briefly describe how he or she will be trained in the specific research techniques.

Dr. [redacted] is a board-certified physician and [redacted] surgeon. [redacted] has worked in the [redacted] Laboratory using the in vivo canine model for the past [redacted] years.

Dr. [redacted] is a [redacted] with specific clinical and research interest in voice disorders.

Dr. [redacted] has experience with all aspects of this protocol, including canine anesthesia, canine surgery, neuromuscular stimulation, intralaryngeal dissection of laryngeal nerves, and in vivo phonation. [redacted] has completed the species-specifc training on canines.

For the past [redacted] years [redacted] has been the co-director of the [redacted] and performed or supervised nearly all canine experiments.

Please list the duties (including specific procedures to be performed, as appropriate) that this person will perform involving live animals under this protocol.

Study design, animal surgery, intralaryngeal dissection, neuromuscular stimulation, high-speed imaging, recording of experimental parameters, data analysis.

Will this person handle radioactive materials or radioactive animals?

No

Co-Investigator

[redacted] View Person Detail

Email:	[redacted]	UID:	[redacted]
Phone:	[redacted]	Degree:	[redacted]
Fax:		Dept:	[redacted]
Status:	[redacted]		

What role will this person be performing in this protocol?

Co-Investigator

Which species will this person handle in this protocol?

Dog

Will this person handle animal tissue in this protocol?

No

Will this person be involved with Survival Surgery Procedures?

No

Will this person handle rDNA and/or infectious materials?

No

Will this person handle highly toxic chemicals and/or carcinogens?

No

Please provide a brief account of the person's qualifications and experience with the animal model(s) and procedures in this protocol. Please include a description of any experience obtained beyond the required ARC/DLAM training courses. If this individual does not have any relevant previous experience, please briefly describe how he or she will be trained in the specific research techniques.

Dr. [redacted] is Associate Professor [redacted] in the Department of [redacted] has special expertise with the electronics, imaging and data collection aspects of this proposal. [redacted] primary duties will be to implement neuromuscular stimulation of canine laryngeal nerves, collect and analyze electronic and imaging data.

Please list the duties (including specific procedures to be performed, as appropriate) that this person will perform involving live animals under this protocol.

[redacted] will not be directly involved in live animal surgery. [redacted] will be primarily responsible for nerve stimulation using custom designed computer hardware and software, data acquisition, and implementation of computer software to control neuromuscular stimulation.

Will this person handle radioactive materials or radioactive animals?

No

Personnel

[redacted] View Person Detail

Email:	[redacted]	UID:	[redacted]
Phone:	[redacted]	Degree:	[redacted]
Fax:		Dept:	[redacted]
Status:	[redacted]		

What role will this person be performing in this protocol?

Personnel

Which species will this person handle in this protocol?

Dog

Will this person handle animal tissue in this protocol?

Yes

Will this person be involved with Survival Surgery Procedures?

No

Will this person handle rDNA and/or infectious materials?

No

Will this person handle highly toxic chemicals and/or carcinogens?

No

Please provide a brief account of the person's qualifications and experience with the animal model(s) and procedures in this protocol. Please include a description of any experience obtained beyond the required ARC/DLAM training courses. If this individual does not have any relevant previous experience, please briefly describe how he or she will be trained in the specific research techniques.

[redacted] is a [redacted] who is spending a year in research in-between [redacted]. [redacted] has taken all required courses for animal handling, including dog species specific training. As part of

[redacted] training in medical school, [redacted] has participated as an assistant in numerous medical and surgical procedures in humans.

Please list the duties (including specific procedures to be performed, as appropriate) that this person will perform involving live animals under this protocol.

All [redacted] duties are under the direct supervision of Dr. [redacted] will assist during animal surgery under direct supervision, including charting of vital signs of the animal, and also perform data gathering and analysis.

Will this person handle radioactive materials or radioactive animals?

No

Personnel

DLAM Staff

View Person Detail

Email:	
Phone:	[redacted]
Fax:	
Status:	Staff

UID:	999999998
Degree:	
Dept:	DIV LAB ANIMAL MEDICINE

What role will this person be performing in this protocol?

Personnel

Which species will this person handle in this protocol?

Dog

Will this person handle animal tissue in this protocol?

Yes

Will this person be involved with Survival Surgery Procedures?

Yes

Will this person handle rDNA and/or infectious materials?

No

Will this person handle highly toxic chemicals and/or carcinogens?

No

Please provide a brief account of the person's qualifications and experience with the animal model(s) and procedures in this protocol. Please include a description of any experience obtained beyond the required ARC/DLAM training courses. If this individual does not have any relevant previous experience, please briefly describe how he or she will be trained in the specific research techniques.

DLAM staff include board certified veterinarians and veterinary technicians who all have previous experience with surgical procedures with dogs.

Please list the duties (including specific procedures to be performed, as appropriate) that this person will perform involving live animals under this protocol.

Assist with pre and intra operative preparation.

Will this person handle radioactive materials or radioactive animals?

Yes

Personnel

[redacted]

View Person Detail

Email:	[redacted]
Phone:	
Fax:	
Status:	

UID:	[redacted]
Degree:	[redacted]
Dept:	[redacted]

What role will this person be performing in this protocol?

Personnel

Which species will this person handle in this protocol?

Dog

Will this person handle animal tissue in this protocol?

Yes

Will this person be involved with Survival Surgery Procedures?

No

Will this person handle rDNA and/or infectious materials?

No

Will this person handle highly toxic chemicals and/or carcinogens?

No

Please provide a brief account of the person's qualifications and experience with the animal model(s) and procedures in this protocol. Please include a description of any experience obtained beyond the required ARC/DLAM training courses. If this individual does not have any relevant previous experience, please briefly describe how he or she will be trained in the specific research techniques.

Dr. [redacted] is an [redacted] who is completing a [redacted] fellowship in [redacted] is involved in daily medical and surgical care of patients with ear/nose/throat diseases. [redacted] currently holds a title of [redacted]

[redacted] in [redacted] [redacted] has taken the required courses.

Please list the duties (including specific procedures to be performed, as appropriate) that this person will perform involving live animals under this protocol.

Under the direct supervision of Dr. [redacted] Dr. [redacted] will assist during animal surgery and also perform data gathering and analysis.

Will this person handle radioactive materials or radioactive animals?

No

Personnel

[redacted] View Person Detail

Email:	[redacted]	**UID:**	[redacted]
Phone:	[redacted]	**Degree:**	[redacted]
Fax:		**Dept:**	[redacted]
Status:	[redacted]		

What role will this person be performing in this protocol?

Personnel

Which species will this person handle in this protocol?

Dog

Will this person handle animal tissue in this protocol?

Yes

Will this person be involved with Survival Surgery Procedures?

No

Will this person handle rDNA and/or infectious materials?

No

Will this person handle highly toxic chemicals and/or carcinogens?

No

Please provide a brief account of the person's qualifications and experience with the animal model(s) and procedures in this protocol. Please include a description of any experience obtained beyond the required ARC/DLAM training courses. If this individual does not have any relevant previous experience, please briefly describe how he or she will be trained in the specific research techniques.

Dr. [redacted] has over [redacted] years experience working with the canine laryngeal model in the Department of [redacted] Laboratory. [redacted] has also worked for the [redacted] years in the Department of [redacted], [redacted] has previously performed all surgical aspects of this protocol including intralaryngeal microdissection. [redacted] is also the caretaker of th [redacted] Laboratory.

Please list the duties (including specific procedures to be performed, as appropriate) that this person will perform involving live animals under this protocol.

[redacted] will assist with all animal handling and surgical aspects of protocol.

Will this person handle radioactive materials or radioactive animals?

No

Contacts

Name:	[redacted]
Contact Type:	Emergency, Administrative
Home Phone:	[redacted]
Mobile Phone:	[redacted]
Email:	[redacted]

Name:	[redacted]
Contact Type:	Emergency
Home Phone:	
Mobile Phone:	[redacted]
Email:	[redacted]

Funding

1. Funding Types (Check All That Apply):

- ☐ Department
- ☑ Extramural
- ☐ UCLA Academic Senate
- ☐ Gift
- ☐ No funding at this time

☐ Other:

Proposals

List all funding agencies to which this animal protocol has been or will be submitted for consideration. Include all pending applications.

For each grant/proposal subm tted to a funding agency, subm t a copy of the grant proposal. If the agency is not listed, please contact the **Office of Contracts and Grants.** Please note that the National Institutes of Health may be found by typing in the keyword "NIH" when searching for an Agency Code.

Please note that the Publ c Health Service (PHS) Policy requires the Inst tution to verify approval of those components of the grant applicat on or proposal related to the care and use of animals. **Therefore, it is strongly recommended that prior to submission, investigators review all of the proposed experiments pertaining to animals in the grant application to ensure congruence with the animal research protocol. Please detail any inconsistencies between the grant and the protocol in the spaces below.**

Agency Name:

[redacted]

Agency Code:

[redacted]

PI of Proposal/Award:

[redacted]

Proposal/Award Title:

[redacted]

Proposal/Award Number:

[redacted]

Please detail any inconsistencies between the grant and the protocol in the space below (e.g., species or activities described in grant not in ARC protocol, projects completed or not begun, etc.):

There are no inconsistencies.

Rationale

1. Provide a non-technical summary of the overall objectives of the study.

With this research we want to understand how the larynx works to produce sound in normal and abnormal conditions, and how to optimally treat abnormal conditions. When speaking, the muscles of the larynx change the three-dimensional shape of the vocal folds in complex ways, and this shape is thought to greatly affect voice characteristics such as pitch, loudness, and quality. Previously, we successfully studied the roles of laryngeal muscles in voice production. Now we want to study the effects of laryngeal muscle activation on vocal fold shape and how to treat vocal weakness and paralysis with vocal fold implants. We will study how laryngeal muscles control vocal fold shape and its consequences on voice production. Then we will design implants that mimic those shapes to see if we can successfully rehabilitate a weakened or paralyzed larynx.

2. Indicate the possible benefits to mankind and/or animals or the advancement of knowledge that may be derived from this study.

The larynx produces voice used in speech, singing, and non-speech communication. Normal voice production requires the two vocal folds of the larynx to vibrate in a symmetric and regular manner. In vocal paresis or paralysis, vibration is irregular due to asymmetry in mass or tension between the vocal folds, and gap between the folds. Laryngeal paresis is now regarded as the most common etiology of dysphonia in humans and is more common than vocal fold paralysis. Common ailments such as a viral upper respiratory infection can weaken the Vagus or laryngeal nerves and lead to paresis. However, there remain significant clinical challenges in the diagnosis and management of vocal fold paresis. In most instances the diagnosis of paresis is missed because of subtle abnormal laryngeal findings. While laryngeal paresis is now felt to be the cause of a majority of cases of neurogenic voice problems, most clinicians have difficulty diagnosing the condition, let alone how to treat it. With this research, we hope to understand how the larynx behaves in laryngeal paresis and paralysis. We also want to understand how best to treat these conditions with vocal implants that mimic the shape of vocal folds during normal phonation.

The most important reason for lack of understanding and consensus in the diagnosis and treatment of laryngeal paresis is that it could not be previously studied in a laboratory model. The major hurdle was that laryngeal paresis could not be modeled in the in vivo setting.
Our proposed research is unique in that we are able to provide "graded stimulation" of the laryngeal nerves to activate the muscles at any level of contraction, from no activation (grade 0, paralysis state) to full activation (maximal grade), to mimic any level of weakness (paresis) from subtle weakness to complete paralysis. We are able to do this because (1) we have developed the unique computer hardware and nerve stimulation software and protocols to provide graded stimulation, (2) we have the surgical expertise to perform distal laryngeal muscle activation by dissecting distal branches of the laryngeal nerves and (3) we are allowed to use the canine model, which has been time tested over nearly a century and is the best in vivo match to the human larynx to study laryngeal physiology (see section 3 below).

Our research work has direct translation into the diagnosis and treatment of human laryngeal diseases. For instance, we recently performed a study to evaluate the effects of superior laryngeal nerve (SLN) asymmetry. The SLN innervates the cricothyroid (CT) muscle, which is the muscle needed to raise the pitch of the voice. The SLN is commonly injured during surgeries in the neck such as thyroidectomy. Many singing careers have ended due to SLN damage. However, it was unclear how unilateral SLN (CT) paresis could lead to dysphonia. This was because no study

of CT muscle paresis had heretofore been performed. We did so, and we found that CT asymmetry leads to reduced lengthening (strain) of the vocal folds, reduced vocal range with reduced pitch, and reduced speed of the vocal fold closure. There were also asymmetries of vibration seen on the surface of the vocal folds. Thus, we were able to show for the first time how laryngeal paresis directly affects voice production and we were able to corroborate some clinical observations made on this topic. Another study from this research protocol showed the differential roles of the two vocal fold adductors (thyroarytenoid and lateral cricoarytenoid) and concluded with determination of the most optimal procedure to treat vocal fold paralysis. Yet another showed how the larynx controls pitch and loudness. These are just a few examples of the studies that have already been performed that has direct translation to clinical medicine. The currently proposed experiments have a even higher translational potential as we want to test implants used to treat human laryngeal paresis and paralysis. Implants are currently the most common surgical treatment for vocal paresis/paralysis, but the results are not always optimal. The proposed experiments will investigate why that is and how to mold the implants appropriately. These experiments are directly translatable and applicable to advancing human medical care by improving our understanding of laryngeal function and how to treat some dysfunctional states.

3. Explain the rationale for the use of animals, including (a) why the chosen species is the most appropriate for the study and (b) why the chosen species cannot be replaced with a phylogenetically lower species. Note that cost cannot be accepted as a justification.

Human laryngeal physiology has been modeled with computer models, static geometric shape systems, excised human larynges, excised canine larynges, and human laryngeal non-invasive measurements. Our [redacted] laboratories have experience with all of these models and currently use them appropriately.

Computer models can be used for predictive modeling of voice production but they cannot yet accurately recreate the mucosal wave of the intact self-oscillating vocal folds, or model the intricacies of multiple laryngeal muscle activation, vocal fold collision, interactions with the nearfield glottal airflow, and possible acoustical interactions with sub- and supra-glottal systems.

Indeed, such computational models require validation with direct in vivo measurements, as proposed in this investigation. Direct measurements are needed to improve computer models, which will ultimately reduce the number of animals needed for voice research.

Excised laryngeal models (post-mortem human or animal larynx) do not have the same measurable mucosal wave movement that occurs from stimulation of the recurrent laryngeal nerve, nor can they model active muscle contraction, including vocal fold stiffening, shortening, and bulging when muscle is activated. Most importantly, the thyroarytenoid muscle action (the most important muscle in phonation) cannot be replicated in excised larynges as it requires active muscle contraction (while other muscles actions can be approximated with sutures). Only in vivo nerve stimulation can activate the muscles into the posture required for voice production.

Finally, the proposed research method employs intricate microsurgery of the larynx and invasive control of laryngeal physiological variables that cannot be obtained in human subjects. Other in vivo animal models (such as pig and sheep) do not share the same neuromuscular anatomy and are not amenable to microsurgical dissection of nerves and the shape of the vocal fold tract needed for phonation. Rabbits and pigs are also used in our labs, however only tissue engineering type experiments or non-specific phonation experiments are possible currently in those animals, as neuromuscular stimulation of individual muscles and recording of vocal fold motion and posture changes in not possible. In addition, in rabbits the larynx is too small to perform the types of experiments proposed in this study such as effects of vocal fold implants on voice. Mice and rats have been used in laryngeal research investigating whole larynx transplants and nerve regeneration experiments, but not to study voice production, again because of the immensely small size of the larynx and neuromuscular structures in these species.

The in vivo canine larynx has dimensions and physiologic properties similar to that of the human larynx. Therefore, only anesthetized canine in vivo phonation allows for the control of independent variables (individual muscle activation) and the ability to measure dependent variables (acoustics, aerodynamics, vibration) invasively. Phylogenetically lower species do not have larynges with similar properties. Moreover, the canine larynx is unique in its ability to allow adequate control and measurements for various aspects of laryngeal physiology, especially with regard to the simultaneous [redacted] contraction, stiffening, and bulging of the medial surface of the vocal fold thyroarytenoid (TA) muscle. It is also the only model in which the ability to dissect and stimulate individual branches to the intrinsic laryngeal muscles has been demonstrated and perfected. Due to near perfect size match to the human larynx, the canine model also allows for investigations of vocal fold implants to treat paresis and paralysis. For these reasons, the canine is the most frequently used in vivo phonatory model, and the most appropriate for this proposed research, as evidenced by nearly a century of voice research using canines.

We continue to need to do these in vivo canine experiments to further understand voice production and treatment of voice disorders. Specifically, we need more data on hemilaryngeal phonation and more information on how vocal fold medial surface shape affects the voice. In the past years, we have made great strides in discovering the intricate roles of laryngeal adductor muscles in facilitating a tremendous variety of phonation possible. These finding could not have been made in an ex vivo or computer models. However, what we learn from in vivo canine experimentation we use to improve the computer models.

Many improvements in surgical treatment of human laryngeal disorders, such as treatment of spasmodic dysphonia, laryngeal papilloma, and vocal fold paralysis (just to name a few disorders), have come directly from canine experiments performed in our labs at [redacted]

Experimental Design & Justification for Requested Number of Animals

1. Provide a two- to four-sentence lay description of the experimental procedures written in language easily understandable to a seventh grade student.

In the laboratory we will stimulate each of the muscles of the larynx, individually and in various combinations, to investigate how each muscle, or combination of muscles (1) controls the shape of the vocal fold for voice production (2) affects the sound that is produced, and (3) conrols the vibratory pattern on the vocal fold surface. We will also test the effects of various surgical implants on voice production. These investigations will help us

understand how muscles of the larynx normally work to produce voice, how they affect voice when they are diseased, and the optimal implants to return the voice.

2. Provide a complete description of: (a) all activities involving the use of research animals; (b) a scientific justification for the total number of animals required to conduct this study. The number of animals justified in this section must match the totals in the Pain Category Assignments. To the extent possible, assign all animals to experimental groups, which can be easily distinguished by the independent variables defining each group (e.g.,drug dosages, time points, controls, etc.). Clearly indicate the number of animals needed per group and explain how group sizes were determined, either(i) by statistical analysis, or (ii) where statistics are not applicable (e.g., teaching labs, feasibility studies, antibody production, etc.), on the basis of other considerations (e.g., student/animal ratio, tissue yield per animal, antigen/animal ratio, prior experience, etc.). If statistical analysis is employed to determine the number of animals required, please specify the statistical method used.

This research is a multi-year comprehensive effort to understand the vibratory, aerodynamic, acoustic, and perceptual sequelae of laryngeal asymmetry, and to generate a scientific basis for optimal treatment of glottal insufficiency. Asymmetric laryngeal activation in laryngeal neuromuscular diseases such as vocal fold paresis and paralysis alter the flow-structure interaction within the glottic channel and affect acoustic and aerodynamic parameters such as fundamental frequency (F0), phonation onset pressure/airflow relationship, stability of the laryngeal vibration to increasing subglottal pressure, and voice quality. Understanding the fundamental mechanisms that control these parameters is necessary to treat vocal fold insufficiency from neuromuscular disease. Unfortunately, these mechanisms are poorly understood, which limits our ability to optimize treatment for this disorder.

In our previous investigations using this unique in vivo canine model, we developed the technique of automated, independent stimulation of multiple distal laryngeal nerves, such that individual laryngeal muscles could be stimulated at any level of activation ("graded stimulation") so that the phonatory effects of any level of muscle activation, from dense paresis to normal, could be investigated. We developed the technique where concurrent stimulation of up to 6 individual laryngeal nerves allowed us to test 320 distinct laryngeal neuromuscular activation states in a single larynx, which greatly reduced the number of animals needed for experimentation.

However, while our previous studies were performed in a normal, symmetrically activated larynx, and increased our understanding of a normally active larynx, patients with laryngeal paresis and paralysis suffer from asymmetrical activation. Currently, significant gaps exist in our understanding of variables such as the degree of left/right asymmetry and glottal channel shape changes that lead to abnormal flow/pressure relationships, abnormal vibratory patterns, and perceptible changes in voice quality. This continuation proposal therefore expands on our previous studies to focus on laryngeal asymmetries and how to treat them.

General Methods:
On the day of the experiment, the canine will be pre-medicated in [redacted] and transported to the surgical suite. After an intravenous (IV) line is placed, the subject will be induced with propofol and endotracheal tube used to intubate the trachea. General anesthesia with isoflurane is then commenced. The animal is placed on the surgical bed and vital signs monitoring devices are connected.

The neck is prepared for surgery then microdissection of the distal laryngeal nerves, placement of nerve-fitting tripolar cuff electrodes, and automated stimulation of the laryngeal nerves are commenced. Our primary focus is on clinically relevant scenarios of laryngeal paresis/asymmetry [laryngeal muscles TA (thyroarytenoid), LCA/IA (lateral cricoarytenoid/interarytenoid), CT (cricothyroid), individually and in combinations). CT activation is implemented by stimulation of
the external branch of the SLN.

A tracheotomy is performed and an endotracheal tube placed through the tracheotomy. A suprahyoid pharyngotomy is then performed to expose the larynx in the neck externally so high-speed video of the vibrating larynx can be performed. The larynx is stimulated via electrodes placed in respective nerves. The following independent variables are tested: (1) graded neuromuscular activation of selected muscles or muscle groups, (2) airflow levels, (3) implant stiffness and (4) implant medial surface shape.

Output variable measured include: (1) High speed imaging of the vibrating vocal folds (2) acoustic signals using a hi-fidelity microphone (3) subglottal pressure. High speed video will be used to analyze vocal fold shape changes due to stimulation (vocal fold length, contour, etc.) and the acoustic signal will be analyzed for frequency, loudness, vocal quality etc.

Data analysis and interpretation:
Due to the limitation in the number of animals that can be ethically used for each experiment and because data of this type are relatively unique, power calculations are not available. Regression analysis will be performed whenever possible on the measured and derived data. Findings will be descriptive and compared with the literature regarding acoustic, aerodynamic, pre-phonatory posturing, vocal fold vibration, and vocal quality.

Specific Aims and justification for the number of animals requested:

A.
Proposed studies towards Specific Aim 1: Quantify the effects of laryngeal asymmetry on acoustics, aerodynamics, vibration, and voice quality.

In vivo canine preparation: Full larynx experiments will be undertaken and high-speed video recording of the vibrating larynx will be obtained from a superior view.
Independent variables: The independent variables and conditions tested are displayed in Table 1 below. For these neuromuscular experiments 4 (four) combinations of muscles will be studied, mimicking clinically relevant paresis conditions: TA only, LCA/IA only, combined TA + LCA/IA mimicking various levels of RLN paresis, and combined TA +LCA/IA + CT mimicking various levels of vagal nerve paresis (Experiments #1-4). Each muscle or muscle combination will be stimulated over 10 levels of graded stimulation from threshold (< 10% activation) to full activation (100%). Three airflow levels will be used (500, 700, 900 ml/s) because a larger glottal gap at the lower muscle activation levels will require higher airflow to achieve phonation. Based on our previous research, we expect this airflow range to result in phonation onset in nearly all activation conditions. Each condition will be tested with all other adductor muscles at 3 activation levels (80, 90%, and 100%), to explore a range of "normal" activation levels, and three CT activation levels (0%, 50%, 90%), to test a range of vocal fold strains. Thus, a total of 270

unique neuromuscular activations states will be tested per canine per each muscle or muscle combination.

Table 1:
Experiment 1. Canines 1,2: Effects of TA asymmetry on acoustics, aerodynamics, vibration, vocal quality
Experiment 2. Canines 3,4: LCA/IA asymmetry
Experiment 3. Canines 5,6: TA + LCA/IA asymmetry
Experiment 4. Canines 7,8: TA + LCA/IA + CT asymmetry

B.
Proposed Studies Towards Specific Aim 2: Perform a comprehensive, quantitative analysis of the neuromuscular control of vocal fold medial surface (glottal channel) pre-phonatory shape and the resulting vibration, using combinations of intrinsic laryngeal muscle activations representing a wide range of paresis states from normal activation to laryngeal paralysis.

In vivo canine preparation: To evaluate the effects of neuromuscular activation on vocal fold medial surface pre-phonatory shape, hemi-larynx preparations will be used. High-speed video recording of posture changes will be obtained from the medial view (looking straight at the vocal folds from the perpendicular axial plane). One-half larynx is removed to fully visualize the medial surface of the vocal fold).
Independent variables: The independent variables and conditions tested are displayed in Table 2. For these neuromuscular experiments, there will be 3 neuromuscular input variables and their combinations: TA, LCA/IA, and CT. For the non-phonatory experiment (#5) designed to capture the static glottal pre-phonatory posture without airflow (Canines 9-10), each muscle will be paired and tested over 7 levels of graded activation from threshold to full activation (8 levels including the no stimulation condition) while the third muscle is activated at a constant level. We can reduce the number of canines needed by performing all non-phonatory posture measurements for all muscle combinations in a single canine, with a second canine tested for assessment of inter-animal variability (Canines 9-10, see Table 2). Experiments 6-8 (canines 11-16) are dynamic experiments with airflow and measurement of phonation which take a significant time to accomplish and all muscle combination cannot be performed in the same animal due to time limitations and combinations of nerves dissected and divided.

Table 2:
Experiment 5. Canine 9, 10: Muscle pairs tested: LCA/IA versus CT
Experiment 5. Canine 9, 10: LCA/IA versus TA
Experiment 5. Canine 9, 10: TA versus CT
Experiment 6. Canine 11, 12: LCA versus CT
Experiment 7. Canine 13, 14: LCA/IA versus TA
Experiment 8. Canine 15, 16: TA versus CT

C.
Proposed studies towards Specific Aim 3: Systematically evaluate the role of implant stiffness and medial surface shape, and their effects on acoustics, aerodynamics, glottal vibration, and vocal quality.

In vivo canine preparation: To evaluate the phonatory effects of implant stiffness and medial surface shape, full-larynx preparations will be used. A rectangular type I thyroplasty window will be made on the left thyroid cartilage at the level of the glottis to allow access to the paraglottic space, and thyroplasty implants will be inserted through the window to a depth of insertion that brings the medial edge of the membranous vocal fold to the glottal midline (a line drawn from the anterior commissure to the midpoint of the posterior commissure) as viewed endoscopically from above. Implants will be fabricated as described previously by mixing a two-component liquid silicone polymer solution in ratios that result in the desired stiffness. Independent variables are implant medial surface shape and implant stiffness. Four implant shapes and four implant stiffness levels will be tested.

In each canine, the efficacy of implants will be evaluated in 4 modeled neuromuscular paralysis states: (1) TA paralysis (2) LCA/IA (3) TA+LCA/IA (RLN paralysis), and (4) TA+LCA/IA+CT (Vagal paralysis). Experiments 9-12 address the question "which implant stiffness is better" by evaluating 4 implants with a fixed medial shape but differing stiffness. Experiments 13-16 address the question "which implant shape is better" by evaluating all four implant shapes with same stiffness (Table 3).

Table 3:
Experiment 9. Canine 17, 18: Divergent implant, 4 stiffness levels
Experiment 10. Canine 19, 20: Convergent implant, 4 stiffness levels
Experiment 11. Canine 21, 22: Rectangular implant, 4 stiffness levels
Experiment 12. Canine 23, 24: Rounded implant, 4 stiffness levels
Experiment 13. Canine 25, 26: 4 implant shapes, 1 stiffness level
Experiment 14. Canine 27, 28: 4 implant shapes, 1 stiffness level
Experiment 15. Canine 29, 30: 4 implant shapes, 1 stiffness level
Experiment 16. Canine 31, 32: 4 implant shapes, 1 stiffness level

Additional Notes:

All drugs used are pharmaceutical-grade.

All animals will be obtained from commercial vendors who have bred the animals specifically for research purposes.

Pain Category Assignments

NOTE: A painful procedure is defined as any procedure that would reasonably be expected to cause more than slight or momentary pain and/or distress in a human being to wh ch that procedure is applied. Examples of potentially painful/distressful procedures include, but are not lim ted to the following: terminal surgery; exuberant inflammation from adjuvants; ocular and skin irritancy testing; food or water deprivation beyond that necessary for normal presurgical preparation; noxious electrical shock that is not immediately escapable; paralysis or immobility in a consc ous animal; extensive irradiation.

Category	Description
C	Momentary or no pain/distress (Examples: injections of non-toxic substances; peripheral blood collections not requiring anesthesia; euthanasia and

	harvesting of tissue only; observing natural behavior; behavioral testing without signif cant restraint or noxious stimuli.)
D	Pain/distress relieved by use of appropriate anesthetics, analgesics, tranquilizers or by euthanasia (Examples: terminal surgery; survival surgery; retro-orbital blood collection; euthanasia of animals showing signs of more than slight or momentary pain and/or distress.)
E	Pain/distress can not be relieved by use of anesthetics, analgesics, or tranquilizers, as the use of these agents would interfere w th the experimental design (Examples: pain research; toxic ty testing.)

Species:	Dog
Strain or Breed (if applicable):	Mongrel
Average Weight:	20 lbs
Sex:	Mixed
Pain Category:	D
Number of Animals Needed for the 3 Year Period:	32

Pain Category

1. If the animals are listed under Pain Category D and/or E, check below all criteria that will be used to assess any potential pain/distress/discomfort in the animals. If applicable, include criteria used to evaluate post-operative pain/discomfort.

- ☐ Restlessness
- ☐ Vocalizing
- ☐ Decreased or impaired mobility
- ☐ Conjunctivitis, corneal edema, photophobia
- ☐ Licking, biting, or guarding a painful area
- ☐ Failure to groom, unkempt appearance
- ☐ Open sores/necrotic skin lesions
- ☐ Loss of appetite
- ☐ Weight loss.
  Percentage weight loss (max allowable 20%):
- ☑ Other: N/A as this is a terminal procedure. Animals monitored as described in the Surgery section.

2. If the animals are listed under Pain Category E, please specify the pain/distress/discomfort experienced by animals as a result of the experimental manipulations and provide scientific justification indicating why pain/distress/discomfort-relieving methods will not be employed in this protocol.

NOTE: Procedures that may cause more than momentary or slight pain or distress to the animals must be performed with appropriate sedatives, analgesics or anesthet cs, unless withholding such agents is justified for scientific reasons and will continue for only the necessary per od of time.

Not applicable

The following questions must be answered for animals listed under Pain Category D and/or Pain Category E. Federal Regulations require that investigators consider alternatives (the 3 Rs - replacement, refinement and reduction) to procedures that may cause more than momentary or slight pain or distress to animals.

3. Consider all the alternatives listed below and explain why each of the following is not an available alternative for the proposed potentially painful/distressful procedure.

A. Replacement of animals with non-animal models (e.g., in vitro procedures, computer model) or a phylogenetically lower species:

As also mentioned earlier, our laboratory has experience with all laryngeal models used for investigations of laryngeal physiology: computer models, static geometric shape systems, excised human larynges, excised canine larynges, and human laryngeal non-invasive measurements.

Computer models cannot yet accurately recreate the mucosal wave of the intact, self-oscillating vocal folds—much less model the intricacies of vocal fold collision, interactions with the nearfield glottal airflow, and possible acoustical interactions with sub- and supra-glottal systems. Computer models are based on in vivo experiments; however such in vivo investigations have never been performed. Therefore, this in vivo study is necessary. However, this study will ultimately improve computer models, which will then lead to reduction in the need for animals for exprimentation.

Excised laryngeal models do not have the same measurable mucosal wave movement that occurs from stimulation of the recurrent laryngeal nerve, nor can they model active muscle contraction, including vocal fold stiffening and bulging.

Finally, the proposed research method employs intricate microsurgery of the larynx and invasive control of laryngeal physiological variables that cannot be obtained in human subjects. Therefore, only anesthetized canine in vivo phonation allows for the control of independent variables and the ability to measure dependent variables invasively.

The in vivo canine larynx has dimensions and physiologic properties similar to that of the human larynx. Phylogenetically lower species do not have larynges with similar properties. Moreover, the canine larynx is unique in its ability to allow adequate control and measurements for various aspects of laryngeal physiology, especially with regard to the simultaneous contraction, stiffening, and bulging of the medial surface of the thyroarytenoid (TA) muscle. It is also the only model in which the ability to dissect and stimulate individual branches to the intrinsic laryngeal muscles has been demonstrated.

For these reasons, the canine is the most frequently used in vivo phonatory model, and the most appropriate for this proposed research.

B. Please discuss why the procedures cannot be further refined in order to minimize potential pain and/or distress to animals:

The experiments will be performed under general anesthesia.
We will monitor the vital signs (blood pressure, oxygen saturation, heart rate, temperature, expired CO2 levels) closely to ensure adequate levels of anesthesia. We will use a warming blanket and a heating pad and monitor core temperature using a rectal thermometer. Eyes will be taped shut to prevent dessication. At the end of the surgery the animals will be humanely euthanized (note that all surgeries are non-survival surgery). Therefore, we will take all known and necessary measures to minimize potential pain and distress to the animals.

As per veterinary recommendation, to decrease the possibility of adverse events caused by blood pressure abnormalities, we will incorporate an invasive blood pressure monitoring equipment provided by DLAM vet staff.

C. Reduction in the number of animals proposed in this application (e.g., fewer animals involved in potentially painful procedures):

Minimum numbers of animals needed to assess validity of each experiment is requested. Thus, two animals are requested per experimental condition, the minimum needed to demonstrate validity of experimental findings between animals. In addition, in each experiment, we will combine as many study questions as possible (i.e. study multiple muscles at once) so fewer animals are needed to answer multiple scientific questions.

Pain Literature Search

The following questions must be answered for animals listed under Pain Category D and/or Pain Category E.

Please note that according to PHS Policy IV.C.1.a, the Guide for the Care and Use of Laboratory Animals (the Guide p. 10) and USDA Animal Welfare Act Regulat ons §2.31(d)(1)(i) "procedures involving animals will avoid or minimize discomfort, distress, and pain to the animals." Further, in order to meet the above-ment oned regulatory requirement and in accordance with UCLA's Animal Welfare Assurance on file w th the National Institutes of Health Off ce of Laboratory Animal Welfare (OLAW), the Committee must ensure that the "principal investigator has considered alternatives to procedures that may cause more than momentary or slight pain or distress to the animals, and has prov ded a wr tten narrative descript on of the methods and sources used to determine alternatives were not available." Please also note that the Comm ttee recommends the use of keywords that are specif c to the painful/distressful procedures you will be conducting and the animal model that will be used.

1. Indicate at least two databases or other sources consulted to support the conclusion that appropriate alternatives are not available.

- [x] Pubmed (Medline)
- [ ] PsychINFO
- [ ] Altweb
- [ ] UC Center for Alternatives
- [ ] Animal Welfare Information Center
- [ ] BIOSIS
- [ ] Current Contents
- [x] Other:

ISI Web of Knowledge

2. Combination of keywords used during the search:

Please specify the keywords used in the box below, including 1) the specific painful procedures that you are conducting, 2) the animal model being used and 3) alternative terms (e.g., animal model, welfare, pain, stress, distress, methods, *in vitro*).

Please see the following examples, noting that the keywords listed only apply to a protocol involving these experimental variables:

Mouse and chronic implant and in vitro model
Mouse and artery ligation and pain
Mouse and sleep deprivation and welfare

Keywords used:

Two Databases:
PubMed (Medline)
ISI Web of Knowledge

Keywords Used:

(dog OR canine) AND voice production AND nerve stimulation AND (pain OR distress OR alternatives OR welfare)

** 0 publications in PubMed

** 1 publications in ISI Web of Knowledge.

[redacted]

Neuromuscular stimulation was applied to the thyroarytenoid or lateral cricoarytenoid muscles in the patients during extended phonation, and measures were made of fundamental frequency and sound pressure level in the stimulated and nonstimulated conditions.

This study was done in humans with simple direct stimulation of two laryngeal muscles via EMG needles. Not applicable to study vocal fold shape changes during phonation or to test efficacy of surgical implants.

3. Date of most recent search (MM/DD/YYYY):

NOTE: The literature search must be updated whenever experiments that may cause potential pain or distress are proposed/modified. The literature search must also be updated at the time of each three-year renewal, and should be conducted within 2 months of submission.

1/31/2019

4. Years Covered (e.g., 1980-2019):

1966 - present (PubMed). 1864-present (ISI Web of Knowledge).

Animal Care

1. Will the experiments involve tumor formation?

The ARC requires daily monitoring of tumor growth.

No

2. Will the experiments involve chronic disease (e.g., diabetes, chronic seizures, infections with disease agents) or a chronic condition (e.g. headcaps, implants)?

No

3. Will the experiments involve other procedures that may lead to potential complications (e.g., surgical procedures, administration of compounds with potential toxic effects)?

No

4. For all types of experiments, if animals may experience complications, please describe the criteria for premature euthanasia below.

The animals may be excluded from undergoing surgery if for any clinical findings that may preclude successful surgery and implementation of experimental protocol (e.g., if behavioral changes or signs of illness such as loss of appetite prior to the surgery are observed). A DLAM veterinarian will be consulted prior to exclusion.

5. Check below all that apply to convey special animal care requirements to the responsible veterinary staff.

- ☐ Temperature Range(s)
- ☐ Humidity
- ☐ Light Cycles
- ☐ Bedding/Litter changing schedules
- ☐ Water (e.g., sterile or deionized)
- ☐ Special diet/Feeding schedule
- ☐ Deprivation of food and/or water for reasons other than surgical preparation

6. If you checked any of the boxes above, explain special care requirements in detail.

N/A

7. Environmental Enrichment: UCLA vivarium staff provide environmental enrichment to all species (please refer to the **ARC Policy on Environmental Enrichment**).

a. If you request to provide additional or alternative environmental enrichment, please describe the environmental enrichment below.

N/A

b. Please provide scientific justification if your research precludes the use of environmental enrichment.

Pair housing is acceptable whenever possible. Pair housing may include other social species, such as pigs.

8. If you will be using transgenic animals in this research, please clarify whether there are any anticipated or suspected phenotypes of the transgenic mice that might cause pain or discomfort to the animals. If any pain, distress, or morbidity is associated with the phenotypes of this line, please indicate the criteria for premature termination of these mice.

N/A

9. PLEASE COMPLETE IF YOU HAVE MICE AND/OR RATS IN DLAM-MANAGED FACILITIES. Please check one response to the following:

I request that the veterinarian (or his/her designee) euthanize animals found to be sick or injured for me:

○ I request that the DLAM veterinarian (or his/her designee) euthanize my animals for me in accordance with his/her veterinary discretion at the time that they are found sick or injured. This decision will only apply to animals in cages that I've marked with a green euthanasia sticker on the cage card. DLAM will notify me of the euthanasia by email after the fact.

I understand that I remain responsible for monitoring of my animals, in accordance with my approved protocol and with the ARC Policy on ***Responsibility for Monitoring Laboratory Animals***.

I will treat or euthanize animals:

◉ I assure the ARC that I will promptly respond to Veterinary Health Case notifications regarding my animals, as required by the ARC Policy on ***Notification of Investigators with Sick or Injured Animals***.

Locations

Please indicate ALL locations where animals will be housed and/or used, including:

1. **Vivarium Housing** (where animals will be housed). Please note that if vivarium housing has not been assigned, select "VIVARIUM" as the building name and "Unassigned" as the room number.
2. **Study Area** (any investigator-maintained facility outside the vivarium where USDA-covered species will be housed for per ods longer than 12 hours, or where non-USDA-covered species will be housed for per ods longer than 24 hours).
3. **Research Area** (where non-surg cal activities, including euthanasia, will be performed).
4. **Surgery Area - Survival** (where recovery surgery will be performed).
5. **Surgery Area - Non-Survival** (where terminal surgery will be performed).

Building	Room	Species	Location Type
[redacted]	[redacted]	Dog	Surgery Area - Non-Survival **Reason:** Surgery, neuromuscular stimulation, induced phonation, high-speed photography, acoustic and aerodynamic recording, will be done in this room. Only non-survival surgery is proposed in this protocol. The study area is the [redacted] that permanently houses all the bulky equipment needed for these experiments (high-speed cameras, phonation apparatus, hi-fidel ty recording dev ces). The experimental apparatus is delicate and sensitive, calibrated to the study area, and cannot be moved to another location. We are unable to use [redacted] surgery area because these studies require equipment and built-in apparatus/structures that cannot be moved from animal lab room [redacted] In fact the [redacted] animal lab room [redacted] There are multiple dedicated lines of data and control cables for stimulation, recording, and automated control of airflow and neuromuscular stimulation. Cables run over the instruments, down a large aluminum cage structure built around the surgical table, and onto the surgical table. Multiple cables run back to the computer to measure acoust cs, aerodynam cs, and high speed v deo. All this apparatus cannot be dismantled and recreated in another locat on. A dedicated laboratory is needed and exists in [redacted]
[redacted]	Unassigned	Dog	[redacted]

Medications and Experimental Drugs

List below all medications/drugs/compounds/agents/etc. that will be given to the animals. Please be sure to include analgesics, anesthetics, antibiotics and all experimental drugs or treatments. Cell lines injected in suspension should be listed here.

The select on of the most appropriate med cation/agent should reflect that which best meets clin cal and humane requirements w thout compromising the scientific aspects of the research protocol. In accordance w th federal regulat ons, consultat on with an attending veterinarian is required in the planning of a research protocol involving procedures that may cause more than momentary or slight pain or distress to the animals. The **ARC Policy on Use of Pharmaceutical-Grade Compounds** requires that investigators use pharmaceut cal-grade compounds whenever they are available, even in acute procedures.

If pharmaceut cal-grade preparations are not available, please dentify wh ch compounds are affected and provide supporting justification in your Experimental Design. All non-pharmaceutical-grade drugs must be filter-sterilized pr or to use.

Please do not list euthanasia drugs in this section.

Drug/Compound Name:	Buprenorphine
Species:	Dog
Medication Type:	Analges c
Dose or Concentration:	0.01 - 0.02 mg/kg
Volume:	
Frequency:	q6h as needed
Route:	im
Length of treatment/administration:	
Purpose:	Pre-Operative/Intra-Operative

Drug/Compound Name:	Isoflurane
Species:	Dog
Medication Type:	Anesthet c
Dose or Concentration:	1-3%
Volume:	
Frequency:	Continuous inhalational during surgery
Route:	other: Via tracheal intubation
Length of treatment/administration:	Intraoperative
Purpose:	Pre-Operative/Intra-Operative

Drug/Compound Name:	Propofol
Species:	Dog
Medication Type:	Anesthet c
Dose or Concentration:	6-8 mg/kg
Volume:	
Frequency:	Once for induction of anesthesia
	iv

Route:	
Length of treatment/administration:	
Purpose:	Pre-Operative/Intra-Operative Other: Induction of Anesthesia

Drug/Compound Name:	Cephazolin
Species:	Dog
Medication Type:	Other
Dose or Concentration:	25 mg/kg
Volume:	
Frequency:	once
Route:	im
Length of treatment/administration:	
Purpose:	Pre-Operative/Intra-Operative

Drug/Compound Name:	Dexamethasone
Species:	Dog
Medication Type:	Other
Dose or Concentration:	0.5 mg/kg
Volume:	
Frequency:	Once after intubation
Route:	iv
Length of treatment/administration:	
Purpose:	Pre-Operative/Intra-Operative

Euthanasia

For each species used, please provide the euthanasia information. Techniques for euthanasia must follow guidelines established in the AVMA Guidelines for the Euthanasia of Animals: 2013 Edition.

1. **Species:**

   Dog

2. **How will animals be euthanized?**

   Non-Physical Method

3. **For animals that will be euthanized by a physical method, please indicate that method (decapitation or cervical dislocation).**

   a. **Please indicate the appropriate physical method.**

   Other:Animals will also undergo removal of the larynx and trachea as confirmatory method.

   b. **Will anesthesia be used prior to use of the physical method of euthanasia?**

   Yes

   c. **If anesthesia cannot be administered, please provide scientific justification.**

4. **For animals that will not be euthanized at the end of the study, please indicate the final disposition.**

Euthanasia Medications

List the drug(s) used for euthanasia on an animal by physical or non-physical methods.

Please note that according to the **AVMA Guidelines for the Euthanasia of Animals: 2013 Edition**, "compressed CO_2 in cylinders is the only recommended source of carbon dioxide because the inflow to the chamber can be regulated precisely. Carbon dioxide generated by other methods such as from dry ice, fire extinguishers, or chemical means (e.g., antacids) is unacceptable."

Drug Name:	Veterinary Grade Pentobarb tal
Species:	Dog
Dose or Concentration:	100 - 200mg/kg
Route:	iv
Purpose of Drug:	Euthanasia

Tissue Collection

Please enter the following information regarding tissue collection for the protocol. See ARC Policy on Blood Collection from Laboratory Animals.

1. Tissue To Be Collected:

☐ Blood

☑ Other Collected: Larynx and trachea; for additional neuroanatomic studies or for use in excised larynx experiments

2. Frequency of blood and/or other tissue collections:

Once after euthanasia.

3. Volume of blood and/or other tissue collected per time point:

One larynx and upper portion of the trachea.

4. Describe techniques that will be used to collect blood and/or other tissue.

The larynx will be removed using standard laryngectomy techniques. It will be extracted with a portion of the trachea attached.

5. Describe how anemia and infection will be prevented.

N/A

Surgical Procedures and Post-Operative Care

Please complete the following questions, noting that any requested exception to ARC Policy must be justified in the space provided.

Note: ARC pol cy requires investigators to employ the following measures to ensure asepsis while conducting survival surgery: asept c surg cal techniques; asept c surg cal field; sterile instruments; clean lab coat/surg cal gown; and sterile surg cal gloves. *For information on surgeries on rodents and birds, please see the **ARC Policy on Survival Surgery in Mice, Rats and Birds***.

Non-survival surgeries of extended durat on or procedures otherwise likely to increase the risk of Intraoperative infection and/or sepsis (e.g. gastrointestinal surgery) will be evaluated on a case-by-case basis to determine whether aseptic techn ques must be used. Refer to the **ARC Policy on Non-survival Surgical Procedures** for further informat on.

Please note that surgical records are required for all animals. These records must include anesthetic administration and intra-operative mon toring, as well as post-operative recovery observations, including administration of analgesics and antib otics and suture/staple removal if appl cable. Additionally, any adverse outcomes must also be recorded.

1. Pre-Operative care will include (check all that apply):

☐ Lab tests

☐ Conditioning

☑ Fasting: Eight (8) Hours

☐ Other:

Please note that a phys cal examinat on is required.

2. Will neuromuscular blocking agents be used (e.g., Pancuronium, Succinylcholine)? Refer to the ARC Policy on Neuromuscular Blocking Agents.

No

3. Select all criteria that will be used to assess the proper level of anesthesia.

The level of anesthesia should be assessed on a continuous basis.

☑ Respiration rate

☑ Heart rate

☐ EEG

☐ EKG

☑ Muscular relaxation

☑ Positive toe pinch

☑ Corneal reflex

☑ Color of mucous membranes

☑ Other:

Pulse oximetry, expired CO2

4. Surgical preparation of all mammalian species must include:

1) Removal of hair w th #40 clipper blade in a wide margin around the incis on site.
2) Three alternating scrubs using a germ cidal scrub and 70% alcohol.
3) Placement of lubricating ointment into the eyes.
4) Covering the animal except the surgery site w th a sterile drape.
5) Placing the animal on an external heat source (water circulating heat pad or heating pad set on "low" with a barrier placed between the animal and the heating pad).

◉ I assure the ARC that surgical preparation will be performed as outlined above.

○ *Not applicable, as this protocol includes only non-survival surgeries for which aseptic technique is not required.*

PLEASE NOTE: Any deviation from the policies above must be detailed and scientifically justified in the space below.

We already follow all of the items listed above although only non-survival surgery is proposed. In the last version of this protocol, the ARC requested the following and we plan to continue to follow this protocol:

1. Prep the animal and perform the surgery aseptically for the animal preparation and surgical exposure portion of the experiment. Animal preparation and surgical exposure of the larynx involves the first half (about 3 hours) of the proposed experiments.

2. For the laryngeal stimulation and data measurement part of the experiments, there are many instruments that cannot be sterilized. For example, the delicate nerve stimulation electrodes, glass prisms used to provide stereoscopic views for the high speed photography, and the subglottal tubes and instrument system used to measure pressure and acoustics cannot be sterilized. These can be cleaned by soaking in alcohol and rinsing under clean water. Sterilizing these equipment places undue burden on the feasibility of this proposal as these instruments are expensive and can break during the sterilization process. Thus we will use clean conditions.

3. The ARC was concerned about risk of intraoperative infection. However, please be aware that our lab has the most experience in the world using this animal model, and we have been routinely performing similar experiments in over [redacted] without development of a single case of intra-operative infection. However, to further allay the concerns of the ARC, I will continue administration of a peri-operative dose of antibiotics and steroids.

i. Cephazolin 1 gram prior to incision.
ii. Dexamethasone prior to incision.
iii. Aseptic animal prepararation and surgery (about 3 hours) on the larynx, then clean experimentatal conditions (clean gloves, gown, cap worn by personnell, all instruments clean) during laryngeal stimulation and data measurement (about 3-4 hours)

5. Indicate the methods to be employed to prevent (a) hypothermia and (b) dehydration (including volume of fluids and route). If this question is not applicable to the proposed surgical procedures, provide a brief explanation.

To prevent hypothermia, the veterinarian recommends the use of water-circulating heading pads over heating lamps and/or electr cal heating pads. The use of heating lamps is strongly discouraged. If not used properly, heating lamps and electrical heating pads may cause thermal injury to the animal. Therefore, describe precaut ons taken to prevent hyperthermia.

(a) A water circulating heading pad is used.
(b) A warm air blanket above body.
(c) Rectal temperature is checked at all times.
(b) Use of normal saline solution 5-10 ml/Kg/hr IV. The rate will be typically 5 ml/Kg/hr and adjusted as needed (e.g. for low blood pressure)
If a higher rate than 10 ml/kg/hr is indicated, a DLAM veterinarian will first be consulted, and his/her directions followed.

6. Surgical preparation of the surgeon must include:

1) Wash hands w th germicidal soap.
2) Sterile gloves.
3) Surgical Mask.
4) Cap and booties (not required for mice and rats)
5) Sterile gown (clean lab coat or gown acceptable for m ce and rats)

◉ I assure the ARC that surgical preparation will be performed as outlined above.

○ *Not applicable, as this protocol includes only non-survival surgeries for which aseptic technique is not required.*

7. Instrument preparation must be performed by:

1) Autoclave sterilization or ethylene oxide (gas) sterilizat on.
2) Either chemical disinfect on (acceptable between multiple surgeries in mice, rats, and non-mammalian species) or
3) Hot bead sterilizer.

◉ I assure the ARC that instrument preparation will be performed using one of the methods outlined above.

○ *Not applicable, as this protocol includes only non-survival surgeries for which aseptic technique is not required.*

8. Duration of Surgical Procedures (Must be completed as applicable):

For non-survival surgery, ind cate the duration from anesthesia induction to euthanasia. For survival surgery, ind cate the durat on from anesthesia induction to recovery from anesthesia.

Survival:
Non-Survival: Up to 8 Hours

9. Provide scientific justification for performing multiple survival surgeries on a single animal.

Multiple survival surgeries will be approved only when they are related components of the experimental design.

N/A

10. Please describe all surgical procedures, including non-survival procedures.

We have proposed that thirty-two (32) research-bred mongrel canines, approximately 12-18 months old and 15-25 kg in weight, be used in this research. Of these thirty-two (32) canines, it is proposed that twenty-four (24) be used for full larynx experiments (Experiments #1-4, 9-16), and eight (8) be used for hemi-larynx experiments (Experiments #5-8; see Research Strategy section). The age range of the

canines span the typical early adult range, in order to obtain canines with healthy adequately sized larynges.

On the day of the experiment, the canine will be pre-medicated intramuscularly with Buprenex in the housing area and then transported to the laboratory. An intravenous line will be placed in the laboratory and Propofol will be used for induction of anesthesia. After induction, the animal will be placed supine on an operating table, and will be intubated through the mouth with an endotracheal tube. General anesthesia will be maintained with isoflurane (1-3%), and intravenous Propofol as needed, to prevent reflexive response to corneal stimulation, or other signs of inappropriate level of anesthesia, as monitored by respiratory rate, heart rate, muscular relaxation, positive toe pinch, color of mucous membranes, and pulse oximetry. Intravenous dexamethasone will be given to decrease possible nerve and vocal fold swelling. Core temperature will be maintained with a warming blanket and measured with a rectal thermometer. Oxygen saturation and expired CO2 will be monitored.

DLAM vet staff will catheterize the femoral artery for placement of blood pressure monitoring system.

After adequate induction of general anesthesia, the anterior neck skin will be shaved, prepped with betadine and draped in a sterile fashion for surgery. A midline skin incision will be made and surgical dissection will be performed to expose the trachea, larynx, the superior laryngeal nerves (SLNs), and the recurrent laryngeal nerves (RLNs). A low tracheostomy will be made and an endotracheal tube will be placed for intra-operative ventilation. The anesthesia machine ventilation circuit will be switched to this tube and the oral endotracheal tube will be removed. The trachea will be transected at ring 3-4 ring and a subglottal tube to provide rostral airflow for phonation experiments will be attached with ring clamps. The subglottal tube also contains ports for a probe microphone (Model 4128 Bruel & Kjaer, Norcross, GA) to measure subglottal acoustics and a pressure transducer (MKS Baratron 220D, MKS instruments, Andover, MA) to measure subglottal pressure. The subglottal tube is attached to the computer controlled mass airflow controller (MCS Series Mass Flow Controller, Alicat Scientific, Tucson, AZ), which will regulate the airflow level. The air supply will be heated and humidified using a medical grade humidifier (HumiCare 200, Gruendler Medical, Freudenstadt, Germany).

A pharyngotomy will be made at the level of the thyrohyoid membrane to slightly exteriorize the larynx in the neck for easy access to place India ink fleshpoints on the vocal fold surface, and for measurement instruments such as high-speed photography and outside microphone. The epiglottis will be trimmed. The RLN will be further dissected and the Individual branches of the RLNs (TA and LCA/IA branches) are exposed as needed for each experimental protocol. Miniature tri-polar cuff electrodes will be placed on each nerve branches for stimulation of the TA, LCA/IA, and CT muscles. The internal (sensory) branch of the SLN and the PCA branch of the RLN will be divided to remove the effects of these nerve/muscle variables on phonation experiments.

For hemilaryngeal experiments, a vertical partial laryngectomy (removal of one vocal fold and false vocal fold, typically on the right) will be performed to create and expose a hemilarynx. India ink will be used to provide visual markers on the medial surface of the vocal fold. High-speed camera will visualize the medial surface of the intact vocal fold through a right-angle prism to record high-speed, stereoscopic imaging of vocal fold posture (static studies) and/or vibration (dynamic studies).

As previously enumerated in the Experimental design/Specific Aims, for each phonatory condition explored, we will determine which intrinsic laryngeal muscles contribute to the measured parameter, the relative contribution of each laryngeal muscle on each parameter (e.g. vocal fold medial bulging, phonation threshold pressure, acoustic intensity, etc.). Our stereoscopic imaging techniques, automated extraction of three-dimensional physical coordinates, spatio-temporal analyses of the data, and automated recording of acoustic and aerodynamic parameters has reached a stage of development such that we are now able to automatically deliver the neural stimulation and concurrently measure the acoustic, aerodynamic, and vibratory parameters in symmetric and asymmetric activation of the larynx. Using newly developed methods of graded stimulation in our laboratory, we will perform graded stimulation of the laryngeal muscles. In particular, we will stimulate three to six independent nerve branches in a graded fashion using tri-polar electrodes, as outlined in the research strategy. In each experiment, we will determine the threshold and maximal activation stimulating current for each muscle. At threshold activation the muscle has a hint of motion while at maximal activation the maximal posture change s obtained. The stimulation pulse rate (100-200 Hz) of the applied pulse trains will be set high enough to prevent single twitch muscle responses. The duration of the pulse train will be kept short enough (1 to 1.5 seconds) and adequate time will elapse between stimulation pulse trains (3.5 seconds) to prevent fatiguing of the nerves and the muscles and we have demonstrated continuous recording of in vivo posture change and phonation for or up to 3 hours.

Based on the empirically determined activation response function of each intrinsic laryngeal muscle we will use 5-10 different graded stimulation levels (as specified in the Research Strategy) to evaluate the effect of each intrinsic laryngeal muscle on measurement parameters (posture, acoustics, vibration, etc.). Our automated neural stimulation and measurement system will concurrently deliver independent stimulation pulse trains to intrinsic laryngeal muscles then automatically record the measured variables of subglottal acoustics, subglottal pressure, supraglottis acoustics, and high-speed video of vocal fold posture and vibration. The stimulation pulse amplitudes corresponding to graded levels for each intrinsic laryngeal muscle activation will be applied in a randomized way to reduce the potentially confounding effects of the order of stimulation.

During each activation condition, we will measure acoustics, aerodynamics, and glottal vibration. For each glottal configuration (paresis or paralysis state), the effects on voice quality, phonation type, aerodynamics, and glottal vibration will be explored (see Research Strategy). For the full larynx the superior surface vibration will be imaged for assessment of posture and vibration. For the hemi-larynx, the medial surface dynamics will be imaged, to further explore mechanisms of vibration along the medial surface of the vocal folds, which is expected to vary substantially as a function of TA muscle activation, and should shed light on the role of medial vocal fold (glottal channel) surface shape changes in phonation.

After completion of the experiment, the animal is euthanized while still under general anesthesia. Euthanasia will be implemented intravenously using sodium pentobarbital. A total laryngectomy is then performed and the larynx

collected as described earlier.

11. Please indicate the suture materials to be used:

- ☐ Internal: absorbable sutures (e.g., Dexon, Vicryl)
- ☐ External: non-absorbable skin sutures (e.g., Nylon, wound clips). *Please note that external skin sutures or wound clips must be removed 7-14 days following surgery.*
- ☑ Other/not applicable (describe below):

Non-survival surgery.

12. During recovery from anesthesia, what indications will be monitored to assure the animals are stable?

In accordance with the Guide for the Care and Use of Laboratory Animals, particular attent on should be given to thermo-regulation, card ovascular and respiratory function, and post-operative pain or discomfort during recovery from anesthesia.

N/A

13. How often will animals be monitored after anesthetic recovery?

The ARC requires that animals be observed continuously by trained personnel during the immediate anesthet c-recovery period (i.e., until the animal is ambulatory) and at least daily after anesthetic recovery. However, post-operative mon toring frequency may be greater depending on the complex ty of procedures involved, administration of post-operative analgesia, and the species of animal used.

N/A

Species Surgery

Species:	Dog
Number of Animals:	32
Surgery Type:	Nonsurvival Surgery
Surgeries per Animal:	
Time Between Surgeries:	

Gas Anesthetic

NOTE: Gas anesthetics like isoflurane, halothane, enflurane, and ethane must be used safely. The Off ce of Environment, Health & Safety (EH&S) requires the use of a certified fume hood or a gas anesthet c machine that contains a scavenging dev ce (e.g., anesthet c gas machine w th charcoal filter; ducted fumehood or ducted b osafety cabinet; Crump WAG System; vaporizer w th a scavenging filter, such as F-air canister) when using gas anesthetics.

1. What gas anesthetic agent(s) will be used?

- ☐ Halothane
- ☑ Isoflurane
- ☐ Other:

2. Gas anesthetic(s) will be scavenged via:

- ☐ Certified Fume Hood:
- ☑ Other: Anesthesia Machine - [redacted]

Scavenging Location

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Principal Investigator Assurance

After you have reviewed and answered yes to the items below, please click "Save" at the bottom of the page. Please note that the PI must complete this section. To determine your eligibility to serve as Principal Investigator of a research protocol, please refer to UCLA Policy 900 (Principal Investigator Eligibility) or contact the ARC administrative office (310-206-6308). If the terms of Policy 900 are not met, faculty sponsorship or principal investigatorship by a UCLA employee with faculty appointment may be required.

Regarding policies governing animal research at UCLA:

Yes	No	
◉	○	I agree to abide by all applicable federal, state, and local laws and regulat ons and UCLA pol cies and procedures.
◉	○	I am aware that deviat ons from an approved protocol or violations of applicable pol cies, gu delines, or laws could result in immediate suspension of the protocol.
◉	○	I understand that the attending veterinarian or his/her designee must be consulted in the planning of any research or procedural changes that may cause more than momentary or slight pain or distress to the animals.
◉	○	I declare that all experiments involving live animals will be performed under my supervis on or that of another qualified scientist. All listed personnel will be trained and certified in the proper humane methods of animal care and use prior to conducting experimentation.
◉	○	I understand that emergency veterinary care will be administered to animals showing evidence of discomfort, ailment or illness.
◉	○	I declare that the information provided in this appl cation is accurate to the best of my knowledge. If this project is funded by an extramural source, I certify

		that this appl cation accurately reflects all currently planned procedures involving animals described in the proposal to the funding agency.
◉	○	Any modificat ons to the protocol will be subm tted to and approved by the ARC prior to initiation of such changes.
◉	○	If this is a renewal application, I assure that appropriate progress has been made to justify the continued use of research animals.
◉	○	The experimental design has been refined in order to minimize the invasiveness of the proposed procedures.
◉	○	I assure that the proposed research does not unnecessarily duplicate previous experiments.

Agreement on electronic submission:

I understand that by submitting this document that this document will be sent to appropriate members for review. I further understand that once submitted for review, this protocol cannot be modified or changed unless so requested by the ARC. In addition, once approved, all changes or modifications must be subm tted for review and approval of the ARC prior to in tiation.

Completed by: [redacted] **1/3/2019**

FS Assurance

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