

OCT 13 2017

College of Natural Resources & Sciences  
Humboldt State University

HUMBOLDT STATE UNIVERSITY

**INSTITUTIONAL ANIMAL CARE AND USE PROTOCOL  
FOR THE HUMANE CARE AND USE OF LIVE VERTEBRATE ANIMALS**

**This box is for the review of the use by the Institutional Animal Care and Use Committee.  
Authors should not write or type inside the borders of the box.**

Date 1<sup>st</sup> Received: 10/13/17 Revision 1 Date: \_\_\_\_\_ Revision 2 Date: \_\_\_\_\_ No. 17/18 P 34-A

- ( ) E- Procedures are exempt from full IACUC review because they are purely observational, non-invasive, and produce no perceptible discomfort or they concern only the use of tissues from dead animals. To be considered exempt, tissues from dead animals must be obtained from animals euthanatized or otherwise killed by means, and for purposes, unrelated to the proposed project. The procedure may be approved by the Chair one additional member of the IACUC.
- ☒ A- Procedures will be minimally invasive or produce relatively little discomfort. Protocols may involve, bleeding, injections, minimal sampling, anesthesia or humane euthanasia without prior invasive manipulation. The procedure may be approved by the Chair and two additional members of the IACUC. Project topics will be reviewed by the IACUC at the next scheduled meeting.
- ( ) B- Procedures will involve prolonged manipulation or be invasive. Protocols may involve surgical or other stimuli inducing pain or distress, but all pain or distress will be mitigated with appropriate anesthetics or analgesics. The procedure may be initially approved by the Chair, the Campus Veterinarian and one additional member of the IACUC. Protocols will be reviewed by the IACUC at the next scheduled meeting.
- ( ) C- Procedures will be invasive and may cause prolonged physiological or psychological stress. Pain, considerable distress, or discomfort may be induced and not mitigated by anesthesia or adequate analgesia (e.g. LD50 experiments, long-term food or water deprivation, etc.). These protocols will be reviewed thoroughly by the IACUC prior to commencement of the project.

Requires Health Assurance ☒ Yes ( ) No

[Signature] 10/23/17  
Signature, IACUC Member Date

☒ Approved ( ) Denied

Chair Dutk for Peggy Wiltzbach 10/23/17  
Signature, IACUC Member (see attached email) Date

☒ Approved ( ) Denied

\_\_\_\_\_  
Signature, Campus Veterinarian (if necessary) Date

( ) Approved ( ) Denied

[Signature] 10-25-17  
Signature, IACUC Chair Date

☒ Approved ( ) Denied

**Final Committee Decision. All protocols must be approved prior to the start of research.**

Protocol Edition 2/15/2017



HUMBOLDT STATE UNIVERSITY  
**INSTITUTIONAL ANIMAL CARE AND USE PROTOCOL**  
**FOR THE HUMANE CARE AND USE OF LIVE VERTEBRATE ANIMALS**  
**INSTRUCTIONS**

Federal animal welfare regulations require that an Institutional Animal Care and Use Committee (IACUC) review and approve all activities involving the use of vertebrate animals prior to their initiation. This includes any animals used for the development of experimental methodologies, instructional purposes, research, etc. Approved protocols for ongoing and recurrent activities must be reviewed by the IACUC on an annual basis. However, extensions and amendments requiring an abbreviated application process may be granted for a total of three consecutive years. Compliance with animal welfare regulations is mandatory and is the responsibility of all individuals (including faculty and students) who choose to work with live vertebrate animals.

To avoid the proliferation of submissions, please provide generic descriptions (including multiple routes of compound administrations, minor procedural variations, similar laboratory exercises from a single course, routine exercises used in several courses, etc). When multiple vertebrate species are to be used, please clearly describe all procedures, and all variations thereof, to be used with each individual species.

Please submit your protocols to the Dean's Office, College of Natural Resources and Sciences, Forestry Bldg, Room 101. All protocols should be submitted on the most recent version of the forms downloaded from the IACUC web page (<http://www.humboldt.edu/iacuc>). You can expedite the review process by following these formatting rules: leave an extra blank line between the questions and your responses; leave questions in bold-face type; type your answers in regular (non-bold) type; and do not delete anything from the questions. Please contact the Campus Veterinarian, Dr. Rick Brown, (by phone, 826-3320, or e-mail, [RBrown@humboldt.edu](mailto:RBrown@humboldt.edu)) or the Chair of the IACUC, Dr. Rick Zechman (by phone, 826-3546, or by email [Rick.Zechman@humboldt.edu](mailto:Rick.Zechman@humboldt.edu)) with questions concerning protocol preparation and submission.

- ◆ Please allow ten working days for review of proposals to conduct minimally invasive procedures and an excess of one month for review of proposals to conduct invasive procedures; note that these time periods are minimal and assume that no revisions will be necessary prior to approval. ALWAYS verify approval (Office of the Chair of the IACUC; 826-3256) before starting your project. Authors of protocols should contact the Campus Veterinarian, the Chair of the IACUC or Violet McCrigler in the CNRS Dean's Office, if they haven't heard any news after 10 days following protocol submission.

**1. Faculty Project Leader:** Ethan Gahtan

**Department Affiliation:** Psychology

**2. Project Title:** Effects of an environmental xeno-estrogen, BPA, on brain aromatase expression, neural proliferation, neural activity, and behavior, in zebrafish larvae

**3. Email address(es) of the Faculty Project Leader and other corresponding applicants:**

Ethan.gahtan@humboldt.edu, rjm126@humboldt.edu, mkb16@humboldt.edu

**4. Names of others handling live animals in the absence of, or not directly supervised by, the faculty project leader, and their qualifications to perform the procedures indicated. (Do not include class rosters here - see 8 below):**

Ryan McAuly, Psychology Master's student. This is Ryan's Master's thesis project. Qualifications: course work in relevant areas of biology and behavioral science; independent study of background research literature; training by the supervisor

McKay Butler, Undergrad Biochemistry major. Qualifications: course work in relevant areas of biology; independent study of background research literature; training by the supervisor

**5. Will the described project be funded?** ☐ Yes ☒ No

**If funded, will the funds be administered by the HSU Sponsored Programs Foundation (SPF)?**

☐ Yes ☐ No

**If funded, but not administered by the HSU SPF, then list the unit that will administer the funds:**

Click or tap here to enter text.

**6. Proposed starting date (the starting date cannot precede date of approval, and all protocols must be renewed or extended annually).** The Annual Protocol Review Form must be approved on or before the anniversary of the approval date to indicate termination of the project or to request extension of the dates of approval.

Date of approval

**7. Provide a brief, non-technical, description of the project. Your response should include the proposed goals, general methods, and educational or scientific objectives that the proposed use is designed to meet.**

Estrogen is a steroid hormone found in all vertebrates. It has many biological effects including well known effects on early brain development. Some environmental chemicals, when ingested, have estrogen-mimicking effects (called xenoestrogens), and can disrupt estrogen-dependent brain development to permanently affect brain structure and behavior. Identifying and characterizing xenoestrogens has medical and public health implications.

Zebrafish larvae are a useful model for this research. They develop quickly and externally and their transparency allows estrogen signaling-related



molecules and processes to be analyzed in intact animals using microscopy and biological labeling methods. Locomotor behavior, a useful index of estrogen disruption, can also be analyzed in zebrafish. Finally, transgenic zebrafish lines are available for studying estrogen related genes and physiological systems.

The Gahtan lab currently maintains two transgenic zebrafish lines proposed for use in this study. Both lines express a fluorescent protein in a specific subset of brain cells. Cyp19alb-GFP zebrafish expresses green fluorescent protein in brain cells that express the estrogen-synthesizing enzyme, brain aromatase. Expression is limited to a subset of forebrain radial glial cells which are neural progenitor cells that generate new neurons through asymmetric cell division. Brain aromatase is activated by estrogens, including the xenoestrogen, bisphenol-A (BPA; Ref 1). Experiment 1: We propose to replicate brain aromatase activation by BPA as measured by fluorescence brightness in cyp19alb-expressing radial glial cells. To do this, larvae will be incubated in BPA or control solution from 1-7 days post fertilization, followed by microscopic imaging of cyp19alb fluorescence.

The second transgenic line, elavl3-GCaMP6s, expresses a calcium-sensitive fluorescent protein in nearly all neurons in a larva's brain. Elavl3 is neuron-specific; it's expressed only when new brain cells differentiate into neurons, so the expression level reflects the rate of neurogenesis in a developing larva. Since BPA treatment modifies radial glial cells which generate new neurons, we hypothesize that the rate of neurogenesis will be altered by BPA treatment. Experiment 2: We will count the number of elavl3-expressing forebrain cells in BPA treated and control larvae to assess BPA effects on neurogenesis. This is a theoretical replication as BPA was previously shown to increase neurogenesis in hypothalamic neurons in zebrafish larvae using a different method for quantifying neurogenesis (Ref 2).

Experiment 3: Since the GCaMP fluorescent protein expressed in these neurons is calcium sensitive (fluorescence gets brighter when calcium levels increase), we will perform time-lapse imaging of elavl3-GCaMP neurons (in the same larvae used for cell counting) to determine whether intracellular calcium dynamics (brightness fluctuations over time) are altered by BPA treatment.

It was previously shown (Ref 3) that BPA produces swimming hyperactivity in larvae. Experiment 4: We will attempt to replicate this reported BPA-induced swimming hyperactivity in order to correlate any observed changes in neural proliferation or calcium dynamics with changes in behavior.

#### References:

1. Le Fol V, Ait-Aïssa S, Sonavane M, Porcher JM, Balaguer P, Cravedi JP, Zalko D, Brion F. In vitro and in vivo estrogenic activity of BPA, BPF and BPS in zebrafish-specific assays. *Ecotoxicol Environ Saf.* 2017 Aug;142:150-156. doi: 10.1016/j.ecoenv.2017.04.009. Epub 2017 Apr 11. PubMed PMID: 28407500.
2. Kinch CD, Ibhaheziebo K, Jeong JH, Habibi HR, Kurrasch DM. Low-dose exposure to bisphenol A and replacement bisphenol S induces precocious hypothalamic neurogenesis in embryonic zebrafish. *Proc Natl Acad Sci U S A.* 2015 Feb 3;112(5):1475-80. doi: 10.1073/pnas.1417731112. Epub 2015 Jan 12. PubMed PMID: 25583509; PubMed Central PMCID: PMC4321238.

3. Saili KS, Corvi MM, Weber DN, Patel AU, Das SR, Przybyla J, Anderson KA, Tanguay RL. Neurodevelopmental low-dose bisphenol A exposure leads to early life-stage hyperactivity and learning deficits in adult zebrafish. *Toxicology*. 2012 Jan 27;291(1-3):83-92. doi: 10.1016/j.tox.2011.11.001. Epub 2011 Nov 15. PubMed PMID: 22108044.

8. Is the primary purpose of the project for ☐ instruction, ☒ research, or ☐ both?

Based on your answer, please address the relevant questions below.

**If the primary purpose is for instruction**, list the course number and write the CRN for this semester (note that this CRN will need to be updated with any future offering of the course covered by this protocol).

Course # (e.g. ZOOL 356): Click or tap here to enter text.

CRN: Click or tap here to enter text.

Will all of the enrolled students in the course denoted by the CRN above participate in the use of animals covered by this protocol? ☐ Yes ☐ No

If no, then provide a list of the students exposed to, or otherwise using, live vertebrate animals.

Click or tap here to enter text.

Describe the learning objectives that justify 1) the use of, and 2) duplication of procedures involving, live animals for instruction.

Click or tap here to enter text.

**If the primary purpose is for research**, explain how you determined that this protocol does not unnecessarily duplicate previously published observations or experiments; please include:

1. **the type of literature searches conducted:**

Pubmed.gov was used with no date limits. Google scholar, ResearchGate, and bibliographies of obtained articles, were also used to locate relevant literature. Several of the proposed experiments are replications but they are not "unnecessary" because the findings to be replicated are recent, not yet replicated by others, and have broader significance related to understanding health effects of xenoestrogens. This is a crowded field with literally hundreds of published studies on directly related topics.

2. **keywords used:**

combinations of the following search terms were used: bisphenol A, BPA, estrogen, aromatase b, neurogenesis, toxicity, cyp19a1b, zebrafish, locomotor behavior, xenoestrogen, hypothalamus

3. **range of dates searched:**

The Pubmed database searched ranged from the 1970's through the most recent 2017 publications.

4. **other resources used:**

see Q1 response

9. Will any of the animals described in this protocol be housed in an animal facility?



☒ Yes ☐ No

If yes, check the appropriate facility below:

- ☐ Biological Sciences Animal Rooms  
☐ Fish Hatchery  
☐ Samoa Aquaponics  
☐ Telonicher Marine Lab  
☐ Wildlife Pens  
☒ Zebra Fish Development Lab  
☐ Other. Please list: Click or tap here to enter text.

9a. Facility managers must be consulted prior to submitting protocol form. Please enter the date the manager was consulted: Ethan Gahtan is the manager and supervisor of this project.

10. Scientific name, common name, and characteristics of all species to be used. List species separately to explain variation in use. Please also list the total numbers of animals to be used or substantially affected by this project.

For field studies, please list all target species and note their status (not protected = NP; protected, including species of special concern or candidate species = P; considered by the state or federal government to be threatened = T, considered by the state or federal government to be endangered = E); also list non-target species that are likely to be impacted. List the range of numbers of individuals to be used for each species.

TARGET SPECIES - please attach additional pages if needed

Latin Binomial(s)	Common name(s)	Sex	Age or Wt Range	Status	Numbers
<i>Danio Rerio</i>	Zebrafish	Unspecified	Larvae up to age 10 days post fertilization	NP	504
<i>Danio Rerio</i>	Zebrafish	Select One	Adults from breeding colonies	NP	30
Click or tap here to enter text.	Click or tap here to enter text.	Select One	Click or tap here to enter text.	Select One	Click or tap here to enter text.
Click or tap here to enter text.	Click or tap here to enter text.	Select One	Click or tap here to enter text.	Select One	Click or tap here to enter text.
Click or tap here to enter text.	Click or tap here to enter text.	Select One	Click or tap here to enter text.	Select One	Click or tap here to enter text.

Click or tap here to enter text.	Click or tap here to enter text.	Select One	Click or tap here to enter text.	Select One	Click or tap here to enter text.
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**NON-TARGET SPECIES** – *please attach additional pages if needed*

Latin Binomial(s)	Common name(s)	Status	Numbers
Click or tap here to enter text.	Click or tap here to enter text.	Select One	Click or tap here to enter text.
Click or tap here to enter text.	Click or tap here to enter text.	Select One	Click or tap here to enter text.
Click or tap here to enter text.	Click or tap here to enter text.	Select One	Click or tap here to enter text.
Click or tap here to enter text.	Click or tap here to enter text.	Select One	Click or tap here to enter text.

11. Explain why a smaller number would not allow you to meet your objectives (please provide justification based on statistical or other logical reasoning). If this is a field project, and you cannot predict the exact number of animals to be sampled, please give your best estimate and an explanation of the variables that will determine your sample size. N/A is an inappropriate response unless the protocol covers only the transportation, use, and/or storage of carcasses or tissues.

The number of larvae needed was based on a statistical power analysis that is attached to this application.

12. Source of the animals (or tissues) to be used for captive studies or the location of study area(s) for field studies. For transportation, storage, and use of tissues from carcasses, explain the circumstances of death. If this information is unknown, provide the name and contact information for the person or company from which the samples are to be obtained.

Adults are maintained in the zebrafish facility in BSS 122 for which there is an IACUC approved SOP. All larvae will be generated by adult mating crosses (mating cross procedures explained in the SOP)

13. Will live vertebrate animals be maintained in captivity for greater than 12 hours?

☒ Yes ☐ No

If yes, describe where and how the animals will be housed (include all relevant husbandry details):



Please see the Zebrafish facility 'standard operating procedures' on file with the IACUC.

**Who will be responsible for their daily care?**

Ethan Gahtan, Ryan McAuley, McKay Butler

**14. List the specific procedures likely to affect the behavior, physiology or wellbeing of live animals.**

1. Larvae will be anesthetized using MS-222 before embedding in agar for microscope imaging.
  2. Larvae will be embedded in agar to constrain them during microscope imaging
  3. Larvae undergoing calcium imaging will be treated with 100 $\mu$ M d-tubocurarine in the larva's water to block neuromuscular transmission. This is necessary because calcium imaging requires the larva to be stable for up to 60 minutes and this drug safely blocks muscle contractions that could move the imaging field.
  4. Incubation in 1 $\mu$ M BPA solution from 1-7 days post fertilization in larvae in the experimental group is expected to increase the larvae's locomotor activity level
  5. Behavioral testing, as being placed individually in a new dish for behavior recording may affect the larva's behavior
- Euthanasia (see response to Q20)

**15. Mark the level of expected pain or distress caused by your methods below.**

- ☐ The methods described are purely observational and non-invasive OR will involve only the tissues or carcasses of dead animals; behavior of live animals will not be influenced intentionally.
  - ☐ The methods will affect behavior, but no animals will be captured or handled (e.g. baiting animals, cameras in close proximity to animals, production of noises within normal limits of volume and frequency)
  - ☐ The methods involve capture or handling without anesthesia, but only for a brief period for measurement or observation. No samples will be collected.
  - ☐ The methods involve capture or handling without anesthesia, and routine samples (hair, blood, etc.) will be collected or euthanasia will be performed; this may involve use of routine pharmaceuticals to promote health (e.g. antibiotics, vitamins, fluids). This work may also involve temporary marking, placement of permanent tags, or fitting with telemetry transmitters or GPS receivers.
- ☒ The methods require use of anesthesia to mitigate distress or facilitate handling, and routine samples (hair, blood, etc.) will be collected or euthanasia will be performed. As above, this work may involve temporary marking, placement of permanent tags, or fitting with telemetry transmitters or GPS receivers.
- ☐ The methods require use of anesthesia to mitigate pain or distress, and procedures will be invasive enough to require pain killing drugs (analgesics) upon revival. Sampling and marking may be performed as above.
- ☐ The methods will cause pain or considerable distress, but analgesics will not be used to mitigate the pain (e.g. surgeries from which animals are revived without provision of analgesics).



- ☐ The methods will be invasive and cause prolonged physiological or psychological stress without adequate mitigation of pain or distress. This may involve allowing animals to progress to death without provision of euthanasia or analgesia (e.g. LD50 experiments or long-term food or water deprivation).

16. Provide a complete and detailed description of all procedures to be performed involving live vertebrate animals. This response should justify comments made in # 13-15 and provide a detailed explanation of all procedures that affect animal behavior, physiology or wellbeing. Your response must address the handling and restraint of animals; deprivation of food or water; use of chemicals or biological agents; sampling methods for removal of biological samples; surgical and post-surgical procedures. N/A is an inappropriate response unless the protocol covers only the transportation, use, and/or storage of carcasses or tissues.

#### Bisphenol-A drug exposure

1. Bisphenol-A [BPA; 2,2-bis(4-hydroxyphenyl)propane] will be purchased from Sigma Chemicals
2. A 10mM (10,000x) stock solution of BPA dissolved in DMSO will be made, stored frozen, and used as needed by diluting in fish water to make fresh 1.0uM BPA solutions
3. From days 1-7 post fertilization, larvae (up to 30) in the experimental group will be raised in 100ml petri dishes containing 1.0uM BPA, and control group larvae will be raised in identical dishes containing the same concentration in of DMSO solvent with no BPA. Half of the volume (50ml) will be replaced with fresh solution daily. This exposure protocol replicates a previous published study (doi: 10.3389/fnins.2016.00112) in which the authors report no signs of harm or behavioral distress in larvae from the same BPA concentration or from a 10x higher concentration (they state: "Treated animals survived until 7-days larval stage and were identical to untreated larvae regarding morphology and motility")
4. Larvae will be removed from treatment or control solutions on day 7 and maintained in clean fish water for subsequent imaging or behavioral testing

#### Imaging procedures

- 1) Larvae will be anesthetized / paralyzed for about 2 minutes using MS-222 for agar embedding
  - a) This allows investigators to position a larva as needed on the microscope slide
  - b) A 0.01% (weight/volume) MS-222 solution will be made fresh for each experiment by dissolving MS-222 and an equal weight of sodium bicarbonate (to maintain neutral pH) in fish water. MS-222 expiration dates will be checked and each usage will be logged.
  - c) Larval fish are first transferred individually by plastic suction pipette from their home tanks to the MS-222 solution and watched closely for the onset of paralysis (usually within 30 seconds), then re-suspended for several seconds in a clean fish water solution to rinse off the MS-222 (they remain anesthetized for several minutes despite the rinse, but the rinse is done to prevent unnecessary prolonged exposure), and then pipetted onto a microscope slide for embedding



2) Agar embedding to constrain larvae during microscope imaging

- a) A drop of low melting temperature agar (1.3% weight/volume, dissolved in fish water, with a safe, neutral pH), is placed to harden on a microscope cover glass, and then a straight channel is cut into it with a razor blade
- b) An anesthetized larva is pipetted into the agar channel so the dorsal surface of the head is in contact with the glass cover slip. A moistened piece of lab tissue paper (Kimwipe) is used to gently move the larva into position and absorb excess water around it
- c) A drop of melted agar (at a safe temperature, not above 32 deg C) is dripped from a pipette onto the positioned larvae and allowed to harden, embedding the larva on the slide
- d) Once hardened, the agar is consistently perfused with fish water, allowing larvae to maintain sufficient blood oxygen through transdermal respiration
- e) After imaging larvae will be de-embedded from the agar by gently dissecting the agar away with a blunt tool under a dissecting microscope, and the freed larva will be transferred to a petri dish containing clean fish water and returned to the incubator.
- f) This is a well-established procedure which Ethan Gahtan has extensive experience using. Larvae typically can survive for many hours before de-embedding. Students will be trained by Gahtan before attempting it themselves.

3) Neuromuscular blockade with d-tubocurarine.

- a) During imaging, embedded larvae may be perfused in fish water containing 100uM d-tubocurarine, pH'd to neutral
- b) d-tubocurarine blocks acetylcholine receptors on muscle fibers
- c) The d-tubocurarine will diffuse through the agar, into the larva, and within 5 minutes will block muscle contractions that could interfere with imaging
- d) Several other studies have used this methods (including doi:10.1016/j.cub.2012.12.040, DOI:10.1523/JNEUROSCI.0880-10.2010, and DOI:10.1523/JNEUROSCI.2179-09.2009) and none have reported harm in zebrafish larvae resulting from d-tubocurarine

4) Imaging laser exposure

- a) Fluorescent imaging will require illuminating cells with a narrow 480nm laser beam that scans across the imaging field.
- b) Laser exposure will be limited to ~30 minutes of intermittent scanning per larvae, using minimum laser power required to get an adequate image
- c) At higher power and longer exposure this type of laser can damage cells but the imaging protocol proposed here will not be harmful to larvae as determined by Ethan Gahtan's experience imaging larvae with this system.

Swimming activity test:

Separate groups of BPA and control treated larvae will be individually transferred, via plastic pipette, into 10mm wells containing 4 mL of fish water. A digital camera below the wells will record swimming activity to be scored later from videos. Post recording, larvae will be pipetted



back into 60mm petri dishes containing clean fish water and housed in the incubator.

- 17. Use of animals for teaching or research requires consideration of alternative procedures to reduce the number of animals used and the pain and suffering caused by animal use. Explain how you determined whether alternative procedures were feasible for your study.**

Please refer to the Altweb website (<http://altweb.jhsph.edu/resources/searchalt/>), which provides links to search engines and provides general information on alternatives, for help in searching for alternatives to animal use.

**1. the type of literature searches conducted:**

Pubmed, google scholar, research gate, bibliographies of articles already obtained

**2. keywords used:**

combinations of: Bisphenol-A, estrogen, aromatase, cy19a1b, brain, radial glia, neurogenesis, zebrafish, development, locomotor activity, calcium, GCaMP

**3. range of dates searched:**

pubmed.gov searches go back to the early 1970's or further

**4. other resources/methods used to determine alternative procedures:**

We have reviewed the literature carefully to be certain the proposed experiments address a useful question and do not needlessly replicate established findings. We reviewed information on altweb.jhsph.edu, in considering alternatives

- 18. Describe alternative procedures that were considered and rejected and a brief explanation of why the alternative procedures were rejected. N/A is an inappropriate response unless the protocol covers only the transportation, use, and/or storage of carcasses or tissues.**

We considered reducing the number of animals required by using shared control groups but there are no duplicated control groups to eliminate. Cell counting and calcium imaging are separate measurements that will be made in the same animals, which does reduce the numbers needed.

We propose a relatively conservative statistical significance criteria, which increases the number of animals needed to achieve adequate statistical power. This was done to avoid a false positive or negative results and maximize the reproducibility of results. But we will re-consider the assumptions of this statistical power analysis and may conclude that fewer animals may be used. Therefore the proposed number of animals should be considered an upper limit. We considered ways to refine the experiment design to minimize the number of animals needed but believe our proposal is the most efficient design.

Cell culture can't be used to replace animals because the questions are about BPA effects on the development of intact brains

- 19. Identify serious human health risks (non-routine exposures to risk, disease agents, toxic chemicals, dangerous environmental conditions, etc., ) to which any participants might be exposed during the routine performance of the duties proposed herein.**

The chemicals, MS-222 and BPA could potentially be harmful to humans, although not in the amounts proposed for use in this study.

**Describe steps taken to mitigate risks.**



Investigators will always wear gloves and eye protection when handling MS-222 or BPA

20. Describe the fate of the animals upon completion of the protocol. Include (1) the procedure for euthanasia whether necessary as an experimental termination or in the case of unanticipated, accidental, injury whenever animals will be confined or handled and (2) the method of verification of death. Chemical euthanasia methods must include an appropriate, pharmaceutical-grade, drug, the route, and the dose to be used. Applicants should review the current Guidelines for Euthanasia (or its replacement in the Code of Federal Regulations), and justify any variations from the approved methods. Note that the Responsible Faculty Member must report unexpected deaths to the IACUC immediately and that N/A is an inappropriate response unless the protocol covers only the transportation, use, and/or storage of carcasses or tissues.

The following AVMA-approved methods will be used (see section S6.2.6.3 of the AVMA Guidelines for the Euthanasia of Animals: 2013 Edition): Adult fish too old for breeding, and larvae age 4 days or older will be euthanized by rapid chilling (hypothermic shock) in a 2-4 deg C ice water slurry. Steps will be taken to ensure contact of the fish's body with chilled water and not the ice itself. Loss of orientation and operculum movements should occur within 20 seconds. Adult zebrafish will be exposed for a minimum of 10 minutes and fry older than 4 days post fertilization for at least 20 minutes following loss of operculum movement. Embryos younger than 4 days old may withstand the above 1-step euthanasia procedures so a 2-step procedure is recommended. Embryos will be euthanized by rapid chilling and then as a second step to ensure death, they will be incubated in a bleach solution (sodium hypochlorite 6.15%, dissolved in water), for at least five minutes. Nervous system mechanisms for pain perception have not developed at this stage so this is not considered a painful procedure.

21. I certify by checking each of the boxes below, that all of the following are true:

- ☒ I have read and agree to abide by the "Principles for the Utilization and Care of Vertebrate Animals Used in Testing, Research, and Training at HSU," and that I will make copies of these principles and other pertinent guidelines available to those persons who work under my supervision, and that deviations from this protocol, including any unanticipated injuries or death of animals, will be reported *immediately* to the IACUC.
- ☒ My level of supervision will be such that the procedures outlined in this protocol will be carried out in a humane and a scientifically acceptable manner as described herein.
- ☒ I take responsibility for the conduct of anyone working under this approved protocol, and I will supervise the research to ensure that no work is conducted that is not covered herein or in a separate approved protocol.
- ☒ I will ensure that no work described in this protocol will begin until the protocol has been fully approved by the IACUC, and that I will adhere to all deadlines and procedure outlined in the HSU ANIMAL WELFARE ASSURANCE in accordance with the PHS Policy for Humane Care and Use of Laboratory Animals.
- ☐ I am aware that my research might require permits from federal and/or state agencies that regulate the harassment, capture, transport, captive maintenance, handling and manipulation of live vertebrate animals.
- ☒ My research will be conducted in accordance with all relevant federal and state laws.
- ☒ My study does not unnecessarily duplicate previous studies using live vertebrate animals, as determined through literature database searches.



☒ I have considered the use of less invasive procedures, use of fewer numbers of animals and have determined that the methods proposed in this protocol are justified for the research and/or instructional objectives described herein.

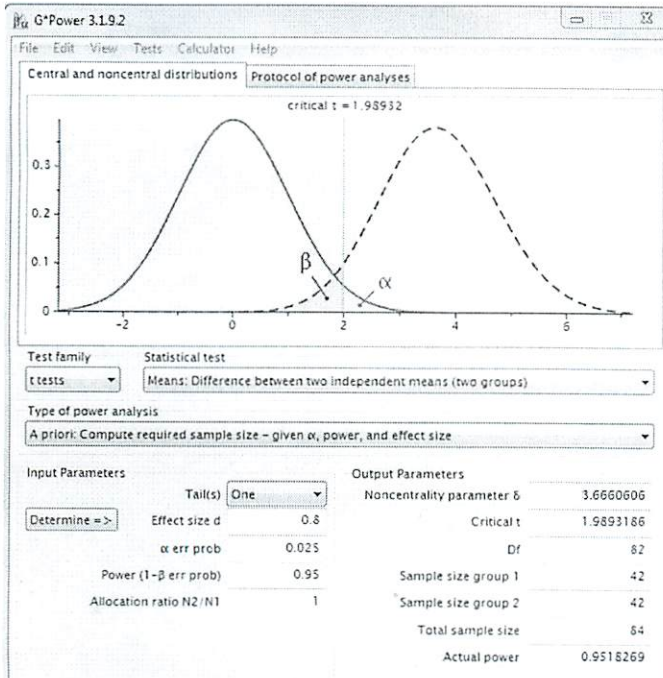
I am aware that the following Acts apply to my study (check all that may apply):

- ☐ Animal Welfare Act
- ☐ State of California Fish and Game Commission (Title 14) - Scientific Collecting Permit(s)
- ☐ Endangered Species Act
- ☐ Fishery Conservation and Management Act
- ☐ Lacey Act
- ☐ Marine Mammal Protection Act
- ☐ Convention on International Trade in Endangered Species of Wild Fauna and Flora
- ☐ **Other: please list** Click or tap here to enter text.

  
Signature, Responsible Faculty Member

10/13/17  
Date

# Addendum to IALUC titled "Effects of an environmental xeno-estrogen, BPA..."

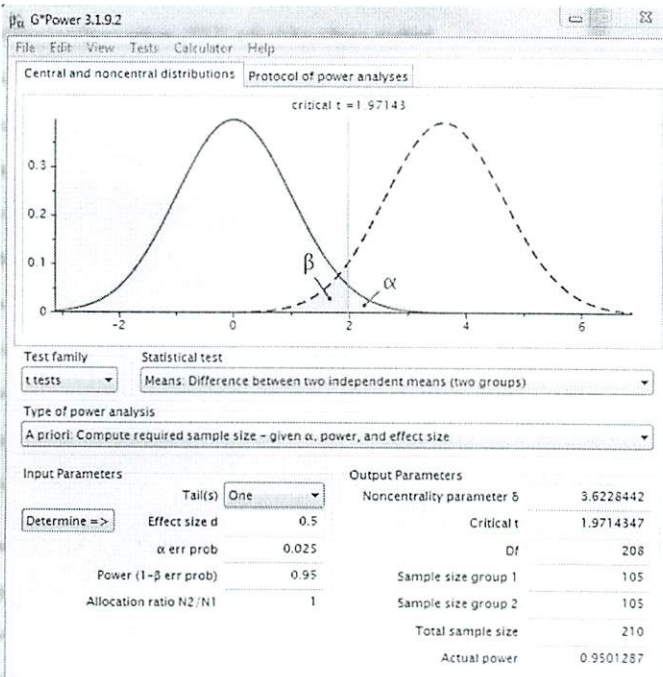


Power analyses for determining sample size requirements. All use a conservation 0.025 alpha error probably.

Power analysis for (exp 1) comparison cyp19a1b fluorescence in BPA and control

Large effect size based on large fluorescence increases with estrogen reported previously in this line. One paper suggested this was because upregulation of aromatase b results in more estrogen synthesis and more aromatase induction- a positive feedback loop

N=42 per group (BPA and control), 84 total



Power analysis for comparisons of (exp 2) cell counts in elavl3, and (exp 3) calcium imaging in elavl3. These experiments use the same larvae.

More realistic moderate effect size predicted. *Can consider changing to large effect size if only large effects are of interest to detect.*

N = 105 per group, 210 total

Same power analysis for comparison of swimming activity (exp 4) but done with different larvae.

N = 105 per group, 210 total

Total of 504 larvae to be used for experimental comparisons. Up to 20 larvae will be needed for

training and piloting procedures. So up to 524 larvae may be used. All larvae will be euthanized after completing experimental procedures, at day 6 post fertilization. *Unless we decide to continue observing behavior such as feeding or adult sexual behavior in the larvae treated for the swimming activity experiment.*

Up to 30 adults will be used to generate clutches of larvae for experiments. These adults will be maintained in the breeding colony.





Claire Roth &lt;cfr70@humboldt.edu&gt;

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**IACUC: New Protocol Arrival - 10/16/17**

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**Rick Zechman** <rick.zechman@humboldt.edu>

Wed, Oct 18, 2017 at 8:45 AM

To: IACUC HSU &lt;iacuc-hsu@humboldt.edu&gt;

The following protocols have just arrived in the office. For your review convenience, please find a scanned copy of each protocol attached to this email. If you would like to review one or more of these protocols but have not been asked to do so, please contact Claire Roth. A summary of each protocol is described below.

Thank you!

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Title: Effects of an environmental xeno-estrogen, BPA, on brain aromatase expression, neural proliferation, neural activity, and behavior, in zebrafish larvae

No: 17/18.P.34-A

PI: Ethan Gahtan

Grant Funded: No

Summary: Using zebrafish larvae as a model for research on how environmental chemicals, when ingested, have estrogen-mimicking effects and can disrupt brain development to permanently affect brain structure and behavior. The larvae are useful models for this research because they develop quickly and externally and their transparency allows estrogen signaling-related molecules and processes to be analyzed in intact animals using microscopy and biological labeling methods.

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Title: Aposematic flash coloring in Pacific chorus tree frogs (*Pseudacris sierra*) populations in Humboldt County

No: 17/18.W.35-A

PI: Aletris Neils

Grant Funded: No

Summary: Quantifying the presence of a brilliant yellow coloring on the inside thighs of the Pacific chorus tree frog, only revealed when the frog is fleeing in order to confuse a predator. No peer reviewed literature describes this in any account of the old *Pseudacris regilla*, any of its subspecies, or the three newly proposed species; this study would be the first to record the presence of these markings in *Pseudacris regilla*. The markings will be measured through digitally photographing frogs in the field. The frogs will be caught, placed in a box with a clear bottom, and photographed. The weight, sex, and length of the frog will be recorded. The frog will then be released at the site of capture after no more than ten minutes of handling.

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Claire Francis Roth

Front Desk - Humboldt State University College of Natural Resources & Sciences

Volunteer - Northcoast Environmental Center's EcoNews

HSU Class of 2017 - Environmental Studies - Media Production Focus



Please consider the environment before printing this email.

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**2 attachments****17-18.W.35-.pdf**

3436K

**17-18.P.34-.pdf**

4714K



Claire Roth &lt;cfr70@humboldt.edu&gt;

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**IACUC: New Protocol no. 17/18.P.34-A**

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Margaret Wilzbach <paw7002@humboldt.edu>  
To: Richard N Brown <richard.brown@humboldt.edu>  
Cc: Claire Roth <Claire.Roth@humboldt.edu>

Mon, Oct 23, 2017 at 1:09 PM

Hi Rick: yes, I'm happy to sign off without need for revision if Ethan corrects Q.15 in person.

Claire: please sign off on this protocol in my name once Ethan corrects his answer to Q. Q.15. Thanks.

Peggy

Sent from my iPhone

On Oct 23, 2017, at 12:55 PM, Richard N Brown <richard.brown@humboldt.edu> wrote:

Hi Peggy,

They are using anesthesia, and I agree with your comments. Would you be able to sign it if we ask him to come in and check the correct box (rather than asking him to submit a revision)? If so, I can sign it today. If not, then let me know and I'll send him a note asking for the revision.

Best Regards,  
Rick

.....  
Richard N. Brown (Rick), Department of Wildlife, 1 Harpst Street, Humboldt State University, Arcata, CA 95521.  
Office, west side of the Game Pens (Building 34); Phone, (707) 826-3320; Fax, (707) 826-4060.

On Thu, Oct 19, 2017 at 4:42 PM, Peggy Wilzbach <wilzbach@humboldt.edu> wrote:  
[Quoted text hidden]



## Routing Slip for IACUC Protocol Reviews