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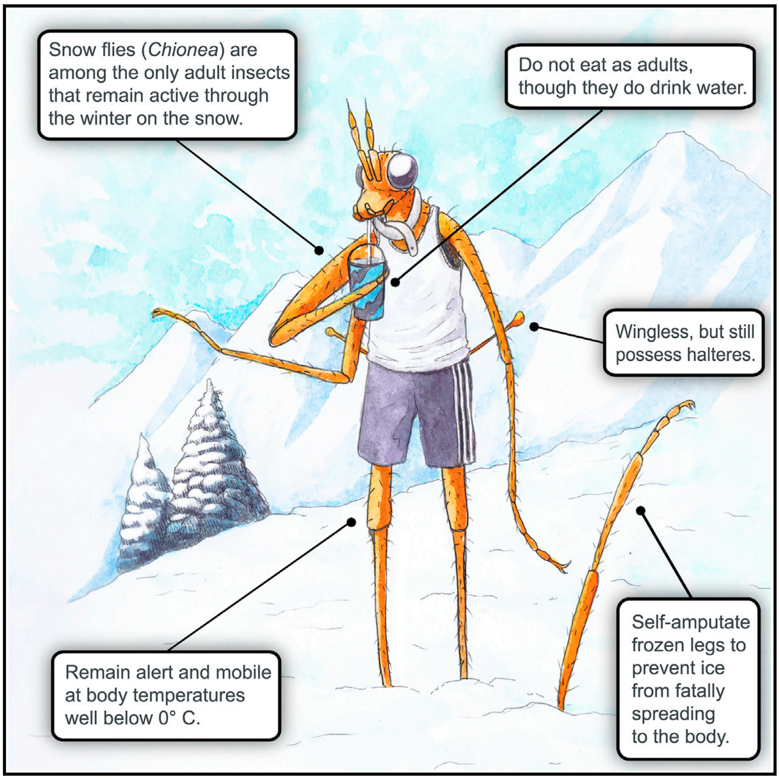
Current Biology

Snow flies self-amputate freezing limbs to sustain behavior at sub-zero temperatures

Graphical abstract



Authors

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In brief

The snow fly, *Chionea*, is one of the only insects that remains active through the winter in snowy environments. Using thermal imaging, Golding et al. show that snow flies walk at sub-zero temperatures that incapacitate other insects. They also discovered that snow flies detect internal ice formation and self-amputate legs to avoid death by freezing.

Highlights

- Snow flies sustain walking at internal body temperatures as low as −10°C
- At this lower limit, the snow fly's precious body fluids freeze, and it dies
- Snow flies self-amputate freezing legs to prevent ice from spreading to the body
- Leg self-amputation prolongs snow fly survival under frigid conditions

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Snow flies self-amputate freezing limbs to sustain behavior at sub-zero temperatures

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SUMMARY

Temperature profoundly impacts all living creatures. In spite of the thermodynamic constraints on biology, some animals have evolved to live and move in extremely cold environments. Here, we investigate behavioral mechanisms of cold tolerance in the snow fly (*Chionea* spp.), a flightless crane fly that is active throughout the winter in boreal and alpine environments of the northern hemisphere. Using thermal imaging, we show that adult snow flies maintain the ability to walk down to an average body temperature of −7°C. At this supercooling limit, ice crystallization occurs within the snow fly's hemolymph and rapidly spreads throughout the body, resulting in death. However, we discovered that snow flies frequently survive freezing by rapidly amputating legs before ice crystallization can spread to their vital organs. Self-amputation of freezing limbs is a last-ditch tactic to prolong survival in frigid conditions that few animals can endure. Understanding the extreme physiology and behavior of snow insects holds particular significance at this moment when their alpine habitats are rapidly changing due to anthropogenic climate change.

INTRODUCTION

Cold temperatures are a significant barrier to animal life in many regions on Earth. Animals that live in polar, boreal, or alpine environments possess physiological and behavioral adaptations to survive and move in extreme cold.[1] Endotherms, such as birds and mammals, generate and conserve body heat to maintain a consistent internal body temperature, even in extreme winter conditions.[2] However, this strategy is energetically expensive. Perhaps for this reason, most animals are ectothermic—their body heat is derived from the environment. Most ectothermic animals have adapted their physiology and behavior to survive across a range of temperatures. Some insect species migrate before winter begins to avoid decreasing temperatures, whereas others overwinter in a state of programmed quiescence called diapause.[3–5] These adaptations are necessary because most insects become paralyzed at temperatures below the melting point of water (0°C). Cold paralysis occurs due to an inability to maintain the membrane potential required for neuromuscular function, which leads to a phenomenon called spreading depolarization.[6–9] Insects in cold environments must also contend with internal freezing of the hemolymph, which is often fatal. The threat of paralysis and death by freezing limits the capacity of most insects to remain mobile and survive at sub-zero temperatures.

The snow fly, *Chionea* spp., is one of the few insects that remains behaviorally active at sub-zero temperatures (Figure 1A). Adult snow flies are found throughout boreal and alpine regions of the northern hemisphere.[10] In the Pacific Northwest (USA), they are active on the snow from October to April and are not typically found in the summer. Snow flies exhibit behavioral preference for temperatures close to −3°C[11]; however, we have observed them running on the snow at ambient temperatures as low as −10°C.

Snow flies belong to the crane fly family, Tipulidae, and resemble other crane flies except for a complete absence of wings and flight musculature. Compared with other crane flies, the life cycle of snow flies is poorly understood. Byers[10] speculated that they deposit eggs in the subnivean zone, beneath the snowpack, in tree litter or moist soil, and that the larvae consume plant detritus or rodent feces during the summer, pupating during late fall or early winter. They have a significantly longer lifespan than other crane flies, living as long as 2 months.[10] Snow flies are not known to eat as adults,[10] which is not unusual for a crane fly species. Rather, it seems that their primary reason for being active on snow is to locate a mate.[14] Two potential advantages of searching for mates in winter are the uniformity of the snow as a substrate and the absence of predators.

The thermal limits and dynamics of snow fly behavior have not previously been characterized, probably because they are challenging to collect from the wild and cannot be bred in the lab. To overcome these obstacles, we created a crowd-sourced science project (www.snowflyproject.org) that leveraged the expertise of backcountry skiers and mountaineers to collect snow flies from remote alpine regions of the Pacific Northwest. We also collected summer-active species of winged crane flies in Western Washington for comparison. We then used thermal imaging to measure their temperature and behavior when subjected to sub-zero temperatures in the lab.

We found that snow flies possess a remarkable ability to sustain locomotion at temperatures well below the paralysis threshold of other crane flies. Our results suggest that snow flies

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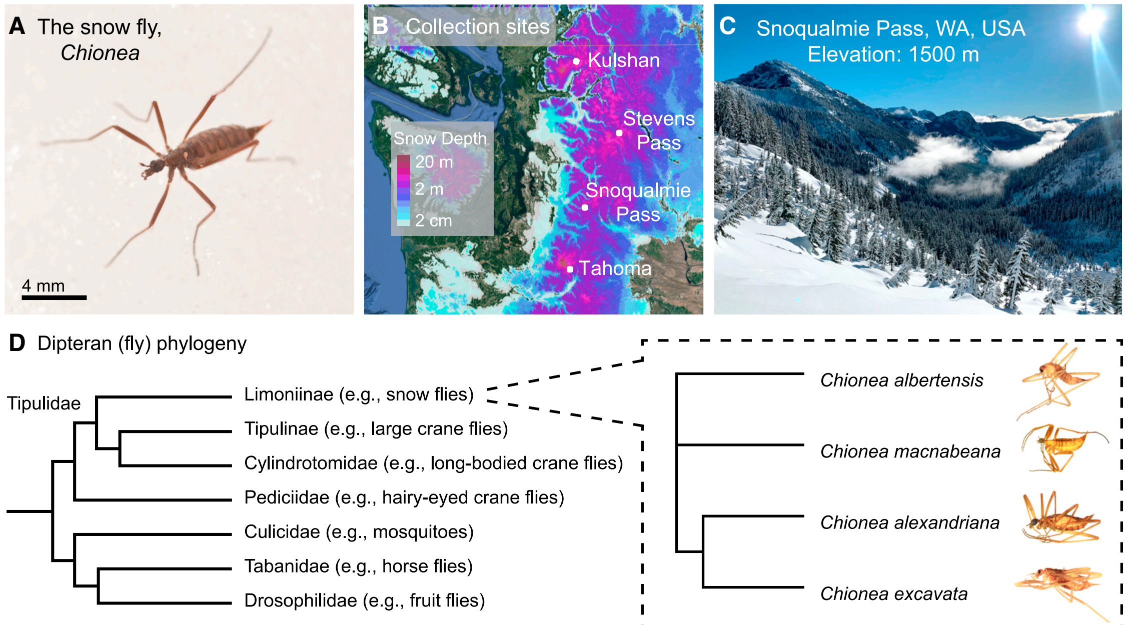

Figure 1. The North American snow fly, *Chionea*

(A) Dorsal view of a female *Chionea alexandriana*. Photo © Bryan Kelly-McArthur.

(B) Collection locations of snow fly specimens used for cold tolerance experiments. The four species used in this study significantly overlapped in distribution across collection sites. Collections of flies used for experiments took place from December of 2020 to November of 2022. Flies were found from late October through early May each year. Snow depth is shown for a typical collection day (February 25, 2022).

(C) An example snow fly collection site, which is representative of the alpine and boreal environments in which snow flies are active throughout the winter.

(D) Phylogeny of snow flies used in this study. Branch lengths do not correspond to the length of evolutionary time between nodes. Snow flies are a genus within Tipulidae, commonly referred to as the crane fly family. Crane flies are one of the largest and oldest families within the Dipteran order, with over 15,270 species identified.[12,13]

See also Video S1 and Data S1A.

possess adaptations that allow their neurons and muscles to overcome thermodynamic constraints on membrane potential maintenance, ion channel gating, and action potential conduction.[15,16] We also discovered that snow flies can self-amputate freezing limbs, which prevents the fatal propagation of ice to other parts of the body. Limb self-amputation is common in crane flies[17] but is typically triggered by mechanical stimuli, such as during predation.[18] We propose that snow flies possess a unique capacity to rapidly amputate legs using thermosensory detection of freezing body fluids.

RESULTS

From 2020 to 2022, we and other volunteers collected 256 adult snow flies, primarily from alpine regions of Washington (Figure 1B), as well as from Colorado, Vermont, British Columbia, and Yukon (Data S1A). In Washington, we typically found snow flies running on freshly fallen snow at, or close to, the tree line (Figure 1C; Video S1), between elevations of 1,200–2,000 m, and at an average daily temperature of 0°C. Collected snow flies were individually housed at 1°C. We conducted cold tolerance experiments on 39 males and 38 females, all collected locally in Washington, which were from four species (Figure 1D): *Chionea alexandriana* (55), *Chionea excavata* (14), *Chionea albertensis* (4), and *Chionea macnabeana* (4). We determined the species of each snow fly using a combination of morphological identification[10] and DNA barcoding.[19] We did not observe differences in the behavior of these four species; hence, we pooled data for further analysis.

We first sought to establish the minimum temperature at which snow flies can sustain behavior. We were unable to use the traditional method of measuring the temperature of the flies with a thermocouple[20] because it prevented the flies from moving. Instead, we used infrared imaging with a thermal camera[21,22] to record the cuticular temperature and behavior of individual snow flies on a cold plate (Figure 2A). We validated our thermal camera measurements with a thermocouple (Figure S1). During each experiment, the temperature of the cold plate decreased by 0.8°C/min over 25 min (Figure 2B). We manually tracked snow fly movement in the thermal imaging video, from which we extracted the insect's walking velocity and temperature (Figures 2B and 2C). The average rate of snow fly cooling was 0.5°C/min and the temperature of the snow fly was typically a few degrees warmer than the cold plate. We attribute this offset to the thermal gradient between the cold plate and surrounding air, which was 5°C (see STAR Methods). We also observed a thermal gradient between the surface of the snow and the snow fly in the wild (Video S1), which is likely due to the absorption of solar radiation by the snow fly's cuticle, as has been shown in other winter-active insects.[23] Therefore, snow flies are not likely to generate substantial heat through muscle contraction, as, for example, bees do.[24]

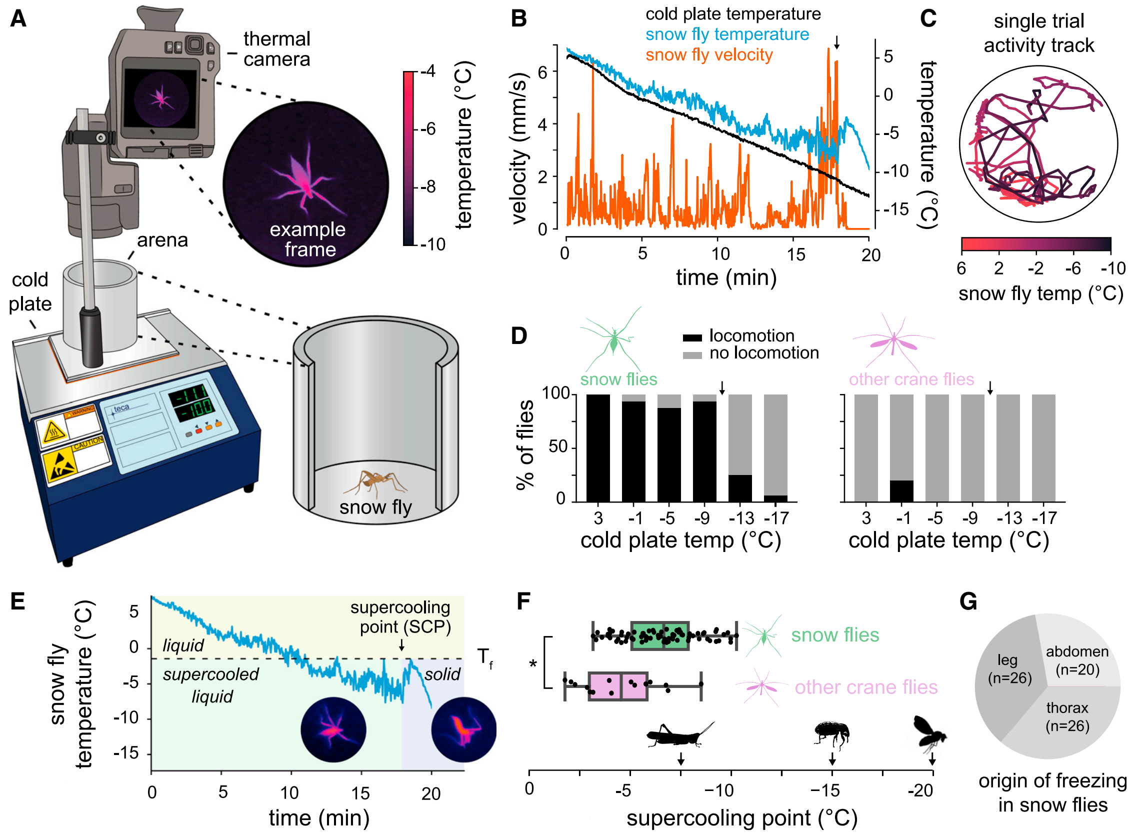

Figure 2. Snow flies sustain locomotor behavior at sub-zero temperatures until they freeze

(A) Schematic of the thermal imaging setup used to record snow fly activity and temperature.

(B) The cuticular temperature of the snow fly (blue), cold plate temperature (black), and fly velocity (orange) during the first 20 min of one experimental trial. The fly sustains consistent motor coordination until 18 min in, when a rapid temperature increase indicates that the fly has frozen. The black arrow indicates the time of freezing. Afterward, all movement ceases.

(C) Position and temperature of the snow fly within the arena over the course of the trial shown in (B).

(D) Snow flies sustain locomotion at sub-zero temperatures (n = 16 trials), while other crane flies do not (n = 5 trials). For each cold tolerance trial (e.g., shown in B), we manually scored fly locomotion within 6 different windows (cold plate temperatures of 3°C, −1°C, −9°C, −13°C, −17°C ± 0.5°C). Black arrows indicate the average cold plate temperatures at which snow flies and other crane flies froze.

(E) A second example trial, illustrating that below the freezing point (T_f), snow flies are supercooled. At the supercooling point (SCP), ice nucleation occurs within the snow fly and ice spreads throughout the body. This is indicated by a rapid increase in the fly's temperature.

(F) Distribution of SCPs across snow flies (green; n = 73 trials) and other crane flies (purple; n = 16 trials). The SCP of snow flies is lower than that of other crane flies (two-sample t test, *$p < 0.001$). The SCP of short-horned grasshoppers (*Stenocatantops splendens*),[25] alfalfa weevils (*Hypera postica*),[26] and fruit flies (*Drosophila melanogaster*)[27] are indicated by black arrows for comparison.

(G) Ice nucleation typically occurred in the snow fly's abdomen, legs, or thorax.

See also Figure S1, Videos S2, S3, and S4, and Data S1A and S1B.

Snow flies continued to move at cold plate temperatures well below 0°C, with most sustaining locomotion even when the cold plate dropped to −9°C (Figure 2D). For comparison, we conducted equivalent experiments on other crane flies that we collected locally from Western Washington during summer months (see STAR Methods). We found that summer-active crane flies were generally unable to sustain locomotion at cold plate temperatures below 0°C (Figure 2D; Video S2). Soon after being placed on the cold plate, they entered a coma-like state in which their movement was minimal and uncoordinated. Thus, snow flies appear to possess a unique ability to remain behaviorally active at sub-zero temperatures that incapacitate other insects, including their relatives in the crane fly family.

We observed that the point at which snow flies ceased movement was preceded by a sudden increase in body temperature (Figure 2E; Video S3). This sudden warming is an indication of ice formation within the snow fly's hemolymph.[20] Freezing is an exothermic process, releasing heat as the liquid molecules rearrange into a crystalline molecular structure.[28] Internal ice formation was almost always lethal; only 3/77 snow flies survived after freezing occurred within their body. This low survival rate indicates that *Chionea* spp. are generally not freeze tolerant.

Cold-tolerant insects that cannot survive internal ice formation often exhibit physiological and behavioral traits that help them avoid fatal freezing events. For example, some freeze-avoidant insects, including snow flies,[29] synthesize sugars and proteins

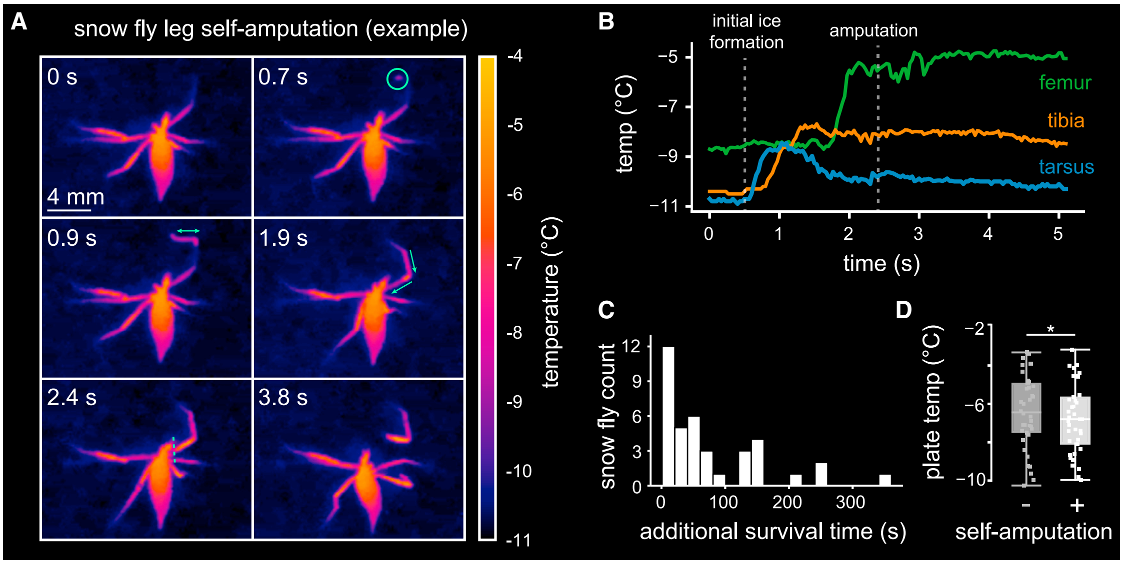

Figure 3. Snow flies evade fatal freezing events by self-amputating limbs (autotomy)

(A) An example of snow fly leg amputation. At 0 s, the tibia and tarsus of the fly's right front leg are at the same temperature as the cold plate (−11°C). At 0.7 s, freezing occurs in the mid tarsus (0.7 s, green circle) and spreads bidirectionally toward the tip of the tarsus and the tibia (0.9 s, green arrow). Ice crystallization propagates through the tibia and reaches the femur (1.9 s, green arrows). Amputation at the femur/trochanter joint occurs at 2.4 s, before freezing can propagate through the trochanter and to the body. The fly pulls away from the detached leg, whereas the leg flexes, with the body of the fly remaining unfrozen (3.8 s).

(B) Time course of freezing in the tarsus (blue), tibia (orange), and femur (green) for the example in (A). The time of detachment (white dotted line) indicates that autotomy is rapid, occurring within 1.7 s of the initial freezing event in the tarsus.

(C) Successful amputation increases snow fly survival time in lab experiments (n = 43 trials). The average additional time of survival was 77 s.

(D) Snow flies that amputate at least one limb (n = 43 flies) survive to a lower cold plate temperature than snow flies that retain all limbs (n = 34 flies) during cold tolerance experiments (two-sample t test, *p = 0.01).

See also Videos S4, S5, and S6.

that act as antifreeze.[28,30] This allows them to take advantage of the phenomenon of supercooling, by which small volumes of water can remain in a liquid state to −40°C or lower.[31] Supercooling is common in small insects, as their hemolymph primarily consists of water and lacks nucleating particles that are required for the ordered conglomeration of water molecules during ice formation. The temperature at which an insect freezes is referred to as its supercooling point or SCP.[20,32]

We measured the SCP of snow flies (n = 73 trials) and crane flies (n = 16 trials) from the same cold tolerance experiments described above (Figure 2F). Because they spend most of their adult lives exposed to sub-zero temperatures, we expected that the SCP of snow flies would be significantly depressed compared with summer-active crane flies. The SCP of snow flies was lower than that of other crane flies (−6.6°C ± 1.9°C vs. −4.6°C ± 2.0°C, $p < 0.001$, two-sample t test), but it was also close to or higher than other insect species that are not cold tolerant, such as grasshoppers, weevils, and fruit flies (Figure 2F). For example, the SCP of *Drosophila melanogaster* has been found to be −20°C, although fruit flies are unable to sustain locomotion below 0°C.[27] Thus, snow flies have a unique ability to sustain behavior until the moment they freeze, although they freeze at similar temperatures as summer-active insects.

We also examined the site of ice formation within the snow fly's body. Although the posterior region of the abdomen and distal segments of the tarsi were typically the coldest regions of the snow fly, this was not always where ice nucleation occurred—28% of terminal freezing events originated in the abdomen, 36% in the thorax, and 36% in a leg (Figure 2G).

When we inspected the thermal imaging videos more closely, we noticed that snow flies frequently self-amputated their legs following internal ice formation (Figure 3A; Video S4). Limb self-amputation, also known as autotomy, was relatively common: over half of the snow flies lost at least one leg during our experiments (43/77 flies). Amputation occurred 31% of the time after freezing originated in a leg (60/194 instances). The median number of limbs lost across trials was one, although flies lost as many as five limbs prior to the rest of the body freezing.

Limb self-amputation in our experimental setup followed a typical pattern. The snow fly's leg often became stuck to the plate due to freezing condensation. As the snow fly struggled to free itself, the tip of the leg froze, indicated by a sudden increase in temperature at the tarsus, radiating proximally toward the tibia and femur (Figures 3A and 3B; Video S4). We quantified the time course of ice propagation within the leg (Figure 3B). The median time it took for ice to propagate from the snow fly's tarsus to the tibia was 0.5 s (n = 44 instances). During successful amputations, the leg detached from the body before the wave of ice reached the trochanter. Amputation occurred at a median delay of 2.5 s following ice formation in the tarsus (n = 60 instances). Failure to detach the leg following ice nucleation in the tarsus resulted in ice propagation to the body, which was fatal for the fly (Video S4).

Leg amputation also appears to occur frequently in the wild: nearly 20% (15/77) of the snow flies we collected were missing

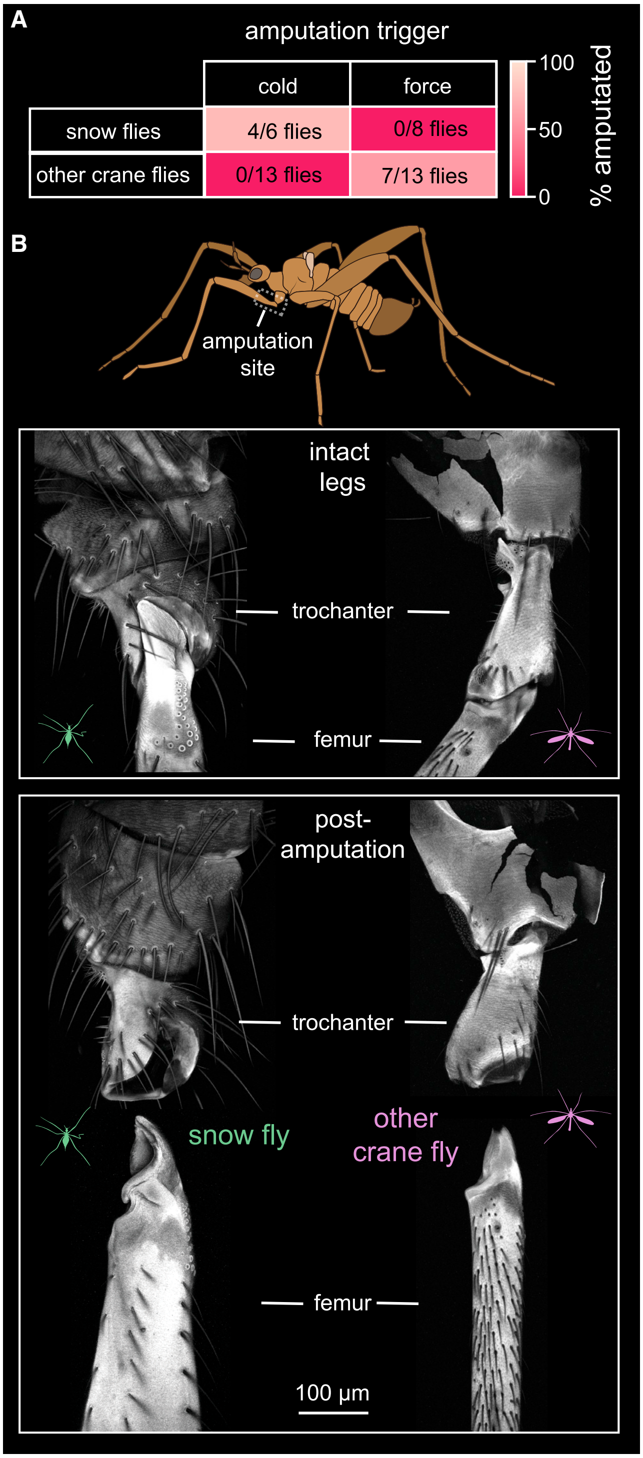

Figure 4. Leg self-amputation occurs at the same site in the leg but is triggered by distinct stimuli in snow flies vs. other crane flies

(A) Snow flies self-amputate legs in response to freezing, but not pulling with forceps. Other crane flies self-amputate legs in response to pulling, but not freezing. The same flies were used for both mechanical and cold plate autotomy experiments. Two snow flies had died prior to the cold plate autotomy experiments.

(B) The morphology and amputation site at the femur/trochanter joint are consistent across snow fly (left) and crane fly (right) legs. Images show cuticle autofluorescence (emission wavelength of 633 nm).

one or more legs. In rare cases, we even observed snow flies effectively navigating complex snowy terrain with only three legs (Video S5).

Since amputation often followed leg freezing, we speculated that this could be a mechanism to prolong snow fly survival under extreme conditions. We found that snow flies that amputated a limb following ice nucleation in the tarsus survived an average of 77 additional seconds before freezing in our experimental setup (Figure 3C). Snow flies that amputated limbs (n = 43 flies) froze at significantly lower cold plate temperatures than those that did not (n = 34 flies) (Figure 3D; two-sample t test, −11.6°C ± 1.2°C vs. −10.7°C ± 2.0°C, p = 0.01). There was no significant difference in SCP between snow flies that amputated legs and those that did not (two-sample t test, −6.8°C ± 1.8°C vs. −6.2°C ± 2.0°C, p = 0.20). Overall, snow flies that amputated frozen legs survived longer and to lower cold plate temperatures.

Crane flies and some other insects can self-amputate legs in response to pulling, such as when they are captured by a predator.[17,18] We tested whether leg self-amputation in snow flies could be initiated by a mechanical stimulus applied to the leg (Figure 4A). We held one of the legs captive using forceps and prodded the captive leg with a mechanical probe (Video S6). The mechanical stimulus failed to produce leg self-amputation in snow flies. However, two-thirds of those same snow flies later lost at least one limb during cold tolerance experiments (Figure 4A). In contrast, over half of the other crane flies self-amputated a limb when we applied the same mechanical stimulus to their legs. We never observed other crane fly species self-amputate legs during cold tolerance experiments (Figure 4A).

Leg amputation in snow flies and other crane flies always occurred at the same point within the leg: the joint between the femur and the trochanter (Figure 4B). The structural similarities across snow flies and other crane flies suggest that amputation is mediated by activation of similar muscles, which have been previously described in other insects.[33] We speculate that this leg self-amputation originally evolved as an anti-predation mechanism in summer-active crane flies and was subsequently co-opted by snow flies to prevent propagation of internal freezing and prolong survival in extreme cold.

DISCUSSION

Using thermal imaging, we found that snow flies can sustain behavior down to the temperature at which they freeze and die. In our experimental setup, fatal freezing occurred at a minimum temperature of −10°C and mean of −6.6°C, which seems to defy the thermal constraints on neuromuscular function that have been well-characterized in other insect species.[34] Unlike snow flies, the other crane flies we tested during summer months were paralyzed above 0°C. The fact that snow flies move and respond to external stimuli under these conditions suggests that they possess adaptations that allow their neurons and muscles to overcome thermodynamic constraints on ion pumps, channel gating, synaptic transmission, and muscle contraction.[35]

Below 0°C, snow fly body fluids are in a supercooled state. Snow flies may produce antifreeze compounds within their hemolymph to depress the SCP.[28,29] However, some summer-active insects are capable of supercooling to −8°C without detectable antifreeze or cryoprotectants.[36] Although we determined that the SCP of snow flies is 2°C lower than summer crane fly species (Figure 2F), this difference suggests that production of antifreeze or cryoprotectants in snow flies is limited. Instead, they likely avoid exposure to temperatures close to their SCP by burrowing into crevices in the snow, where temperatures are consistently close to 0°C. We also found that snow flies are not able to survive freezing, unlike some species of overwintering insects.[37]

Although snow flies are not exceptional in their ability to prevent or survive freezing, they have a unique capacity to maintain neuromuscular function at sub-zero temperatures, a trait shared by few other insect species. Some other species that are known to sustain locomotion at similar minimum temperatures are the snow scorpionfly, *Boreus* spp.,[11] the Tasmanian snow scorpionfly, *Apteropanorpa* spp.,[21] the Himalayan glacier midge *Diamesa* spp.,[38] the ice crawler, *Grylloblatta* spp.,[39,40] and several other species of insects and arachnids.[41–44] Notably, ice crawlers also live in alpine regions of the Pacific Northwest, where they are the only known predator of snow flies.[40,45]

What is the advantage for snow flies in maintaining constant motion until the moment they freeze? In the wild, snow flies may improve their chances of surviving cold night-time temperatures if they search for an insulated place to take refuge under the snow. Air temperature typically fluctuates on a predictable daily cycle but can also change more rapidly during winter storms. The snow fly's ability to sustain movement below freezing may provide them with additional time to locate shelter following sudden changes in weather. Another advantage of sustaining locomotion close to their supercooling limit is that it allows snow flies to disperse and reproduce in an environment that is mostly devoid of other animals, including predators. We and others[46] have observed snow flies audaciously mating in full view on the surface of the snow for 30 min or more.

We found that snow flies rapidly self-amputate freezing limbs to prevent ice from spreading to the rest of the body. To our knowledge, amputation has not been previously described as a mechanism to avoid freezing and prolong survival at cold temperatures. Limb self-amputation occurs in many species, including reptiles, amphibians, mammals, birds, fish, echinoderms, crustaceans, spiders, and insects.[18,47] It is typically used to avoid capture by predators, reduce cost of injury to a limb, escape non-predatory entrapment, or survive complications during molting.[47–50] In snow flies and other crane flies, self-amputation consistently occurs at the joint between the trochanter and femur (Figure 4B). Past researchers have also collected snow flies from the wild with legs broken off at the trochanter.[14] Specialized muscles that control amputation at this joint have been described in stick insects and crickets.[33,51,52] Based on similarities in the breakage plane (Figure 4B) and snow fly leg musculature,[10] snow flies appear to use a similar amputation mechanism.

Although we have never directly observed snow fly leg amputation occur in the wild, nearly 20% of snow flies we collected were missing one or more legs, suggesting that this phenomenon is common. We have also observed dead snow flies that appeared to have frozen as a result of exposure, particularly when an icy crust has coated the surface of the snow. Crust can form when wind, sun, or rain melt the top layer of the snowpack, then cold temperatures make it freeze solid again. We speculate that snow flies may be especially susceptible to leg freezing in cold, crusty conditions, when they are unable to burrow down into the snow for insulation and their legs must come into direct contact with the icy crust.

The key difference between leg amputation in snow flies and other insects appears to be the triggering stimulus. Many insects, including other crane flies, self-amputate legs in response to mechanical stimuli, such as pulling on the leg.[53] The receptors responsible for sensing mechanical stimuli and triggering leg amputation in other insects are likely campaniform sensilla.[33,51] However, we found that mechanical manipulations never triggered leg amputation in snow flies (Figure 4A). We hypothesize that leg self-amputation in snow flies may instead be triggered by thermosensory neurons that detect the temperature increase following ice crystallization of the hemolymph. The rate of ice propagation (0.5 s from the tarsus to the tibia) would provide ample time to execute this leg amputation reflex.

We propose that the ability of ancestral crane flies to self-amputate limbs may have predisposed snow flies to adapt to their unique lifestyle of wandering cold, snowy environments. However, these ecosystems are rapidly changing due to anthropogenic climate change. Washington is on track to lose 46% of its end-of-winter snowpack by the 2040s and 70% by the 2080s, compared with the 20th century average.[54] Loss of snowpack is predicted to increase thermal variability and decrease temperatures within the subnivean layer,[55,56] the thermally stable environment under the snow where snow flies are thought to lay their eggs. Lower temperatures, combined with increased freeze-thaw cycles, can also harm plant life[57] that snow fly larvae consume during spring and summer months. The decrease in snowfall and increased climate variability in the Pacific Northwest and across the planet will likely imperil snow flies and other animals that rely on snow for survival.[58] We may have limited time to study these species before they disappear altogether.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.cub.2023.09.002.

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AUTHOR CONTRIBUTIONS

D.G., J.C.T., and K.L.R. designed and conducted behavioral experiments; A.S. performed confocal imaging of snow fly and other crane fly legs; D.G. and A.S. performed snow fly species identification; D.G. and B.P. developed code for motion tracking and quantification of locomotion; D.G., B.P., and J.C.T. analyzed and interpreted data; D.G. and J.C.T. wrote manuscript with input from all other authors.

DECLARATION OF INTERESTS

The authors declare no competing interests.

INCLUSION AND DIVERSITY

One or more of the authors of this paper self-identifies as an underrepresented ethnic minority in their field of research or within their geographical location. One or more of the authors of this paper self-identifies as a gender minority in their field of research.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
2X Phusion Master Mix	New England Biolabs	https://www.neb.com/products/m0531-phusion-high-fidelity-pcr-master-mix-with-hf-buffer#
Deposited data		
Raw and analyzed data	This paper	https://doi.org/10.5061/dryad.7sqv9s4xz
Experimental models: Organisms/strains		
Chionea spp.	This paper	N/A
Crane flies	This paper	N/A
Oligonucleotides		
LCO1490 forward primer	Integrated DNA Technologies	https://www.idtdna.com/pages
HCO2198 reverse primer	Integrated DNA Technologies	https://www.idtdna.com/pages
Software and algorithms		
FLIR ResearchIR	Teledyne FLIR	https://www.flir.eu/support/products/researchir#Overview
MUSCLE	Edgar[59]	https://doi.org/10.1093/nar/gkh340
FIJI	Schindelin et al.[60]	https://imagej.net/software/fiji/
Data analysis code	This paper	https://github.com/tuthill-lab/Golding_2023
Other		
FLIR T860	Teledyne FLIR	https://www.flir.com/instruments/t-series/

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, John Tuthill (tuthill@uw.edu).

Materials Availability

This study did not generate new unique reagents.

Data and code availability

- Data has been deposited on Dryad: https://doi.org/10.5061/dryad.7sqv9s4xz
- Analysis code is available on Github: https://github.com/tuthill-lab/Golding_2023
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

Male and female adult specimens of *Chionea* spp. and other winged crane fly species were collected from Washington state for use in our experiments, with permission from the US Forest Service. Species of *Chionea* were identified based upon morphology and DNA barcode sequences. All snow flies were individually kept within 5 mL snap-cap centrifuge tubes, provided a drop (about 0.05 mL) of maple syrup diluted in water, and housed in a refrigerator at 1 °C. All other crane flies were individually housed at room temperature (approximately 20 °C) in plastic food storage containers lined with wetted paper towels.

METHOD DETAILS

Chionea collection and species identification

256 adult specimens of *Chionea* spp. were collected from October of 2020 to late November of 2022. Location coordinates for each collected snow fly are included in Data S1A. Generally, specimens were found from 10 am to 4 pm during overcast and sunny days after snow had fallen the previous night. On average, flies were captured at a temperature of 0 °C, based on weather data from the Visual Crossing Weather Query Builder (https://www.visualcrossing.com/weather/weather-data-services). Specimens were collected at an average elevation of 1485 m (minimum of 224 m, maximum of 3428 m). Snow depth data in Figure 1B is from the US National Operational Hydrological Remote Sensing Center (https://www.nohrsc.noaa.gov/earth/). After completing experimental trials, dead snow flies were preserved in 95% ethanol at -20 °C. We examined the morphology of 101 dead flies to identify species as described in Byers[12]. We subsequently used DNA barcode sequencing to confirm our species identifications by comparing a 640 bp region of cytochrome c oxidase I subunit I (COI-5P) from each fly. We extracted genomic DNA from leg tissue (n = 245) using 10.1 μL of 100 mM of Tris-Cl (pH 8.0), 1 mM EDTA, 25 mM NaCl, and freshly added 2 mg/mL proteinase K. Each sample was incubated at 37 °C overnight, followed by 5 minutes at 95 °C to inactivate proteinase K. Extracted DNA was then amplified using PCR. We accomplished this by adding 1 μL of DNA after extraction to a solution of 8.8 μL H_2O, 2X Phusion Master Mix, and 0.1 μL of forward primer (LCO1490) and 0.1 μL of reverse primer (HCO2198). Amplified DNA was sent to the Genewiz laboratory in Seattle, Washington for Sanger sequencing. Traces were uploaded to the Barcode of Life Data System (BOLDSystems) online database where a taxon ID tree was generated using the MUSCLE alignment algorithm.[59] The taxon tree included five branches that correlated with our morphological species identifications: *C. alexandriana*, *C. albertensis*, *C. jellisoni*, *C. excavata*, *and C. macnabeana*. Of these species, *C. alexandriana* was most common (68% of captured flies), followed by *C. excavata* (18%), *C. albertensis* (4%), *C. jellisoni* (4%), and *C. macnabeana* (3%). Behavioral experiments used a subsample of 77 flies from four species in Washington State collected from December of 2020 to November of 2022 (*C. alexandriana*, *C. albertensis*, *C. excavata*, *and C. macnabeana*; Figure 1D).

Collection and species identification of other crane flies

Adult crane fly specimens (n = 26) were collected from late May to early September of 2022 in Seattle, Gig Harbor, and Toledo, Washington (Data S1B). Species identification for the crane fly depicted in Figure 4B was determined using the snow fly DNA barcode sequencing protocol described above and the BOLDsystems identification engine. From the cytochrome c oxidase subunit 1 (COI) full database of barcoding sequences, we found a 99.68% match in sequence similarity to a specimen identified as *Austrolimnophila* spp. (specimen ID MPG2107-22) sequenced at the Centre for Biodiversity Genomics at Guelph, Ontario CA.

Infrared imaging and temperature measurements

We assessed the cold tolerance ability of snow flies (n = 77 flies) and other crane flies (n = 16 flies) by placing them on a TECA Model AHP-301CPV cold plate preset to cool at a rate of 0.80 °C/min for 25 minutes, after which the temperature increased to 2 °C. Each trial took place in a cold room at an ambient temperature of 5 °C. To prevent escape, all flies were held within an aluminum ring coated with Rain-X or liquid graphite lubricant. On occasion, a paint brush or canned air was used to keep the fly within the ring. Each trial was recorded using a FLIR T860 infrared camera (30 FPS) elevated 22 cm from the surface of the cold plate. The object and atmosphere parameters were adjusted from factory settings to account for the distance of the lens to the surface of the cold plate, the reflected temperature of the cold plate, the atmospheric temperature of the cold room, and the relative humidity of the cold room. This was necessary to ensure accuracy of temperature readings. To validate the accuracy of the thermal camera, we attached a Physitemp MT-29/1HT needle microprobe to 10 crane flies using UV glue or Vaseline and recorded their surface temperature using an Onset HOBO UX120-014M 4-Channel thermocouple data logger throughout the trial (Figure S1). After each trial was complete, the supercooling point (SCP) was determined using the FLIR ResearchIR program with a 1x1 pixel ROI centered on the abdomen of each fly. The supercooling point was indicated by a rapid temperature increase of at least 1 °C within the fly's body. Visually, the fly was observed to rapidly change color when monitoring the video in real time or using the ResearchIR software, as indicated in Figures 2B and 2E. The survival of the fly and number of legs lost was recorded after the trial. In Figures 2B and 2E, we determined the temperature of a representative snow fly (SF0181; Data S1A) in a downsampled thermal video (0.5 fps) by finding the minimum temperature of the abdomen within a 3x3 pixel ROI based on its 2D position. Note that the temperature values were extracted from frame-specific temperature maps, which we downloaded from the FLIR ResearchIR software.

Inducing autotomy by mechanical stimulation of the leg

To provoke leg autotomy, we briefly anesthetized each fly using CO_2 and gently grasped the tarsal segment of one leg using pean forceps. Anesthetization was necessary to slow the fly and ensure accurate capture of the leg by forceps. Based upon previous observations that all limbs are capable of detachment, we grasped any available limb for the experiment. When the fly had regained locomotor ability after anesthetization, the tibia and tarsus of the captive limb was repeatedly prodded using thumb forceps or a fine paint brush for one minute. This was repeated every five minutes for 15 minutes. Snow fly trials (n = 8 flies) took place in the same cold room used for thermal imaging (5 °C). Crane fly trials (n = 13 flies) took place outside of the cold room (21 °C). Additionally crane fly wings were clipped to prevent flight. Some flies autotomized their legs during the initial grasping of the leg with forceps. These were recorded as instances of autotomy. Afterward, we conducted cold tolerance experiments using the same flies to see whether freezing initiated autotomy. Two snow flies had died between experiments, reducing the number of flies used for the cold tolerance experiments (n = 6).

Motion tracking and quantification of locomotion

To determine body velocity and trajectory of the snow fly in Figures 2B and 2C, we manually tracked the abdomen of a representative snow fly (SF0181, Data S1A) in a thermal video that was downsampled from 30 fps to 0.5 fps. We converted the position of the abdomen in each frame from pixels to millimeters using a conversion factor based on the measured diameter of the aluminum ring in pixels and millimeters (600 pixels and 70 millimeters, respectively). Instantaneous body velocity was calculated in python using the following equation:

$$\textit{Instantaneous Velocity} = \frac{\sqrt{\Delta X^2 + \Delta Y^2}}{\Delta t}$$

Where ΔX and ΔY are the frame-by-frame change in the 2D position of the fly and Δt is the time interval between frames.

Locomotor ability of snow flies and other crane flies was assessed by examining whether snow flies (n = 17 flies) and crane flies (n = 5 flies) engaged in coordinated movement at 4 °C cold-plate temperature intervals during a 25-minute imaging session. The temperature was assessed using a 3x3 ROI cursor fixed on the cold plate. We manually scored fly locomotion within 6 different windows (cold plate temperatures of 3, -1, -9, -13, -17 +/- 0.5 °C).

Confocal imaging of intact and detached legs

Intact and autotomized legs were mounted in VECTASHIELD media (Vector Laboratories) and imaged with a 20x objective on a Confocal Olympus FV1000 using a far-red laser (633nm) to collect z-stacks of cuticle auto-fluorescence. We processed maximum-projection images in FIJI.[60]

QUANTIFICATION AND STATISTICAL ANALYSIS

We used scripts written in Python to perform analyses used for Figures 2 and 3. We tested for statistically significant differences between the two groups using two sample t-tests (Figures 2 and 3), performed using Python and Google Sheets.