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DATE: February 19, 2019

TO: Spencer Nyholm, Ph.D.
Department of Molecular and Cellular Biology, Unit 3125

FROM: Randall Walikonis, Ph.D. Randall Walikonis

SUBJECT: Notice of IACUC Approval for Protocol No. A18-029

This letter serves as written notice of animal use **APPROVAL** by the IACUC on February 19, 2019. Please note that at the June 20, 2018 meeting, the IACUC determined that the protocol required modifications to secure approval that now have been addressed. Please refer to the assigned protocol number for all animal orders and future correspondence with the IACUC; cage cards should contain this protocol number. Please advise us in the future of any necessary corrections or revisions by completing the appropriate form at http://research.uconn.edu/iacuc/iacuc-forms/.

Please Note: All investigators are required to make study records available for inspection during normal business hours. If study records are kept in locked facilities, a member of the research staff must be designated as the official contact person for record inspection.

If you have added new personnel (students, post-docs, or faculty members) to the protocol, you must contact ACS Veterinary Staff (acstraining@uconn.edu) to schedule the appropriate training in Basic Animal Handling and if assisting or conducting surgery, training in Anesthesia and Aseptic Technique, etc.

This institution has an Assurance of Compliance on file with the Office of Laboratory Animal Welfare, National Institutes of Health (Assurance #A3124-01, 2/28/2020).

Funding Source:

1. NSF: "Characterizing the Role of a Bacterial Consortium in a Host Reproductive Organ", IOS-1557914
2. OVPR UConn Seed Grant: "A Unique Host-Microbe Symbiosis as a Novel Source of New Antifungal Drug Leads"

Office of the Vice President for Research
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compliance.uconn.edu

All animal use protocols must be reviewed annually from the date of IACUC approval; you will receive an e-mail notice requesting an annual update. Thank you for your efforts to help keep the University in compliance with all animal welfare regulations.

Title:	**"The Hawaiian Bobtail Squid, Euprymna scolopes, as a Model for Host-Microbe Research"**
Species:	**Bobtail Squid**
Date Approved:	**February 11, 2019-February 10, 2022**
Protocol #:	**A18-029**

c: Michael Lynes, Department Head, Dept. of Molecular and Cellular Biology
IACUC Designated Reviewer
Curtis Schondelmeyer, Animal Care Services
Sheryl Lohman, Animal Care Services
William Field, Environmental Health and Safety

University of Connecticut

IACUC-1 Protocol Application for Live Animal Care and Use

Institutional Animal Care and Use Committee, Office of Research Compliance

Whetten Graduate Center, Rm #214, 438 Whitney Road Ext., Unit 1246 Storrs, CT 06269-1246 860-486-8802

Office Use Only
IACUC Protocol # A18-029
Approval Date: 2/19/19
Expiration Date: 2/18/22
Species: Squid
Guide exceptions: Y__ N✓
Hazards: Y__ N✓

Sample language and additional guidelines for completing protocol forms are listed on the Protocol Instructions page.

Section I: PI (Principal Investigator) and Laboratory Information

Principal Investigator (PI) Spencer Nyholm
Department: Molecular and Cell Biology
E-mail: spencer.nyholm@uconn.edu
Unit #: 3125
Phone #: 860-486-4886
Emergency contact information (please provide cell phone#): 617-921-7575

DEC 31 2018
Office of Research Compliance

Section II: General Protocol Information

Project Title: The Hawaiian bobtail squid, Euprymna scolopes, as a model for host-microbe research
Submission type: ☒New ☐ Three year Renewal or ☐Modification of approved submission #
Type of project: ☒ Research ☐Teaching (course #:) ☐Public Service ☒Field research Check All That Apply
Anticipated project start date: ongoing since August 2007
(If different than above) When will animal work on this project begin?
Species: Euprymna scolopes (bobtail squid)
Total animal number: 90-180 field-caught adult squid/yr; 60 juvenile wild-caught juvenile squid/yr; 5000-20000 embryos-juvenile squid/yr; 500 raised squid/yr

Section III: Funding and Collaboration

A. Project Funding Status
Funding sources may include internal (department, internal grants) or external (NIH, USDA, NSF etc.)

Check all that apply:

	Status	Name of granting agency or other source (department, institution, etc.)	Title of award (s) *or* FRS# (if internally funded)
X	Fully or partly funded by federal grant	NSF	IOS-1557914, "Characterizing the role of a bacterial consortium in a host reproductive organ"
	Pending funding by federal grant		
X	Funding to be provided by University of CT		A Unique Host-Microbe Symbiosis as a Novel Source of New Antifungal Drug Leads
	Funding provided by other organization		
	Other: describe how animal expenses will be covered:		

B. Collaboration:
Will any live animal work for this project be performed at a facility or institution other than the University of Connecticut Storrs campus (excluding the receipt of animals or tissues from approved vendors)?

No X Yes If Yes, complete the following:

1. Which institution/facility will have ownership of the animals?
   ▶ University of Hawaii, Manoa (Kewalo Marine Laboratory)
2. What is the nature of the collaborative work being performed, including where and with whom work will be performed?
   ▶ The Hawaiian bobtail squid, Euprymna scolopes, is collected from the wild on Oahu, HI. Animals are stored in tanks at Kewalo Marine Laboratory in the labs of Drs. Margaret McFall-Ngai and Edward Ruby before shipment to the Nyholm Laboratory at the University of Connecticut, at which point UConn takes ownership of the animals.
3. If the collaborative work is being performed as part of an approved IACUC protocol at another institution, please include a copy of the approval letter with this protocol submission.
   ▶

Section IV: Project Overview

A. **Please summarize your project. It may be helpful to think about how you would describe your project to the Media, or to someone with a high school education. Technical language from grant submissions is not acceptable in this section. Be sure to include how live animals will be used to contribute to your teaching or research goals and how this project will advance scientific knowledge or otherwise benefit human or animal health or well-being.**

▶ The Hawaiian bobtail squid serves as a model animal host for studying relationships with beneficial bacteria. The Nyholm lab studies interactions between the immune system of the squid host and a bioluminescent (light producing) bacterium that resides in a specialized light organ. Female members of this and many other squid species also harbor bacteria in their reproductive system in a specialized organ. The function of these bacteria is unknown but has been hypothesized to assist with protecting externally laid eggs from being fouled by other microorganisms. The Nyholm lab is using genetic, biochemical, and proteomic analyses to test the ability of these beneficial bacteria and compounds that they produce to inhibit fungi and other microorganisms. Furthermore, the ability of these bacteria to induce development of the squid's reproductive organ is being tested.

B. **Is your project an extension of previous work? If this project is an extension of your previous work or the work of others, briefly explain why additional work is needed.**

▶ The Hawaiian bobtail squid, *Euprymna scolopes*, has been used a an animal model for understanding influences of beneficial bacteria on animal development and interactions with the innate immune system for close to 30 yrs. PI Nyholm has been studying this organism since 1995 and his laboratory has been using this animal as its primary research organism since the beginning of his faculty appointment at the University of Connecticut in 2007. At this time, no virtual or non-animal model exists for these symbiotic relationships and the squid-bacteria model is one of the best systems to study the influences of beneficial bacteria on animal development.

Section V: Description of Procedures:

A. **Please provide a timeline, flowchart, or other depiction of the course of the stud(ies), experiment(s) or activit(ies).**
▶
Timeline: 2018-2020 A) Animal collections (ongoing) 3-4 times/per yr as needed. Adult *E. scolopes* will be collected from Hawaii 3-4 times/yr typically in January, May, September but months may vary depending on experimental needs and environmental conditions in Hawaii (e.g., weather and sea water temperature).

B) Raising experiments will also be ongoing (3-4 times/yr) with cohorts raised from juveniles obtained from the egg clutches of wild-caught squid for developmental experiments related to understanding the maturation of the squid immune system and how a symbioitic reproductive organ develops in female squid. A typical raising experiment lasts 3-4 months from hatched squid to sexual maturity.

B. **Please provide a complete description of the procedures that will be performed in live animals. You should provide sufficient detail to allow the IACUC to discern what is happening to the animals every day the animals are on study; from arrival to endpoint, and ensure that the terminology and nomenclature used to describe groups is consistent here and in other sections. Reference to an IACUC approved written ACS/ANSCI/IACUC Standard Operating Procedure (SOP), when performing the procedure exactly as stated in the SOP, is acceptable. Include copies of all referenced SOP's. Note when surgical procedures are performed but describe them in detail in Appendix G. DO NOT REPEAT INFORMATION GIVEN IN LATER SECTIONS OR IN APPENDICES.**

▶ **Live-animal experiments**

Many experiments are performed within the first 3-5 days after hatching when the squid still have egg yolk reserves on which to survive. Adult squid are also used for experiments where blood will be collected or organs harvested (after anesthesia; see below)

To maintain squid in the aposymbiotic state after collection (non-colonized), the animals are transferred to individual 20 mL scintillation vials containing 3 mL FSIO.

Hatchlings used in colonization experiments are transferred and pooled (50 squid maximum) in a plastic cup containing 100 mL of FSIO with 3,000-10,000,000 CFU/mL of *Vibrio fischeri*. After 24 h, the animals are transferred to individual scintillation vials as described above. Scintillation vials are placed in racks and loosely covered with shrink-wrap plastic film to limit evaporation. Racks are stored in the incubation room, so that the hatchlings experience the same light-dark cycle and temperature as they did as eggs during incubation. Whenever possible, juvenile animals are co-housed until sexual maturity.

On a daily basis until the experiment is complete, hatchlings are checked for colonization and luminescence. The scintillation vial is put into a Berthold luminometer, and the light produced by the animal is recorded.

Hatchlings or eggs/embryos may be used for photography through a dissecting microscope. Such use in not lethal, and does not require anesthetic. Hatchlings may also be used for live imaging, and are anesthetized before observation.

Experiments commonly expose squid to:

- *Vibrio* species in FSIO
- Chemical reagents/inhibitors/antibodies diluted in FSIO
- 4% paraformaldehyde in marine PBS (after anesthesia)
- RNAlater for RNA extraction (after anesthesia)
- Flash frozen (after anesthesia) for protein extraction

Imaging:

- Many experiments (live imaging, fixed-animal imaging (confocal and epifluorescence microscopy), collection of light-organ contents, collection of hemolymph, *etc.*) require prior anesthesia of the squid. Animals are transferred to a vessel with filter-sterilized sea water or instant ocean salt mix (FSSW or FSIO) containing either 2% ethanol or 0.12 – 0.15 M $MgCl_2$. Anesthesia is considered complete when the animal begins to drift or become ataxic. Chromatophore control will also relax and the animals will appear pale.
- Squid used for live imaging are anesthetized as described, and then their central nervous system is disrupted by applying pressure with forceps quickly between the eyes. The mantle can then be dissected open, and the animal is mounted in FSSW or FSIO for imaging. These procedures are terminal and animals are disposed of as described below (See Section XI).
- Hemolymph can be collected from adult squid and juvenile animals. For adult animals, squid are anesthetized in FSSW or FSIO containing 2% ethanol as described above. Hemolymph is withdrawn from the cephalic blood vessel located between the eyes using a sterile 1-cc syringe and 28½-gauge needle. Animals are either euthanized (see below) or subsequently revived by placing in FSSW or FSIO. Survivability after bleeding is high and most animals can sustain multiple bleeds over several days. For juvenile animals, squid are anaesthetized in 2% ethanol as described above and euthanized before blood is removed by severing the anterior and posterior portions with a razor blade. Unless otherwise noted, the following procedures are conducted in the dark under a dim red light during the animal's nocturnal period. To collect expelled light organ contents, animals are anaesthetized, ventrally dissected to expose the light organ, and then exposed to a light cue to induce 'venting' of the light-organ contents.

- To collect light-organ contents *in situ*, adult animals are anaesthetized either in FSSW or FSIO containing 2% ethanol or 0.12 – 0.15 M $MgCl_2$, or in 250 mL of FSSW or FSIO. When the animal is pale and cannot correct its position when oriented dorsally, the animal is transferred to a dissecting tray, and covered in sufficient FSIO (2% ethanol, or 0.12 – 0.15 M $MgCl_2$) to ensure ventilation by the gills. A small primary incision is made in the animal's mantle. If, at this point, the squid does not respond by moving tentacles or dilating pupils, then a medial incision is made through the ventral mantle tissue to expose the animal's light organ. A medial incision is also made in the funnel to fully expose the light organ. To induce expulsion of the light-organ contents, incandescent white light is directed at the squid's eyes. The expelled material is extruded out of the lateral openings of the light organ, and is collected with a 50-µl capacity Gilson Microman negative-pressure pipet. Expulsion of the crypt contents may occur as much as 30 min following initial light exposure, depending on the method of anesthesia used, among other factors.

Section VI: Justification of Animal Use and Numbers

A. Justification of Animal Use:

1. **Rationale for using live vertebrate animals in this project:**
   What is the reason that live vertebrate animals are necessary for this project? (Complete Table)

Check all that apply:

X	The complexity of the processes being studied cannot be replicated, duplicated, or modeled in simpler living systems, such as in plants, insects, or other invertebrates.
X	There is not enough information about the process being studied to design in-vitro or non-living models
X	Existing in-vitro or non-living processes cannot produce the required results (e.g., cell culture for monoclonal antibody production, computer modeling of protein synthesis, etc)
	Preclinical studies in living vertebrate animals are necessary prior to human testing
X	This is a behavioral, learning, or development study: a whole living system is required
X	This is an ecological or field study
X	The animals will be used for teaching/ demonstration purposes
	Other- please describe:

2. **Appropriateness of species / strain selected:**
   For each species and strain listed in Section V.A.2, please describe the rationale in the table below:

Enter species name across top, then check all rationale that apply for that species	Species/ strain 1: Euprymna scolopes	Species/ strain 2:	Species/ strain 3:	Species/ strain 4:
This is a new model with untested properties				
A large database exists for this species/ strain which will allow comparisons to previous data	X			
The anatomy, genetics, physiology, phenotype, or behavior of the species is uniquely suited to the proposed study	X			
This is the phylogenically least complex model that will provide adequate tissue, size, or anatomy for the proposed study	X			
The results will be directly applicable to the health or care of this species	X			
Other: please describe additional rationale used to select the species and strain requested				

B. Justification of Animal Numbers:

1. What was the method(s) used to determine how many live animals are required for this study? Check all that apply, and supply additional information where asked to do so:

	This is a field study (e.g. a mark/recapture study for estimating population size/trends or survival) in which the nature of the research requires as many animals as can be located)	
	Numbers were mandated by FDA or other government agency (e.g. GLP work)	Which agency?
	Numbers were based on results of a pilot study	Please reference study:
X	Numbers were based on previous research or experiment by self or others	Please reference experiment: Please see publication list from Nyholm lab (attached: nyholmlab.uconn.edu)
	Numbers were calculated using a statistical formula	Please reference the name of the formula(S):
	Numbers were calculated via consultation with a statistician	Who was consulted, and on which date(s):
	Numbers are based on expected student enrollment: reflects animal/student ratio required for effective teaching	
X	This is a breeding or holding protocol, and numbers represent the estimates of offspring that will be produced and/or animals that will otherwise need to be held while not on study	
	This is a pilot project which will be used to refine future experiments	
	None of the above methods could be used to determine numbers, and the numbers requested represent the best estimates in the PI's professional judgment	Please explain why none of the above methods could be used, and how the final numbers were determined:

C. **Animal Number Calculations: Please describe how you calculated the animal numbers, either through a narrative or using a simple table that tabulates across studies and/or groups. IF THIS IS A FIELD STUDY IN WHICH NUMBERS ARE OPEN ENDED, SKIP THIS STEP. Make SURE the numbers and terminology for individual studies/groups is consistent with the description you provided in Section V, and confirm that numbers match totals provided in Section II. Also confirm numeric consistency with percentages in the Pain and Distress table below (Section VIII.A), including ALL animals used (e.g., culled pups and spent breeders, typically category B). Again, culls and breeders MUST be reflected in Total Animal Number, here and elsewhere. (Suggest use Microsoft Word Insert Table feature or similar quick tool.)**

▶ For our breeding colony, we require (and can easily house) 90 adult females and 90 adult male wild-caught *E. scolopes* in any given year (up to 270 females and 270 males over a 3-yr period). These adult animals live out their natural lifespan, typically in 2-4 months, and field collections generally occur every 3-6 months to replace this breeding stock. The Nyholm lab has consistently used these numbers of squid since the PI Nyholm started his position at UCONN in 2007. This ratio has proved to be successful in providing 2-6 clutches weekly which, in turn, release about 5000 - 20000 hatchling squid/yr. We can house up to 80 egg clutches at a time, but usually have about 20-30 at various stages of incubation. These number of eggs yield anywhere from 5000 to 20000 embryos annually (up to 60,000 over a 3-yr period). Roughly 500 hatchlings (annually, 1,500 over a 3-yr period) are used for long-term experiments lasting 4 days to 6 months. All other hatchlings are for used in experiments within the first 3-5 days of hatch. The Nyholm Lab has the capacity to raise up to 500 squid/yr or 1500 over a 3-yr period separate from the adult wild-caught breeding colony. In addition, up to 60 sexually immature wild-caught animals/yr (180/ 3 yrs) may be collected for development studies.

Section VII: Request for Animal Use

A. Species Information:

1. Privately owned animals. If you are using any privately owned animals for research, teaching, or demonstration purposes, you must *submit a completed copy of Appendix B* with this protocol submission.

2. Species, location and source. To list additional species or strains, add lines to Table
**Occasionally, it is not feasible to list all species and/ or strains that may be associated with a project (such as in field studies where other species may be unintentionally captured, or if a large number of transgenic mouse models will be used). Please provide a brief explanation if all species/strains cannot be listed:*

Species/Strain	Source(s) of animals
Euprymna scolopes	Field caught, Hawaii

3. Age and sex of animals on study: Indicate the sex and approximate age(s) of animals to be used in your research, if possible. Also identify in your description use of any breeder animals, neonates, feti, and all culled animals:
▶ The Nyholm lab typically houses up to 90 adult females and 90 adult males in any given year. This ratio has proved to be successful in providing 2-6 clutches weekly which, in turn, release about 5,0000 - 20,000 hatchling/yr for experimental procedures. We can house up to 80 egg clutches at a time, but usually have about 20-30 at various stages of incubation. Embryogenesis takes 3 weeks. Roughly 500 hatchlings (annually) are used for long-term experiments lasting 4 days to 6 months. All other hatchlings are for used in experiments within the first 3-5 days of hatch.

4. Animals with special requirements: Do any of the animals have a phenotype that may cause a known painful or distressful physical, behavioral, or physiological condition (e.g. susceptibility to a particular illness, weakened immune system) or that may require special precautions to be taken by caretaker staff for the animal's benefit (e.g. requiring vaccination of staff, special hygiene precautions)?
☒ No ☐Yes: If "Yes", describe condition(s) and any special caretaker requirements
▶

5. Genetically modified animals: Will this project use any transgenic, knock-out, knock-in, floxed, cloned, or otherwise genetically modified stock or strain of animal?
☒No ☐Yes: If "Yes," complete the following:
   a. Identify which ones:
   ▶
   b. Do any of the genetic modifications make the animals susceptible to a known disease condition, or otherwise contribute to conditions of pain or distress?
   ☐No ☐Yes: If "Yes", describe:
   ▶
   c. List all known phenotypes of this strain (e.g. prone to seizure, splayed legs, etc.)
   ▶
   d. Ensure you have completed all necessary documentation for use of genetically modified animals as required by the Institutional Biosafety Committee (IBC) www.ibc.uconn.edu .

Section VIII: Pain and Distress Classification* by Species

A. Categories:

Insert the common names of each species used and enter the approximate percentage of Animals for each pain classification Category. Count each animal only once. For example, if you are working with rabbits, and some will undergo procedures classified as Category B at some point during the experiment, but then the *same animals* will undergo Category D classification procedures at a later time point, classify 100% of these animals as Category D.

**A detailed guide for using the USDA's Pain Classification system can be found at: http://oacu.od.nih.gov/ARAC/documents/USDA_Reports.pdf*

Common name of species used: (Place animals in "highest" USDA category.)	Species 1: Euprymna scolopes	Species 2:	Species 3:	Species 4:
% Category B: Animals being bred, conditioned, or held for use in teaching, testing, experiments, research or surgery, but not yet used for such purposes.				
% Category C: Animals upon which teaching, research, experiments, or tests will be conducted involving either no pain/distress, or only momentary/transient pain/distress.	50%			
% Category D: Animals used for teaching, research, experiments, testing or surgery that will involve accompanying pain or distress and for which appropriate anesthetic, analgesic, or tranquilizing drugs will be used.	50%			
% Category E: Animals used for teaching, research, experiments, testing or surgery that will involve accompanying pain or distress for which the use of appropriate anesthetic, analgesic, or tranquilizing drugs will adversely affect the procedures, results, or interpretation of the teaching, research, experiments, testing or surgery.				

1. Category D or E:
If any of the above procedures fall into Category D or E, then you must *complete Appendix E*

2. Category E:
If any of your classifications above include Category E procedures, you must *scientifically justify* below, the withholding of analgesia, anesthesia, tranquilizers, medical treatments, or other methods to alleviate pain/distress; include scientific references:
▶

B. Overall Consideration of Sources of Stress and Distress:

According to the principles of Refinement, the IACUC must identify all potential sources of stress and distress. *(These may include but are not limited to, stressors such as: food or fluid restriction, painful or distressful blood or tissue collection, noxious stimuli, painful surgery, separation of pre-weans or fledged animals from their mother, environmental distress, forced exercise, disease conditions, deliberate exposure to predator scents, exposure of non-acclimated animals to human handling, excessive noise, etc.)*

Will animals in the proposed study be exposed to any factor(s) that may induce physiological or behavioral stress or distress above and beyond that which is associated with normal pet ownership or basic husbandry procedures or natural behaviors defined for the species?
☐No ☒Yes: if "Yes," *complete table below:*

Stressful and Distressful Procedures

Check all that apply	Procedures	If you checked this item, you must complete and submit the appropriate Appendix form or include additional information as identified below.
☐	Restrainers: metabolism cages, vests, harnesses, or slings, etc.	*Describe:▶*
☒	Potential stressors: food or water restriction, noxious stimuli, electrical shock, environmental stress, forced exercise, etc.	*Describe:▶ Wild specimens will be collected, held and transported for use in experiments.*
☐	Invasive methods of animal identification (tattoos, brands, implants, ear tags, ear notches or punches, toe clipping, tail biopsy genotyping etc.)	*Describe:▶*
☐	Injections or Inoculations (drugs, substances, vaccines etc.)	*Compounds and procedure(s) used must be described in Section VIII.A.1 below*
☒	Blood Collection	*Procedure(s) used must be described in Section VIII.A.4*
☒	Terminal Tissue Harvest: e.g. perfusion, exsanguination under anesthesia	*Ensure procedure is referred to by name in Section VIII.C below and is included as the method of euthanasia in Section X.C.*
☐	Non-Survival Surgery: i.e. surgical procedures in which an animal will be maintained in a plane of anesthesia for more than a few minutes from which it will not recover (e.g. non survival neuronal recordings, teaching surgical technique)	*Describe in APPENDIX G Ensure procedure is referred to by name in Section VIII.C below*
☐	Survival Surgery (Minor)	*Describe in APPENDIX G Ensure procedure is referred to by name in Section VIII.C below*
☐	Survival Surgery (Major)	*Describe in APPENDIX G* *Ensure procedure is referred to by name in Section VIII.C below*
☐	Exposure to radiation, lasers, or radioisotopes	*APPENDIX D* *Describe:▶*
☐	Exposure to Toxins (biological or chemical)	*May require an APPENDIX D* *Describe:▶*
☐	Injections with or exposure to human cells (e.g. ESC)	*APPENDIX D* *Describe:▶*
☐	Injections with or exposure to biological materials of animal origin (e.g. murine cells)	Describe:▶

☐	CRISPR or other Gene editing technology *(e.g. CRISPR/Cas9, ZNF, TALENS, Meganucleases)*	*May require an* APPENDIX D Describe:▶
☒	Injections with or exposure to Infectious Agents (e.g. human or animal pathogens, non-pathogenic biological microorganisms, viral vectors, etc.)	*May require an* APPENDIX D Describe:▶ Vibrio fischeri ES114 Leisingera sp. ANG1/JC1
☐	Transgenic or Knockout Animals	Ensure that animals are described in Section V.A.5 Contact the IBC to complete the necessary documentation

C. Recognition and Assessment of Stress and Distress: **For all procedures, including those listed above: Please identify any expected potential indicators of stress and distress for this project, and explain how the indicated conditions will be monitored. Examples include, but are not limited to: reduced activity, weight loss, abnormal posture, etc.**

▶ Signs of stress include failure to bury in the sand during daytime hours, failure to eat, and failure to respond to gentle touch. Animals deemed to be ill are euthanized (see below). For obtaining blood, animals are transferred to a vessel with filter-sterilized sea water or filtered instant ocean salt mix (FSSW or FSIO) containing either 2% ethanol or 0.12M-0.15M $MgCl_2$. Anesthesia is considered complete when the animal begins to drift or become ataxic. Chromatophore control will also relax and the animals will appear pale. If these conditions are not observed, the animal will either be allowed to stay in the anesthetic until complete or else revived by placing in FSSW or FSIO until normal chromatophore control and behavior (being able to right itself) are observed. The animal will then be returned to its tank in the Nyholm lab squid facility. For terminal harvesting of organs, animals are first anesthetized. Anesthetization is performed as described above: exposure to either 2% ethanol or 0.12-0.15M $MgCl_2$ in FSSW or FSIO. Anaesthetized animals may be put into a dry, sealed container (a plastic bag or vial), and dropped into a bath of liquid nitrogen. Within a few seconds, the animal is completely frozen, and the tissue stored at -80° C. If organs need to be harvested after an experiment squid are first anesthetized as described above and the brain is quickly pithed and head severed with a scalpel or razor blade.

D. Refinement: **For all procedures, including those listed above: Describe how you have incorporated the principle of refinement in order to reduce pain and distress. Examples include, but are not limited to: use of analgesia, acclimation and training of animals, catheterization for frequent blood collection, etc.**

▶ For all procedures described above animals are first anesthetized in either 2% ethanol or 0.12-0.15 M $MgCl_2$ in FSSW or FSIO. Non-lethal blood collection is only performed once a day and animals are allowed to recover in their tanks overnight to several days (typically 48+ hours) before another blood collection is attempted. However, on occasion blood samples are required daily over a 4-6 day period.

Section IX: Specific Procedures and Studies

A. Invasive procedures:

1. **Substance Administration: Will any research material, drug, chemical or compound, including anesthetics or analgesics, be given to the animals?**

☐ No, If no, SKIP TO QUESTION 4.
☒ Yes: List EACH research material, drug, chemical or compound in the table below. Add additional rows to the table if needed. Complete the information for each column, including names, dosages and administration details, including route, needle gauge ranges, frequency of application and maximum volumes.

Name of research material, drug, chemical or compound	Dose	Administration (Route, needle gauge range, frequency of application and maximum volume provided)
Ethanol	2% in filter-sterilized seawater (fssw)	Animals are placed in the solution until the required depth of anesthesia is achieved.
Magnesium Chloride (MgCl)	.12-.15 M in 500 ml fssw	Animals are placed in the solution until the required depth of anesthesia is achieved.
Vibrio fischeri ES114	10^2-10^7 CFU/ML in FSSW	Animals are exposed to V. fischeri, the natural light organ symbiont of E. scolopes for 3-12 hours post-hatching
Leisingera sp. ANG1/JC1	10^2-10^7 CFU/ML in FSSW	For some trials, animals will be exposed to these reproductive symbiotic bacteria during raising experiments daily for 1-4 months

2. Non-Pharmaceutical Grade Compounds: *The 2011 Guide (pg. 31)* states, "*[Pharmaceutical grade chemical compounds] should be used, when available, for all animal related procedures".* USDA APHIS and OLAW define pharmaceutical grade compounds as "a drug or compound that can be purchased from a medical or veterinary drug supplier in a formulation that is ready-to-use in living vertebrate animal subjects; including those intended for use as investigational agents for clinical purposes and in terminal studies". "USP" is not equivalent to "pharmaceutical grade."

   a. Does this study involve injections of non-pharmaceutical grade chemicals /substances?

   X No All chemical compounds used will be human or veterinary pharmaceutical grade.

   Yes Non-pharmaceutical grade chemicals or substances will be used because:

   (Check all that apply)

	Pharmaceutical grade compound is not available from a veterinary or medical supplier
	Pharmaceutical grade compound is not available from a veterinary or medical supplier in the needed concentration or formulation
	The compound is required in order to produce data that is comparable to previous year's data
	Reagent grade compound is more pure than pharmaceutical grade compound
	Non-pharmaceutical grade compounds are necessary to meet the scientific goals of the study. Briefly explain: ▶

b. Please provide a written SOP (either below, or attach as an appendix) describing how the compound is prepared and stored. Be sure to include the following in your description:

- Chemical compound concentration (in units or %)
- Vehicle used
- How sterility is achieved (e.g. filtered, autoclaved)
- How labeled to include: Date compounded, by whom, shelf life and expiration date
- Storage requirements
- Assessment of pH (pH test paper is appropriate)

▶

3. Controlled Substances: Does the proposed work include use of any federal or state controlled substances?

☒No
☐Yes, under the handling and direction of a veterinary professional only
☐ Yes and the PI or named protocol personnel are licensed and store and/or administer the substances

DEA/CT license numbers of licensee:▶

Date issued:▶

The state of CT now requires you to provide a list of research personnel who are authorized by you to be named as handlers of the drugs under your license. You may email them this list at: drug.control@ct.gov

4. Blood collection: Does the proposed study involve survival collection of blood from a live animal?

☐No ☒Yes: **Describe procedures below, including route, site of collection, any use of catheterization, frequency, volume, and animal recovery methods that will be used.**

▶ Hemolymph can be collected from adult squid and juvenile animals. For adult animals, squid are anesthetized in filter-sterilized sea water or instant ocean salt mix (FSSW or FSIO) containing 2% ethanol. Anesthesia is considered complete when the animal begins to drift or become ataxic. Chromatophore control will also relax and the animals will appear pale. Hemolymph is withdrawn from the cephalic artery located between the eyes using a sterile 1-cc syringe and 28½-gauge needle. For a non-lethal bleed, 5-10 microliters of hemolymph are recovered. For a lethal bleed, approximately 100-200 microliters of hemolymph are recovered. Animals are either euthanized or subsequently revived by placing in FSSW or FSIO. Survivability after bleeding is high and most animals can sustain multiple bleeds over several days. Animals are typically allowed to recover in their tanks 48+ hours before another bleed is attempted, although on occasion animals are bled consecutively once a day over a 6 day period to monitor daily changes in hemocyte response. For juvenile animals, squid are anaesthetized in 2% ethanol as described above and euthanized before blood is removed by severing the anterior and posterior portions with a razor blade.

5. Tissue collection: Does the proposed study involve survival collection of tissue(s) from a live animal?

☒No
☐Yes, for genetic biopsy such as ear punch or tail snip with anesthesia – *Describe in Appendix G* if different from NIH defined practices for rodent genetic biopsy (link referenced below)
http://oacu.od.nih.gov/ARAC/documents/Rodent_Genotyping.pdf

☐Yes, for other survival collection of tissue under anesthesia Ensure procedures are *listed by name* in Section VIII.C and *complete Appendix G* .

6. Surgical procedures: Does the proposed study involve any incisions, or abrading, or other surgical procedures on live vertebrate animals?

☐ No

☐Yes, for survival procedures (animal is expected to recover consciousness following the procedure) Ensure that procedures are *listed by name* in Section VIII.C, and *complete Appendix G.*

☒Yes, for non-survival surgeries where animals will be maintained in a plane of anesthesia for more than a few minutes (e.g. non-recovery neuronal recordings): Ensure procedures are *listed by name* in Section VIII.C and *complete Appendix G: Sections I,II & III only).*

☐Yes, for terminal tissue harvest procedures (e.g. perfusion, terminal tissue harvest, cardiac puncture): Ensure procedures are *described in* Section VIII.C. *DO NOT complete Appendix G*

B. Specific types of studies:

1. Does the proposed study involve the experimental modification and/or observation of animal behavior?
   ☒ No
   ☐Yes, if "Yes," ensure the methodology and time line is covered in Section VIII.C.

2. Does the study involve water restriction, food restriction, shock, restraint or other stressors?
   ☒ No
   ☐Yes, if "Yes," ensure that procedures were described in the table in Section VIII.B, and that measures taken to assess pain and distress as a result of these procedures were described in Section VIII.C

3. Does the proposed study include the capture, manipulation, observation, housing, tracking, identification, or collection, of any free-living, non-domestic animals?
   ☐ No
   ☒Yes, if "Yes" ensure the methodology and time line is covered in Section VIII.C. Be advised that all necessary permits must be obtained prior to the start of work, and that those permits should be available to the IACUC upon request. No permit is required for collection of *E. scolopes*, and the population is not considered threatened or endangered either locally or at the state level. No special permits are required for the transport/shipment of this species and they are transported via air cargo services.

4. Does the proposal include teaching or demonstration as a protocol objective?
   ☐ No
   ☒Yes, if "Yes" ensure that methodology and procedures are covered in Section VIII.C below.

C. Location of Live Animal Procedures: Identify, in the table below, where each of the procedures identified above will be performed. Check all that apply, and provide the additional information requested. ONLY INCLUDE AREAS WHERE LIVE ANIMALS WILL BE BROUGHT; DO NOT INCLUDE FIELD WORK:

	Type of activity	Building	Room(s)	Duration of stay*
X	Animal Housing*	TLS	82 and 57	From collection until death

X	Non-surgical procedures	BPB	419, 420	<12 h
X	Surgical procedures	BPB	419, 420	<12 h
X	Recovery Procedures	BPB	419, 420	<12 h
X	Terminal procedures, including euthanasia	BPB	419,420	<12h
X	Other- describe:	BPB	302, moving to G05	<12h

**If any animals will be maintained outside of the primary housing facility for more than 12 hours, provide location and rationale here*

▶

Justification for keeping animal facilities in TLS:

1. There is no direct route to move a cart between PI Nyholm's main lab on the 4th floor of BPB and the aquatics facility in pathobiology and remain inside. We routinely move seawater, animals and sand for experiments from our main lab in BPB and the animal rooms. During inclement weather it would not be possible to move a cart without having to carry items up a large flight of stairs between the basement of PBB and the ground floor outside of Pathobiology where we would then access another elevator and still have to walk outside. Since we are often transporting heavy carboys of seawater and animals in buckets back and forth between my lab and the squid rooms, carrying these up stairs would be very difficult and may result in injuries.
2. Another big concern is the distance from our lab in BPB. My students need to check on the animals every day. Since the squid are nocturnal, we do all water quality checks before 1 pm when the lights are on and feed the animals during their nocturnal period (in our system 1 pm – 1am). When we are raising juvenile squid to adults (a major objective of my current NSF grant), we monitor the animals throughout the day (typically 4-5 times). In addition, we currently transport juvenile animals to my lab in BPB to monitor light production daily during the first 5 days after they hatch to measure colonization by the symbiont *Vibrio fischeri*. This is done with a luminometer that I can't store in the animal husbandry room as the electronics may be damaged by corrosion in a high salt environment. The added distance will make monitoring our animals much more challenging.
3. Moving the animals from the aquatics facility to our lab in BPB for experiments would add additional levels of stress for the animals. The squid are very sensitive to rough handling/movement and excessive changes in light. This would occur if we were to use a cart outside the buildings, especially on uneven or rough pavement.
4. In addition to our animal experiments, we use the artificial seawater system in TLS 82 to make up all of our marine media for growing the numerous strains of marine bacteria that we study. It would not be possible to set up a similar system in BPB. I would not want large volumes (typically 200-300 gallons) of artificial seawater in my main lab near sensitive electronic equipment. Therefore, if we were to move to the aquatics facility, this system would have to be duplicated.
5. If we were to move our husbandry operation to the aquatics facility, I would have to move the whole system, including tanks for holding shrimp, mysids and the egg nursery as it would not be feasible to have the non-IACUC regulated activities (egg nursery and shrimp) separated by a long distance from the IACUC-regulated squid.

Section X: Animal Husbandry and Transportation

A. Animal Housing:

Please select one answer from the following table:

	This section is not applicable because animals will not be housed for any period of time on this protocol (Proceed to Section IX.C)
	I am familiar with the standard husbandry practices (including environmental enrichment) established by the University for the species I am using, and agree that the practices, will meet my research objectives (Proceed to section IX.C)
X	The species I am using will require routine care practices not yet identified by University staff (Proceed to Section IX.B)

	The objectives of my research will require modifications to standard husbandry practices established by the University, e.g. individual housing of social species (Proceed to Section IX.B)

B. Special Husbandry Requirements:

1. **If the handling and care of your species requires husbandry procedures not yet identified by the University staff (i.e., you are using a novel species or strain with unique characteristics that necessitate additional care), either reference or attach an IACUC approved SOP or describe below. Be sure to include information regarding who will be expected to provide the special husbandry (e.g. ACS, lab staff)**

   ▶ All husbandry of E. scolopes will be carried out by PI Nyholm as well as graduate and undergraduate students in the Nyholm lab. Please see attached SOP.

2. **If your research objectives will require modifications to existing or identified routine husbandry practices (requires single housing, opting out of environmental enrichment, a different cage change frequency, food/ fluid restriction, custom diets, sterilized food, bedding or enclosures etc.), describe and provide scientific rationale below:**

   ▶ The Hawaiian bobtail squid E. scolopes is not a typical animal found in most animals facilities being that it is a marine invertebrate. Please see attached Nyholm lab sop for husbandry practices that are used.

3. **Will any experimental conditions or manipulations have anticipated effects on the normal physiology, anatomy, or behavior of the animals on study?**
   ☒ No ☐Yes, If "Yes", describe the effects in detail:

   ▶ The Hawaiian bobtail squid has a symbiosis with the light organ symbiont *Vibrio fischeri*. During our experiments with hatchling squid we will expose them to their normal symbiont *V. fischeri*. Control animals will not be exposed to *V. fischeri* and will be raised aposymbiotically. In addition, some raised female animals will be exposed to a natural bacterial consortium (consisting of cultured strains of *Leisingera* sp.) during development. Control animals will not be exposed to these bacteria. Because the light organ symbiont is used in antipredatory behavior (counter illumination) there may be effects on host behavior for those animals that don't receive the proper bacteria. However, the Nyholm lab does not expose these animals to predators so differences in this behavior are not observed.

4. **Will any aspects of the study require animals to be observed more frequently than once a day; or require additional training of the animal care staff to properly identify and monitor experimentally induced conditions?**
   ☐No ☒Yes, If "Yes", describe observation requirements (include frequency, special requirements, and if research personnel or ACS will perform; do not include methods required for surgical recovery, as these are covered in detail in *Appendix G*).

   ▶ All animals are observed at least once a day and the PI and all members of the PI's lab are trained in identifying unusual behaviors such as cessation in eating and not burying in sand when lights are on in the animal facility. A daily checklist for animal care will also be used to ensure that all tasks are carried out daily.

C. Animal Tracking
Briefly describe how animals will be tracked throughout the experiment. **Such as use of cage cards, ID tags, photographs, censuses, etc. :**

▶ Animal identifiers are designated by number and/or letter and monitored for the duration of the animal's life. Animal death dates are recorded, and if the animal is euthanized, that is recorded as well. Mean longevity in captivity can thus be calculated. Each egg clutch is assigned a unique identifier, which includes the cohort alphabet designation. For each clutch, the lay date, the approximate number of eggs, the female, and the PVC cave identifier are recorded. From each clutch, the number of hatchlings is recorded. This information includes the number of hatchlings that hatched overnight ("earlies") and the number of hatchlings that hatched

at known times ("normals"). Any dead, sickly, or premature hatchlings are also noted. From these data, we can compute the number of hatchlings per clutch total, the number of hatchlings per time period, the number of hatchlings per female, and the clutch's range of incubation time.

D. Animal Transportation

1. Will animals be transported in accordance with IACUC Policy #AW-02-2012?
☒ Yes ☐No, If "No", please complete the table below:

	This protocol will require animals to be transported:	Briefly describe transportation methods and any containers being used. In addition please note who will transport the animals (i.e. ACS or protocol personnel):
	This protocol will not require any transportation of animals outside of the housing facility	
X	Within a facility but outside of animal housing areas (i.e., from the housing facility to your laboratory)	Animals are sometimes moved between tanks in the Nyholm lab squid facility. This is done during the animal's daylight hours when they are quiescent. Animals are gently scooped in a smooth glass, non-abrasive cup and transported to another tank
X	Between facilities on the Storrs campus	Adult, animals are sometimes moved to the Nyholm lab in BPB from the animal facility in TLS. Juvenile animals are monitored daily for bioluminescence and are transferred to the PI Nyholm's lab for measurement in a phtotometer. This is done during the animal's daylight hours when they are quiescent. Animals are gently scooped in a smooth glass, non-abrasive cup. The cup/container is covered with foil to not disturb the animals and they are carried to the lab in BPB.and transported
X	To other facilities off campus and/or out of state	*Euprymna scolopes* is collected from Hawaii and transported to PI Nyholm's lab. Please see attached sop for procedures.
	Other not described	

Section XI: Endpoint Criteria, Disposition and Euthanasia

A. Endpoints In Study Design *"Endpoint" is defined as the moment at which an animal:*

- *Is euthanized, and its heart and respiratory functions cease*
- *Has its ownership transferred elsewhere, such as sending livestock for sale or slaughter*
- *Is released back into its natural habitat, such as for field studies*
- *The study ends, without animals having been handled*

1. Experimental Endpoints: What are the identified endpoints in the study design?
(Experimental endpoints are defined as the point at which scientific aims and objectives have been reached)
There may be more than one endpoint, list all:

▶ For some experiments, animals are euthanized before tissues are harvested (please see attached sop).

2. Humane Endpoints: What animal welfare or behavioral criteria will be used to assess the need to either prematurely remove an animal from the study, or consult with ACS veterinary staff? Examples of criteria could include weight loss, decreased activity, tumor burden, etc. If a field study of free-living animals captured for research, address whether injury from capture process, markers, etc. is likely, and possible responses to observed injury.

► Animals will be observed for signs of severe distress and senescence. This includes inability to control chromatophore activity, not burying in sand during daylight hours and not feeding. Such animals exhibiting such conditions will be euthanized.

Note: In the event that an animal is found to be in severe pain or distress, all attempts will be made to contact the PI for guidance. However, the veterinarian has authority to use appropriate treatment or control measures, including euthanasia if indicated, following diagnosis of an animal disease or injury. The veterinarian's authority is exercised with the concurrence of the IACUC and the Institutional Official.

B. Disposition Other than Euthanasia: Will any study animals be alive at the completion of this study?

☒No
☐Yes identify the intended disposition of any live animals at the completion of this study:

	This is a field study in which animals are either never handled, or will be released back into their natural environment (wild-caught animals only)	How many animals?
	Animals will be transferred to another approved protocol for the same PI	How many animals, and which protocol(s)?
	Animals will be transferred to another PI at the University of Connecticut	How many animals, and which PI and protocol?
	Animals will be transferred to another PI outside of the University	How many, and where will they be going?
	Animals will be transferred from a breeding protocol to a holding, teaching, or research protocol	How many animals?
	Other not specified ►	Describe in detail below ►

C. Euthanasia/Method: Complete this section to describe euthanasia methods, perfusion and terminal harvest, and carcass disposal procedures being used for this protocol. Note that all methods of euthanasia should be congruent with the AVMA guidelines on euthanasia. IF THIS IS A FIELD STUDY IN WHICH EUTHANASIA IS NOT AN ENDPOINT AND/OR IS PROHIBITED BY THE TERMS OF YOUR PERMIT, SKIP TO SECTION XI.

(Check here)	Chemical Euthanasia Method	Species (indicate all that apply)	Indicate defined study endpoints this method will be used for (IE end of experiment, brain tissue harvest, etc.)	Will be used for non-experimental purposes (IE culling of animals that cannot be used on experiment, humane endpoints, etc.)	Agent and exposure route to be used (IP. IM, SQ, IV, etc.):	Dosage or flow rate:	Other information:
	Overdose of injectable anesthetic						
	Inhalation of carbon dioxide from a				[Inhalation chamber gas CO2 is		

	compressed gas cylinder				only acceptable method for this agent]		
	Overdose of inhalant anesthetic						
	Overdose of injectable barbituate						
	Other- Indicate here:						

(check here)	**Physical/ Combined Euthanasia Method**	**Species (indicate all that apply)**	**Indicate defined study endpoints this method will be used for** (IE end of experiment, brain tissue harvest, etc.)	**Will be used for non-experimental purposes** (IE culling of animals that cannot be used on experiment, humane endpoints, etc.)	**Agent and exposure route to be used (IP. IM, SQ, IV, etc.):**	**Dosage or flow rate:**	**Other information:**
	Cervical dislocation without anesthesia				N/A	N/A	Justification required
	Cervical dislocation with anesthesia						
	Decapitation without anesthesia				N/A	N/A	Justification required
X	**Decapitation with anesthesia**	Euprymna scolopes	End of experiment, tissue harvest		2% EtOH in filter sterilized seawater or 0.12-0.15M magnesium chloride		After anesthesia, animals are pithed and head is severed with razor blade or are flash frozen in liquid nitrogen
	Exsanguination under anesthesia						
	Perfusion under anesthesia						
	Other- describe:						

1. <u>Justification to use physical euthanasia methods alone:</u> ***(complete only if using a physical method as the primary euthanasia method).*** **The use of physical methods as the sole method for humanely euthanizing animals requires scientific justification. What is/are the reason(s) that anesthetic cannot be used prior to the use of the selected physical method(s)?**

▶

2. <u>Verification of Death:</u> **How will animal death be verified prior to disposal of animal carcasses? (check all that apply)**

	Thoracotomy under anesthesia		Prolonged exposure to CO2 (>5 minutes)
	Cervical dislocation	X	Observation of vital signs for 5 minutes
X	Rapid freezing (neonatal rats and mice)	X	Decapitation
X	Exsanguination under anesthesia		Other:

3. Carcass Disposition:

a. Will animal tissues or fluids be potentially contaminated with chemical or biological agents of known or unknown toxicity, reactivity, or infectivity?
☒No ☐Yes, if Yes, you must complete Appendix D

b. Will tissues or fluids be harvested from the euthanized animals?
☐No ☒Yes

c. Will carcasses be disposed of by ACS or Veterinary Pathobiology services?
☐Yes ☒No, if No, describe what will be done with animal carcasses:

► After an experiment, or at the end of an adult's life, the tissues (hemolymph, light organ, testes, eyes, *etc.*) are often harvested, and frozen for future use. Otherwise, after experiments and euthanasia, juvenile squid are placed in plastic bags and disposed of through Inserv animal waste via a collection site in Torrey Life Sciences; similarly, after experiments and euthanasia, the entire carcass of adult/reared animals is put into labeled and sealed plastic bags, and stored in a dedicated freezer for future use or reference. Alternatively, carcasses of adult animals will be disposed of through Inserv animal waste via a collection site in Torrey Life Sciences.

D. Euthanasia/Training: Please describe the training program for ensuring the procedures are performed humanely and appropriately:
►All new students are trained by PI Nyholm or trained graduate students in how to properly anesthetize and euthanize bobtail squid. PI Nyholm and all students will also comply with all UCONN IACUC training guidelines

Section XII: Occupational Health and Safety Considerations

Hazards Associated With Animal Research: The PI is responsible for ensuring that exposure of humans to physical, chemical, environmental, biological, and radiation hazards is minimized to the greatest extent possible during the course of active research. All people with animal exposure must enroll in the OHS Program for animal handlers. All people with animal exposure are required to complete the necessary EHS training as indicated based on hazard/exposure type. Please review and complete the following:

☒ I have read and understand the Occupational Health and Safety Program for Animal Handlers https://ehs.uconn.edu/

☒ I have completed the Workplace Hazard Assessment (WHA) form for my lab/workplace https://ehs.uconn.edu/

☒ I will submit to EHS an Employee Safety Training Assessment (ESTA) form for each person working under this protocol https://ehs.uconn.edu/

☒ I understand that all animal work can potentially expose personnel to allergens derived from animal fur, feathers, dander or fluids. Each person working under this protocol will either take the EHS Biosafety in Animal Research Training or will be trained by the PI on this topic.

☒☐ I will ensure that all personnel working with chemicals will complete the annual lab safety training provided through EHS and will read and understand the Chemical Hygiene Plan for the University of CT.

A. Physical risks:

1. Will the nature of the study potentially expose personnel to animal bites, kicks, scratches, punctures, etc.? Ensure that any risks noted below are described in your WHA.
   - ☒No
   - ☐Yes, for rodents or other traditional laboratory species only
   - ☐Yes, for non-traditional species whose use poses minimal physical or biological risk to personnel or animals
   - ☐Yes, for large or non-traditional/ exotic species whose handling may increase risk to personnel.

2. Does the work covered by this protocol increase the likelihood of personnel acquiring injuries due to needle sticks, falls, slips and trips, bruising, or other physical hazards (including risks encountered in the field as a direct result of the research)? Ensure that any risks denoted by a "yes" below are described in your WHA.
   - ☐No
   - ☒Yes

3. Does the work covered by this protocol expose personnel to industrial or occupational hazards, such as loud noise, dangerous machinery, heavy lifting, etc.? Ensure that any risks denoted by a "yes" below are described in your WHA.
   - ☐No
   - ☒Yes

B. Chemical risks: Chemicals or compounds in use

CHEMICAL TYPE	DESCRIPTION	USED IN/WITH LIVE ANIMALS	APPENDIX D REQUIREMENT
Acute Toxins/Toxicants	A toxic chemical is defined as any chemical with an LD_{50} <500 mg/kg (oral, rats); LD_{50} <1,000 mg/kg (skin, rabbits); or LC_{50} <20,000 mg/m^3 for 1 hr. (inhalation, rats).	Yes	Yes
Carcinogens	A chemical listed as a Category 1A, 1B, or 2 carcinogen in the safety data sheet.	Yes	Yes
Reproductive Toxins/Toxicants	A chemical listed as a Category 1A, 1B, or 2 reproductive toxin/toxicant in the safety data sheet	Yes	Yes
Novel Chemicals with Unknown Risk/Hazards	Newly synthesized chemicals for which safety data may be unavailable	Yes	Dependent on EHS Risk Assessment
Chemicals Not Otherwise Specified	Chemicals not known to be toxins, carcinogens, or reproductive toxins but still may be hazardous.	Yes	Dependent on EHS Risk Assessment
Drugs/ Medicines	A compound or preparation used for the treatment or prevention	Yes	No- If used as prescribed Yes- If not used as prescribed

	of disease or anesthetic agent		
Chemicals NOT used in Live animals	Chemicals that are not used in live animals are covered by the Chemical Hygiene Plan. (Such as fixatives)	No	No

1. Will the study potentially expose the animals PI, research staff or ACS staff to any chemicals or compounds: (refer to the table above for categories and their definitions)

☐No

☐Yes, for chemicals or compounds KNOWN TO BE acute toxins/toxicants, that WILL BE USED IN/WITH LIVE ANIMALS: *Complete and submit Appendix D.* PLEASE LIST CHEMICALS OR COMPOUNDS BELOW and provide link to vendor(s) SDS:
▶

☐Yes, for chemicals or compounds KNOWN TO BE carcinogens (including chemotherapeutics) that WILL BE USED IN/WITH LIVE ANIMALS: *Complete and submit Appendix D.* PLEASE LIST CHEMICALS OR COMPOUNDS BELOW and provide link to vendor(s) SDS:
▶

☐Yes, for chemicals or compounds KNOWN TO BE reproductive toxins/toxicants, that WILL BE USED IN/WITH LIVE ANIMALS: *Complete and submit Appendix D.* PLEASE LIST CHEMICALS OR COMPOUNDS BELOW and provide link to vendor(s) SDS:
▶

☐Yes, for novel chemicals or compounds of unknown risk: *Appendix D may be required.* PLEASE LIST NOVEL CHEMICALS OR COMPOUNDS BELOW and provide either the vendor/supplier information:
▶

X☐Yes, for other chemical or compounds used in animals not otherwise specified. PLEASE LIST COMPOUNDS BELOW and provide link to vendor(s) SDS:
▶0.12-0.15 M Magnesium Choloride

☐Yes, for drugs or medicines used as prescribed/in accordance with product insert that WILL BE USED IN/WITH LIVE ANIMALS. Please note that while medications can be hazardous if used as prescribed, they do not need inclusion in Appendix D; however, the PI is responsible for communicating any hazards to all users and to supply the SDS for the compound. **NOTE: chemotherapeutics do not fall into this category and must not be listed here.** PLEASE LIST DRUGS/MEDICINES BELOW and provide link to vendor(s) SDS:
▶

X☐Yes, for preservatives or fixatives: Please note that while these compounds can be hazardous, they do not need inclusion in Appendix D and are covered by the chemical hygiene plan. PLEASE LIST COMPOUNDS BELOW.
▶ Preservatives such as 4% paraformaldehyde will be used only with harvested tissues and euthanized animals

*** For administration of agents that do not require an orange hazard card, green substance administration cards must be used. The compound, route of administration, and other pertinent information must be listed on the card. Cards should stay in front for 72 hours.**

C. Biological risks:

1. Will the study potentially expose the animals PI, research staff or ACS staff to any biological agent
☐No

☐Yes for recombinant or synthetic DNA/RNA. PLEASE LIST AGENTS BELOW and complete Appendix D (note: AAVs and some synthetic nucleic acids may not require inclusion in Appendix D, contact Biosafety for guidance):
▶

☐Yes for CRISPR or other Gene editing technology *(e.g. CRISPR/Cas9, ZNF, TALENS, Meganucleases)* PLEASE LIST CONSTRUCTS BELOW:
▶

☐Yes for human, plant or animal pathogens. PLEASE LIST AGENTS BELOW and complete Appendix D:
▶

☒Yes for non-pathogenic biological microorganisms. PLEASE LIST AGENTS BELOW; Appendix D may be required:

▶ For live animal work, animals will be exposed to Vibrio fischeri ES114 and Leisingera sp. ANG1/JC1. These are both BSL 1 organisms and are covered under PI Nyholms IBC Registration #806C

☐Yes for human material (cells, tissues, organs, body fluids). PLEASE LIST AGENTS BELOW and complete Appendix D:
▶

You may also be required to submit a protocol/project proposal for review and approval by the Institutional Biosafety Committee (IBC), contact the IBC Coordinator for further clarification.

**** For administration of agents that do not require a hazard card, green substance administration cards should be used. Note compound, route of administration, and any pertinent information about the compound. Cards should stay in front for 72 hours. (Same under bio section.)**

D. Radiation Hazards:

1. Will the nature of the experimental procedures require handling or manipulation of sources of ionizing radiation, including but not limited to x-rays*, magnetic field generators, tomography scans, isotopes, irradiation of cells or animals, lasers, etc.? PLEASE NOTE THAT LICENSURE AND SPECIALIZED TRAINING IS REQUIRED FOR THIS TYPE OF WORK. CONTACT EHS FOR FURTHER GUIDANCE. **Ensure that any risks denoted by a "yes" below are described in your WHA.**

X☐ No
☐Yes Complete and ***submit Appendix D.***

*Use of approved IVIS imagers, while it does not expose personnel to x-rays and does not need inclusion in Appendix D, any user will need to comply with SOP-03-2016.

Section XIII: Personnel and Training

Please complete Appendix C, listing all personnel, including the PI, who will be assigned to work on this project in its first year. If personnel are added or dropped, resubmit Appendix C as often as necessary to keep it current.

Section XIV: Record-Keeping

Attach copies of, or provide links to, all record forms you plan to use (as defined below):

A. Where will animal husbandry records be maintained for animals on this protocol?

☐ N/A, Animal will not be housed under this protocol
☐ All records will be kept in the ACS facility where the animals are housed.
☒ Other- Describe building and location where records can be found:
▶ All records will be maintained in PI Nyholm's laboratory BPB 419 and office BPB 405.

B. Where will individual animal medical records be maintained?

☐ N/A, Animal will not be housed under this protocol
☐ Animals are housed at ACS facility. Individual animal medical records are kept by ACS staff with the animals. Research activities that impact the animals are documented in the records by PI and/or PI's staff.
☒ Other- Describe building and location where records can be found:
▶ All records will be maintained in PI Nyholm's laboratory BPB 419 and office BPB 405.

C. Where will research/experimental records be maintained? Describe & provide building and room:

▶ All records will be maintained in PI Nyholm's laboratory BPB 419 and office BPB 405.

D. Where will surgical records be maintained? Describe & provide building and room:

☒ N/A, No surgeries will be performed under this protocol
☐ Other- Describe building and location where records can be found:
▶

Section XV: Statement of Literature Search and Duplication of Research

Duplication of Research Statement: Federal regulations require that no unnecessary duplication of research be performed. Please complete the following statement, indicating, in your own words, why you feel that the research or teaching goals of this protocol are not unnecessarily duplicative:

As the Principal Investigator responsible for the information contained in this protocol, I have performed the following literature searches, bibliographical searches, and consultation(s) with colleagues in my field of research, on the procedures and objectives contained in this submission:(indicate any that apply)

Check all that apply, *using at least one source,* and supply additional information:

X	Literature search was conducted	Which database(s): PubMed	Key words used to search: Euprymna scolopes	Years that were searched: 1990-present	Date the search(s) was/were completed: 8/29/18
	Consultation with in-house colleagues	Who was consulted?		What date was the consultation performed?	

	Consultation with external colleagues	Who was consulted?		What date was the consultation performed?	
	Journals and/or Bibliographical sources were used	What titles were used?		What year(s) were the publications?	
	Other source(s) were used	Describe:			

I have carefully considered the need to perform the described procedures in live vertebrate animals. I certify , based on my 22 years of experience in the field, careful consideration of the research or teaching objectives, and consideration of the literature review, consultations, and conferences referenced, that the proposed work is not unnecessarily duplicative. I believe this to be true because I am very familiar with all research that has been conducted with this species. My lab is one of 16 in the country studying this model system. Based on the aforementioned, I believe that using Euprymna scolopes is the most appropriate method for reaching my teaching or research goals.

Section XVI: PI Responsibilities and Signature

I certify that:

I will comply with IACUC Policy SI-05-2011: "Principle Investigator Responsibilities". A copy of this policy is available at the IACUC website.

The information provided within this application is accurate to the best of my knowledge. I understand that, should I use the project described in this application as the basis for a proposal of intramural or extramural funding, it is my responsibility to ensure that the description of animal use in such funding proposal is identical in principle to that contained in this application.

Principal Investigator:

Name: Signature:_____

Date:

Student (if student project):

Name: Signature:_____

Date:

Comments:

Section XVII: Department Head/Chair Signature

I certify that I have reviewed the content of this protocol.

Name: Signature:_____

Date:

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IACUC Protocol # A18-029
Approval Date: 2/19/19
Expiration Date: 2/19/22
Species: Squid
Guide exceptions: Y__ N✓
Hazards: Y__ N✓

University of Connecticut

Appendix C: Personnel Assigned To Work on Animal Research Protocols

Institutional Animal Care and Use Committee, Office of Research Compliance

Whetten Graduate Center, Rm #214, 438 Whitney Road Ext., Unit 1246 Storrs, CT 06269-1246 860-486-8802

Instructions: This form is a companion form to Section XII of form IACUC-1, "Animal Care and Use Protocol Submission Form". The completion and submission of this form is required for all protocol submissions, and describes:

- **The name, background, training, and qualifications of all persons assigned to work on the aforementioned animal research or teaching protocol**

Section I: PI (Principal Investigator) and Protocol Information

Principal Investigator (PI)
Project Title: The Hawaiian bobtail squid, Euprymna scolopes, as a model for host-microbe research

Section II: Qualifications of PI

A. **Please include a paragraph summarizing the qualifications of the PI; including brief description of background and expertise, animal use training, years of experience with laboratory animals, and other relevant University of Connecticut or external training received:**

▶ PI Nyholm has extensive experience studying host/microbe interactions and has used a number of invertebrate models in his research. He has made significant contributions to understanding the mechanisms by which specificity is ensured during initiation and maintenance of the association between the Hawaiian bobtail squid *Euprymna scolopes* and the bacterium *Vibrio fischeri.* His laboratory has also pioneered characterizing a reproductive symbiosis in *E. scolopes* and has experience in conducting genomic, transcriptomic and proteomic analyses. He first began studying *E. scolopes* as a model organism in 1995 and has almost 22 years of experience of researching this animal. He has been involved with animal collection and husbandry during this time. His laboratory first described the procedures to obtain and study hemocytes from blood collection in *E. scolopes.* Since starting his faculty position at UCONN in 2007, his laboratory has been using *E. scolopes* as its primary research organism. Since 2007, the Nyholm lab has collected and maintained *E. scolopes* in the laboratory and has established an active breeding program for this species. He has published 20 peer-reviewed publications on the Hawaiian bobtail squid. Since cephalopods have recently been added to AAALAC for accreditation purposes, PI Nyholm's research has not been the subject of IACUC approval. PI Nyholm and all lab personnel will obtain IACUC training through UCONN.

Section III: Project Personnel

A. **Please complete the chart on the following page to describe the qualifications and training of each individual who will be assigned a role for work on this project (excluding PI and unlisted students below)**

Section IV: Unlisted Students and Personnel

A. Will any students or other personnel not listed below in Section VI be assigned to work with animals on this protocol?

X ☐No ☐Yes, if Yes, qualify below

1. Who will supervise or oversee these individuals?
   ▶

2. Describe the training these individuals will receive prior to working on this protocol:
   ▶

3. Describe the types of activities these individuals will be engaged in with regards to animal procedures:
   ▶

Section V: Occupational Safety and Health Registration

A. Have all personnel listed in Section V been enrolled in the University's Occupational Health and Safety (OHS) Program for Animal Handlers, by completing and submitting the appropriate forms from the EHS web site?

☐Yes X ☐No (Note that individuals will not be approved to work under this protocol until all required OHS documentation is complete)

Special note regarding ethics in research training:

The Office of Research Compliance would like to remind Principal Investigators that effective January 4, 2010, changes in federal regulations mandate that the University of Connecticut must certify that a plan is in place for providing the appropriate training and oversight in the responsible and ethical conduct of research (RCR) to all technicians, undergraduate students, graduate students, and postdoctoral researchers who participate in federally funded research (renewals or new applications). It is not yet required of faculty members; however they are encouraged to take the course. The RCR curriculum will be offered in a variety of formats (in-person and on-line) and must be completed during the course of the individual's involvement in the federally funded project. It is NOT required that the training be completed prior to involvement in a project. More information about RCR is available at http://vprge.uconn.edu/rcr.cfm.

This training is not required for IACUC review, but is required for the procurement of federal funds for research.

Name of individual (last, first, MI)	Status (student, etc.)	Procedures this individual will perform	Species this individual will work with	# of years and type of experience working with this species	Type of experience working with these procedures	If this person has worked w/ species or procedure <1 year, describe training that will be provided prior to beginning work on this protocol	Who will provide this training?	List date and type of most recent IACUC training (Initial classroom session, on-line, one-on-one, etc.)	Describe any other relevant training and experience
Sarah McAnulty	PhD student	Animal husbandry, blood collection, tissue collection	Euprymna scolopes	5	Has performed and mastered all laboratory techniques and extensive experience with husbandry procedures			IACUC, June 7, 2016 retraining on 5/4/18	Two summer internships working with cephalopods at the Marine Biological Laboratory
Andrea Suria	PhD student	Animal husbandry, tissue collection	Euprymna scolopes	5	Has performed and mastered all laboratory techniques and extensive experience with husbandry			IACUC (CITI), retraining April 17, 2018	
Lydia Abini-Agbosom	undergrad	Animal husbandry only	Euprymna scolopes	1 yr 8 months	Has performed animal husbandry procedures under supervision		PI and PhD students in the Nyholm lab	IACUC, March 7, 2017), retraining Oct. 8, 2018	
Elise Huysman	undergrad	Animal husbandry only	Euprymna scolopes	2 yrs	Has performed animal husbandry procedures			IACUC, March 7, 2017, retraining 10/8/18	
Abishek Arokiados	undergrad	Animal husbandry only	Euprymna scolopes	8 months	Has performed animal husbandry procedures Under supervision	All animal husbandry training has been and will continue to be performed and supervised by trained graduates students and the PI	PI and PhD students in the Nyholm lab	Classroom session 2018	
Hope Dieffenbach	undergrad	Animal husbandry and egg experiments only	Euprymna scolopes	1 yr 8 months	Has performed animal husbandry experiments			Classroom, April 17, 2018	
Sarah Cleveland	undergrad	Animal husbandry	Euprymna scolopes	10 months	Has performed animal husbandry procedures Under supervision	All animal husbandry training has been and will continue to be performed and supervised by trained graduates	PI and PhD students in the Nyholm lab	IACUC, April 9, 2018	

						students and the PI			
Colin Schleicher	undergrad	Animal husbandry, tissue collection	Euprymna scolopes	8 months	Has performed animal husbandry and experiments under supervision	All animal husbandry training has been and will continue to be performed and supervised by trained graduates students and the PI	PI and PhD students in the Nyholm lab	Classroom session 2018	
Courtney Marren	undergrad	Animal husbandry only	Euprymna scolopes	1 yr 6 months	Has performed animal husbandry under supervision	I		Classroom April 17, 2018	
Nidhi Vijayan	PhD student	Animal husbandry, tissue collection	Euprymna scolopes	4 months	Has performed animal husbandry and experiments under supervision	All animal husbandry training has been and will continue to be performed and supervised by trained graduates students and the PI	PI and PhD students in the Nyholm lab	TBD (will take next available class)	
Derrick Kamp	PhD student	Animal husbandry, tissue collection	Euprymna scolopes	4 months	Has performed animal husbandry and experiments under supervision	All animal husbandry training has been and will continue to be performed and supervised by trained graduates students and the PI	PI and PhD students in the Nyholm lab	Classroom session 2018	
Spencer Nyholm	PI	Animal husbandry, blood collection, tissue collection	Euprymna scolopes	22	Has performed and mastered all laboratory techniques and extensive experience with husbandry procedures			IACUC, March 7, 2017	

Section VI: Listed Protocol Personnel:

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IACUC Protocol # A16-029
Approval Date: 2/19/19
Expiration Date: 2/19/22
Species: Squid
Guide exceptions: Y__ N✓
Hazards: Y__ N✓

University of Connecticut

Appendix G: Use of Surgery in Animal Research Protocols

Institutional Animal Care and Use Committee, Office of Research Compliance

Whetten Graduate Center, Rm #214, 438 Whitney Road Ext., Unit 1246 Storrs, CT 06269-1246 860-486-8802

Instructions: This form is a companion form to Section VIII.A.6 of form IACUC-1, "Animal Care and Use Protocol Submission Form". The completion and submission of this form is required if your protocol submission contains:

- The cutting, abrading, suturing, laser or otherwise physical alteration of body tissues and organs of live vertebrate animals.
- Survival surgical procedures, i.e. the animal must be expected to regain consciousness after the procedure is performed.
- Non-survival surgical procedures, i.e. the animal will not regain consciousness following the procedures: **FOR NON-SURVIVAL PROCEDURES COMPLETE THIS APPENDIX THROUGH SECTION III ONLY.**

AUG 29 2018
Office of Research Compliance

Section I: PI (Principal Investigator) and Protocol Information

Principal Investigator (PI):Spencer Nyholm
Project Title: The Hawaiian bobtail squid, Euprymna scolopes, as a model for host-microbe research

Section II: Providers of Care: Who will be performing the surgical procedures described in this protocol? Check all that apply:

	Animal Care Services (ACS) Staff
	Agricultural animal care staff
X	Principal Investigator (PI) and/or research staff
X	Students under the direction/ instruction of the PI
	Other (describe)

Section III: Identification of NON-SURVIVAL Surgical Procedures

A. Describe non-survival surgery below, including methods of anesthesia, checking for depth of anesthesia, and surgical preparation and procedures. (n.b., the *Guide 2011* states that for non-survival surgeries, at a minimum, the surgical site should be clipped, the surgeon should wear gloves and the instruments and surrounding areas should be clean).

► To collect light-organ contents *in situ*, adult animals are anaesthetized either in FSSW or FSIO containing 2% ethanol or 0.12-0.15 M $MgCl_2$,. When the animal is pale and cannot correct its position when oriented dorsally, the animal is transferred to a dissecting tray, and covered in sufficient FSSW or FSIO (2% ethanol, or 0.12-0.15 M $MgCl_2$) to ensure ventilation by the gills. A small primary incision is made in the animal's mantle. If, at this point, the squid does not respond by moving tentacles or dilating pupils, then a medial incision is made through the ventral mantle tissue to expose the animal's light organ. A medial incision is also made in the funnel to fully expose the light organ. To induce expulsion of the light-organ contents, incandescent white light is directed at the squid's eyes. The expelled material is extruded out of the lateral openings of the light organ, and is collected with a 50-µl capacity Gilson Microman negative-pressure pipet. Expulsion of the crypt contents may occur as much as 30 min following initial light exposure, depending on the method of anesthesia used, among other factors. All animals are euthanized following this procedure. All staff performing procedure wear gloves and all instruments are cleaned with 70% ethanol prior to and after the procedure.

Section IV: Identification of SURVIVAL Surgical Procedures

A. Minor surgical Procedures: Minor surgical procedures are surgical procedures that *do not expose a body cavity AND that cause minimal or no physical impairment*

1. Will any minor surgical procedures be performed in this protocol?

☐No ☐Yes (qualify below)

a. Identify all minor surgical procedures used in this protocol:
►

b. Which species will receive these procedures?
►

c. What is the purpose of the procedures identified (briefly describe in layman's terms)?
►

B. Major surgical procedures: Major surgical procedures are surgical procedures that *expose a body cavity AND/OR cause substantial physical or physiological impairment*

1. Will any major surgical procedures be performed in this protocol?

☐No ☐Yes (qualify below)

a. Identify all major surgical procedures used in this protocol:
►

b. Which species will receive these procedures?
►

c. What is the purpose of the procedures identified (briefly describe in layman's terms)?
►

Section V: Surgical Preparation: Answer the following questions related to all survival surgery procedures identified in Section IV:

A. Preparation of surgical location:

1. Where will the surgery or surgeries be performed? Specific location(s):
   ▶

2. Is this location a dedicated surgical suite? ☐Yes ☐No

3. How will this location be prepped for the surgery (surfaces cleaned, disinfected, or sanitized; surfaces reorganized to accommodate surgery, dedicated hood prepped with UV light, etc.):
   ▶

4. Animal preparation

   a. Will animal preparation require fasting or fluid restriction? ☐No ☐Yes How long?
      ▶

   b. Will animal preparation include use of a pre-anesthetic or early administration of analgesia? ☐No ☐Yes

   c. Are any additional animal preparations necessary?
      ▶

5. Surgical instrument preparation

   a. How will surgical instruments be prepped for surgery?
      ▶

   b. How will sterility or asepsis of surgical instruments be maintained throughout the course of the surgery?
      ▶

6. Surgical site preparation:

   a. How will the surgical site be prepped for surgery?
      ▶

Section VI: Conduction of Surgery

A. Anesthesia: Complete the following questions regarding the use of anesthesia for surgical procedures described in this protocol.

1. Will any surgical procedures be performed without anesthesia?

   ☐No
   ☐Yes, if Yes, justification MUST be provided below:

   ▶

2. Please complete the following regarding any primary and secondary surgical anesthesia that will be used for this protocol:

Species	Surgical Procedure	Anesthetic used	General or local	Dose	Route of administration	Timing of administration (pre-

			anesthetic?			surgery, during surgery, etc.)

3. For any general anesthetics listed above, please indicate how depth of anesthesia will be monitored (check all that apply- at least 2 methods must be checked for surgery that requires a surgical plane. One method is acceptable for surgery requiring the numbing of an area)

	Blood pressure/ EKG readings		Respiratory rate
	Corneal reflexes		Tail/toe pinch (pedal reflexes)
	EEG		Other: specify
	Heart Rate		Other: specify
	Pinching or pricking of incision site (local anesthesia only)		

B. NMB: Neuromuscular Blocking Agents

1. Will any neuromuscular blocking agents, muscle relaxants, or paralytic drugs be used in this protocol? ☐No ☐Yes (qualify below)

   a. What species will receive the agent(s)?
   ▶

   b. What drug and dosage?
   ▶

   c. How will respiration and anesthesia depth be monitored while animals are under the influence of these agents?
   ▶

C. Surgical techniques

1. Describe, in detail, the procedures for any minor surgical techniques identified in Section IV, or provide copy of SOP (or reference to IACUC approved SOP#). If more than one species is used in the protocol, indicate which one(s) are undergoing the procedure:
   ▶

2. Describe, in detail, the procedures for any major surgical techniques identified in Section IV, or provide copy of SOP (or reference to IACUC approved SOP#). If more than one species is used in the protocol, indicate which one(s) are undergoing the procedure:
   ▶

3. Describe any types of sutures, wound clips, staples, glue, or other tissue closure method that will be used:
   ▶

4. How will bleeding be controlled during surgery?
   ▶

Section VII: Medications

A. Withholding Analgesia: Complete the following regarding the use of analgesia for this protocol:

1. Will any animals have analgesic use withheld for surgical procedures?

☐No ☐Yes, if Yes:

a. What species and procedures?
▶

b. What is the justification for withholding analgesia?
▶

c. What non-analgesic measures will be taken to alleviate pain or discomfort?
▶

B. Administration of medications:

1. Please complete the following table regarding medications that will be administered to animals due to surgical procedures for this protocol (examples include analgesics, antibiotics, anti-rejection drugs, fluids, etc.):

Species	Surgical Procedure	Medication used	Dose	Route of administration	Timing of administration (pre-surgery, during surgery, post-surgical, peri-surgical)	Frequency and duration of reapplication

3. If medication other than analgesia or antibiotic, please describe its purpose and function:
▶

Section VIII: Recovery

A. Post-operative monitoring

1. Who will provide immediate post-operative care and observation to the animals?
▶

2. Will animals be provided a warming blanket or other heat source to aid recovery from anesthesia?
▶

3. How often will animals be checked, and by whom, prior to fully awakening?
▶

4. Will animals require any specialized post-operative care? Describe:
▶

5. Where will post-operative recovery occur?
▶

B. Return to regular housing

1. What are the criteria for determining an animal is ready to be returned to a regular housing facility (or otherwise released back into its environment)? ▶

2. Will animal require any housing or environmental modifications upon its return? (e.g. single housing, dietary supplement, food placed on floor of cage, etc.) Describe:
▶

3. Will animal require sutures or staples to be removed?
☐No ☐Yes, if Yes: When, and by whom:
▶

C. Surgical Impairment

1. Describe the anatomical and/or physiological effects the surgery is expected to have on the animals:
▶

D. Post-surgical maintenance of animals

1. Will the effects described above require any long-term maintenance or monitoring of the animals' condition, or will procedures decrease the animal's normal lifespan?
☐No ☐Yes, if Yes, describe:
▶

E. Surgical Complications

1. What are the anticipated and potential surgical complications that may be seen with the surgical procedures outlined in this protocol, and what is the plan for dealing with these complications should they arise?
▶

2. What is the expected animal attrition due to surgical complications for this procedure when it is performed by a skilled individual? Ensure that attrition has been considered in the numbers justification section of the main protocol form.
▶

3. Can any of the surgical procedures utilized by this protocol be described as unfamiliar, novel, or infrequently used [by personnel in your laboratory]?
☐No ☐Yes, if Yes, briefly describe training that will be provided to acclimate research associates:
▶

Section IX: Multiple Major Survival Surgery

A. Will any animals identified in this document be receiving a second (or more) major survival surgery during the course of this protocol?
☐No ☐Yes, if "Yes":

1. What is the nature of the two (or more) major survival surgeries?
▶

2. Why are multiple surgeries necessary for meeting the research goals and objectives? (Scientific or animal welfare-related justification is required for this response)

▶

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Approval Date: 2/19/19
Expiration Date: 2/18/22
Species: Squid
Guide exceptions: Y__ N✓
Hazards: Y__ N✓

University of Connecticut

Appendix E: Considerations of Alternatives to Painful or Distressful Procedures in Animal Research Protocols

Institutional Animal Care and Use Committee, Office of Research Compliance
Whetten Graduate Center, Rm #214, 438 Whitney Road Ext., Unit 1246 Storrs, CT 06269-1246 860-486-8802

Instructions: This form is a companion form to Section VII of form IACUC-1, "Animal Care and Use Protocol Submission Form". The completion and submission of this form is required if your protocol submission contains:

- Live vertebrate animal research procedures classified as USDA Pain Category D or E

AUG 29 2018
Office of Research Compliance

Section I: PI (Principal Investigator) and Protocol Information

Principal Investigator (PI) Spencer Nyholm
Project Title: The Hawaiian bobtail squid, Euprymna scolopes, as a model for host-microbe research

Section II: Background

A. USDA Policy #11 provides guidance on the use of potentially painful or distressful procedures in animal care and use

B. USDA policy # 12 requires that procedures involving live animals will be designed in such a way as to minimize discomfort, distress, and/or pain to that which is absolutely necessary to meet research goals, and requires a written narrative of the consideration of alternatives to painful or distressful procedures

C. The *Guide for the Care and Use of Laboratory Animals* requires that a veterinary consultation *must* occur when pain or distress is beyond the level anticipated in the protocol description or when interventional control is not possible

Section III: Search for Alternatives

A. For *each* protocol procedure that is likely to produce pain and/or distress, please complete the following table for any literature search that was conducted. At least one database or other source must be referenced:

Name of procedure	Date literature search was performed (month/year)	Years covered by search	Database(s) searched	All Keywords Used to search (or search strategy) (must contain the following search terms: "alternative", "[procedure]", and "[species]"	Number of "hits" returned
Blood/hemolymph collection	08/18	1991-2018	pubmed	Euprymna scolopes, blood hemolymph hemocytes	17
Non-survival	08/18	1991-2018	pubmed	Euprymna	74

surgery for tissue for harvest				scolopes, tissue	

B. It is also acceptable to utilize current material from other resources. If you used any of the following to evaluate procedures on your protocol, please supply the following information:

Name of Procedure:	Method used to search for alternatives:	Source utilized:	Summary of Topics, searches, strategies, discussions, etc.	Date
	Consultation with in-house colleagues	Who was consulted?		What date was the consultation performed?
	Consultation with external colleagues/ experts	Who was consulted?		What date was the consultation performed?
	Journals and/or Bibliographical sources were utilized	What titles were utilized?		What year(s) were the publications?
	Other source(s) were utilized	Describe:		

C. Evidence of Veterinary Consult: (Provide the date that you or your staff consulted (phone, email or in person is acceptable) with a member of the ACS veterinary staff to discuss Category D or E procedures)

▶

Section IV: Written Narrative

A. For *each* search identified in Section III above, please draft a narrative statement that summarizes the relevance of the "hits" your search provided to the context of the objectives and goals of the study. You must include information in your narrative as to why any alternatives found cannot be used for this protocol:

▶

Hemolymph collection: PI Nyholm was the first investigator to describe the procedures for blood collection and hemolymph from *Euprymna scolopes* (Nyholm et al., 2009). His laboratory currently has 7 publications addressing hemocytes from E. scolopes. His work has also been cited in AAALACs "Guidelines for the care and welfare of cephalopods in research-a consensus based on an intiative by CephRes, FELASA and the Boyd Group" Laboratory Animals (2015) Vol. 49 (S2) 1-90. There are no suitable alternate procedures to currently obtain hemolymph from *E. scolopes*. Blood can only be collected as described in the Nyholm lab SOP.

Relevant primary publications from the Nyholm lab related to the collection of hemolymph:

- Nyholm SV, Stewart JJ, Ruby EG, McFall-Ngai, MJ (2009) Recognition between symbiotic *Vibrio fischeri* and the haemocytes of *Euprymna scolopes*. *Environ. Microbiol.*, 11: 483-93.
- Collins AJ, Nyholm SV (2010) Obtaining hemocytes from the Hawaiian bobtail squid *Euprymna scolopes* and observing their adherence to symbiotic and non-symbiotic bacteria. *JoVE.* 36. doi: 10.3791/1714
- Collins AJ, Schleicher TR, Rader BA, Nyholm SV (2012) Understanding the role of host hemocytes in a squid/*Vibrio* symbiosis using transcriptomics and proteomics. *Frontiers in Immunology* 3:91.
- Schleicher TR, VerBerkmoes NC, Shah, M, Nyholm SV (2014) Colonization state influences the hemocyte proteome in a beneficial squid-vibrio symbiosis. *Mol. Cell Proteomics* 13: 2673-86.

Tissue harvesting: PI Nyholm has over 20 years of experience studying E. scolopes. He has been author or co-author on 20 publications related to this animal host. Currently there are no suitable alternatives for harvesting tissues from *E. scolopes* other than through anesthesia and euthanasia as describes in the Nyholm lab SOP. Publications related to tissue harvesting from PI Nyholm:

- Kerwin AK, Nyholm SV (2017) Symbiotic bacteria associated with a bobtail squid reproductive system are detectable in the environment and stable in the host and in eggs throughout development. *Environ. Microbiol.* 4:1463-1475 doi: 10.1111/1462-2920.13665

- Gromek SM*, Suria A*, Fullmer MS, Garcia JL, Gogarten JP, Nyholm SV♯, Balunas MJ♯. (2016) *Leisingera* sp. JC1, a bacterial isolate from Hawiian bobtail squid eggs, produces indigoidine and differentially inhibits vibrios. *Front. Microbiol.* 7: 1342 doi:10.3389/fmicb.2016.01342 (* contributed equally; ♯ co-corresponding)

- Collins AJ, Fullmer MS, Gogarten JP, Nyholm SV (2015) Comparative genomics of *Roseobacter* clade bacteria isolated from the accessory nidamental gland of *Euprymna scolopes*. *Front. Microbiol.* 6: 123 doi:10.3389/fmicb.2015.00123.

- Schleicher TR, VerBerkmoes NC, Shah, M, Nyholm SV (2014) Colonization state influences the hemocyte proteome in a beneficial squid-vibrio symbiosis. *Mol. Cell Proteomics* 13: 2673-86. (featured on cover)

- Albertin CB, Bonnaud L, Brown CT, Crookes-Goodson WJ, da Fonseca RR, Di Cristo C, Dilkes BP, Edsinger-Gonzales E, Freeman RM Jr, Hanlon RT, Koenig KM, Lindgren AR, Martindale MQ, Minx P, Moroz LL, Nödl MT, Nyholm SV, Ogura A, Pungor JR, Rosenthal JJ, Schwarz EM, Shigeno S, Strugnell JM, Wollesen T, Zhang G, Ragsdale CW. (2012) Cephalopod genomics: a plan of strategies and organization. *Stand Genomic Sci.* 7: 175-188.

- Nyholm SV, Graf J. (2012) Knowing your friends: how invertebrate innate immunity fosters beneficial symbioses. *Nature Reviews Microbiol*ogy 10: 815-27. doi: 10.1038/nrmicro2894

- Rader BA, Nyholm SV (2012) Host/Microbe Interactions Revealed through "Omics" in the Symbiosis Between the Hawaiian Bobtail Squid, *Euprymna scolopes*, and the Bioluminescent Bacterium, *Vibrio fischeri*. *Biological Bulletin* 223: 103-111.

- Fidopiastis P, Rader BA, Gerling D, Gutierrez N, Watkins K, Frey M, Nyholm SV, Whistler CA (2012) Characterization of a *Vibrio fischeri* aminopeptidase and evidence for its influence on an early state of squid colonization. *Journal of Bacteriology* 194: 3995-4002.

- Collins AJ*, Schleicher TR*, Rader BA, Nyholm SV (2012) Understanding the role of host hemocytes in a squid/*Vibrio* symbiosis using transcriptomics and proteomics. *Frontiers in Immunology* 3:91. (*co-first authors)

- Collins AJ, LaBarre BA, Wong Won BS, Shah MV, Heng S, Choudhury MH, Haydar SA, Santiago J, Nyholm SV (2012) Diversity and partitioning of bacterial populations within the accessory nidamental gland of the squid *Euprymna scolopes*. *Appl. Environ. Microbiol.* 78: 4200-4208.

- Schleicher TS, Nyholm SV (2011) Characterizing the host and symbiont proteomes in the association between

the bobtail squid *Euprymna scolopes* and the bacterium *Vibrio fischeri. PLoS ONE* 6(10) e25649.

- Collins AJ, Nyholm SV (2011) Draft genome of *Phaeobacter gallaeciensis* ANG1, a dominant member of the accessory nidamental gland of *Euprymna scolopes. J. Bacteriol.* 193: 3397-3398

- Collins AJ, Nyholm SV (2010) Obtaining hemocytes from the Hawaiian bobtail squid *Euprymna scolopes* and observing their adherence to symbiotic and non-symbiotic bacteria. *JoVE.* 36. doi: 10.3791/1714

- Wier AM, Nyholm SV, Mandel MJ, Massengo-Tiassé RP, Schaefer AL, Koroleva I, Splinter-Bondurant S, Brown B, Manzella L, Snir E, Almabrazi H, Scheetz TE, Bonaldo Mde F, Casavant TL, Soares MB, Cronan JE, Reed JL, Ruby EG, McFall-Ngai MJ. (2010) Transcriptional patterns in both host and bacterium underlie a daily rhythm of anatomical and metabolic change in a beneficial symbiosis. *Proc. Nat. Acad. Sci., USA,* 107, 2259-64

- McFall-Ngai MJ, Nyholm SV, Castillo MG (2010) The role of the immune system in the initiation and persistence of the *Euprymna scolopes-Vibrio fischeri* symbiosis. *Semin. Immunol.* 22: 48-53.

- Nyholm SV, Stewart JJ, Ruby EG, McFall-Ngai, MJ (2009) Recognition between symbiotic *Vibrio fischeri* and the haemocytes of *Euprymna scolopes. Environ. Microbiol.*, 11: 483-93.

- Nyholm SV, Nishiguchi, MK (2008) The evolutionary ecology of a sepiolid squid-*Vibrio* association: from cell to environment. *Vie et Milieu* 58: 175-184.

- Nyholm SV, McFall-Ngai MJ (2004) The Winnowing: Establishing the Squid-*Vibrio* Symbiosis. *Nature Rev. Microbiol.* 2: 632-642.

- Nyholm SV, McFall-Ngai MJ (2003) Dominance of *Vibrio fischeri* in secreted mucus outside the light organ of *Euprymna scolopes*: the first site of symbiont specificity. *Appl. Environ. Microbiol.* 69: 3932-7.

- Nyholm SV, Deplancke B, Gaskins HR, Apicella MA, McFall-Ngai MJ (2002) Roles of nonsymbiotic and symbiotic bacteria in the dynamics of mucus secretion during symbiont colonization of the *Euprymna scolopes* light organ. *Appl. Environ. Microbiol.* 68:5113-5122.

- Nyholm SV, Stabb EV, Ruby EG, McFall-Ngai MJ (2000) Establishment of an animal-bacterial association: recruiting symbiotic vibrios from the environment. *Proc. Nat. Acad. Sci., USA* 97:10231-10235.

- Nyholm SV, McFall-Ngai MJ (1998) Sampling the light-organ microenvironment of *Euprymna scolopes*: description of a population of host cells in association with the bacterial symbiont *Vibrio fischeri. Biol. Bull.* 195: 89-97.

University of Connecticut

Appendix K: Occupational Health Assessment of Protocol Submissions

Institutional Animal Care and Use Committee, Office of Research Compliance

Whetten Graduate Center, Rm #214, 438 Whitney Road Ext., Unit 1246 Storrs, CT 06269-1246 860-486-8802

Office Use Only
IACUC Protocol # A18-029
Approval Date: 2/19/19
Expiration Date: 2/19/22
Species: Squid
Guide exceptions: Y__ N✓
Hazards: Y__ N✓

Instructions: This form is to be used to formally capture risk assessment and evaluation of potential hazards in protocol submissions for animal care and use. It is to be completed by the IACUC Administrator and appropriate representatives of the University Occupational Health program

Section I: PI (Principal Investigator) and Laboratory Information

Principal Investigator (PI) Spencer Nyholm
Department: Molecular and Cell Biology
E-mail: spencer.nyholm@uconn.edu
Unit #: 3125 FEB 13 2019
Phone #: 860-486-4886 Office of Research Compliance
Emergency contact information (please provide cell phone#): 617-921-7575

Section II: General Protocol Information

Project Title: The Hawaiian Bobtail Squid, Euprymna scolopes, as a Model for Host-Microbe Research
Submission type: ☒New ☐ Three year Renewal ☐ Modification of approved submission #
Type of project: ☒☐☐ ☐Public Service ☐Field research Check All That Apply
Anticipated project start date: ongoing
Species: squid

Section III: Providers of Care: Who will be performing the procedures described in this protocol? Check all that apply:

	Office of Animal Care (OAC) Staff
	Agricultural animal care staff
X	Principal Investigator (PI) and/or research staff
X	Students under the direction/ instruction of the PI
	Other (describe)

Section IV: Risks Identified by IACUC Office that we are requesting assessment of: (Indicated below)

☐ status of DEA license are checking the status of DEA license

☐ ☐ PI has current license that will need to be renewed on [date]

☒ Chemical Safety assessment

Notes from IACUC: The PI describes the use of magnesium chloride to be administered to squid as an anesthetic agent. This work is described in the IACUC-1 and the SOP associated with the protocol. Please let us know what, if any, additional information is requested of the PI, and whether or not an appendix D is necessary.

Notes from Chemical Safety	
Name of Reviewer:	Brent Lewchik
(check one below)	
	I have reviewed the proposed procedures for minimizing the risk to animal handlers and other personnel assigned to work on this project, and have found them acceptable for the nature of the work being conducted
X	The following additional comments require attention and consideration:

- **Magnesium chloride does not meet the definition of "carcinogen," "reproductive hazard," or "toxic" defined in the protocol, is not required to be listed in Appendix D, and does not require chemical isolation. Appropriate engineering controls and personal protective equipment listed in the safety data sheets must be worn during the handling of the chemical.**
- **Page 18- Magnesium chloride must be listed under "Yes for chemical KNOWN NOT TO BE toxic...."**

☒ Biological Safety assessment

Notes from IACUC: The PI describes experiments in which squid will be exposed to *Vibrio fischeri*, *Leisingera* spp and *Verrucomicrobia* spp. This work is described in the IACUC-1 and associated SOP. Please let us know what, if any, additional information is requested of the PI, and whether this will require additional review/approval from the IBC.

Notes from Biological Safety	
No additional information is required at this time. The PI has submitted an IBC registration renewal for review at the next meeting.	
Name of Reviewer:	David J. Cavallare
(check one below)	
X	I have reviewed the proposed procedures for minimizing the risk to animal handlers and other personnel assigned to work on this project, and have found them acceptable for the nature of the work being conducted
	The following additional comments require attention and consideration:

☐ Radiation Safety assessment

Notes from IACUC:

Notes from Radiation Safety	
Name of Reviewer:	
(check one below)	
	I have reviewed the proposed procedures for minimizing the risk to animal handlers and other personnel assigned to work on this project, and have found them acceptable for the nature of the work being conducted
	The following additional comments require attention and consideration:

☐ Occupational Safety assessment

Notes from IACUC:

Notes from Occupational Safety

Name of Reviewer:	
(check one below)	
	I have reviewed the proposed procedures for minimizing the risk to animal handlers and other personnel assigned to work on this project, and have found them acceptable for the nature of the work being conducted
	The following additional comments require attention and consideration:

☐ **Occupational Health assessment**

Notes from IACUC:

Notes from Occupational Health	
Name of Reviewer:	
(check one below)	
	I have reviewed the proposed procedures for minimizing the risk to animal handlers and other personnel assigned to work on this project, and have found them acceptable for the nature of the work being conducted
	The following additional comments require attention and consideration:

A15-029

On arrival at UCONN, the water temperature of the bag is measured, and the squid are allowed to acclimate to the aquarium seawater temperature in the bags (open to air circulation) before release. Acclimation is conducted over a 2 h period with 200-300 ml water exchange every 20-30 min.

Almost all squid survive shipment, but a 10% loss within the first week is not unusual. The mortality may be attributed to stress of capture, transport, or failure to acclimate to captivity, or age of animal.

Long-term holding

Annual animal use

Animal Type	Number (3 yr period)	Use
Adult female	270	Breeding colony
Adult male	270	Breeding colony
Other wild-caught juveniles	180	Experimental
Hatchlings squid	60,000	Experimental
Raised squid	1500	Experimental

The lab has room to house 90 adult females and 90 adult males per year. This ratio has proved to be successful in providing 2-6 clutches weekly which, in turn, release about 5,000-20,000 hatchlings/yr for experimental procedures. We can house up to 80 egg clutches at a time, but usually have about 20-30 at various stages of incubation. Roughly 500 hatchlings (annually) are used for long-term experiments lasting 4 days to 6 months. All other hatchlings are for used in experiments within the first 3-5 days of hatch. Up to 60 juvenile wild-caught squid/yr are also collected for developmental studies.

Containment of exotic species

E. scolopes is a marine species, and is not native to Connecticut. As previously mentioned, they are found only within the Hawaiian Islands. However, as it is a tropical, salt-water species, introduction to any natural body of water locally would be fatal, due to temperature stress. We do not have any special precautions in place to reduce risk of introduction of this squid species locally. No special treatment is taken with effluent water.

Animal areas

The area in which the aquaria are kept is within the Nyholm laboratory in 2 aquarium rooms located in Torrey Life Sciences (TLS 82 and TLS 57). The PI, graduate students and/or trained undergraduates monitor the animals at least once each day.

The adult aquaria are on a 12h light/12 h dark cycle (lights on 0100 h; lights off 1300 h) This interval is equivalent to what the animal experiences in the wild, and provides ample time for lab maintenance and cleaning without disturbing these nocturnal animals. The air temperature is ambient for the lab space, about 24°C, which is also an environmentally natural temperature for the animals.

The egg-incubation aquarium is in a separate, light- and temperature- controlled aquarium with the same light/dark schedule and temperature.

Aquarium parameters

The main squid aquarium room in TLS 082 holds 2 rack systems (each system has 10 (15 gallon, 57 liter) tanks with a 50 gal (189 liter) reservoir for re-circulating seawater. A 300 gallon (1135 liter) reservoir tank is connected to each system. In addition, there is nursery tank (200 gallons; 757 liters) with its own recirculating system. Water temperature is ambient, about 24°C. Each system and nursery

contains a sump with a protein skimmer, particle and carbon filters through which the seawater passes. The seawater also runs through a UV filter before returning to the animal-containment tanks. Artificial seawater is prepared to a desired 34 PPT salinity from Instant Ocean salts-mix and deionized water, and allowed to sit at least 24 h before use. Ammonia and nitrite levels are monitored and kept at zero (below detection). Nitrates are kept as low as possible, and are always <10 PPM. Each of the 2 adult and nursery aquaria is a closed system, so should an emergency arise, not all animals are lost. In fact, each system could be used as a separate quarantine system should the need arise.

Each tank is made of either darkened or opaque acrylic. The bottom of each tank is covered with approximately 2 cm of calcium-carbonate (marine) sand, and halved 4-in PVC pipes are placed in the tanks, within which the females lay eggs. The lids of each cube are transparent acrylic and allow light to penetrate, so that the squid experiences a natural light-dark cycle.

Each adult animal is housed in a separate tank and labeled with its own number. On occasion, 2-3 animals are kept per tank with no apparent increase in stress to the animals.

Should a power outage occur, the volume of water on the tables is sufficient to keep the squid immersed and oxygenated for several hours.

Feeding

Live freshwater glass shrimp (*Palaemonetes kadiakensis* or *P. paludosus*) are fed *ad libitum* to squid each afternoon, during the animals subjective "dark" cycle. Shrimp are enriched with a nutritional fish-flake food shortly before use. The glass shrimp are obtained through a licensed wholesale fish farm in Florida. In the lab, the shrimp are maintained in separate 50-gal aquaria.

When available, squid are fed live saltwater white shrimp, *Penaeus vannamei. P. vannamei.* These can be collected from Long Island Sound during summer months.

Cleaning

On a daily basis, dead and dying feeder shrimp are removed from the tanks first thing in the morning. This prevents buildup of nitrogenous waste in the system and in the sand layer.

Each individual tank is cleaned between squid collections or when noticeably dirty. The squid are transferred to a separate holding tank for the duration of cleaning (< 20 minutes). Sand is removed and washed with very hot tap water, followed by a thorough rinsing with deionized water. Algae, leftover feed, and other detritus is removed from the sand layer through this process. The sides, bottom, and top of the tanks are also scrubbed.

Annually, the tanks are completely drained, the filters are changed, the UV light bulbs are changed, and deionized water is pumped through the table for a few days. The tanks are then refilled with seawater and allowed to run for at least a week before a new shipment of squid comes in or before new eggs are placed on the nursery.

Breeding of adults

The mantle lengths (ML) of all squid are measured in the first week of arrival after collection. A single female is matched with a similarly sized (or slightly smaller) male. During each breeding event (once every 2 weeks for each female), the male is moved into the female's tank for 12 h overnight, and returned to its own tank the next morning for recovery. Each male has at least 2 days of recovery between mating events.

Record Keeping

Electronic animal records are maintained in the Nyholm lab for ease of input and access. Facility records, such as water parameters and purchasing receipts, are maintained in paper and electronic form. Temperature and salinity are recorded daily. Nitrate, nitrite and ammonia levels are checked 2x per week or more often if animals display signs of distress. Animal identifiers are designated by number and/or letter and monitored for the duration of the animal's life. Animal death dates are recorded, and if the animal is euthanized, that is recorded as well. Mean longevity in captivity can thus be calculated.

Each egg clutch is assigned a unique identifier, which includes the cohort alphabet designation. For each clutch, the lay date, the approximate number of eggs, the female, and the PVC cave identifier are recorded.

From each clutch, the number of hatchlings is recorded. This information includes the number of hatchlings that hatched overnight ("earlies") and the number of hatchlings that hatched at known times ("normals"). Any dead, sickly, or premature hatchlings are also noted. From these data, we can compute the number of hatchlings per clutch total, the number of hatchlings per time period, the number of hatchlings per female, and the clutch's range of incubation time.

Incubation and hatching

Aquarium parameters

When a clutch is laid in an adult tank, it is recorded and moved (while immersed in a glass bowl filled with saltwater) to the incubation table. At no time is the clutch exposed to air.

The nursery can accommodate up to 20-30 containers that each can house of to 3 clutches each. The nursery has 200 gal of circulating seawater. The water temperature is kept at 23-24° C through the use of a chiller and heater attached to the system. Levels of ammonia, nitrite, and nitrate are in the water are maintained at zero (*i.e.*, below detection), and salinity is kept at 34 PPT. The circulating water is exposed to UV illumination. Because constant aeration of the eggs is essential for their normal development, should a power outage occur, the egg table pump is attached to an outlet that receives power from the building's emergency generator.

Hatching

Most eggs (70-80%) hatch within 2 h of dark, that is, between 1100 and 1400 h. Animals are collected regularly from the egg table using a 5 mL disposable plastic pipette to transfer them to a plastic beaker. During the day, animals are collected within 30 min of hatch and are placed into filter-sterilized (0.22 μM pore-sized filter membrane) Instant Ocean water (FSIO). Animals are washed 3 times in this filtered water to prevent any contamination from the egg table water. Overnight animals (the "earlies"), may remain on the table as long as 16 h, and are used for experiments that do not require either aposymbiosis (no colonization of the symbiotic light organ) or knowledge of the exact time of hatch.

Anesthesia and euthanasia

Recognition of stress or illness

Adult and raised squid are observed at least[SN1] once per day. Signs of illness or distress include failure to bury in the sand during daytime hours, failure to eat, and failure to respond to gentle touch. Animals deemed to be ill are euthanized (see below). Lesions and scars may appear on females immediately after mating, but do not appear to affect the short- or long-term overall health of the animal, and usually disappear within a few days.

During cleaning, breeding and tank maintenance, it is sometimes necessary to remove the squid from its sand cover. This may evoke a temporary stress response (e.g., inking), but care is taken to minimize these occurrences, and to proceed as carefully and gently as possible. Because the viscous ink may clog gills, it is removed immediately from the water with a turkey baster.

Termination of experiments and euthanasia

Adult animals die either naturally, at the termination of an experiment, or are euthanized. Hatchlings and reared animals are euthanized at the termination of an experiment. Euthanasia is performed through over-anesthetization followed by a quick-freeze method. Anesthetization is performed as described above: exposure to either 2% ethanol or 0.12-0.15M $MgCl_2$, in FSSW or FSIO..

Anaesthetized animals may be put into a dry, sealed container (a plastic bag or vial), and dropped into a bath of liquid nitrogen. Within a few seconds, the animal is completely frozen, and the tissue stored at -80° C. If organs need to be harvested after an experiment squid are first anesthetized as described above and the brain is quickly pithed and head severed with a scalpel or razor blade.

Carcass disposition and storage

After an experiment, or at the end of an adult's life, the tissues (hemolymph, light organ, testes, eyes, *etc.*) are often harvested, and frozen for future use. Otherwise, after experiments and euthanasia, juvenile squid are placed in plastic bags and disposed of through Inserv animal waste via a collection site in Torrey Life Sciences; similarly, after experiments and euthanasia, the entire carcass of adult/reared animals is put into labeled and sealed plastic bags, and stored in a dedicated freezer for future use or reference.

Emergency procedures

In case of an emergency power outage or natural disaster, squid tanks are connected to TLS emergency power. In the event of building generator failure, backup stand-alone generators will be provided by UCONN facilities and used to maintain aquaria. In the event of total generator failure, battery operated aerators will be used to maintain oxygen delivery to squid until power is restored. Manual water changes will be conducted in the event that sweater chemistry monitoring determines that ammonia levels are high. Animal health and signs of stress will be monitored during outages at least once daily. In the event that power can't be restored for a prolonged time (greater than 3 days) animal health will be monitored and if animals appear distressed (unburied, lack of chromatophore function, unresponsive to feeding) animals will be euthanized as described above.