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Freezing Reptiles and Amphibians In  
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# Anesthesia and Euthanasia of Amphibians and Reptiles Used in Scientific Research: Should Hypothermia and Freezing Be Prohibited?

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*Current research guidelines for ectothermic tetrapod vertebrates prohibit the use of cold as an adjunct to gaseous anesthesia, and they prohibit freezing as a means of euthanasia of these same animals. Here, we argue that those guidelines merit re-evaluation. Under natural conditions, numerous amphibians and reptiles experience large variations of body temperature, and life at low temperatures is natural. In tropical species less tolerant of cold, nociception is likely to be extinguished at low temperatures because of the anesthetizing actions of cold on membranes and cold block of nerve conduction. Physical principles and physiological data suggest that smaller ectothermic vertebrates do not experience pain attributable to ice crystals that form during freezing. Therefore, whole-body cooling, followed by freezing, should be a humane form of euthanasia for numerous smaller ectothermic species. In addition, we believe that cooling offers a humane and useful means of supplementing currently acceptable methods of anesthesia.*

*Keywords:* euthanasia, freezing, amphibians, reptiles, animal ethics guidelines

**W**hereas low temperatures and freezing were formerly accepted means of anesthesia and euthanasia for ectothermic tetrapods used in scientific research, Institutional Animal Care and Use Committees (IACUCs) and Ethics Committees (ECs) around the world have recently adopted guidelines that, with few exceptions, ban such practices. Opinions vary concerning the humane uses and efficacy of cold as a supplement to conventional methods of anesthesia, or of hypothermia followed by freezing for euthanasia, and there has been little discussion of the scientific evidence underlying current guidelines. With regard to amphibians and reptiles, we believe that current guidelines are based on a mammalian-biased perspective and need to be reevaluated. We believe there is a widespread lack of understanding of the natural history, physiological diversity, and evolutionary adaptations of these ectothermic vertebrates. Therefore, we hope this article will promote a better understanding of the variable and unique physiological

attributes of these animals that should be considered by the authorities responsible for creating and implementing guidelines for choices of anesthesia and euthanasia for amphibians and reptiles used in research.

## Background and perspective

Anthropogenic and climate change-induced extirpations and extinctions of wildlife currently present massive challenges to society and to the capacity of scientists to investigate, understand, and mitigate these phenomena. Biological research produces benefits for humanity and biodiversity alike. Whereas approvals for research by members of ECs (hereafter to include IACUCs and ECs) have improved the overall well-being of animals used in research, disagreements sometimes arise between ECs and investigators concerning appropriate humane practices. Here, we discuss one such area of controversy. Our aim is to promote better understanding that could lead to further discussion and

improve relations between ECs and investigators working with ectothermic tetrapods: amphibians and reptiles.

The use of cold and freezing for anesthesia or euthanasia of ectothermic tetrapods used in scientific research is controversial. In recent years it has been a source of multiple disagreements between ECs and investigators (e.g., Shine et al. 2015). Previously, for more than a century, low temperatures were used as an acceptable method of anesthesia of ectothermic vertebrates, and freezing formerly was considered an acceptable and humane method for euthanasia (Blair 1971). Although various opinions are held regarding advantages or disadvantages of using cold as a means of anesthetizing or euthanizing ectothermic vertebrates, these procedures are now considered unacceptable and are prohibited by many ECs worldwide. Possibly the earliest formal recommendation against the use of cold and freezing as a means of restraint or euthanasia of amphibians and reptiles is the report of a joint Universities Federation for Animal Welfare–World Society for the Protection of Animals Working Party that stated, “freezing is generally contraindicated since the formation of ice crystals on the skin and in the tissues of an animal is likely to cause pain or distress” (Cooper JE et al. 1989). Numerous Internet-based sources of advice concerning euthanasia of amphibians or reptiles repeat the statement that formation of ice crystals causes pain and distress. Unfortunately, these sources do not provide authoritative references, and no such source has cited research that indicates this is actually true. Indeed, it is physically impossible for nerve conduction of pain signals to occur when freezing blocks diffusion of ions across membrane channels. Nevertheless, regulatory guidelines banning the use of hypothermic anesthesia in ectotherms were given further legitimacy by the American Veterinary Medical Association (AVMA) and Institute for Laboratory Animal Research (ILAR) in the early 1990s, although various guidelines were not well referenced and were sometimes contradictory (Martin 1995). Here, we set forth reasons why we believe the justifications behind current guidelines (and likely the guidelines themselves) need to be reevaluated.

#### Concerns Regarding Current AVMA Guidelines for the Euthanasia of Amphibians and Reptiles

The following statements are taken from the AVMA Guidelines for Euthanasia of Animals, 2013 edition, section S7.3 Captive Amphibians and Reptiles, S7.3.7 Unacceptable Methods:

*Hypothermia*—Hypothermia is an inappropriate method of restraint or euthanasia for amphibians and reptiles unless animals are sufficiently small (<4 g) to permit immediate and irreversible death if placed in liquid N<sub>2</sub> (rapid freezing). Hypothermia reduces amphibians’ tolerance for noxious stimuli and there is no evidence that it is clinically efficacious for euthanasia. In addition, it is believed that freezing can result in the formation of ice crystals in tissues that

may cause pain. Consequently, because amphibians and reptiles lack behavioral or physiologic means of demonstrating pain or distress while hypothermic, generalized prohibitions on hypothermia for restraint or euthanasia are appropriate. Localized cooling in frogs may reduce nociception, but this localized effect is not appropriately applied to the whole body as a part of euthanasia procedures. Freezing of deeply anesthetized animals may be justified under circumstances where human safety could be compromised.

Our understanding of amphibians’ and reptiles’ nociception and responses to stimuli is incomplete; therefore, many recommendations for minimizing pain and distress are extrapolated from information available about mammals. Where uncertainty exists, erring to proactively alleviate potential pain and suffering is recommended as an appropriate approach to euthanizing amphibians and reptiles.

Here, we comment on the aspects of these guidelines that we think merit review and reconsideration by the scientific and veterinary community.

We agree that hypothermia by itself is inappropriate for the restraint and anesthesia of amphibians and reptiles, but we believe it does have value as an adjunct to currently acceptable methods (see below). On the basis of rodent models, the guidelines indicate that “rapid freezing” is acceptable euthanasia provided that the animal is very small (less than 4 grams) and is immersed in liquid nitrogen. This must produce “immediate and irreversible death.” However, if entire animals are cooled to the freezing point of body fluids, ice crystals rapidly “seed” the body water, and freezing likely occurs rapidly in small animals (Cziko et al. 2006, but see Claussen and Costanzo 1990 and below). If this is the case, according to the guidelines, the cooling and freezing of small amphibians and reptiles should be an acceptable means of euthanasia. We do not propose that freezing is an appropriate method of euthanasia for larger species such as crocodilians, pythons, or many turtles, but animals that might be euthanized by a procedure of cooling and freezing could be considerably larger (up to several kilograms) than those currently recommended for immersion in liquid nitrogen.

The statement that hypothermia “reduces amphibians’ tolerance for noxious stimuli” is not strictly correct with respect to the literature cited to justify the statement (Stevens and Pezalla 1989, Machin 1999). These references discuss a decrease in nociceptive *threshold* in summer frogs that are “adapted” to cold (4 degrees Celsius, °C) and thus address sensitivity and not tolerance to pain. Moreover, if this statement were correct, it would contradict two statements that appear further on in the same paragraph, which read, “amphibians and reptiles lack behavioral or physiologic means of demonstrating pain or distress while hypothermic,” and “localized cooling in frogs may reduce nociception.” Most importantly, the “noxious stimuli” in these studies

refer to acetic acid or other forms of insult, but not cold. The cited papers are not relevant to whole-body thermal pain or hypothermia *per se*. This distinction is centrally important and is discussed further below.

The guideline's prohibition of freezing as a means of euthanasia is based largely on the widely held "belief" that freezing causes the formation of ice crystals in tissues and that these cause pain. The references cited by the AVMA, J. E. Cooper and colleagues 1989 and Close and colleagues 1997, appear to be speculative and without empirical evidence. Pain is physically impossible upon freezing, and to our knowledge, no one has demonstrated that ice crystals cause pain in amphibians or reptiles. This is discussed in greater detail below.

An important statement in the quoted guidelines is the following: "because amphibians and reptiles lack behavioral or physiologic means of demonstrating pain or distress while hypothermic, generalized prohibitions on hypothermia for restraint or euthanasia are appropriate." The justification for this statement is not clear, and it represents only an opinion. Furthermore, the statement is not correct because nociception has, in fact, been demonstrated in frogs at low temperatures (Stevens and Pezalla 1989, Machin 1999). If the statement were correct, it would (again) contradict the previous statements claiming that hypothermia "reduces amphibians' tolerance for noxious stimuli." Invoking parsimony, we believe that amphibians and reptiles simply do not experience thermal pain or distress from cold while they are hypothermic near freezing, and it is less likely that under these conditions they lack the physiological or behavioral ability to demonstrate pain and distress (according to the statement).

Finally, the statement "many recommendations for minimizing pain and distress are extrapolated from information available about mammals" reflects a lack of understanding how mammals (and birds) differ physiologically from ectothermic tetrapods. Mammals are endothermic, and much of the information about pain in mammals relates to regional cooling, in which cold peripheral parts respond to various stimuli while the central nervous system and peripheral conducting pathways are at normal or near-normal functional temperatures. This situation is entirely different from that in which the whole body of a small ectothermic organism is cooled uniformly to low temperatures, including the central nervous system (Shine et al. 2015).

The proscriptions in the AVMA's guidelines are not isolated. Similar recommendations occur in other publications. For example, in a review of anesthesia in amphibians and reptiles, Ross and Ross (2008) stated that hypothermia is not a "real" anesthetic and provides no analgesia, but they do not provide any evidence or references. They say that the technique should be used with care to avoid mortality in amphibians but that recovery is rapid and deaths are rare in reptiles—again with no data or references.

### The measurement of pain

Many people have subjective impressions of pain when an animal that is being observed or studied experiences it. Such

anthropomorphism must be critical, however, and based on the latest scientific evidence in order to be used in policy (Morton et al. 1990). Symptoms of distress are most likely to be recognized in animals with which investigators are familiar. However, the sensation of pain is difficult to objectively quantify. This remains true even though we know a lot regarding the mechanisms of nociception and the transmission and processing of painful stimuli (Defrin et al. 2002, Paul-Murphy et al. 2004, Foulkes and Wood 2007, Sneddon 2015). *Nociception* is a term that is often used interchangeably with pain and is defined as the neural process of encoding noxious stimuli. However, the word "pain" is generally accepted to imply something more: a sensory and emotional experience associated with actual or potential tissue damage (Millan 1999, Paul-Murphy et al. 2004). Although it is difficult or impossible to accurately assess emotion in any other animal, including people, in 2002, a workshop on pain developed a consensus statement that all vertebrates and some invertebrates experience pain (Paul-Murphy et al. 2004). It is often assumed that amphibians and reptiles perceive pain similarly to, or analogous with, mammals because they possess appropriate anatomical, physiological, and pharmacological components, as well as antinociceptive mechanisms, to modulate behavioral responses to a noxious stimulus (Machin 2001, Mosley 2011).

Although we agree with these general comparative statements, we caution that interpretations of the perception of pain are controversial and that nociception in various vertebrates may not be comparable to the perception of "pain" by humans. For example, differences of opinion concerning whether fish feel pain arise largely from interpretations of a limited repertoire of reflex behaviors to noxious stimuli in context of the neuroanatomy and physiology of mental states (Burghardt 2016, Key 2016). Moreover, some teleosts lack receptors that respond to cold and are therefore unlikely to experience pain associated with cold (Ashley et al. 2006). In context of the present discussion, we adopt the conservative position that amphibians and reptiles (indeed all vertebrates) experience some form of "pain" and distress (Paul-Murphy et al. 2004, Mosley 2011, Guénette et al. 2013). This assumption does not specify the modality of stimulus or the nature of the aversive state that is induced.

Most attempts to measure or detect perception of pain in animals depend on the application of various noxious stimuli eliciting withdrawal reflexes or other defined behavioral or physiological responses (e.g., Martin 1995, Suckow et al. 1999). Experiments performed include the use of thermal stimulators and probes to evoke subjective descriptions of response from human subjects (Defrin et al. 2002), as well as the interpretation of neuronal patterns within the brain (Brown et al. 2011, Shine et al. 2015). There has been no demonstration that ectothermic tetrapod vertebrates experience "cold" thermal pain when the entire body is cooled to low temperatures or when ice crystals form during freezing.

Pain is notably absent among ten neurologic symptoms of mild, moderate, and severe general hypothermia in humans,

including ataxia, dysarthria, and confusion, leading to loss of consciousness and coma (Kempainen and Brunette 2004). Rather, general hypothermia has anesthetic effects (Taylor 1988) and in fact has been used to control intractable pain (Guly 2011). Judgments about thermal pain based on mammalian studies in which only the appendages are chilled (regional heterothermy) while the body remains normothermic are not relevant here, and any application of such results to cold amphibians or reptiles is neither appropriate nor scientifically justified.

#### Natural history and the evolution of tolerance of cold

Many kinds of well-studied amphibians and reptiles undergo large variations of body temperature, both on seasonal or daily bases, and the range of such changes in body temperature may exceed 30°C (e.g., Carey 1978, Navas 1996). Tuataras, as well as north-temperate and south-temperate amphibians and reptiles, function at low temperatures, and extreme cold and winter dormancy are a regular part of the annual environment of numerous other species. These forms often exhibit the capacity for movement as well as sensory responses to the environment at remarkably low temperatures approaching freezing (Ultsch 1989, Costanzo et al. 1999). Amphibians and reptiles living in temperate or montane habitats select overwintering sites that provide some protection from freezing while they become dormant for weeks or months. Many species of amphibians and aquatic turtles overwinter beneath ice in ponds or ground surfaces, and a variety of snakes spend winters in dormancy underground where hibernacula and body temperatures may range from about 2°C to 7°C (e.g., Macartney et al. 1989, Ultsch 1989, Costanzo et al. 1999, Bernstein and Black 2005). Many tropical species of amphibians and reptiles also experience cold, which can reach freezing temperatures at higher elevations, and this can happen on a daily basis at elevations higher than 3500 meters (e.g., Navas 1996, Navas et al. 2013). Moreover, various species of amphibians with aquatic life stages initiate breeding activities in water at temperatures well below 10°C (movements, calling, and mating; e.g., Brattstrom 1999).

Amphibians and reptiles adaptively alter their functional responses to low temperatures, and these adjustments are related to behavior and survival unrelated to "pain" (e.g., Brattstrom 1968). Species that overwinter in sites where freezing occurs require particular biochemical and physiological adaptations to protect living processes. Such species either resist freezing at subfreezing temperatures, or they tolerate the freezing and thawing of their body fluids without damage (Layne and Lee 1995, Costanzo and Lee 2013). Some terrestrial amphibians and reptiles tolerate days or weeks in a state with as much as two-thirds of their total body water in the form of ice (Storey and Storey 1992, Costanzo et al. 1993). Wood frogs living in interior Alaska remain frozen for prolonged periods of up to 6 months and experience minimum temperatures ranging from -9°C to -18°C with 100% survival (Costanzo et al. 2013, Larson et al. 2014). It is

well known that some Arctic marine teleost fishes, as well as notothenioid fishes living in the Antarctic, possess antifreeze proteins that prevent freezing of body fluids at subfreezing temperatures (Fletcher et al. 2001, DeVries and Cheng 2005, Cziko et al. 2006). These fishes live their entire lives in a supercooled state in continuous contact with ambient water having a freezing point around -1.9°C.

Amphibians and reptiles that overwinter near or below freezing temperatures have evolved mechanisms that depress metabolism and change the mass and structure of body tissues (Storey and Storey 2004, Storey 2015). These changes are reversible, and no one doubts that they are adaptive traits because they permit survival for prolonged periods at low temperatures with minimum harm. Exposure to cold—even freezing—is part of their natural life, so it is incomprehensible that these animals experience pain or distress during seasonal or even diurnal exposure to near-freezing temperatures. On the other hand, it is adaptive for animals to remain sensitive to environmental noxious insults, albeit movements or activity are slowed at low temperatures. For example, turtles and frogs have been observed moving either in cold burrows or beneath ice during wintertime, with possible significance related to physiological benefits from following gradients of oxygen tension or temperature (Ultsch 1989). Moreover, it must be important for these overwintering animals to retain responsiveness to detect the arrival of spring (Madsen et al. 2013). Indeed, sensitivity to external stimuli might well be fine-tuned to existence in cold: For example, endogenous opioid systems in Northern grass frogs (*Rana pipiens*) are down-regulated during autumnal hibernation (Stevens and Pezalla 1989).

We emphasize, however, that the retention of functional sensitivity to the external environment in overwintering amphibians and reptiles does not suggest that they experience thermal pain attributable to cold body temperatures *per se*. This does not make biological sense. Similarly, mammals and birds that naturally experience extreme cold during hibernation or regional cooling while walking on snow or ice (Kilgore and Schmidt-Nielsen 1975) cannot be expected to be in pain during these natural behaviors that may continue for many months. It is unlikely (and contrary to natural selection) that Arctic and Antarctic fishes thriving at freezing temperatures are in constant pain throughout their entire lives. These are crucial aspects of physiology and natural history when considering the cooling of entire animals to low temperature. No one can demonstrate—and we doubt anyone would suggest—that normal behaviors and situations in natural environments are painful.

#### Cold or freezing as appropriate anesthesia or euthanasia for amphibians and reptiles

Here, we discuss important points related to the issue of whether cold and freezing are, under certain conditions, humane methods for either supplementing recognized methods of anesthesia or as a primary method of euthanasia of small ectothermic tetrapod vertebrates.

**Hypothermia and anesthesia.** Hypothermia has been used as a primary or supplemental method for anesthesia and analgesia for more than a century, both in ectotherms and endotherms (Blair 1971, Phifer and Terry 1986, Martin 1995, Suckow et al. 1999). In the sciatic nerves of toads, low temperature exerts the same structural and electrophysiological effects on myelinated nerves as various local anesthetics (Luzzati et al. 1999). However, the molecular events associated with anesthesia remain controversial (Mullins 1991, McKemy 2005, Foulkes and Wood 2007), as do opinions about guidelines for whether hypothermia is a clinically efficacious method for anesthesia (Martin 1995). This is because low temperature may lead to apparent paralysis without having fully blocked nociception. Data from studies of fishes suggest that rapid cooling can provide rapid anesthesia, potentially low mortality rates, improved safety, and an effective anesthetic method for scientific research (Matthews and Varga 2012, Chen et al. 2014). Although we do not recommend low temperature as a sole mode of anesthesia, we believe it can be very useful as a supplement to other methods (see below).

An important issue related to transient hypothermia for supplemental anesthesia is the potential for immunosuppression and the subsequent development of infectious disease. The immune responses of amphibians and reptiles are temperature sensitive—often with their strongest responses occurring at “optimal” temperatures specific to a species (Zimmerman et al. 2010)—and can be suppressed by periods of hypothermia or variations of temperature (Cooper et al. 1992, Maniero and Carey 1997, Raffel et al. 2006). Temperature can also affect the prevalence and life cycles of pathogens in addition to immune defense. Problems attributed to hypothermia have been discussed in several publications related to commercially farmed fish and stranded, “cold-stunned” sea turtles (e.g., Bly and Clem 1992, Innis et al. 2009). In the case of sea turtles, only a portion of populations succumb to “cold-stunning” and their preconditions are not known. Conversely, it has been suggested that amphibians can tolerate, or even benefit from, natural patterns of rapid warming and cooling (Terrell et al. 2013). Cooling is not logically implicated to impose long-term health problems for individuals or populations of species that naturally experience seasonal or daily bouts of exposure to low temperature (Storey and Storey 1992). Most importantly, there are no studies that indicate that very short-term hypothermia (e.g., less than 1 hour of supplemental anesthesia) negatively affects the subsequent health of ectothermic tetrapods. Snakes representing several species that were cooled to low temperature as supplemental anesthesia during cardiovascular surgeries recovered normal behaviors rapidly and survived well for periods of months until they were released or transferred to other projects (personal observations by HBL and RSS). We suggest the possible effects of very short-term hypothermia (less than 2 hours of supplemental anesthesia) would be an interesting and profitable area of further research.

**Temperature and conduction of nerve signals.** The conduction velocities for neuronal action potentials decrease with decreasing temperature and may cease at temperatures close to 0°C. There is a clear linear relationship between temperature and the velocity of nerve conduction in tortoises, and nerve blockage occurs at temperatures of 1°C–3.5°C (Rosenberg 1978). Similarly, conduction by peripheral nerves is blocked at about 0°C–2°C in bullfrogs (Roberts and Blackburn 1975). Therefore, the excitable membranes of some ectothermic vertebrates experience cold block of action potentials at low temperatures near freezing. In addition, blockage of nociceptive C fibers occurs at higher temperatures than does blockage of neuromuscular A fibers in bullfrogs (Roberts and Blackburn 1975). In general, the blockage of nerve conduction in mammals occurs at higher temperatures compared with that in ectothermic vertebrates (e.g., Rossi and Britt 1984). For example, anesthetized goats do not respond to peripheral painful stimuli when they are cooled to about 20°C and the anesthetic is removed (Antognini 1993).

Still, cold-resistant and freeze-tolerant species retain functionality at very low temperatures, with the variability of response depending on the thermal adaptations of species (Daló et al. 1995, Costanzo et al. 1999, Madsen et al. 2013). Cold-resistant amphibians and reptiles are capable of coordinated movements down to freezing temperatures and can readily respond to stimuli such as a leg pinch. However, their response to continued lowering of temperature alone does not appear to be one of stress or panic (personal observations by KBS and HBL). Notably, there are no indications of pain withdrawal or distress in the response to simple chilling or freezing when amphibians or reptiles are not touched, which implies that cooling or freezing *per se* is not a painful or distressful stimulus.

Therefore, we believe that a sensation of pain attributable strictly to hypothermia is not present during cooling of whole ectothermic animals before ice crystals begin to form, and this reflects their natural history and assures that these animals retain mobility and other functions at low temperatures. Some will correctly suggest, therefore, that cold temperatures do not readily induce unconsciousness in these animals (Martin 1995, Madsen et al. 2013). The point here, however, is that a trajectory of lowering whole-body temperature to freezing eliminates the possibility of pain that might be associated with freezing of the entire animal. Again, the possibility of pain in freezing tissues is physically impossible.

**Temperature and brain function.** Normal brain function depends on temperature and ceases at low temperatures approaching 0°C in cold-sensitive ectothermic vertebrates. There is a close relationship between brain temperatures and cerebral metabolism, which is reduced dramatically at low temperature (LaManna et al. 1980, Mrozek et al. 2012). The restoration of neuronal membrane potential following depolarization requires energy and suggests a direct

link between temperature and neuronal activity (Sokoloff 1999). Moreover, temperature also influences the passive properties of neuronal membranes, synaptic transmission (including release, reuptake, and diffusion of neurotransmitter) and cerebral blood flow (Mrozek et al. 2012). Electroencephalographic studies demonstrate near-zero activity in recordings from the brains of amphibians or reptiles having body temperatures close to 0°C (Hunsaker and Lansing 1962, Shine et al. 2015). Although only a small number of cold-tolerant species have been studied (Daló et al. 1995, Madsen et al. 2013), one might expect near-zero brain activity to be characteristic of numerous taxa.

Bispectral analysis is a statistic applied to EEG waveforms to monitor the depth of anesthesia. Research in humans indicates that the bispectral index (BIS) decreases by 1.12 units for every °C decrease in body temperature (Mathew et al. 2001). It would be useful to develop a bispectral index for use in monitoring the effects of hypothermia in amphibians and reptiles.

**Formation of ice crystals.** Physical laws contradict the assumption that ice crystals cause pain. The formation of ice crystals will block the movement of all charged particles (freezing them in place) and thereby disrupt any activity of excitable membranes and thus inhibit neural transmission as well as any central integration of "pain." Therefore, any pain experienced concurrent with the initiation of ice formation could be near instantaneous and quickly blocked as ice penetrates through the body and impairs nerve function. In anurans, ice crystals form at -1°C to -4.3°C (Storey and Storey 1986, Hillman et al. 2009). As we have discussed above, peripheral nerves are unlikely to transmit signals when tissues of cold-sensitive species are below 0°C, and the brain is likely to be inactive as well. Thus, nociception or perception of pain is not possible at temperatures that induce formation of ice crystals in tissues, and the creation of a crystal lattice involving neural tissue would further prevent any neural activity. Therefore, we conclude that the transmission of pain associated with freezing is not possible. Note also that cryoprotectants identified in some cold-resistant and freeze-tolerant amphibians would delay but not stop the disruption of excitable membrane function associated with the formation of ice crystals. Moreover, cooling and freezing for euthanasia would not engage time courses sufficiently long to produce effective amounts of cryoprotectants in those freeze-avoiding species that are capable of such protection.

When freezing at any particular location seeds growth of ice crystals, the subsequent change of water to ice can be very rapid at the freezing point (Packard et al. 1993, Cziko et al. 2006, Cheng and Detrich 2007), although the freezing kinetics of organisms can vary considerably depending on the mass of the organism, its water content, and the cooling capacity of the microenvironment (Claussen and Costanzo 1990). Moreover, the rate of nucleation (crystal growth) is faster in tissues than in water, depending on colloidal or macromolecular inclusions (Stephenson 1956). Therefore,

when smaller amphibians or reptiles are cooled and the entire body approaches freezing, the end result is likely to be a very rapid formation of ice. When larval polar fish having low concentrations of antifreeze proteins are cooled to an organismal freezing point causing ice crystals to form (-0.99°C to -2.63°C), complete freezing occurs within 1–2 seconds of the onset of the growth of crystal (Cziko et al. 2006).

Freezing might occur much more slowly in freeze-tolerant species or in animals that cool very gradually or intermittently under natural conditions in the field (Storey and Storey 1992). Again, however, it seems contrary to natural processes that such "slow freezing" would render animals to be in pain for prolonged periods while subject to normal conditions in natural environments. Alaskan wood frogs sometimes undergo multiple, repetitive cycles of freezing and thawing in their natural environment (Costanzo et al. 2013, Larson et al. 2014).

**Cooling of tropical species.** Cooling of low-elevation tropical species to temperatures approaching 0°C can kill tropical species without formation of ice crystals in body fluids or tissues (Wilson et al. 2009). Therefore, we consider either rapid cooling in an ice bath or slower cooling in a refrigerator and/or freezer (Shine et al. 2015) to be a humane method of euthanasia for small tropical amphibians and reptiles because low temperatures will suppress nerve and brain function as discussed above. Moreover, the use of chemical agents for euthanasia might induce equal or greater levels of distress (see below). Generally, freezing of zebrafish is allowed by current guidelines, and we suggest this practice also should be extended to small species of tropical amphibians and reptiles.

**Pain and distress associated with chemical agents.** The injection of euthanizing chemicals can be painful and induce neural and behavioral effects that are highly variable in different species (e.g., Wilson et al. 2009). Arguably, the humane use of chemical agents to induce anesthesia or death in animals depends on appropriate parameters of delivery such as volume, concentration, site and rate of injection, and appropriate buffering. Whereas zebrafish display signs of distress both to buffered and to unbuffered tricaine methanesulfonate (MS222; Wilson et al. 2009, Mathews and Varga 2012), Conroy and colleagues (2009) outlined a two-stage protocol for euthanasia of small reptiles with MS222 suitable for use in both the laboratory and the field. Because of the interspecific variation of responses to chemical agents, the judicious and humane use of chemical anesthetics potentially requires clinical trials and training that is appropriate for any species of wildlife in question. One of us (ERJ) has continued to modify euthanasia methods over 46 years of research and clinical experience with amphibians and reptiles.

**Field research.** We propose that freezing can provide a humane and practical means of euthanasia for amphibians and reptiles

that might be investigated in remote or particular field situations in which the appropriate chemical agents required for euthanasia by current guidelines are not available. Moreover, there are many locales where the transport of controlled substances places the investigator in legal or physical jeopardy, thereby also threatening the viability of the project and the humane treatment of animals that are involved.

#### Validation of the endpoint

The important question arises with respect to euthanasia: How does one determine whether a frozen amphibian or reptile is actually dead, especially if it is a freeze-tolerant species? This issue needs to be addressed when freezing is considered for euthanasia. We recommend that investigators work in cooperation with their respective EC to verify death for any given species that is euthanized. The first approach might be to determine whether animals recover following freezing. If recovery occurs, the frozen animal could be kept in a  $-80^{\circ}\text{C}$  freezer for some period (24–72 hours) subsequent to freezing or be subjected to some other secondary means of physical euthanasia while frozen (e.g., pithing and decapitation). If the animals are not required for further study, they could simply be kept frozen until discarded (e.g., incineration). We suggest that simple tests and observations can be used to determine whether animals euthanized by freezing are indeed “dead,” and we suspect the vast majority of species (especially tropical and subtropical species) will not recover from freezing to temperatures that are characteristic of most commercially available freezers ( $-10^{\circ}\text{C}$  to  $-20^{\circ}\text{C}$ ). Indeed, even freeze-tolerant species would undoubtedly die at these temperatures. Everyone must be open to the possibility that rare exceptions might be found (e.g., the Alaska wood frog; Larson et al. 2014). We also note that confirmation of an end point is required for the use of chemical agents, equally as for freezing.

#### Conclusions

Considerations of physiology and physical principles based on the available literature lead us to conclude that amphibians and reptiles are incapable of experiencing pain at temperatures when ice crystals form in tissues during whole-body cooling, or in the case of cold-tolerant and freeze-tolerant species, there is no pain attributable to whole-body hypothermia *per se*. Nociception is diminished and extinguished at very low temperatures because of the anesthetizing actions of cold on membranes, the cold-block of nerve conduction, and the lack of brain function in cold-sensitive species. Moreover, once ice crystals are seeded and begin to grow, the process is likely to be rapid. Therefore, if whole-body cooling is without pain, cooling to the freezing point and beyond should not induce any painful stimuli other than what is possibly (but unlikely) experienced when ectotherms are flash-frozen in liquid nitrogen, which is allowed by current guidelines.

We propose that cooling to low temperatures is acceptable as supplementary anesthesia when other gaseous (e.g.,

isoflurane) or local (e.g., lidocaine) anesthetics are also used, provided that such bouts of cooling do not compromise the immune competency of the animal. Advantages include clarity of the visual field during surgery owing to the diminishment of blood flow in tissues (reducing surgical “errors”), the reduction of handling time, easier maintenance of the appropriate plane of anesthesia, and potentially more rapid recovery from anesthesia (particularly useful during field work). We also propose that rapid cooling and freezing is a humane form of euthanasia for numerous species of smaller amphibians and reptiles. The advantages include elimination of pain that is sometimes associated with the use of certain chemical agents, minimizing the stresses associated with the handling time of animals, reduced risks to health and safety of investigators (e.g., bites from venomous snakes), the efficiency of costs, and the research-associated advantages of leaving animals without gross damage to body form and without the chemical contamination of body fluids.

Finally, we encourage an open dialogue between investigators who have extensive experience with ectothermic vertebrates and those involved in oversight, including a willingness of both parties to respect each other's opinions and to value what is actually known about the biology of animals (wildlife) that might not “fit” well with current guidelines. Research guidelines for the health and welfare of amphibians and reptiles should include understanding of the evolution and natural histories of species that are investigated, as well as consideration of the “evolutionary value of pain as an essential element for survival” (Paul-Murphy et al. 2004).

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