Post-freeze recovery is delayed with repeated freeze-thaw cycles in Cope's gray treefrog *Dryophytes chrysoscelis*

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Background and Introduction

Cope's gray treefrog *Dryophytes (Hyla) chrysoscelis* is a freeze tolerant anuran capable of freezing and thawing multiple times per year.

- A period of cold acclimation is required for freeze tolerance.
- Accumulation of cryoprotectants like glycerol prevents intracellular freezing. (Fig. 2) [1].
- Specialized proteins called aquaglyceroporins enable movement of water and glycerol across the membrane before freezing and during thawing [2].
- All main physiological functions are stopped during freezing, but cardiac, respiratory, neurological, and muscular functions are recovered within 24 hours of thawing [3].
- **Though animals naturally experience repeated freeze-thaw cycles, repeated freeze-thaw is unstudied to date in *D. chrysoscelis*.**

Fig. 1: Cope's gray treefrog *Dryophytes chrysoscelis*. Photo by Elizabeth Evans.

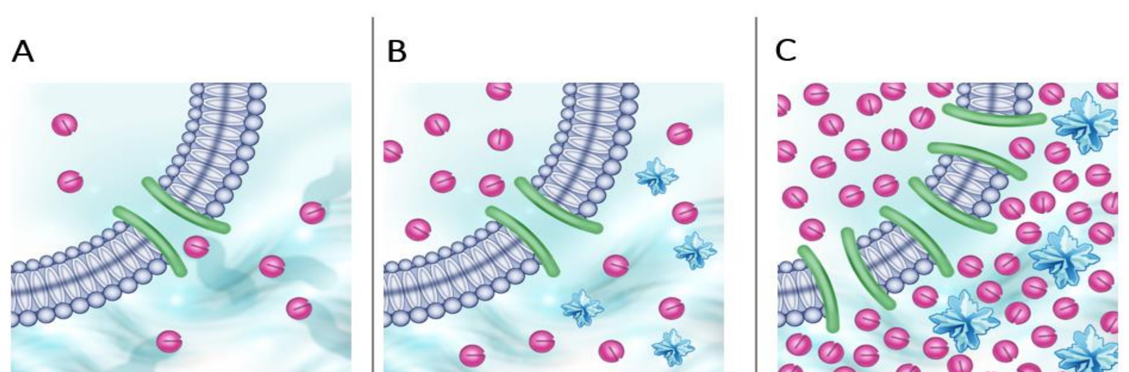

Fig. 2: Intracellular accumulation of cryoprotectants such as glycerol (pink) and evacuation of water molecules by aquaglyceroporin proteins (green) (a) enhances freeze tolerance in *D. chrysoscelis* at very cold and freezing temperatures (b, c).

Methods

- Cold-acclimated animals were frozen by gradually reducing environmental temperature over a period of 6 days (first thaw) or 12 hours (second and third thaw), inoculated with ice to induce freezing, then thawed for 24 hours (first freeze-thaw, N=10, second and third freeze-thaw, N=4) (Fig. 3).
- Digital photography and personal observation were used to document changes in skin color, physical thaw, respiration, movement, etc. (Fig. 4).
- Digital image analysis was performed in ImageJ.

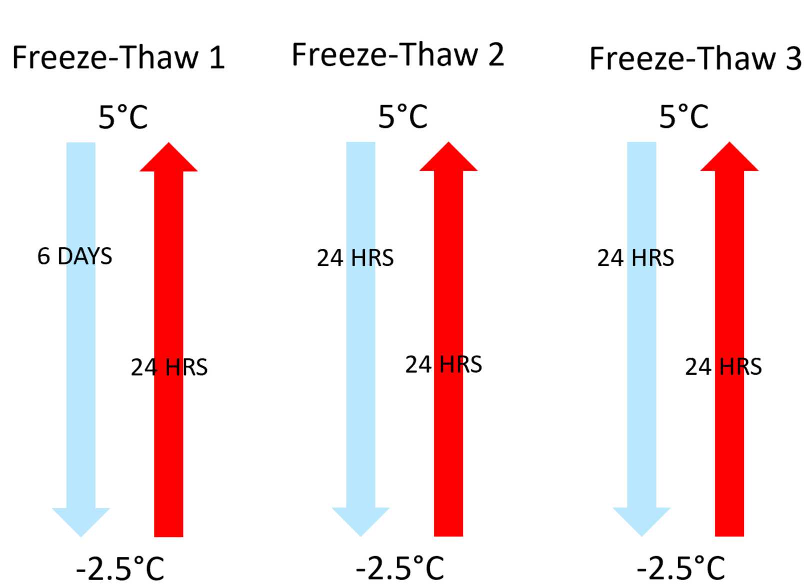

Fig. 3: Experimental design for repeated freeze-thaw protocols.

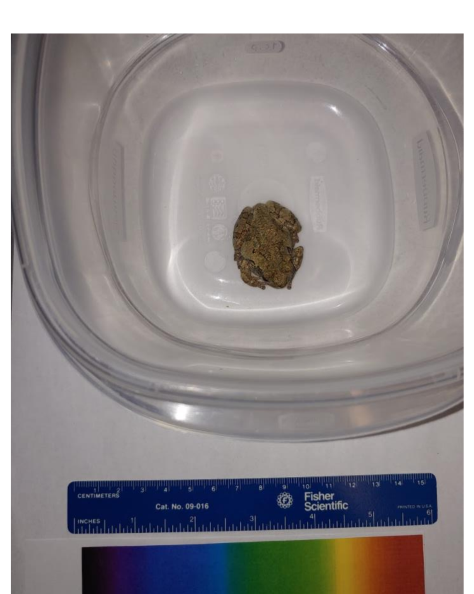

Fig. 4: Animal photographs document traits such as skin color, size, and body positioning.

Objectives and Hypothesis

1. Document physiological milestones with each freeze-thaw cycle.
2. Identify morphological and behavioral variation associated with repeated freeze-thaw cycles.

- Hypothesis: *Repeated freezing and thawing contributes to a delay in post-freeze recovery of respiratory, neurological, and muscular functions.*

Results

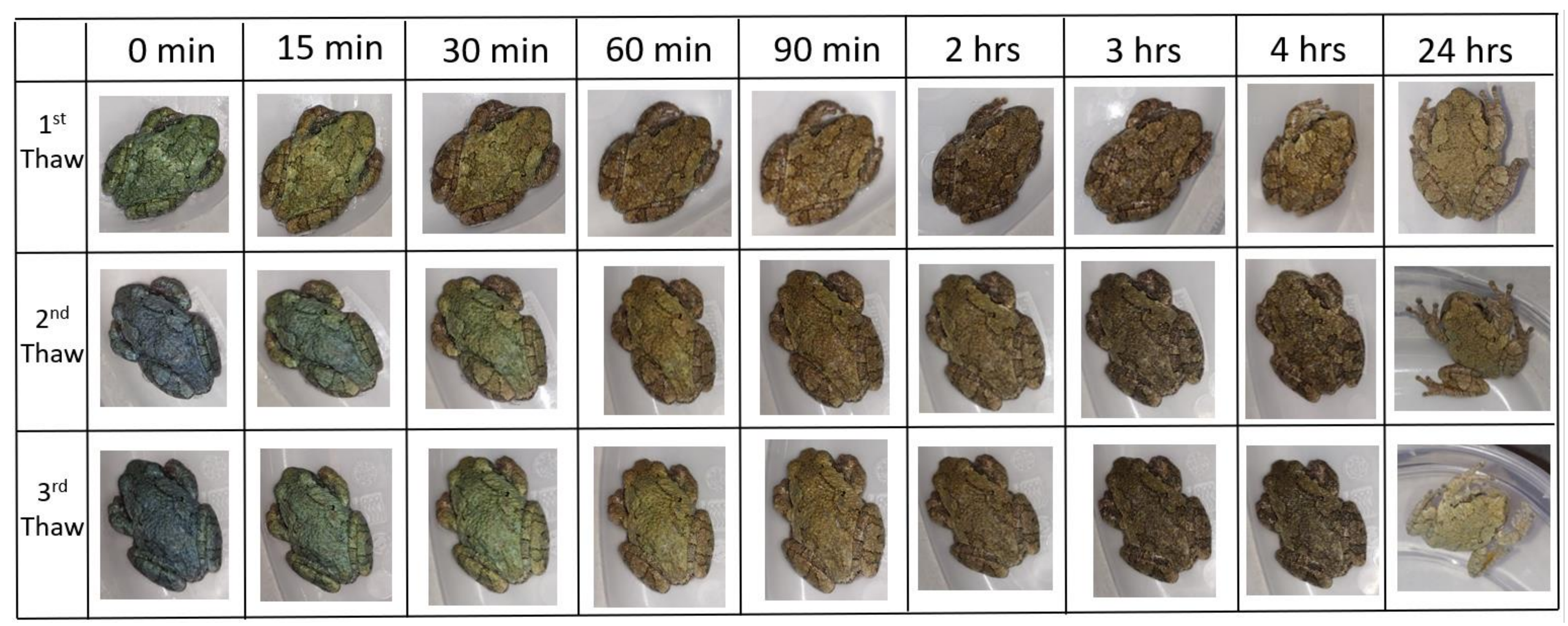

Fig. 5: Initial color at time of thawing and rate of color change throughout thawing varies with repeated freeze-thaw.

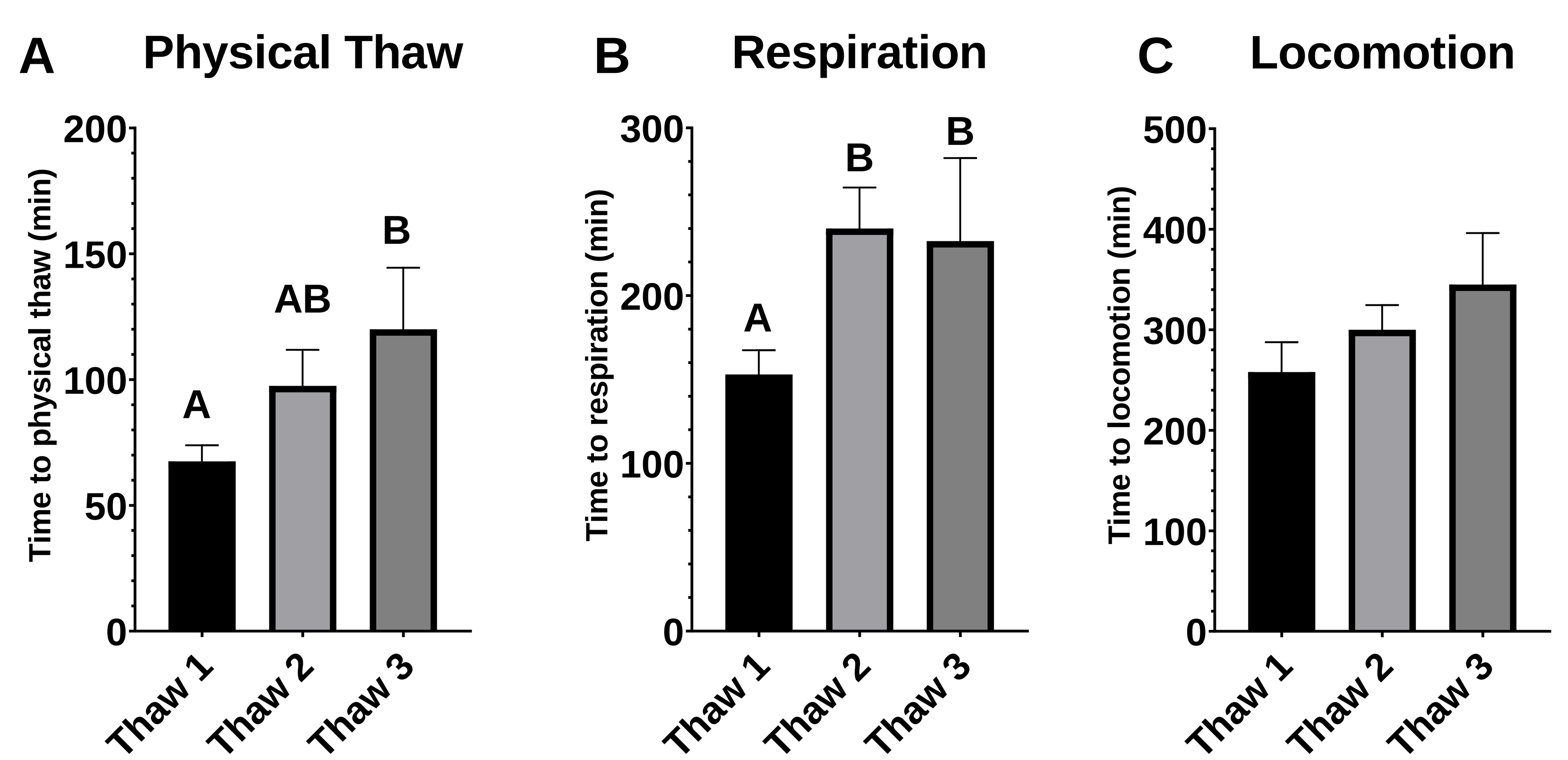

Fig. 6: Variables associated with thawing and post freeze recovery, a. Physical thawing time is delayed with repeated freeze-thaw, b. recovery of respiration is delayed with repeated freeze-thaw, c. post-freeze recovery of locomotion appears to be delayed with repeated freeze-thaw, but is not significantly different. Significant differences are represented by different group letters. $P < 0.05$. (N=8 warm, N=8 cold, N=10 single freeze-thaw, N=4 multiple freeze-thaw).

Results Continued

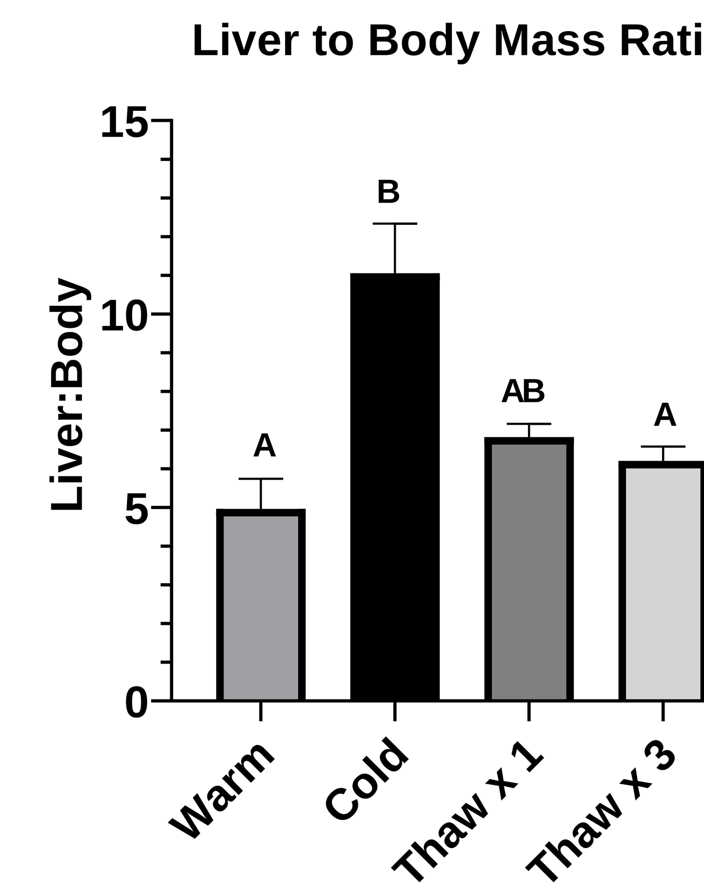

Fig. 7: The liver:body mass ratio in cold-acclimated animals is different than warm-acclimated and repeated freeze-thaw, but not single freeze-thaw animals. Significant differences between groups are represented by different letters. $P < 0.001$. (N=8 warm, N=8 cold, N=10 single freeze-thaw, N=4 multiple freeze-thaw).

Conclusions

- ❖ Previous literature demonstrates an increase in cryoprotectant accumulation with repeated freeze-thaw in the wood frog *R. sylvatica* [4].
  - Change in initial skin color and rate of change of skin color (Fig. 5) in combination with an increased time for physical thawing (i.e. disappearance of ice crystals on the skin) suggests there may be delays in recovery of circulation with repeated freeze-thaw (Fig. 5, Fig. 6a).
  - Significant increase in physical thawing time and recovery of respiration and a trend that suggests an increase in recovery of neuromuscular function may indicate differential cryoprotectant accumulation (Fig. 6).
  - The decrease in liver mass relative to body mass with repeated freeze-thaw may suggest that body water content and/or liver fuel stores change with repeated freeze-thaw (Fig. 7).

References

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[3] Costanzo and Lee (2013) *JEB*, 216: 1961-1967.
[4] Larson and Barnes (2016) *Phys. Biochem. Zool.*, 89(4): 340-346.