Table S1. Summary of literature data on maximum ice fraction in various invertebrates.

Species and taxonomic affiliation	Geographic origin and acclimation status	IF_{max} * (% TBW)	Reference
Insecta			
Eleodes blanchardi (Coleoptera)	Norway, field-collected, overwintering adult	75	Zachariassen et al., 1979
Eurosta solidaginis (Diptera)	Ohio, New York, field-collected, overwintering larva	64	Lee and Lewis, 1985
Hemideina maori (Orthoptera)	New Zealand, field-collected, cold acclimated	82	Ramlov and Westh, 1993
Melasoma collaris (Coleoptera)	Norway, field-collected, cold acclimated	77	Gehrken and Southon, 1997
	ibid., warm-acclimated in lab.	84.5	
Celatoblatta quinquemaculata (Blattodea)	New Zealand, field-collected, overwintering adult	74	Block et al., 1998
Heleomyza borealis (Diptera)	Arctics, field-collected, overwintering larva	81	Worland et al., 2000
Tardigrada			
Adorybiotus coronifer	Greenland, field-collected in winter	79	Westh and Kristensen, 1992
	Greenland, field-collected in summer	89	
	ibid., cold-acclimated in lab.	83	
Amphibolus nebulosus	Greenland, field-collected in summer	88	
Halobiotus crispae	Denmark, field collected, active stage	69	Halberg et al., 2009
	ibid., diapause P1 stage	59	
Macrobiotus sapiens	Croatia, lab culture, active, starved	84.5	Hengherr et al., 2009
	ibid., cold-acclimated	81.1	
Paramacrobiotus richtersi	Germany, lab culture, active, starved	85.7	
	ibid., cold-acclimated	82.5	
Macrobiotus tonollii	Oregon, lab culture, active, starved	86.4	
	ibid., cold-acclimated	81.2	
Milnesium tardigradum	Germany, lab culture, active, starved	85.1	
	ibid., cold-acclimated	80.5	
Echinscus granulatus	Germany, field-collected, active, fed	83.8	
	ibid., cold-acclimated	78.0	
Echinscus testudo	Germany, field-collected, active, fed	84.3	
	ibid., cold-acclimated	80.5	
Nematoda			
Panagrolaimus davidi	Antarctics, lab culture, warm-acclimated,	82	Wharton and Block, 1997
Anelida			
Buchholzia apendiculata	Austria, field-collected, spring active	18.1	Block and Bauer, 2000
Buchholzia simplex		60.1	
Enchytraeus buchholzi		17.0	
Enchytraeus albidus		64.3	
Henlea ventriculosa		40.1	
Fridericia tubulosa		65.6	
Enchytraeus albidus	Germany, lab culture, acclimated to 5°C	61.4	Patricio Silva et al., 2013
	Greenland, lab culture, acclimated to 5°C	58.8	

* The IF_{max} was measured by gradual layer calorimetry (Zachariassen et al., 1979), or by a custom-made calorimeter registering heat absorbed during melting of frozen sample (Lee and Lewis, 1985), or by differental scanning calorimetry (DSC, most other studies). The ^{1}H nuclear magnetic resonance was used to determine the amount of unfrozen water in one study (Gehrken and Southon, 1997).

Table S2. Optimization of cryopreservation protocol for *Chymomyza costata* larvae.

Assay	Parameter value	n (replicates)	Survival (%)		
			larvae	puparia	adults
Control 1:	manipulation only	233 (4)	100	92,0	85,6
Control 2:	freezing only: -30°C / 1h	100 (2)	100	87,0	76,0
Cryopreservation protocols with variable values of assessed parameters:					
Rate r_1	1,5°C / min	80 (1)	55,0	0	0
	1°C /min	60 (1)	35,0	0	0
	0,5°C / min	60 (1)	73,4	13,3	6,7
	0,25°C / min	60 (1)	76,7	13,3	11,7
	0,17°C / min	60 (1)	95,0	28,3	23,3
	0,10°C / min	440 (7)	96,8	53,6	39,3
	0,05°C / min	60 (1)	83,3	28,3	13,3
Rate r_2	2 - 4°C / sec	440 (7)	96,8	53,6	39,3
	10 - 20°C / sec*	50 (1)	56,0	22,0	10,0
	20 - 60°C / sec**	20 (1)	45,0	0	0
Rate r_3	not assessed, maintained invariably between 1 - 2°C / sec				
Rate r_4	0,1°C /min	300 (3)	97,4	55,7	39,8
	0,2°C / min	60 (1)	98,4	43,3	30,0
	0,6°C / min	440 (7)	96,8	53,6	39,3
	1,2°C / min	60 (1)	92,4	15,0	0
Temperature T_1	-5°C	60 (1)	0	0	0
	-5°C*	30 (1)	0	0	0
	-10°C	60 (1)	0	0	0
	-15°C	80 (1)	0	0	0
	-20°C	269 (4)	16,3	0	0
	-22°C	120 (2)	68,4	7,5	5,9
	-24°C	220 (3)	79,5	10,6	7,8
	-26°C	220 (3)	92,6	13,4	8,9
	-28°C	120 (2)	98,4	10,0	5,9
	-30°C	440 (7)	96,8	53,6	39,3
	-35°C	160 (2)	100,0	45,7	28,2
	-40°C	340 (4)	98,1	23,7	15,2
Temperature T_2	-30°C	440 (7)	96,8	53,6	39,3
	-20°C	60 (1)	100,0	31,7	23,3
	-10°C	280 (3)	95,4	63,8	39,9
	-5°C	60 (1)	96,7	5,0	1,7
	0°C	60 (1)	78,3	0	0
	r.t.	40 (1)	65,0	0	0
Storage of *C. costata* larvae in LN_2 (cryopreserved using the optimal protocol) for various periods of time:					
Assay	Storage time	n (replicates)	larvae	puparia	adults
Optimal protocol	1 hour	440 (7)	96,8	53,6	39,3
	2 months	40 (1)	97,5	57,5	42,5
	4 months	80 (1)	95,0	22,5	10,0
	6 months	40 (1)	97,5	55,0	42,5
	9 months	180 (1)	97,2	20,6	13,3
	18 months	160 (1)	92,5	13,8	5,6

n, total number of larvae assessed. Number of biological replicates is shown in parentheses. Generally, variation in adult survival was relatively high not only between generations (biological replicates) abut also between replicated tubes of the same generation (technical replicates). For instance, the optimal protocol assay was replicated in (7) different generations with the following results (mean±S.D., range): larvae, 96.8±1.9, 93.3-98.8; puparia, 53.5±12.3, 41.7-70.0; adults, 39.4±13.5, 11.7-58.3. Because most other treatments were replicated only once or few times, we show final survival as a percentage of survivors from all individuals (generations pooled).

***,** larvae placed into copper capillary (5 cm long, 1.2 mm inner diameter, filled with distilled water), plunged to LN_2

****,** larvae placed into copper capillary, plunged to liquid propane held at -196°C

All parameters were assessed one by one. The values of the assessed parameter varied as shown in the Table (left column), while all other parameters were kept optimal. The optimal protocol is highlighted in blue.

Table S3. Metabolomic profiles in *Chymomyza costata* larvae of four acclimation variants.

	LD		SD		SDA		LD Pro50	
3-Alanine	1,19	0,00	t		t		t	
Alanine	6,69	0,54	6,98	0,48	3,42	0,12	4,26	0,67
Arginine	6,68	0,75	6,43	0,14	7,84	0,62	6,19	0,81
Asparagine	4,91	0,24	20,92	1,97	17,38	1,70	15,52	1,15
Aspartate	0,27	0,03	0,24	0,01	t		0,71	0,10
Cysteine	0,34	0,05	t		t		t	
Glutamine	19,43	1,24	120,61	10,05	24,96	5,12	153,89	57,83
Glutamate	8,63	0,47	5,47	0,35	6,26	0,26	7,55	0,76
Glycine	1,69	0,17	0,60	0,10	1,35	0,09	0,49	0,07
Histidine	7,23	0,79	1,53	0,23	2,07	0,32	t	
Isoleucine	1,10	0,18	0,64	0,03	0,59	0,05	0,74	0,04
Leucine	1,21	0,17	0,76	0,08	0,67	0,06	0,76	0,06
Lysine	1,72	0,30	3,50	0,46	1,57	0,14	1,06	0,24
Methionine	0,36	0,05	t		t		t	
Phenylalanine	0,88	0,02	0,57	0,05	0,63	0,04	0,75	0,08
Proline	31,41	2,11	161,75	12,75	339,08	19,31	487,02	22,95
Serine	1,91	0,25	2,12	0,31	1,48	0,15	1,22	0,19
Threonine	3,31	0,48	0,62	0,13	0,64	0,05	1,17	0,15
Trytophan	0,62	0,07	1,32	0,16	0,72	0,06	0,52	0,08
Tyrosine	5,55	0,93	3,17	0,04	2,58	0,20	2,11	0,37
Valine	2,18	0,26	1,01	0,04	0,84	0,13	1,08	0,23
Ornithine	0,26	0,02	0,31	0,06	t		t	
Sarcosine	0,34	0,03	0,25	0,05	0,38	0,04	0,00	0,00
Citrate	3,87	0,36	6,96	0,29	4,46	0,50	6,31	1,16
Ketoglutarate	t		0,73	0,14	t		t	
Malate	2,17	0,19	1,60	0,06	0,99	0,11	2,51	0,16
Succinate	1,23	0,35	t		t		t	
Lactate	2,95	0,18	2,29	2,24	0,58	0,10	t	
Glycerol	0,19	0,01	0,16	0,02	0,23	0,04	0,32	0,13
Erythritol	0,04	0,00	0,14	0,01	0,18	0,04	0,29	0,08
Mannitol	t		0,05	0,01	0,04	0,00	0,18	0,08
Sorbitol	t		0,47	0,04	0,60	0,03	2,28	0,81
chiro-Inositol	0,21	0,02	0,16	0,01	0,30	0,02	0,23	0,05
myo-Inositol	0,13	0,01	0,09	0,01	0,12	0,01	0,22	0,02
Fructose	0,37	0,02	0,68	0,02	0,77	0,08	1,43	0,37
Glucose	2,05	0,32	0,40	0,06	0,37	0,04	0,61	0,10
Trehalose	23,54	3,25	22,87	1,31	52,36	3,61	50,55	1,01
Total	143,62	9,76	374,29	27,73	473,48	24,85	749,98	80,54

Each value represents mean ± S.D. of four biological replicates (each consisting of a pool of 5 larvae).
All data are expressed as mmol.kg^{-1} TBW. Three most abundant metabolites are highlighted in yellow fields.
t, traces of metabolite present (below the threshold for reliable quantitation).

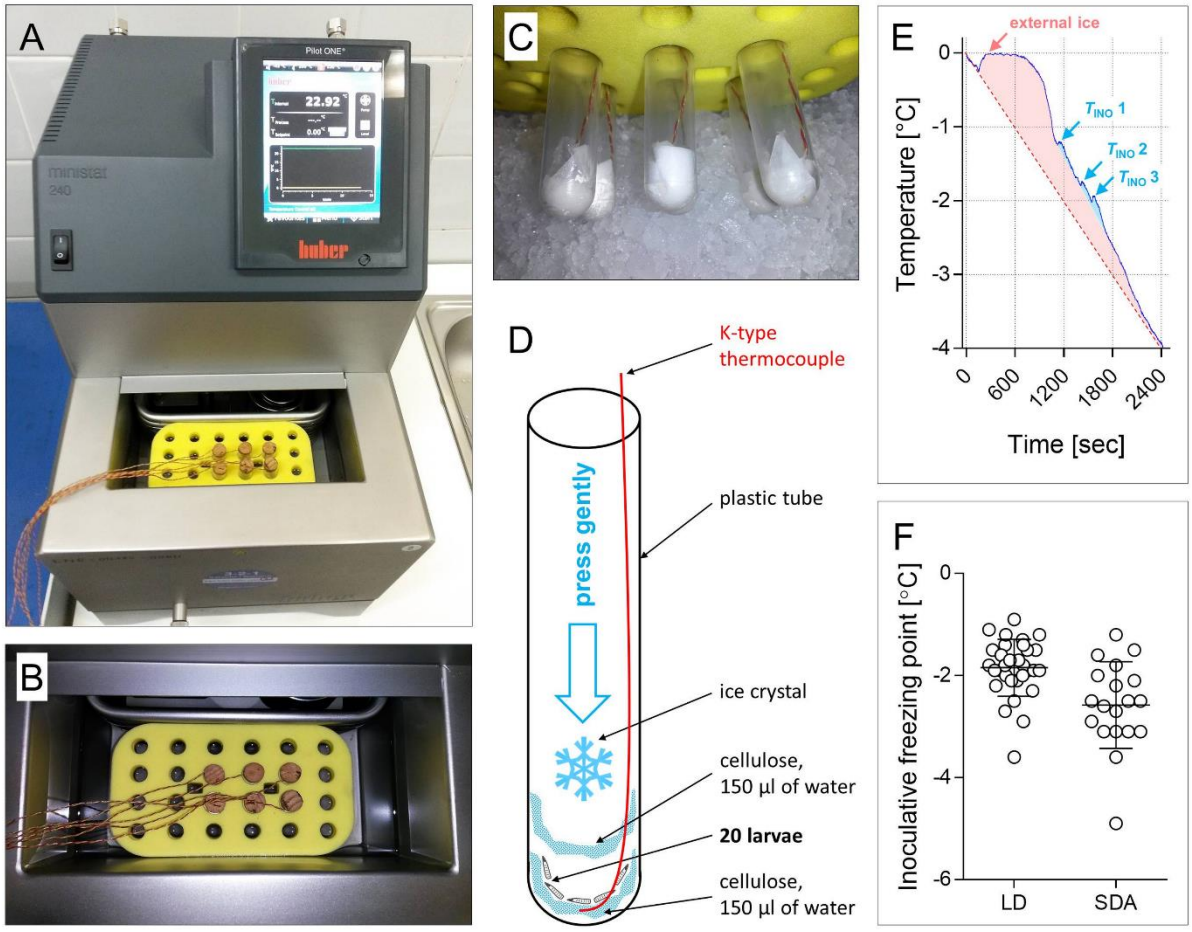

Fig. S1. Ice nucleation of larval body fluids with external ice crystals in Ministat 240 cooling circulator, Huber.

(A-C) Photographs show placement of six plastic test tubes (1 cm in diam., 5 cm long), each containing different number of larvae (5 – 20) depending on experiment, inside the Huber Ministat. The tubes were inserted into holes of a floating island and submerged to cooling medium (Thermofluid SilOil, M60 115/200.05, Huber). (D) Schematic view of the arrangements inside each plastic test tube. Larvae were placed on a small piece of cellulose (75 mg) that was moistened with 150 uL of distilled water. Similar piece of moistened cellulose was placed over the larvae and slightly pressed, which ensured that all larvae were in a tight contact with moisture. The thermocouple was mounted in between the two pieces of cellulose. A small ice crystal was added on top of wet cellulose, the tube was closed using cork plug and temperature program was started in Huber Ministat. (E) An example of temperature record (PicoLog TC-08 datalogger) in an experiment where five larvae of *Chymomyza costata* (acclimation variant LD) were slowly cooled (cooling rate 0.1ºC min^{-1}) in the tube arranged as described on D). Large freeze exotherm (red area) belongs to external water in cellulose. Three small freeze exotherms (blue areas) were detected, which belong to three larvae seeded by external ice crystals. (F) Results of replicated experiments as described in E). The *Chymomyza costata* larvae of two acclimation variants were measured: LD (n = 40), SDA (n = 25) (see Table 1 for detailed description of acclimation variants). All recorded larval freeze exotherms (inoculation freezing points, T_{INO}) are shown: LD (n = 31; mean = -1.85; S.D. = 0.56), SDA (n = 19; mean = -2.58; S.D. = 0.85), the means of LD and SDA group are statistically different (t-test, t = 3.680, P = 0.0006).

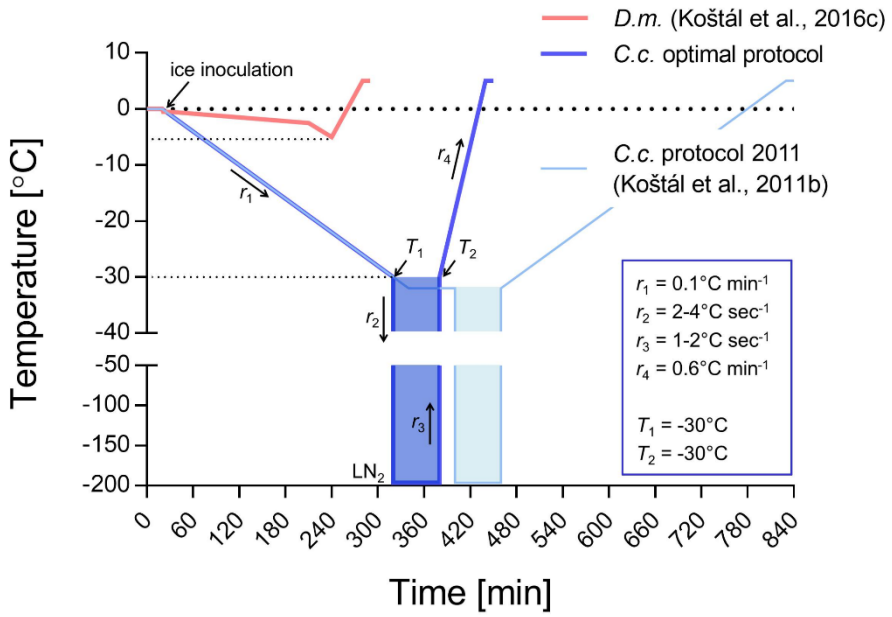

Fig. S2. Freezing and cryopreservation protocols.

In all protocols, larval freezing was initiated by contact with external ice crystals (ice inoculation) at relatively high sub-zero temperatures (see Fig. S1). The protocol used for larvae of *Drosophila melanogaster* (*D.m.*, red line) was previously optimized and published in Koštál et al. (2016). The protocol used previously for cryopreservation of larvae of *Chymomyza costata* (*C.c.*, pale blue line, Koštál et al., 2011) was further optimized in this study (Table S1) and the optimum parameters (ensuring the highest survival of adults) are listed in the blue frame and shown graphically as dark blue line (the rates of cooling/heating are shown as r_1, r_2, r_3 and r_4. At temperature T_1, frozen larvae are plunged into liquid nitrogen (LN$_2$), and, later, returned to Huber Ministat pre-set to T_2 temperature).

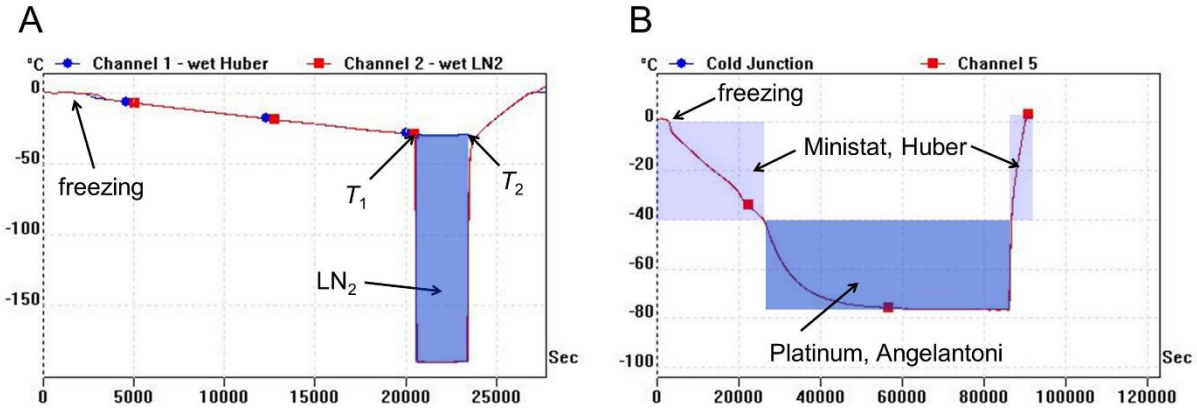

Fig. S3. Examples of temperature record from PicoLog TC-08 datalogger.

(A) Freezing to -30°C / 1h (Channel 1, blue line) and freezing to a T_1 = -30°C followed by 1h in liquid nitrogen (LN$_2$, Channel 2, red line). Note that both channels show freeze exotherms (water in the cellulose wrapping) extending over approximately 1st hour of slow cooling (rate 0.1°C min^{-1}). External ice crystals inoculate larvae inside the wrapping (see Fig. S1 E). (B) Slow freezing to -40°C in Ministat (Huber) followed by transfer to Platinum freezer (Angelantoni), where the sample gradually cooled to -75°C (Channel 5, red line).

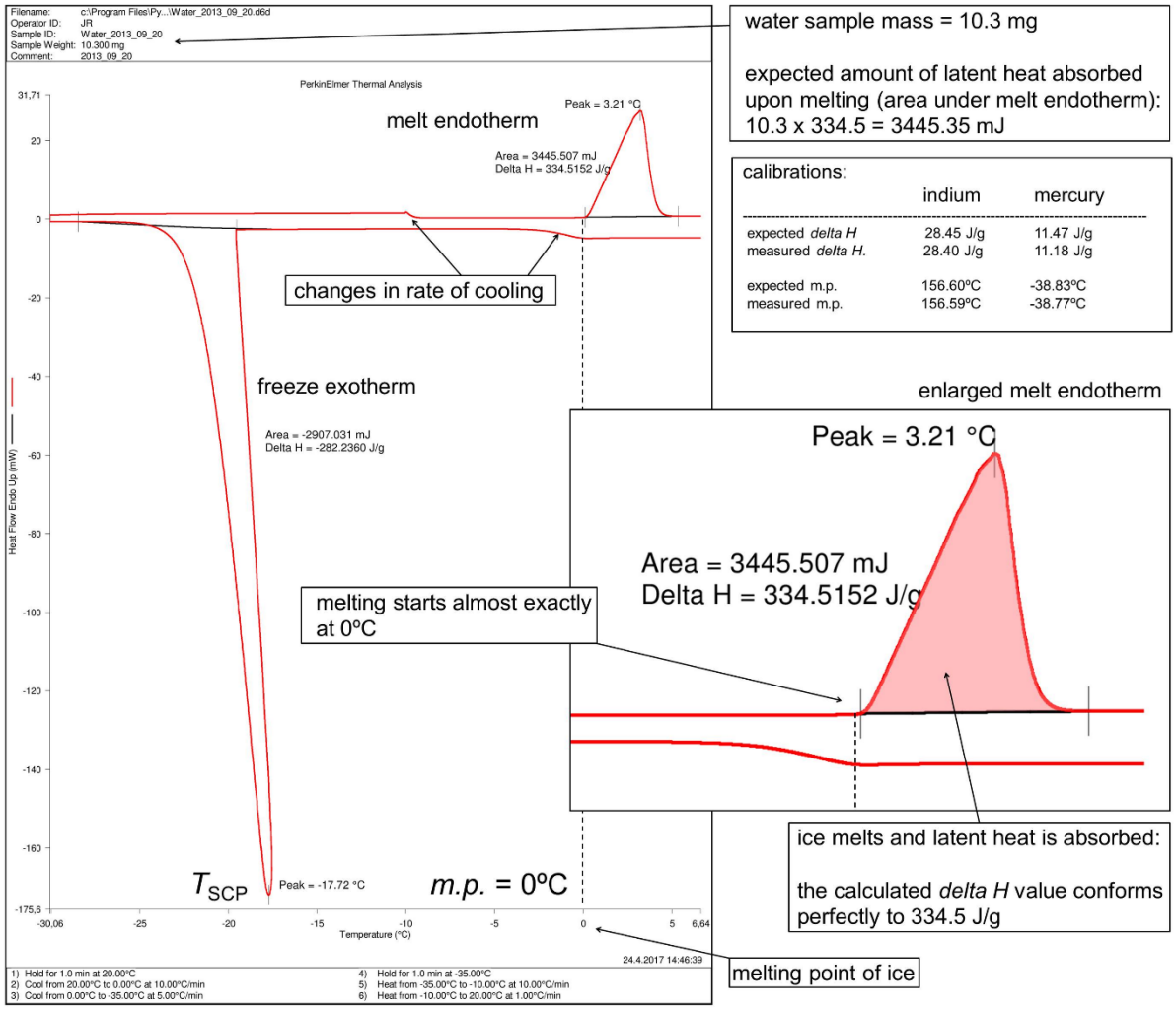

Fig. S4. DSC calibration: example protocol (distilled water).

The temperature scale and the heat flow of DSC4000 instrument were calibrated using indium, mercury and distilled water standards. Calibration parameters for indium and mercury are shown in the frame (right). An example protocol (left) shows that running the thermal analysis of distilled water sample (10.3 mg) returns expected values of melting point (m.p., 0ºC) and specific enthalpy of ice/water transition (*delta H*, 334.5 J g^{-1}) (Wang and Weller, 2011).

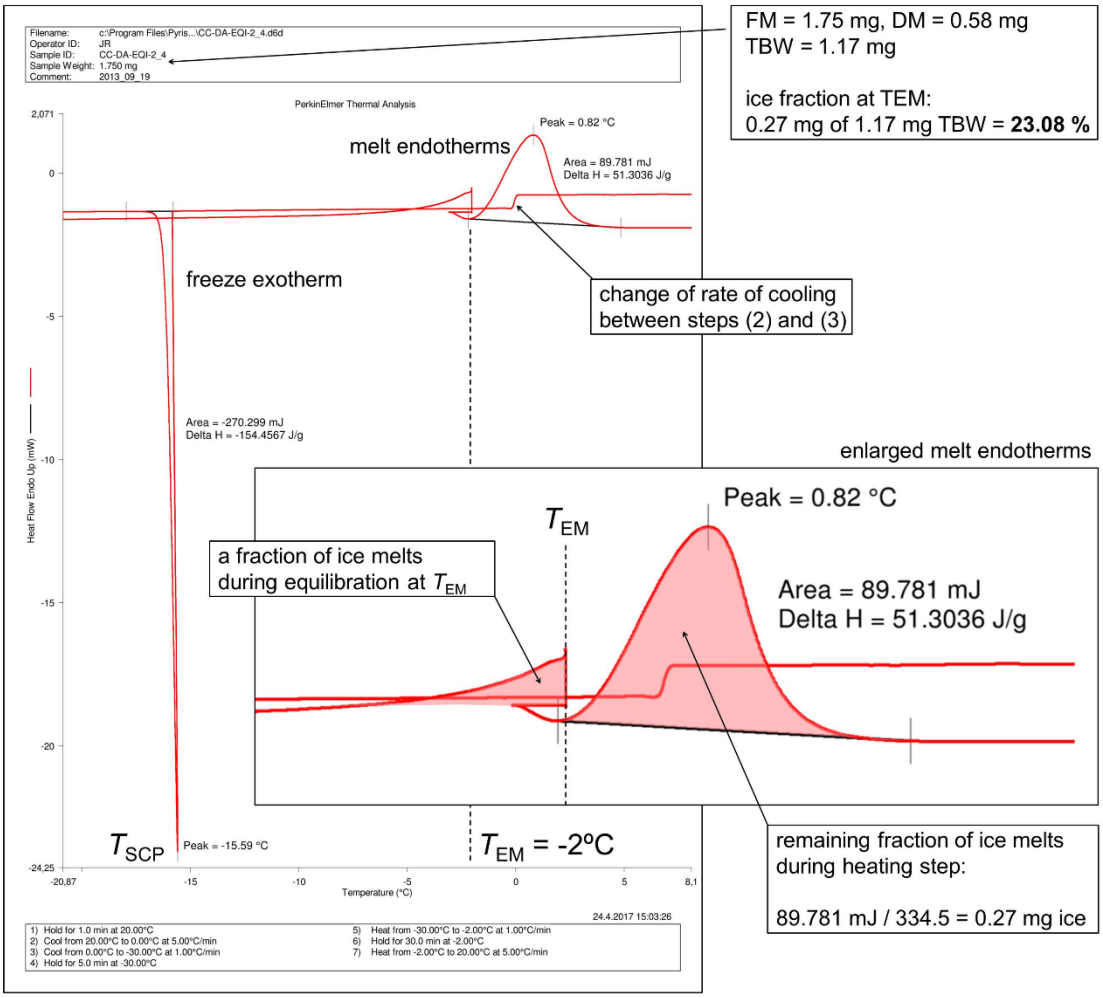

Fig. S5. *Equi-melt* thermal analysis: example protocol (*C. costata*, SDA).

The *Equi-melt* temperature programme: (1) hold for 1 min at 20ºC; (2) cool to 0ºC at a rate 5ºC min^{-1}; (3) cool to -30ºC at a rate 1ºC min^{-1}; (4) hold for 5 min at -30ºC; (5) heat to *Equi-melt* temperature (T_{EM}) at a rate 1ºC min^{-1}; (6) hold for 30 min at T_{EM}; (7) heat to 20ºC at a rate 5ºC min^{-1}.

Notes:

steps (3 and 4): the temperature of -30ºC was sufficient to reach maximum ice fraction in all treatments. We verified in preliminary experiments that neither exposing the larvae to lower temperature (down to -70ºC), nor extending the time of exposure at -30ºC (up to 48 hours) did further increase the ice fraction;

steps (5 and 6): the ice fraction equilibrates to specific T_{EM} that varied from -30ºC to -0.5ºC (in addition, T_{EM} of -50ºC was analyzed for *C. costata* SDA variant, see Fig. S7 C);

step (7): the ice fraction was calculated from the area under melt endotherm using the value of *delta H* = 334.5 J g^{-1} as the enthalpy of ice/water transition.

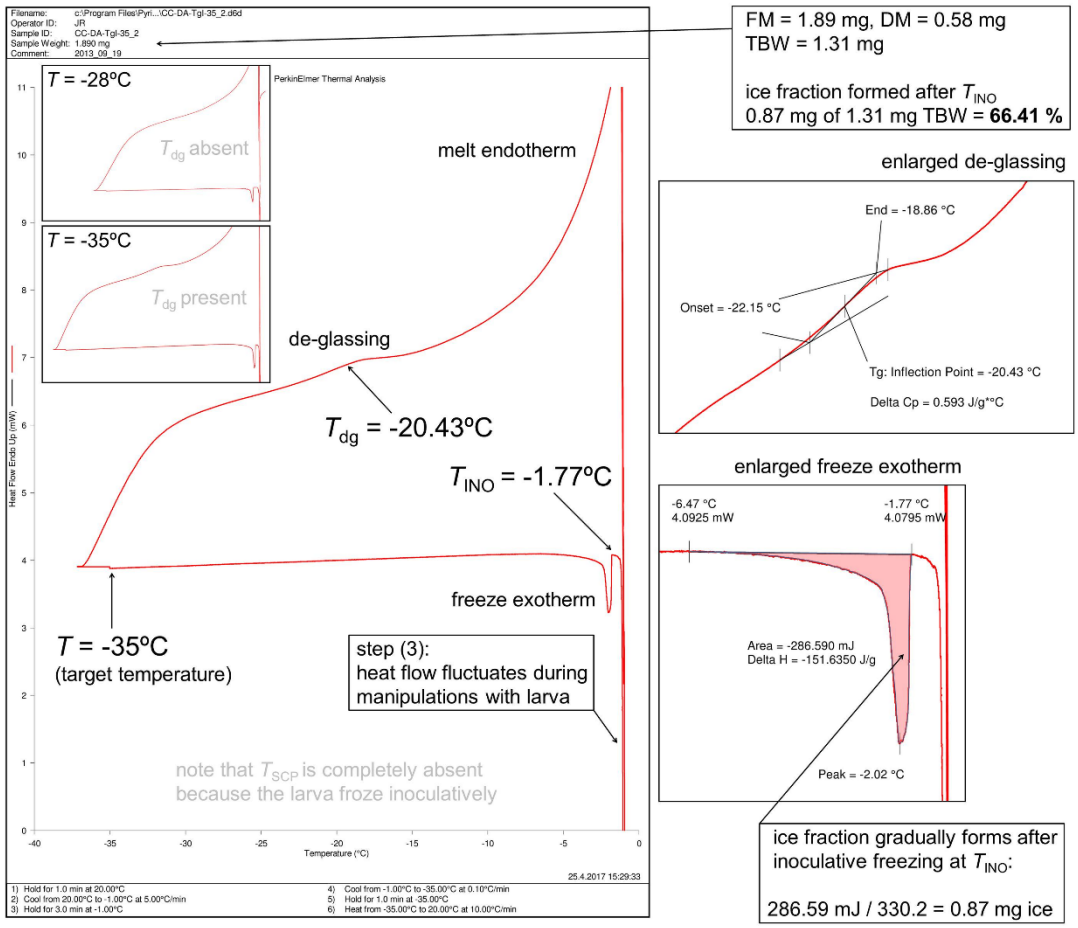

Fig. S6. *Ino-freeze* thermal analysis: example protocol (*C. costata*, SDA).

The *Ino-freeze* temperature programme: (1) hold for 1 min at 20°C; (2) cool to -1°C at a rate 5°C min^{-1}; (3) hold for 3 min -1°C and, meanwhile, open the instrument and insert aluminum pan bottom lined with filter paper disc (SS-033, Wescor) to which 10 uL of distilled water was applied and then submerged in liquid nitrogen to freeze it. Next, after the bottom of test-pan equilibrates to programmed temperature of -1°C (approximately within 10 sec), add larva, cover the bottom loosely with lid and close the instrument; (4) cool to target temperature at a rate 0.1°C min^{-1}; (5) hold for 1 min at target temperature; (6) heat to 20°C at a fast rate 10°C min^{-1}.

Notes:

steps (1 and 2): performed with the reference pan only inside instrument;

step (3): the test pan and larva were added. When performed carefully, this critical step ensures that all larvae are exposed to external ice crystals at exactly -1°C;

steps (4 and 5): the target temperature can be varied in order to see whether or not the de-glassing transition (T_{dg}) occurs upon rapid heating back to 20°C [when it occurs, it indicates that the vitrification transition (T_g) must have occurred during previous slow cooling to target temperature. Otherwise, glass trasition is not observable by DSC method at slow cooling rates]. However, observing only the specific aim of inoculative ice fraction analysis, the target temperature of -10°C would be sufficient as all larval freeze exotherms ended between -3°C and -5°C (see data in Fig. S11). The ice fraction was calculated from freeze exotherms and the enthalpy of water/ice transition (*delta H*) was modified according to the exact peak temperature of inoculative freezing (T_{INO} = -2.02°C in this example) using the formula:

$delta\ H = 334.5 + 2.12\ T_{INO} + 0.0042\ (T_{INO})^2 = 330.2$ (Wang and Weller, 2011)

step (6): the fast rate of heating (10°C min^{-1}) facilitates the observation of de-glassing (T_{dg}) transition.

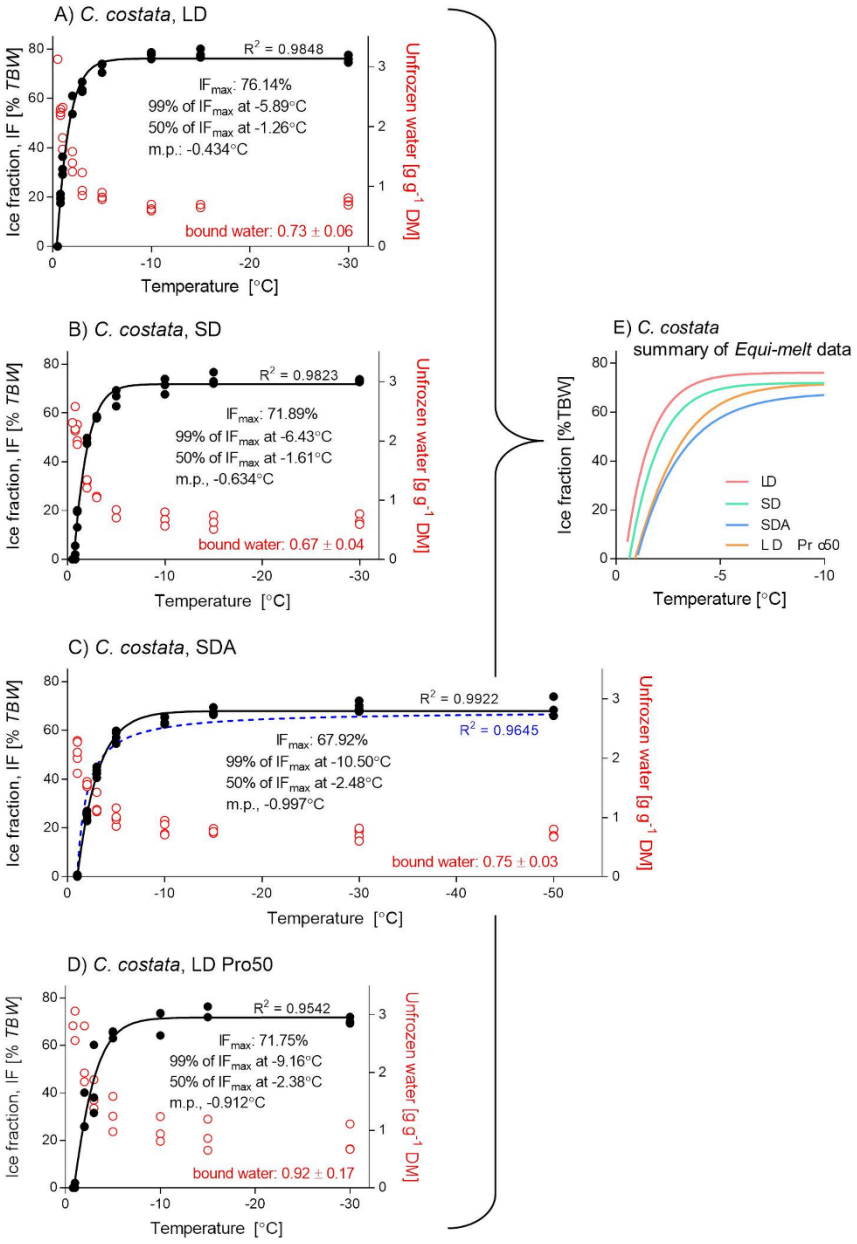

Fig. S7. Summary of *Equi-melt* data for *Chymomyza costata*

The *Equi-melt* thermal analyses were run with different target temperatures (*T*) ranging between -0.5°C and -30°C (or -50°C in SDA variant). At least three larvae were analyzed for each experimental variant (A-D) and target temperature combination (three data-points). The Boltzmann sigmoids were fitted to empirical data (black solid lines). For comparison, an example of a theoretical freezing curve (dashed blue line) *vs.* Boltzmann sigmoid fitting is shown in (C):

theoretical freezing curve (Wang and Weller., 2011): $IF = OAW*(1 - (m.p./T)$

Boltzmann sigmoid: $IF = OAW + (top – OAW)/(1 + \exp((V50-x)/slope))$

All other parameters (IF_{max}; 99% of IF_{max}; 50% of IF_{max}; m.p.) were derived from Boltzmann sigmoids. The amount of unfrozen water per mg DM for each larva is shown as red circle. Bound water (OIW) is calculated as a bottom of a Boltzmann sigmoid fitted to unfrozen water data.

(E) A summary figure showing only the *Equi-melt* curves for each treatment (these lines are used in Fig. 2A).

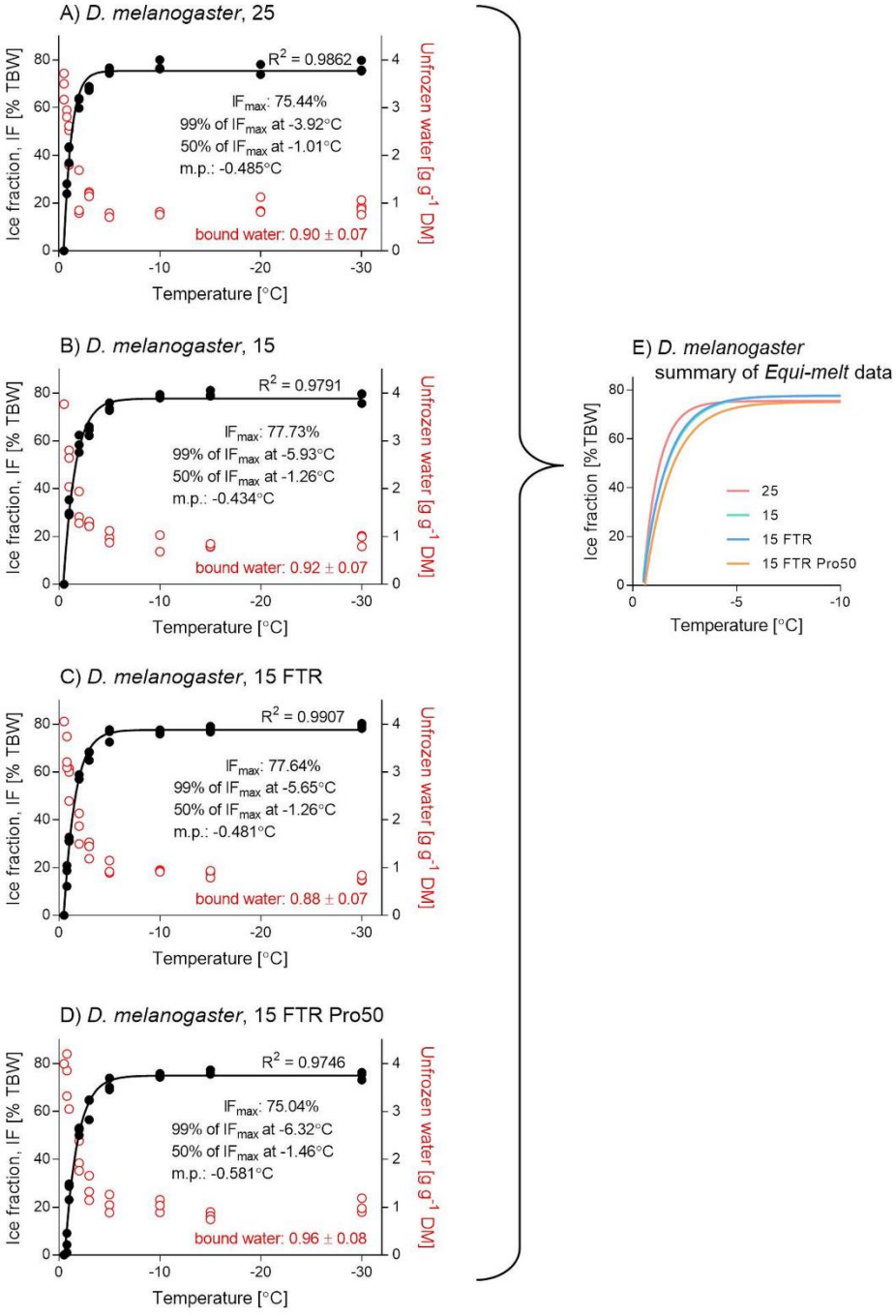

Fig. S8. Summary of *Equi-melt* data for *Drosophila melanogaster*.

All descriptions as in Fig. S7**.**
(E) A summary figure showing only the *Equi-melt* curves for each treatment (these lines are used in Fig. 2E).

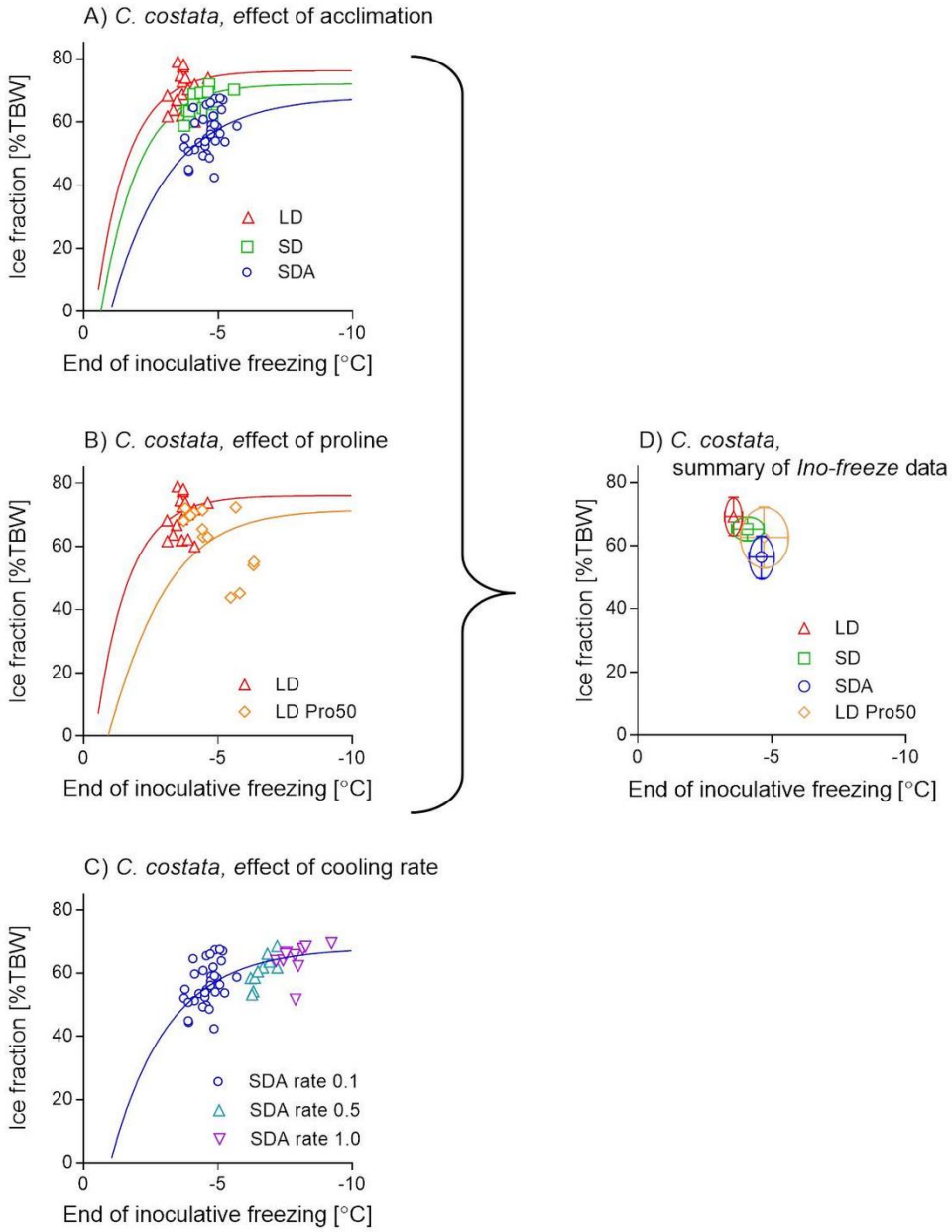

Fig. S9. Summary of *Ino-freeze* data for *Chymomyza costata*.

The *Ino-freeze* thermal analyses were run for individual larvae (points). Detailed results of *Ino-freeze* analyses are summarised in Fig. S11. *Equi-melt* curves (taken from Fig. S7) are also shown in order to allow direct comparison of the ice fraction analyzed by two methods. Note that *Ino-freeze* points and *Equi-melt* lines match very well. (A) Three acclimation variants (treatments LD, SD, SDA). (B) The effect of proline augmented diet (LD *vs.* LD Pro50). (C) The effect of cooling rate during the step (ii.) of freezing protocol. Three different cooling rates were compared: 0.1°C min^{-1}; 0.5°C min^{-1}; 1°C min^{-1}. The points express the ice fraction calculated from area under inoculative freeze exotherm at a temperature corresponding to the end of inoculative freezing (see Fig. S6).
(D) A summary figure showing ellipses based on mean x ± S.D. and mean y ± S.D. values of all *Ino-freeze* data points for each treatment (these ellipses are used in Fig. 2A).

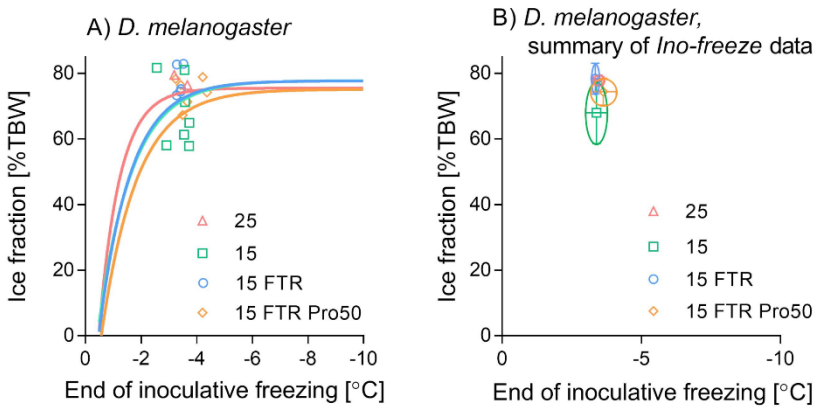

Fig. S10. Summary all *Ino-freeze* data for *Drosophila melanogaster*

All descriptions as in Fig. S9**.**
(B) A summary figure showing ellipses based on mean x ± S.D. and mean y ± S.D. values of all *Ino-freeze* data points for each treatment (these ellipses are used in Fig. 2E).

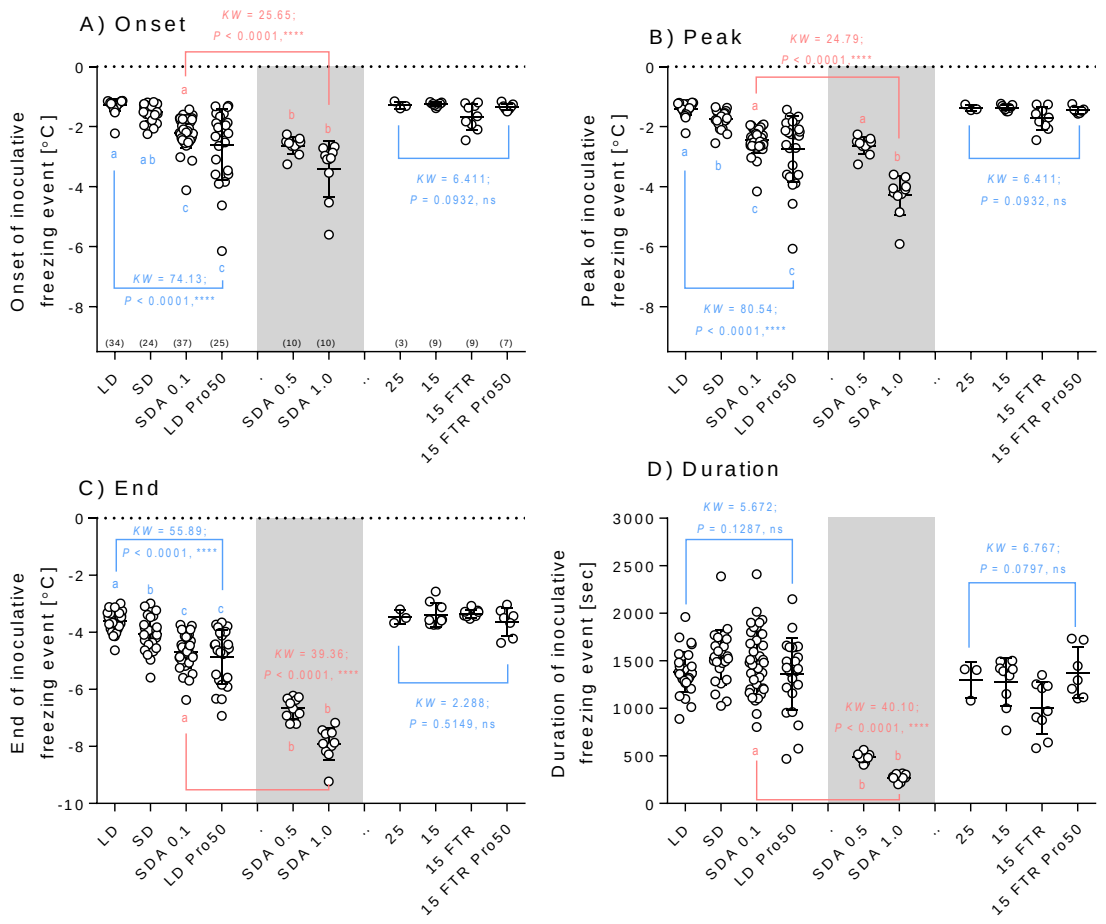

Figure S11. Summary of data obtained by *Ino-freeze* thermal analyses.

The parameters of inoculative freezing event [onset (= T_{INO}), peak, end, and duration; see Fig. S6 for more explanations] in variously treated larvae of *C. costata* (LD, SD, SDA, LD Pro50) and *D. melanogaster* (25, 15, 15 FTR, 15 FTR Pro50). In addition, the effect of cooling rate during the step (ii.) of freezing protocol was analyzed in *C. costata*. Three different cooling rates were compared: 0.1°C min^{-1}; 0.5°C min^{-1}; 1°C min^{-1}. Each point represents single thermal analysis (single larva). Number of larvae analyzed in each treatment (n) is shown in parentheses in (A). Note that there is a good match between the data on onset of inoculative freezing analyzed by DSC (Fig. S11 A) and similar data recorded directly by thermocouples in freeze-tolerance assays (Fig. S1 F). The differences between treatments (shown by blue and red lines) were assessed using Kruskal-Wallis nonparametric test (*KW* statistics is shown) followed by Dunn's multiple comparison test. Treatments flanked by different letter were statistically different according to the Dunn's test.

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