

Evaluation of Euthanasia Techniques for an Invertebrate Species, Land Snails (*Succinea putris*)

Cody R Gilbertson¹ and Jeffrey D Wyatt^{2,*}

The euthanasia of invertebrates used in scientific investigations poses unanswered questions regarding the rapid induction of unconsciousness with minimal distress and pain. Relative to vertebrates, invertebrates' sensory experience of pain, nociception, and physiologic response to aversive stimuli are poorly characterized. The scientific communities in the European Union, Canada, United States, Australia, and New Zealand join in consensus regarding the need to address alleviation of pain and distress in cephalopods (octopus, squid, and so forth), which have the best-characterized nervous system among invertebrates. In the current study, we evaluated various euthanasia techniques in a terrestrial gastropod species, with priority on animal wellbeing, scientific variability, feasibility in both field and laboratory settings, and acceptability by personnel. In addition, we demonstrated that the 2-step method of euthanasia described in the *AVMA Guidelines* as acceptable for aquatic invertebrates is effective for terrestrial snails and meets all welfare and scientific requirements. This 2-step method first induces anesthesia by immersion in 5% ethanol (laboratory-grade ethanol or beer) followed by immersion in a euthanizing and tissue-preserving solution of 70% to 95% ethanol or 10% neutral buffered formalin. Furthermore, alternative methods of euthanasia for terrestrial snails commonly used in field research, such as live immersion in concentrated ethanol or formalin, were shown to be unacceptable.

Abbreviation: RO, reverse-osmosis-purified

As expense, space, conservation, and humane treatment issues involved with keeping large vertebrates continue to increase, invertebrate animals are receiving increasing consideration by research and educational facilities as animal subjects for scientific investigation. The space and resources needed for keeping invertebrates are generally low,³³ making invertebrates good candidates for in situ and ex situ research, educational, and conservation efforts.

Few published practices for euthanizing invertebrates in the wild or captivity are available, most likely due to lack of scientific assessment of invertebrates' potential to experience pain or distress. Therefore, humane practices and animal wellbeing are often overlooked regarding invertebrate species.^{9–12,17,18,43} Human bias toward larger animals¹³ and more charismatic vertebrate groups, such as birds, mammals, and fish,¹⁵ may also influence the lack of attention to humane practices for anesthetizing or euthanizing captive invertebrates. Furthermore, measuring pain and stress in invertebrates is difficult simply because of our lack of knowledge regarding their behavior, nervous system, and nociception.

An increasing diversity of invertebrate species, including snails, are being studied in situ or housed at academic research centers and zoos for scientific investigative or conservation programs. Wild populations of apple snails (*Ampullariidae*) have proven useful as bioindicators of environmental polychlorinated biphenyls and polybrominated diphenyl ethers, with elevated tissue levels correlated with soil and water contamination.¹⁹ Laboratory-housed freshwater snails (*Marisa cornuarietis*) are

now considered as environmentally relevant bioindicators of endocrine disruptive (xenoestrogen) chemicals such as bisphenol A and octylphenol.³⁰ In addition, understanding population health in wild or captive colonies of snails enrolled in conservation-based scientific studies aids malachologists and zoologists to evaluate health trends, contributors to morbidity and mortality, and preventive and therapeutic opportunities as well as risk assessment for captive-bred animals considered for repatriation to restored habitat.¹⁴ All of these programs require consideration of euthanasia techniques.

Best practices in euthanasia have expanded across all taxa in great part due to the 2013 *AVMA Guidelines*,³ which now include wildlife, research, zoo, agricultural, and companion animals. Acceptable 2-step euthanasia methods—with anesthesia followed by euthanasia—for aquatic invertebrates are described in the updated guidelines. Only conditionally acceptable methods involving injectable and inhalant agents or physical and chemical methods are described for terrestrial invertebrates. The guidelines acknowledge that euthanasia is a process that considers the feelings of the person performing or witnessing the technique as well as the wellbeing, pain, and distress of the animal being euthanized and the potential for artifacts when unaltered cytoarchitecture is scientifically required. However, little is known about the anatomic sequelae of techniques for euthanizing terrestrial or aquatic invertebrates, especially when artifact-free tissue preservation is necessary.

For example, the endangered Chittenango ovate amber snail (*Novisuccinea chittenangoensis*, family Succineidae) is being studied in its only known home in the wild (Chittenango Falls, NY) and is housed in a research laboratory for the purpose of developing husbandry techniques as part of the United States Fish and Wildlife Service recovery plan.⁴¹ The partula tree snail species *Partula nodosa*, which is extinct in the wild, is another example where decades of successful captive propagation and

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¹Department of Environmental and Forest Biology, College of Environmental Science and Forestry, State University of New York, Syracuse, New York, and ²Department of Comparative Medicine, University of Rochester, Rochester, New York, and Department of Wildlife Health and Conservation, Seneca Park Zoo, Rochester, New York.

*Corresponding author. Email: Jeff_Wyatt@URMC.Rochester.edu

selected habitat restoration provided an opportunity for reintroduction in the wild. Captive practices have kept *P. nodosa* species thriving, with the ultimate goal for release back to their original habitat in Tahiti. Comprehensive health assessments of captive-bred Chittenango ovate amber snails and Polynesian tree snails require a feasible, effective, and artifact-free method of euthanasia for tissue processing for prerepatriation health assessment as well as postrelease monitoring in upstate New York and Tahiti, respectively. Conservation programs have been developed for *Partula* species^{28,32,40} and are under development for Chittenango ovate amber snails. To release captive-bred animals, the viral, bacterial, fungal, and helminth disease and environmental disorders (for example, nutritional, chemical contaminant) in captive and wild populations must be characterized and monitored closely so that transmission to wild or 'spill over' at-risk species is minimized and that environmental contributors to morbidity and mortality are identified.

Using another terrestrial but common and invasive European succineid snail (*Succinea putris* Linnaeus, 1758) to answer the research question of best and most feasible euthanasia technique for endangered Chittenango ovate amber snail and extinct *Partula nodosa* tree snails may be particularly useful. According to nucleic acid sequencing, species B (our study snail) was most closely identified with and differs slightly from (that is, 1.8%) *S. putris*.⁸ For identification of our study species, this level of difference is within the range of intraspecific changes in Succineidae.³⁵ In addition, similar to the Chittenango and partulid snails, *Succinea putris* lacks an operculum covering over the opening to the shell. Sharing this anatomic characteristic across all 3 terrestrial snail species supports the selection of *S. putris* as our clinical translational model.

In several recently published laboratory research studies, the euthanasia of gastropods was accomplished through diverse methods including freezing,⁴⁴ live dissection,^{16,31} and lidocaine gel for anesthesia before dissection.³⁴ Another study did not state exact processes used for euthanasia, because of a waiver of an animal ethic statement or formal approval for research on gastropods (that is, it was not required).⁷ Regardless, dropping mollusks directly into formalin or 95% ethanol is commonly practiced and is an endorsed technique by museums for preservation. The Bishop Museum and University of Hawaii's preservation of specific taxa states that preserving mollusks directly into formalin can help relax specimens and is a simple accepted technique.⁶ The 2010 US Forest Service Region 6 and Oregon-Washington US Bureau of Land Management Interagency Special Status-Sensitive Species Program (ISSSSP) mandatory guidelines for collecting, processing, and shipping mollusk voucher specimens state that preserving mollusks directly in 95% ethanol "will provide a greater chance of future successful molecular analysis."⁴² Both of these methods are accepted for euthanasia and preservation of terrestrial gastropods.

Identifying the euthanasia method that is humane, least aversive for the animal and personnel, and best for tissue preservation for diagnostic and scientific purposes will enhance the science behind snail conservation and research. In the current study, we assessed the aversive behaviors and tissue effects in the pulmonate land snail *S. putris* due to various euthanasia solutions of ethanol. For captive or wild population health surveillance purposes (that is, undamaged preserved specimens), the goals of this study were to (1) maintain cellular structure and minimize artifacts (dermal irritation, hemorrhage, edema and so forth) during euthanasia to reduce scientific variability, (2) assess snail wellbeing through behavioral observations, and (3)

identify a technique that does not require chemicals that might be controlled or unavailable in field and international settings.

Materials and Methods

All research and housing activities were approved by the Seneca Park Zoo Research Committee, which promotes the humane care and use of animals in science according to the ILAR *Guide for the Care and Use of Laboratory Animals*.²⁵

Subjects. *Succinea putris*, a terrestrial, invasive European snail species also known as 'species B' in a New York State and Federal recovery plan⁴¹ for the endangered Chittenango ovate amber snail *N. chittenangoensis*, were collected from Chittenango Falls State Park (Madison County, NY; latitude, 75° 50' 30" W; longitude, 42° 58' 45" N). Mature snails measuring more than 9 mm in shell length were collected from a variety of vegetation within the riparian zone downstream of the park's waterfall. Snails ($n = 63$) were transported in food-grade plastic sandwich containers in a climate-controlled (70 to 75 °F [21 to 24 °C]) vehicle to the Conservation Science Center of the Seneca Park Zoo (Rochester, NY), which is accredited by the Association of Zoos and Aquariums. Snails were acclimated at 70 to 72 °F (21.1 to 22.2 °C) for 48 h in their transport containers with access to fresh romaine lettuce rinsed in reverse-osmosis-purified (RO) water. All research activities were approved by the Seneca Park Zoo Research Committee.

Euthanasia solutions. Each of 5 groups of snails ($n = 5$ per group) was exposed at 72 °F (22.2 °C) to 1 of 5 solutions. Three solutions of ethanol (5%, 70%, and 95%) were made by diluting 95% laboratory-grade ethanol in RO water as needed. The fourth ethanol-based solution was 4.74% alcohol by volume and was undiluted, uncarbonated (that is, flat) beer (Pabst Brewing, Los Angeles, CA). The fifth solution, RO water, served as a control.

Euthanasia techniques. Each cohort of 5 snails was gently placed as a group into 1 of the 5 solutions (300 mL solution in a 400-mL Pyrex laboratory beaker). The time required for all 5 snails to stop all movement was recorded. After 30 additional minutes, all snails were removed from the solution, rinsed with RO water, evaluated for reaction to a 25-gauge hypodermic needle gently scraped across and inserted 1 to 2 mm into the foot, and then placed in a culture dish for 2 h of observation for potential recovery. As a control, 2 unanesthetized snails each were evaluated for behavioral reaction to a 25-gauge needle scraped across or inserted into the foot.

Behavior. Behavior was assessed by observing snails during immersion in 4 ethanol-based euthanasia solutions and the control (RO water) solution. Reactions including the production of bubbles, body retraction, defecation, and expulsion of mucus were characterized as potential behavioral indications of aversion, discomfort, distress, or pain.

Histopathology. Two additional snails each were immersed into 5%, 70%, or 95% laboratory-grade ethanol or into beer (4.7% ethyl alcohol by volume) until completely immobilized and immediately placed in 10% neutral buffered formalin for fresh fixation for histopathology. Baseline health assessments and potential indicators (cell lysis or membrane disruption, hemorrhage, edema, mucus, and so forth) of a potentially painful irritant or unintended tissue artifact related to method of euthanasia were evaluated through histopathologic analyses by board-certified pathologists (Northwest ZooPath, Monroe, WA).

Results

Behavior (Figure 1). Response to various euthanasia solutions. Group 1 snails were placed in RO water. Three of the 5 snails immediately sank to the bottom; the remaining 2 floated. There

Group	Solution	Reaction
1	Reverse-osmosis-purified water	Normal behavior: Snails climbed out and moved around regularly; no retraction of tentacles or body; no defecation; no bubbles or mucus on contact.
2	5% ethanol	Normal behavior until immobile: No retraction of tentacles or body; no defecation; no bubbles or mucus on contact.
3	4.74% decarbonated beer	Normal behavior until immobile: Snails moved about regularly; no retraction, defecation, bubbles, or mucus.
4	70% ethanol	Aversive behavior until death: Retracted into shell; immediately expelled feces; produced mucus and bubbles on contact.
5	95% ethanol	Aversive behavior until death: Retracted into shell; immediately expelled feces; produced mucus and bubbles on contact.

Figure 1. Summary of reactions of *Succinea putris* snails ($n = 5$ per group) in 5 solutions.

was no retraction of the body into the shell; neither air bubbles nor feces were excreted nor mucus discharged. All 5 snails slowly climbed out of the solution within 13 min of immersion.

Group 2 snails were placed in 5% ethanol. Three of the 5 snails immediately floated, and the remaining 2 snails sank. There was no body retraction, gas bubbles expelled, fecal excretion, or mucus production. All 5 snails moved their bodies slowly without apparent purposeful direction for 10 min, with no attempt to climb out of the beaker. When no movement was observed after 30 min in all snails, they were removed from the solution, rinsed in RO water, and placed in a culture dish containing 3 mm of RO water to prevent desiccation. None of the snails reacted to the scrape of a 25-gauge hypodermic needle across or its insertion into the foot. All 5 snails slowly recovered over a 2-h period and climbed out of the culture dish.

Snails in group 3 were placed in beer. Three of the 5 snails immediately sank, and the remaining 2 floated. There was no body retraction, gas bubble or fecal excretion, or mucus production. All 5 snails slowly moved their bodies in an uncoordinated manner for as long as 10 min. After 30 min of inactivity, the snails were removed from the solution, rinsed in RO water, and placed in a culture dish flooded with 3 mm of RO water to prevent desiccation. None of the 5 snails showed any reaction to the scrape of a 25-gauge hypodermic needle across or its insertion into the foot. All snails recovered within 2 h and climbed out of the culture dish.

Group 4 snails were placed in 70% ethanol. All 5 immediately retracted, secreted cloudy white mucus and gas bubbles for 2 min, and sank within 30 s of contact with the solution; 3 of the 5 snails defecated immediately. After 30 min of inactivity, all 5 snails were rinsed with RO water and placed in a culture dish flooded with 3 mm of RO water to prevent desiccation. The snails did not respond to a scrape by or insertion of a 25-gauge needle. The snails did not recover by 2 h; after 24 h, they were confirmed dead due to no reaction to dissection from their shells. The body tissue was firmly fixed.

Group 5 snails were placed in 95% ethanol and exhibited the same characteristics as group 4. The snails retracted immediately, expelled bubbles, secreted cloudy white mucus, and sank within 5 s of contact with the solution; 3 of the 5 snails defecated immediately. When the snails were removed from the solution after 10 min and rinsed with RO water, they remained motionless; there was no recovery over 2 h, and death confirmed at 24 h in light of dissection from shells. The body tissue was firmly fixed.

Formalin fixation of snails. The additional snails ($n = 2$ each) immersed in 5% ethanol solutions (laboratory-grade or as beer) remained in relaxed body position, with no reaction or change in body posture when placed in 10% neutral buffered formalin. The snails immersed in 70% and 95% ethanol remained deeply retracted, with no motion or reaction when placed in 10% neutral buffered formalin.

Response to needle stimulus in unanesthetized, control snails.

The 2 unanesthetized snails retracted the foot when a 25-gauge needle was gently scraped across it. The same reaction occurred in 2 additional snails after a 25-gauge needle was inserted 1 to 2 mm into the foot.

Pathology. Gross observations. Gross examination documented that the bodies of snails first placed in 5% ethanol solution (laboratory grade or as beer) and subsequently immersed in 10% neutral buffered formalin were relaxed but not prolapsed. The bodies of the snails first placed in 70% and 95% ethanol remained deeply retracted when placed in formalin, with a cloudy white mucus layer at the shell opening. Tissue quality was firm, rigid, and appeared fixed.

Histopathology. Microscopic examination of tissues across all groups of euthanized snails including epidermis, dermis, digestive and reproductive systems identified no evidence of disease, mucus secretion, or cytoarchitectural disruption or artifact. All groups were preserved equally well in formalin for histopathologic evaluation.

Discussion

Ideally, euthanasia methods for animals enrolled in scientific studies should be standardized across institutions to facilitate comparison of results, be feasible, and easily trained across research staff and programs globally. Euthanasia methods should not introduce variables compromising scientific interpretation, should be acceptable to personnel, and most importantly should be humane.

Emerging scrutiny on the pain and distress of all animals used as subjects of scientific investigation requires a more precautionary approach, especially when interpreting invertebrate animal reactions during euthanasia. *ILAR Guide for the Care and Use of Laboratory Animals* acknowledges that the established principles to promote the responsible, humane, and ethical care and use of vertebrate animals in research may provide guidance for invertebrates as well.³⁷ AAALAC endorses flexibility in the review of mission-driven research using invertebrates, with an expectation that the IACUC, ethics committee, or oversight body will consider the species of invertebrate used and invasiveness of the research when providing guidance to researchers (www.aaalac.org/accreditation/faq_landing.cfm#A2). The European Directive, Canadian Council on Animal Care, Australian Code for the care and use of animals for scientific purposes (8th edition), and the New Zealand Animal Welfare Act all promote regulation or oversight of scientific investigations of cephalopod and other invertebrates.^{1,2,29,38} Under these oversight requirements and the conditions of accreditation or funding, the alleviation of pain and distress of select invertebrates must be addressed for experimental procedures such as surgery and euthanasia.

The *AVMA Guidelines on Euthanasia* are no longer silent regarding the euthanasia of aquatic or terrestrial invertebrates.⁴ A 2-step method where the aquatic invertebrate is first anes-

thetized by immersion in an accepted anesthetic followed by a secondary confirmatory method is consistent with techniques recommended for vertebrate species. Anesthetic overdosing with magnesium salts, clove oil, eugenol, or ethanol (1% to 2%) is recommended for aquatic invertebrates, with a cautionary note that ethanol produces an initial excitement or aversion phase in cephalopods. The immersion of conscious animals in 70% ethanol or 10% neutral buffered formalin is not acceptable and is reserved for secondary confirmation of death in an anesthetized aquatic invertebrate with added benefit of tissue fixation. However, this one-step immersion technique is commonly taught and used in field situations for terrestrial and aquatic invertebrates.^{5,42}

Like vertebrates, invertebrates sense noxious or aversive stimuli, and a known reaction to these (that is, noxious chemicals, electric shock, temperature changes beyond normal range) is withdrawing (or retracting, in the case of succineids).³⁷ Gray garden slugs (*Derocerus reticulatum*) show mucus that is clear and low in viscosity normally, but when the animal is disturbed, the mucus is thick and white or, in the case of land slugs (*Arion subfuscus*), the mucus is yellow.³⁹ Mucus production in abalone (*Haliotis iris*) is considered a sign of major excitation during induction of anesthesia.⁵ However, it is difficult to tell whether the bubbles or opaque mucus expelled by species B (*S. putris*), the subject of the current study, is a strong indicator of pain compared with a triggered defense mechanism to aversive stimuli, thus introducing a level of uncertainty here. Previous studies on mollusks and pain evaluated the presence of nociceptors in the animal studied. Nociceptors are defined as high-threshold sensory receptors of the peripheral somatosensory nervous system that are capable of transducing and encoding noxious stimuli, according to the International Association for the Study of Pain.²³ From a phylogenetic perspective, many mollusks are known to have nociceptors. The land snail *Cepaea nemoralis*,²⁵ the sea slug *Tritonia diomedea*,²⁰ and the mollusk *Aplysia californica*²⁴ all show behavioral and neurophysiologic evidence of nociceptor presence. The lack of scientific data regarding invertebrates' ability to distinguish between the sensory experience of pain and simply nociception, a physiologic response to aversive or painful stimuli, complicates the interpretation of behaviors of invertebrates during euthanasia.

Defecation is an indicator of stress in many vertebrate animals. Examples include anxiety in mice,^{27,36} but it is difficult to tell whether defecation is a result of stress, anxiety, or pain in invertebrates. Evoked defecation and regurgitation or excitement in vertebrates experiencing distress or anxiety, especially during euthanasia may not be relevant when seen in invertebrates. However, in the absence of scientific data, a precautionary approach seems prudent.³⁸

Another study promoting the immersion of garden snails, *Cryptomphallus aspera*, in an anesthetic concentration of 5% ethanol to provide a smooth induction followed by a terminal procedure such as immersion in 95% ethanol, formaldehyde, or the physical destruction of the nervous system^{22,42} supports findings of the current study. The immediate retraction of all of the snails' bodies into their shell and the excretion of feces, bubbles and cloudy mucus after being immersed in high concentrations of ethanol (70% and 95%) suggest an aversive reaction and inadequate depth of anesthesia for surgery.⁴² In stark contrast, our succineid snails that were immersed in the lower concentration (4.7% to 5%) of ethanol demonstrated no aversive behaviors and became anesthetized, as shown by the lack of a withdrawal response to a painful stimulus (hypodermic needle scrape and insertion into their foot); similar procedures

and responses have been described regarding pond snails (*Lymnaea stagnalis*).⁴²

Whereas laboratory-grade 5% ethanol may not be readily available, especially when conducting field work, a globally available alternative, beer, was shown in our current study to be equally effective as an anesthetic before confirmatory euthanasia by immersion in 70% ethanol or 10% neutral buffered formalin. Another commercially available alternative to laboratory-grade ethanol, a 10% solution of Listerine (21.9% ethanol; Johnson and Johnson, New Brunswick, NJ) has been reported to anesthetize snails effectively,^{22,42} but their behavior at induction was not described.

In addition to animal welfare considerations, growing emphasis is placed on the psychologic and emotional welfare of personnel performing or witnessing euthanasia. A distressful appearing end-of-life animal experience or even a series of uneventful euthanasias may, over time, negatively affect personnel, leading to undesirable coping mechanisms, compassion fatigue, and job dissatisfaction.³ Attention to staff interpretations and impressions of snails' behavior during euthanasia is important to consider, especially when aversive reactions (for example, body withdrawal, defecation, bubble and mucus production) are observed.

The least invasive method of euthanasia from our behavioral observations resulted from submerging terrestrial snails in a solution of 4.7% to 5% ethanol at room temperature for 10 min. This practice effectively anesthetized the animal; subsequent immersion in 10% neutral buffered formalin or 70% to 95% ethanol resulted in tissue preservation and euthanasia. Anesthetizing animals before their euthanasia eliminates personnel concern regarding unnecessary animal pain or distress. Preserving research subjects in their unretracted state promotes the anatomic investigation of specimens that are in a relaxed position, reducing confounding interpretation from snail to snail. Although our histopathologic assessment revealed no cytoarchitectural artifacts regarding any of the methods of euthanasia used, the relaxed bodies of the snails first anesthetized in 4.7% to 5% ethanol suggests a less aversive or potentially distressful experience compared with immediate retraction inside shells.

Recognizing that aversive reactions in snails and higher invertebrates might be either simple reflexes or behavioral indications of something more distressful or painful, we conclude that until scientific data support one interpretation or the other, the 2-step method of euthanasia recommended for terrestrial snails that we used in the current study presents no scientific disadvantages, errs on the side of animal wellbeing, is readily available globally, and causes no aversive behaviors in the subjects being euthanized.

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