living close to the equator. Those that are ectotherms, including insects, are generally unab

(7; 2)% =ᵢ The metric temperature scale introduced by Anders Celsius is based on prominent phase changes of water; freezing at 0 °C and boiling at 100 °C at 1 atmosphere pressure. (**F**) Water phase changes, solid from vapour, vapour from liquid, and liquid from solid, reflect temperature and pressure. These meet at a single point called the triple point, where all three phases can coexist. (**C**) However, supercooling of water occurs when temperature reduction below 0 °C is not accompanied by crystallization. In biological systems the formation of ice decreases the density by nearly ~9%, leading to an increase in volume of the crystalline structure (ice) that can disrupt tissues. Density changes are illustrated over the ~40 °C range likely to be experienced by New Zealand alpine insects. (**9**) Droplet size influences the temperature at which water phase change occurs (liquid to ice) as demonstrated by Heverly (1949) [14] who cooled water droplets of different sizes to measure their tempe

(7;2)% !@ Insects that are freeze

contribution of gut microbes to cold adaptation is now being recognized in a range of animal hosts [57]. For example, brown bears benefit from their gut microbes during hibernation, and mice inoculated with bear microbes gain a similar metabolic phenotype [58]. In insects, freezing is typically initiated in the gut [24,30,59], and ice^{M} activity is greater in the gut contents of many insects than in their haemolymph [60,61] (Figure 3). Thus, the gut and its microbiome may be the key to understanding freeze-tolerance in a range of invertebrates (Figure 3).

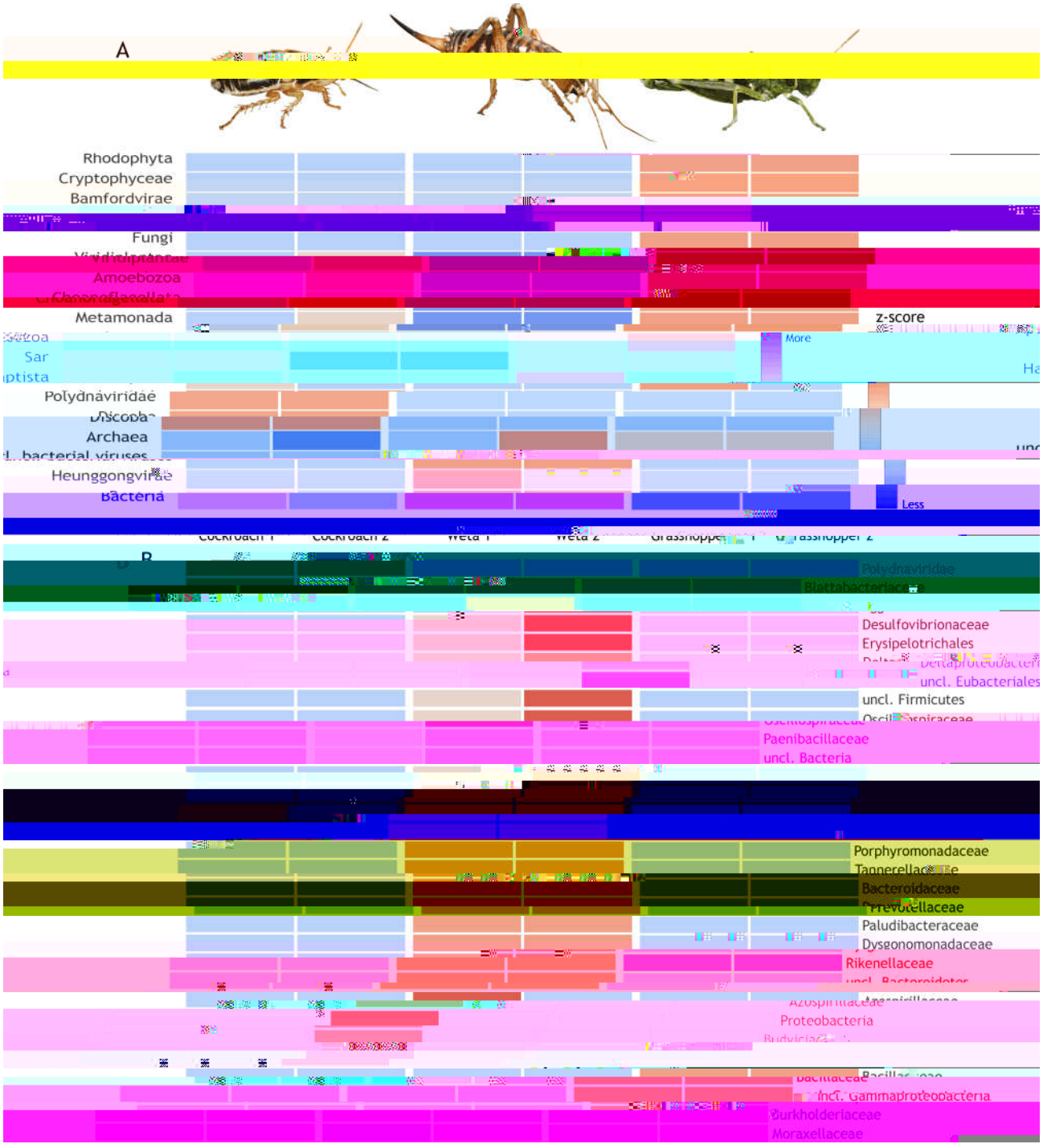
Insects can shape their gut microbiota by ingesting food containing beneficial microbes or ice^{+}

(7; 2)% T Three freeze-tolerant endemic Aotearoa-New Zealand insect species and their phylogenetic relatives. (**F**) The Otago alpine cockroach ( ) is a nocturnal omnivore. Alpine lineages are not monophyletic within New Zealand (denoted with blue star on tree). (**C**) The mountain tree wētā ( ) is a nocturnal omnivore. Alpine lineages are not monophyletic within the wētā clade. (**9**) The southern alpine grasshopper ( ) is a diurnal herbivore within a cold-adapted endemic lineage. Phylogenetic relationships were reconstructed from (**F**) unpublished mtDNA genome sequences, (**C**) 755 transcriptomes [81].

Ice formation in this cockroach is thought to be intercellular, but gut cells of this species are unusual in their ability to survive intracellular freezing [18,59,90,91]. Because the gut is a closed system, the avenues available for ice to reach the haemolymph are limited, but a possible pathway is through the cells forming the wall of the gut. When freezing, 74% of an alpine cockroach's body water is converted into ice [76]. Both thermal-hysteresis (the lagging of freezing) and ice recrystallisation-inhibition activities are absent from the haemolymph of , although both types of chemical activity occur in its gut tissue [59]. Preliminary analysis of whole cockroach ice shell extracts showed evidence for three groups of ice-binding proteins; two small (8.4 kDa and 9.3 kDa) and one larger (>50 kDa; unpublished data).

, /8.% =@ Comparison of critical temperatures for three alpine insects from Aotearoa-New Zealand.

, /R-#	DI%&7%$	, %HI%)/'2)% -0 9):$'/..7*/'7-# X9	Y-1%) Y%'5/. , %HI%)/'2)% X9



(7;2)% U Gut microbiome of six New Zealand freeze-tolerant insects inferred from shotgun DNA sequencing. Heatmaps generated using MEGAN v6.24.1 [105] and visualised as a Z-score for the two samples from cockroaches ( ), wētā ( ), and grasshoppers ( ) from Central Otago. Data are clustered by sample composition similarity (above) and proportions of the data (right) showing all assigned nodes. The six samples were analysed using the Kaiju webserver (using the nr-euk 2021_03 database), downloaded and processed with a custom Perl script. Results are (**F**) visualised at the taxonomic level of kingdom with only data above a normalised cut-off of 1 read per million sequences shown, and (**C**) visualised at the taxonomic level of family with only data above a normalised cut-off of 100 reads per million sequences shown.

<%0%)%#&%$

1. Lisovski, S.; Ramenofsky, M.; Wingfield, J.C. Defining the degree of seasonality and its significance for future research. . =\, , 934–942.
2. Denlinger, D.L. Regulation of diapause. . , , 93–122.
3. Tougeron, K. Diapause research in insects: Historical review and recent work perspectives. =], , 27–36.
4. Fielding, D.J. Diapause traits of and (Orthoptera: Acrididae). ^, , 439–448.
5. Sinclair, B.J. Insect cold tolerance: How many kinds of frozen? . =]]], , 157–164.
6. Graether, S.; Kuiper, M.; Gagné, S.M.; Walker, V.K.; Jia, Z.; Sykes, B.D.; Davies, P.L. β-Helix structure and ice-binding properties of a hyperactive antifreeze protein from an insect. , , 325–328.
7. Toxopeus, J.; Sinclair, B.J. Mechanisms underlying insect freeze tolerance. =^, , 1891–1914.
8. Poikela, N.; Tyukmaeva, V.; Hoikkala, A.; Kankare, M. Multiple paths to cold tolerance: The role of environmental cues,

36. Wharton, D.A.; Selvanesan, L.; Marshall, C.J. Ice-active proteins from New Zealand snow tussocks, and . = , , 239–48.
37. Lee, R.E.; Costanso, J.P.; Mugnano, J.A. Regulation of supercooling and ice nucleation in insects. =]]U, , 405–418.
38. Wilson, P.W.; Heneghan, A.F.; Haymet, A.D.J. Ice nucleation in nature: Supercooling point (SCP) measurements and the role of heterogeneous nucleation. !, , 88–98.
39. Hill, T.C.J.; Moffett, B.F.; Demott, P.J.; Georgakopoulos, D.G.; Stump, W.L.; Franc, D. Measurement of ice nucleation-active bacteria on plants and in precipitation by quantitative PCR. =Q, , 1256–1267.
40.

67. Hosokawa, T.; Kikuchi, Y.; Nikoh, N.; Shimada, M.; Fukatsu, T. Strict Host-Symbiont Cospeciation and reductive genome evolution in insect gut bacteria. U, , e337.
68. Kikuchi, Y.; Hosokawa, T.; Nikoh, N.; Meng, X.-Y.; Kamagata, Y.; Fukatsu, T. Host-symbiont co-speciation and reductive