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Research Article

# Freeze Tolerance as a Pleistocene Relic in Cave-Dwelling *Stygobromus allegheniensis* or adaptation to local conditions?

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## Abstract

Pleistocene glaciers profoundly shaped the distribution and evolutionary history of many subterranean organisms in the northeast United States such as *Stygobromus allegheniensis*. During the last glacial maximum, many of these organisms were subject to sub-zero conditions associated with the Laurentide Ice Sheet. Such conditions would explain why *S. allegheniensis* from the Ice Caves of Sam's Point area of New York are able to survive being encapsulated by ice. The Ice Caves at Sam's Point experience seasonal freezing and prolonged ice formation during winter, whereas Clarksville Cave is warmer and maintains thermally stable liquid water year-round. To determine whether freeze tolerance is a retained ancestral trait in *S. allegheniensis* or a locally derived adaptation, we compared cold tolerance in amphipods from these two cave systems. Amphipods from Clarksville Cave showed some tolerance to near-freezing temperatures and partial ice contact, despite such conditions never occurring in their habitat, indicating probable retention of a vestigial physiological trait. However, they were highly susceptible to complete ice encasement, in stark contrast to Ice Cave specimens, which tolerate prolonged freezing conditions. Survival differed significantly between the two populations under complete ice encasement. Our results suggest that ancestral cold-tolerance mechanisms have persisted in *S. allegheniensis* since the Pleistocene, with populations from caves that naturally freeze nowadays showing further enhancement of freeze resistance through local adaptation.

## Introduction

Cave ecosystems are typically characterized by perpetual darkness, limited nutrient availability, and high humidity, all of which impose strong selective pressures on subterranean organisms [1]. Many obligate cave species exhibit troglomorphic traits, including reduction or loss of eyes and pigmentation, often accompanied by constructive adaptations such as elongation of appendages and enhanced non-visual sensory systems [2]. In contrast, cave fauna is generally not exposed to thermal extremes and rarely encounter freezing conditions. Most caves maintain internal temperatures near the mean annual surface temperature, with substantially lower thermal variability than surface environments [3]. Even in alpine and pre-alpine limestone caves, water temperatures generally remain below 10 °C but rarely reach freezing [4]. Consequently, freeze-tolerance adaptations are uncommon among subterranean species. In fact, only a handful of cave-dwelling crustaceans worldwide are known to survive subzero conditions [5]. Exceptions exist, however. The Ice Caves at Sam's Point in New York function as cold-air traps, retaining winter air and snow, producing ice on walls and floors into the summer [6]. These caves expose aquatic subterranean fauna to conditions rarely encountered elsewhere in the northeastern United States. One resident species, the Allegheny Cave Amphipod (*Stygobromus allegheniensis*), is fully depigmented and eyeless (Figure 1), with a broad geographic range (~600 km north-south) spanning Maryland, Pennsylvania, and New York [7]. While most populations inhabit thermally stable, liquid-water environments, the Ice Caves at Sam's Point periodically reach subzero temperatures, raising the question of whether cold tolerance is a retained ancestral trait across the species or a locally adapted feature of this population.

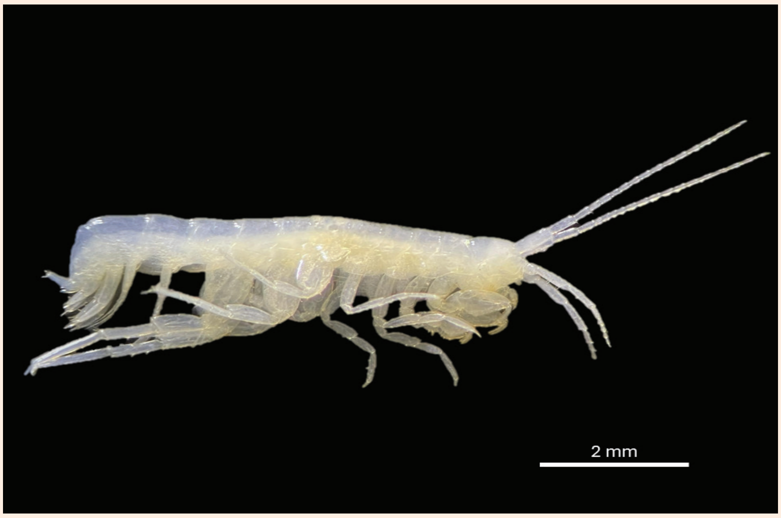


**Figure 1:** Adult *S. allegheniensis* from Clarksville Cave, NY.

To address this, freeze tolerance in *S. allegheniensis* from the Ice Caves at Sam's Point was compared to a population from a non-freezing cave, Clarksville Cave, New York. Clarksville Cave is a limestone cave with year-round flowing water and no ice formation inside the cave. The cave is about 1.5 km long and consists of a series of horizontal passages with puddles, pools, and slow-moving water (Figure 2). Genetic analyses indicate high similarity between the two populations as they have identical H3 histone DNA sequences [8], consistent with recent postglacial colonization (~12,000 years) [6]. Likewise, both populations have been shown that their light-entrainable circadian rhythms controlling motor activity have undergone incipient regressive evolution [9]. Comparisons between these two populations will allow evaluations of whether freeze tolerance reflects a broadly retained ancestral trait in the species or a local population adaptation.

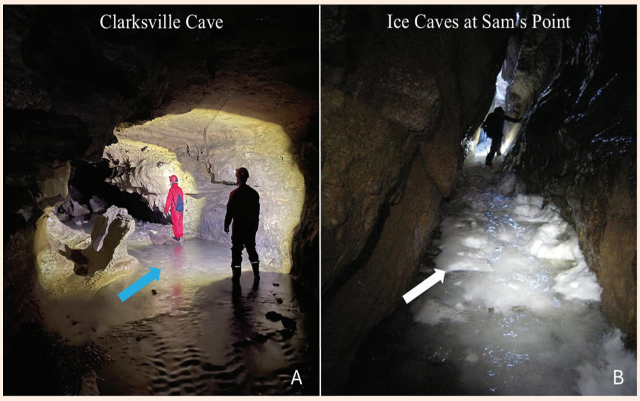


**Figure 2:** Comparison of cave conditions; (A) Clarksville Cave and (B) Sam's Point. Blue arrow highlights that during the winter and spring, water is liquid at Clarksville Cave while on the contrary, water may freeze in the Ice Caves at Sam's Point (White arrow).

## Materials and Methods

### Field Study

Permission from the New York State Office of Parks, Recreation, and Historic Preservation (Application # 2015-MIN-001) was given for the "Amphipods from the Ice Caves" project. Field work was conducted on May 2, 2025, at Clarksville Cave preserve (42º34'36"N, 73º57'56"W). Description of Clarksville Cave and its map can be found in [10]. Clarksville Cave is a limestone cave with a flowing stream year-round. Unlike the Ice Caves at Sam's Point preserve (41º40'20"N, 74º20'47"W), the interior of Clarksville Cave never experiences freezing conditions, and water is always liquid, even during the winter. At the time of the study, the air temperature inside the cave was 11.2 °C and the water temperature was 9.7 °C. Specimens were gently collected from a lateral pool located about 180 m from the nearest entrance (Ward's entrance) in Perry Avenue's gallery. All collected specimens were individually transferred into 2.5 cm diameter vials with about 7.5 ml of cave water. The vials were left uncapped. The specimens were divided into four experimental groups based on the temperature they were exposed to and the duration at which they were frozen. To expose the vials to 0.5 °C temperatures while in the field, the vials were immersed in crunched ice in an ice box. To expose the vials to -14 °C freezing temperature conditions also while in the field, they were immersed into a cooling mixture prepared by mixing a 3:1 ratio of crunched ice to salt, plus water until it turned into slush. Salt-driven freezing-point depression lowers the melting point of ice below ambient temperature. As the ice melts, the endothermic process absorbs heat from the surroundings, maintaining subzero conditions of -14 °C during field work. Specimens were exposed to either of the following conditions:

Two specimens inside a vial were exposed to -14 °C until water temperature inside their vial was 0.5 °C (about 3 min). Vial was then transferred to 0.5 °C for 1.5 hours. Ice formation never developed inside the vial.

Two specimens were exposed to -14°C until water temperature reached 0 °C (about 6 min) and a thin layer of ice coated the walls. A couple of legs of the specimens were caught in the ice, but the rest of the body remained free. Vial was then transferred to 0.5°C for 1.5 hours.

Four specimens were exposed to -14 °C until all water was frozen solid (about 15 min). Vial was then defrosted.

Four specimens were exposed to -14 °C until all water was frozen solid (about 15 min). Vial was then transferred to 0.5 °C for 20 minutes and then defrosted.

### Laboratory Study

Four specimens were transported to the laboratory. After 6 hours from being collected, specimens were individually placed in 5 cm diameter Petri dishes, each filled to a depth of 0.5 cm with cave water and exposed to 2°C conditions for 24 hours. They were then assessed for their ability to maintain active movement following prolonged exposure to the low, above-freezing temperatures. The specimens that were still actively moving (three) were then subjected to -18°C until 95% of the water was frozen (approximately 15 minutes). Movements of appendages not yet encased in ice were assessed. Afterward, the specimens were exposed to -2°C for 1 hour, during which they were fully encased in ice. They were then defrosted at room temperature and assessed for Motility. Survival of amphipods subjected to whole-body ice encasement under freezing conditions for durations of ≤2 hours were compared between individuals from Clarksville Cave (this study) and the Ice Caves [6, 11] by pooling all available field and laboratory data. Differences in survival between populations were evaluated using a two-tailed Fisher's exact test, with the null hypothesis predicting equal survival probabilities between populations.

## Results

*Stygobromus allegheniensis* inhabiting the Ice Caves at Sam's Point Preserve exhibits substantial tolerance to extreme cold conditions. Individuals from this population can survive complete ice encasement for up to 4 hours, partial encasement for at least 27 hours, and prolonged exposure to 2 °C for a minimum of 120 hours [11]. A video on their ability to survive freezing conditions can be seen in youtube.com/watch?v=MgajTnWVl3s. In contrast, specimens from Clarksville Cave -a cave that does not experience freezing conditions- are markedly susceptible to freezing stress. Field observations indicated that none of the eight Clarksville specimens that became fully encased in ice survived (Figure 3). In contrast, both individuals that were not subject to complete ice encasement under short-term freezing and low above-freezing conditions survived.

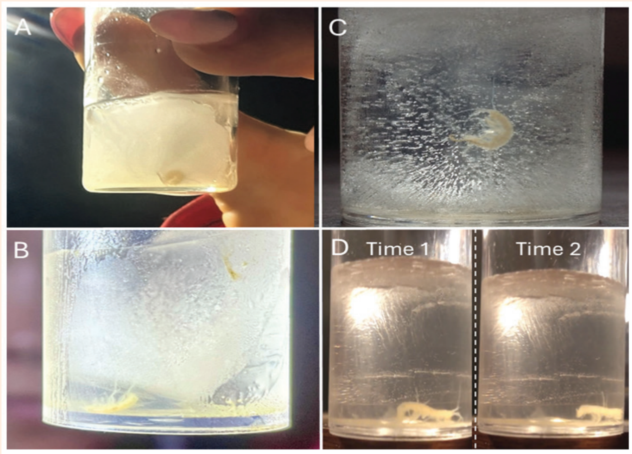


**Figure 3:** Freezing exposure and viability of specimens. (A) Clarksville Cave specimen fully encased in ice. (B) Specimen of Clarksville Cave after thawing, showing they failed to survive. (C) Sam's Point specimen fully encased in ice. (D) Sam's Point specimen after thawing, showing they were alive and immediately started crawling and swimming. Notice changes of position in the vial at D-time 1 and D-time 2

Laboratory experiments provided additional resolution to the cold-induced effects. Following 24 hours of exposure to 2 °C of the Clarksville specimens, 25% of individuals (N=4) lost the ability to actively swim or crawl, exhibiting only appendage twitching and intermittent body contractions. When approximately 95% of the body was encased in ice, 66.6% (N=3) of them showed some movements of their appendages, suggesting at least some partial withstanding to freezing conditions (Figure 4). The remaining 33.3% did not survive. After 1 hour of complete ice encasement, two of the three remaining specimens did not survive; the single alive individual displayed only minimal motor activity (appendage twitching and body jerking) and subsequently died 72 hours later.

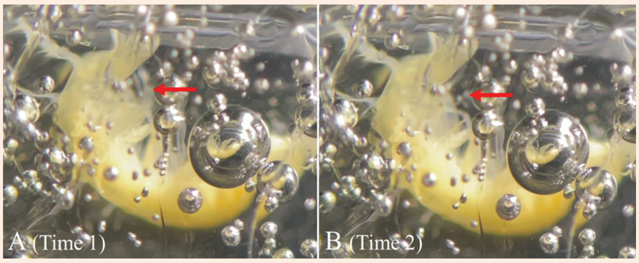


**Figure 4:** Clarksville Cave amphipods, despite not being as freeze tolerant as those of the Ice Caves, show here their partial adaptation to cold environment. A specimen from Clarksville Cave partially encased in ice is still alive and moving one of its appendages (red arrows). (A) Initial position of the appendage at time 1 and (B) subsequent position of appendage at time 2.

Across both field and laboratory datasets, survival of *S. allegheniensis* from Clarksville Cave was strongly influenced by the extent of ice encasement. Only one of the 11 (9.1%) Clarksville specimens survived complete ice encasement for 15 or less minutes, whereas 7 out of 9 (77.7%) of the specimens from the Ice Cave population (combined data from [6, 11]) remained capable of active swimming immediately after thawing, even following up to 2 hours of complete ice encasement (P=0.0045; Table 1). Even the single survivor from Clarksville was severely impaired and, as noted above, was unable to swim or crawl after thawing, and died three days after the experiment. Similarly, the Clarksville population exhibited markedly lower tolerance to partial freezing of appendages and brief exposure to low, above-freezing temperatures compared with the Ice Cave population.

**Table 1:** Effects of being encased in ice for a maximum of 2 hours. The two populations differ in their resistance to freezing conditions (P=0.0045).

| Outcome | Ice caves | Clarksville |
|---|---|---|
| Died | 2 | 10 |
| Survied | 7 | 1 |

## Discussion

These results indicate that freeze tolerance in *S. allegheniensis* varies among populations and is strongly influenced by local environmental conditions. While some Clarksville Cave individuals survived brief exposure to near-freezing water or partial ice contact, none survived full ice encasement. In contrast, Ice Cave individuals tolerated complete ice encasement for up to four hours [11], supporting an enhanced physiological capacity for extreme cold. Is cold tolerance a retained ancestral trait across the species or a locally adapted feature? Findings from this study support both scenarios. The species retains vestigial cold tolerance. Clarksville specimens show partial resistance to near-freezing water or partial ice contact, conditions which are never encountered in their natural habitat. But extreme freeze resistance, surviving up to several hours fully encased in ice, is expressed primarily in populations naturally exposed to ice during most winters, such as in the Ice Caves at Sam's Point. The Ice Cave population likely represents a locally adapted lineage in which selection has strengthened pre-existing physiological traits. This adaptation may involve cellular and metabolic modifications, including cryoprotective compounds that mitigate ice formation and cellular damage. Such localized specialization is rare in subterranean ecosystems, which are generally thermally stable and lack selective pressure for freeze tolerance [3].The hypothesis that the species retains vestigial cold tolerance, which has then been enhanced and selected for in the Ice Caves population, is plausible given the relatively recent deglaciation of the Appalachian Plateau [12], which occurred approximately 12,000 years ago. During the Pleistocene glacial period, ancestral populations of *S. allegheniensis* would have sustained exposure to freezing environments, suggesting that tolerance to low temperatures may have been a Pleistocene-era selectively favored trait that has since become selectively neutral or only weakly selected for in most populations.Freezing tolerance has also been documented in the subterranean amphipod *Niphargus rhenorhodanensis* from France, where cold-acclimated individuals can survive inoculative freezing via accumulation of cryoprotectants such as glycerol and free amino acids [13,14]. Although the species does not currently inhabit freezing environments, these traits are interpreted as remnants of survival during Quaternary glaciations in periglacial refugia or nunataks [15], when glacial meltwater near 0°C [16] likely exposed subterranean habitats to near-freezing conditions. These results illustrate how isolated cave environments can drive population-specific adaptation. In *S. allegheniensis*, a vestigial physiological trait-potentially originating during Pleistocene glaciations has been retained across the species but further enhanced in populations subjected to subzero conditions. The Ice Cave population exemplifies how subterranean organisms can not only persist in stable environments but also evolve highly specific adaptations to extreme, localized ecological niches.

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