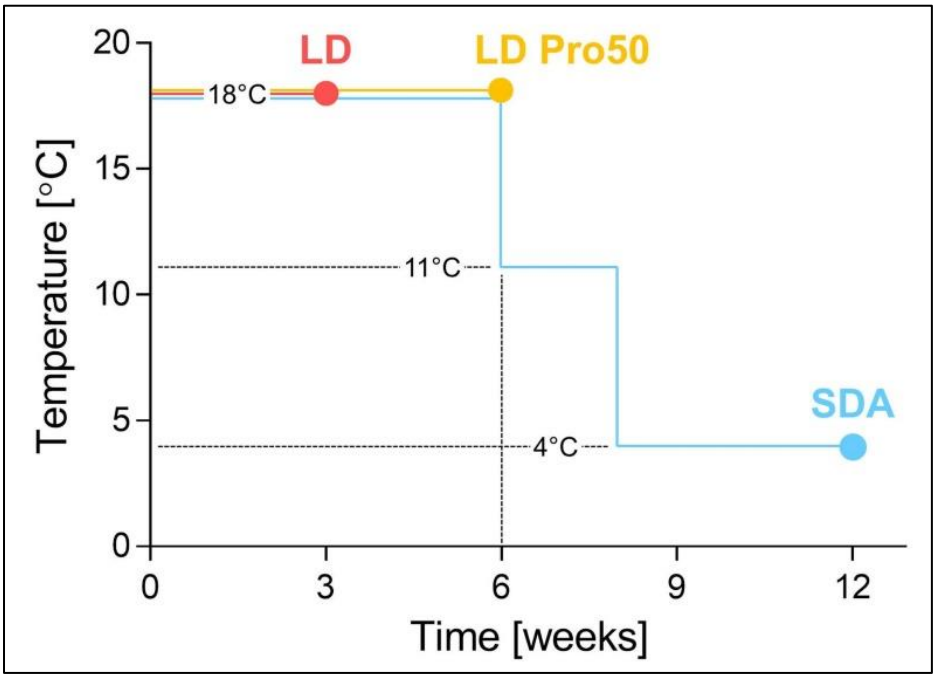

Figure S1. Acclimation of *Chymomyza costata* larvae.

The larvae were reared under constant 18°C and two different photoperiods (LD and SD). Under long day length (LD, 16 h/8 h light/dark cycle) the larvae continue direct development (to pupariation) but under short day length (SD, 12 h/12 h light/dark cycle) they enter diapause (hormonally regulated developmental arrest) (Koštál et al., 2000 **JIP**, 2016 **PE**). **LD**, long day-reared, non-diapause, warm-acclimated larvae in the late 3rd instar (pre-wandering); **LD Pro50**, same as LD but fed L-proline-augmented diet (50 mg of L-proline added per g of diet), which delays their development; **SDA**, cold-acclimated (under constant darkness), diapausing larvae, which spontaneously accumulate large amounts of L-proline in their body. Both treatments, LD Pro50 and SDA, result in dramatic increase of freeze tolerance according to our previous studies (Koštál et al., 2012 **PNAS**; Rozsypal et al. 2018 **JEB**).

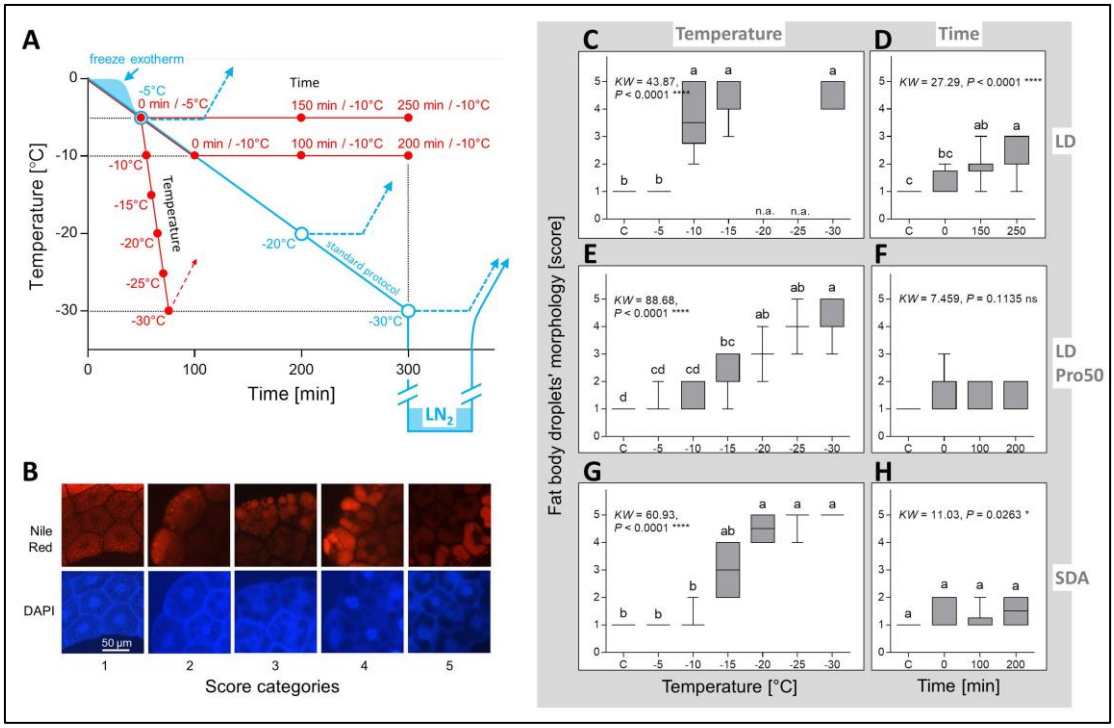

Figure S2. Freezing protocol and lipid droplet coalescence in the fat body of *Chymomyza costata* larvae exposed to cold stress. Fluorescence microscopy.

(**A**) Shows standard freezing and cryopreservation protocol (blue line) (according to Rozsypal et al., 2018 **JEB**) and also the specific protocols used to assess the effects of either low temperatures or time spent at -5°C or -10°C on lipid droplet coalescence (red lines). All larvae were inoculated by external ice crystals and frozen at relatively high subzero temperature (freeze exotherm). The cooling rate was set to 0.1°C min^{-1} in programmable Ministat 240 circulator (Huber, Offenburg, Germany). **Standard protocol:** blue empty circles show three temperatures (-5, -20, and -30°C) to which the larvae were cooled and then, after spending 1 hour at the target temperature, heated to +5°C (0.6°C min^{-1}), dissected and processed for confocal or TEM microscopy. Some SDA larvae, pre-frozen to -30°C, were plunged to liquid nitrogen (LN_2) for 1h. **The effect of low temperatures:** larvae were rapidly (1°C min^{-1}) cooled from -5°C to different target temperatures ranging between -10 and -30°C. Immediately after reaching the target temperature, the heating step started and the melted larvae were immediately processed for fluorescence microscopy observation. **The effect of time:** after reaching -5°C (LD) or -10°C (LD Pro50, SDA), the larvae were maintained at the target temperature for up to 250 min, then heated and processed for fluorescence microscopy.

(**B**) Immediately upon melting, fat body was dissected in PBS (8.0 g NaCl; 0.2 g KCl; 1.8 g $Na_2HPO_4.2H_2O$; 0.24 g KH_2PO_4 in 800 mL water, set pH to 7.4, fill up to 1L), placed on microscopic slide (Superfrost Plus, Thermo Scientific), and covered with 40 µl of Nile Red (Sigma) working solution (WS). The WS was prepared fresh daily by, first, diluting the Nile Red stock (5% in DMSO) 1:50,000 with PBS:glycerol (1:1) solution, and second, adding 1/5 volume (40 µl to 200 µl) of ProLong Gold antifade reagent with DAPI (Molecular Probes, Life Technologies). The fat bodies (five per slide) were covered with cover slips and incubated at room temperature for 15 min (protected from light). Next, the fat body cells were photographed under Karl Zeiss Axioplan 2 fluorescence microscope using Cy3 (red, Nile Red signal) and DAPI (blue signal) filters. The coalescence of lipid droplets in each fat body was arbitrarily scored to five categories from 1 (normal state, no coalescence) to 5 (full coalescence) according to scale shown in (B).

(**C**) Results of lipid droplets coalescence scoring in larvae acclimated according to LD, LD Pro50, and SDA protocols (see Fig. S1). Each box shows 25 and 75 percentiles and median value (horizontal bar in the box), whiskers represent min and max values. Scores were taken from 15 fat bodies (three microscopic slides). The differences between boxes were assessed using Kruskal-Wallis rank test (*KW* statistics is shown) followed by Dunn's multiple comparisons test. The columns flanked by different letters are significantly different.

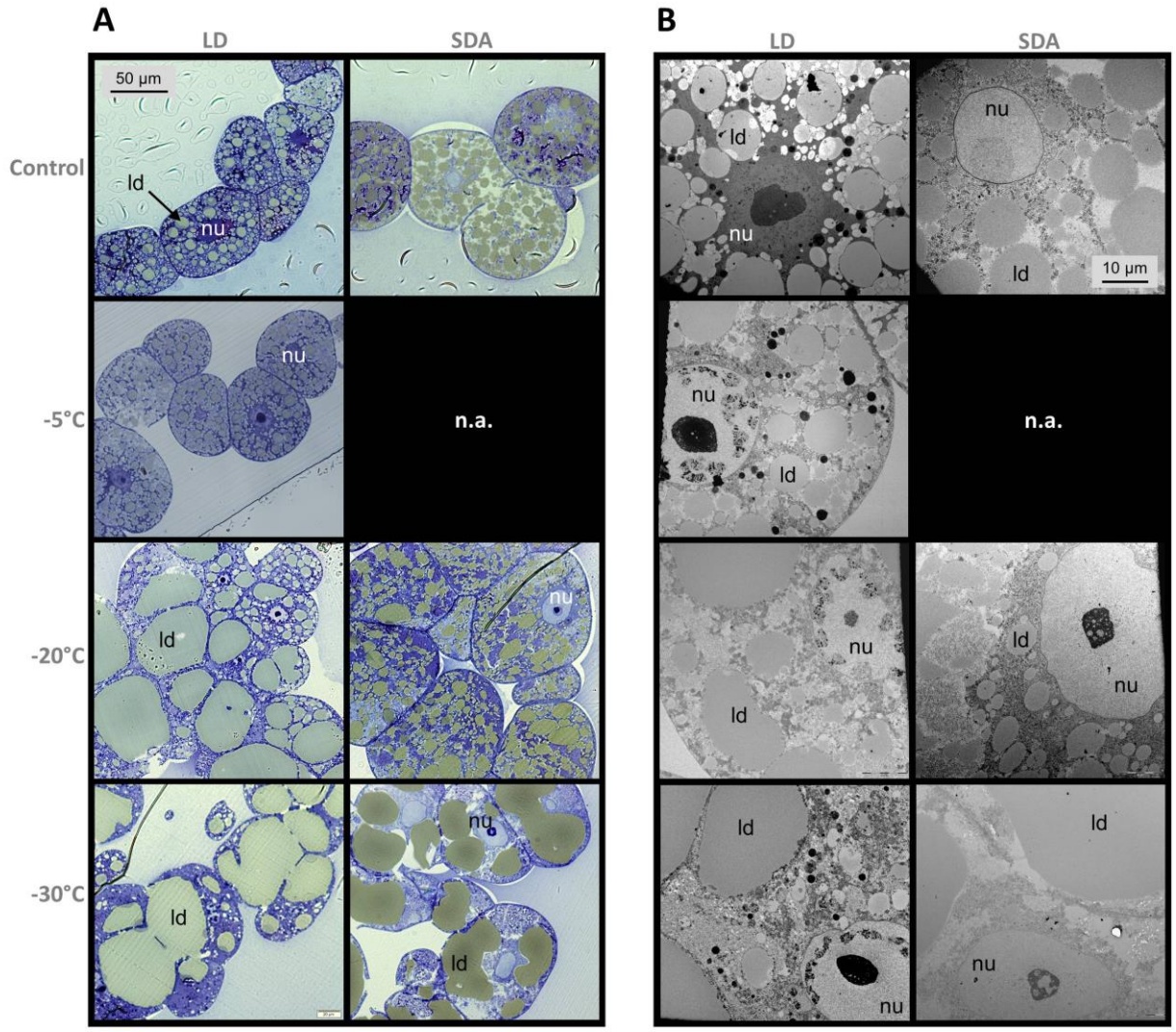

Figure S3. Lipid droplet coalescence in the fat body of *Chymomyza costata* larvae exposed to cold stress. Transmission electron microscopy (TEM).

The larvae acclimated to LD or SDA (see Fig. S1) were frozen to different target temperatures (-5, -20, and -30°C) using standard freezing protocol (see Fig. S2 A, blue line) and, upon melting, dissected and processed for TEM observation. (**A**) semi-thin sections (0.5 µm). The scale bar shown in upper left corner applies to all micrographs in the panel), (**B**) ultra-thin sections (70 nm). The scale bar shown in upper right corner applies to all micrographs in the panel). nu, nucleus; ld, lipid droplet.

Processing of fat body tissue for TEM: Fat bodies were dissected (under ice-cold PBS) into 1.5 mL microvials containing 500 µL of 2.5% glutaraldehyde in PBS. Tissues were fixed overnight, then embedded in 2% agarose gel for easier manipulation, washed in PBS (3 x 15 min), fixed in 2% OsO_4 (EMS, Hatfield, Pennsylvania) in PBS for 2 h, and finally washed in PBS (3 x 15 min). The samples were then dehydrated using grading acetone solutions (30%, 50%, 70%, 80%, 90%, 95%, 100%) in 15 min intervals. Dehydrated samples were saturated by grading solutions of resin Embed-812 (EMS, Hatfield, Pennsylvania) mixed with 100% acetone (resin:acetone at 1:2, 1:1, and 2:1 ratio, respectively) in 1 h intervals, then transferred into pure resin for 24 hours in desiccator. After complete dehydration, the samples were embedded in silicone forms containing pure resin and left for another 24 hours in 60°C for polymerization. Resin blocks containing fat body samples were sectioned using ultramicrotome Leica UC6 (Leica microsystems GmbH, Wetzlar, Germany) using DiATOME diamond knife (EMS, Hatfield, Pennsylvania). **The semithin sections** (500 nm) were placed in a droplet of 10% acetone on 24 x 60 mm glass slides, left on heater at 40°C

until dry, and then left in r.t. overnight for perfect drying. The semithin sections were stained by 1% Toluidine Blue (Sigma-Aldrich, Saint Luis, MO, USA) for 3-5 min and micrographs were taken by a bright-field microscope, Zeiss Axioplane 2 (Carl Zeiss Microscopy GmbH, Jena, Germany). **The ultrathin sections** (70 nm) were placed on 300-mesh-Cu grids. The samples were counter-stained by saturated ethanolic uranyl acetate for 30 min, followed by lead citrate for 20 min. The grids with sections were then coated by carbon film using JEOL JE 4C coater (JEOL, Tokio, Japan) and micrographs were taken by a transmission electron microscope, JEOL JEM - 1010 1 (JEOL, Tokio, Japan).

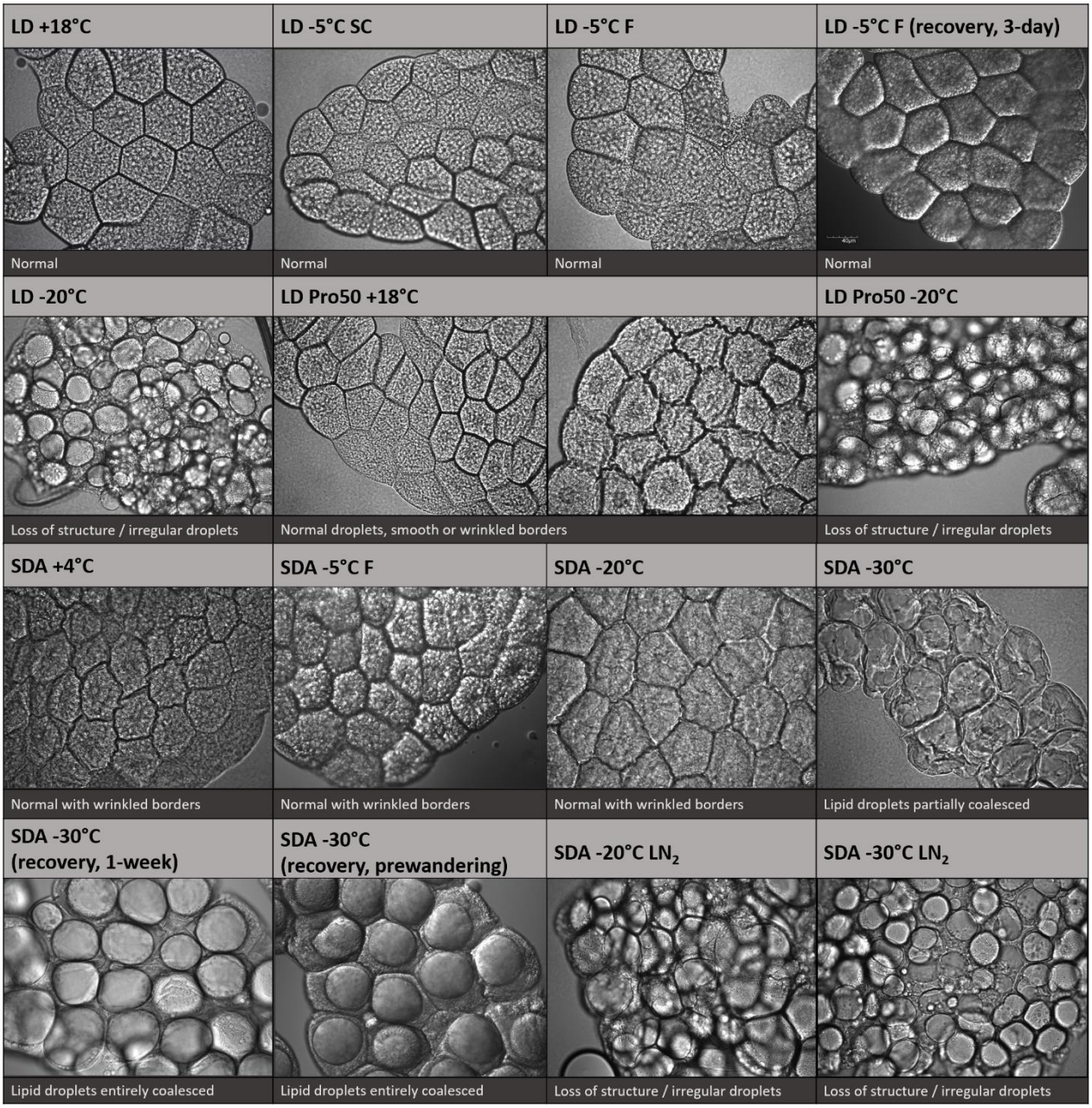

Figure S4. Representative brightfield images of *Chymomyza costata* larval fat bodies for all freeze tolerance variants and treatments, indicating extent of lipid droplet coalescence and cell border morphology (confocal microscopy).

LD: long-day reared, active variant, SDA: diapausing, cold-acclimated variant, Pro50: LD larvae supplemented with 50 mg proline per g diet, SC: larvae supercooled to -5°C without freezing, F: larvae frozen at -5°C. Larvae were cooled gradually and held at target temperatures for 1 hour before rewarming (details in Figure S2). Tissues were dissected and fixed immediately after cold treatments. Larvae exposed to LN_2 were first pre-frozen to either -20°C or -30°C. Prewandering SDA larvae had recovered for approximately 3 weeks.

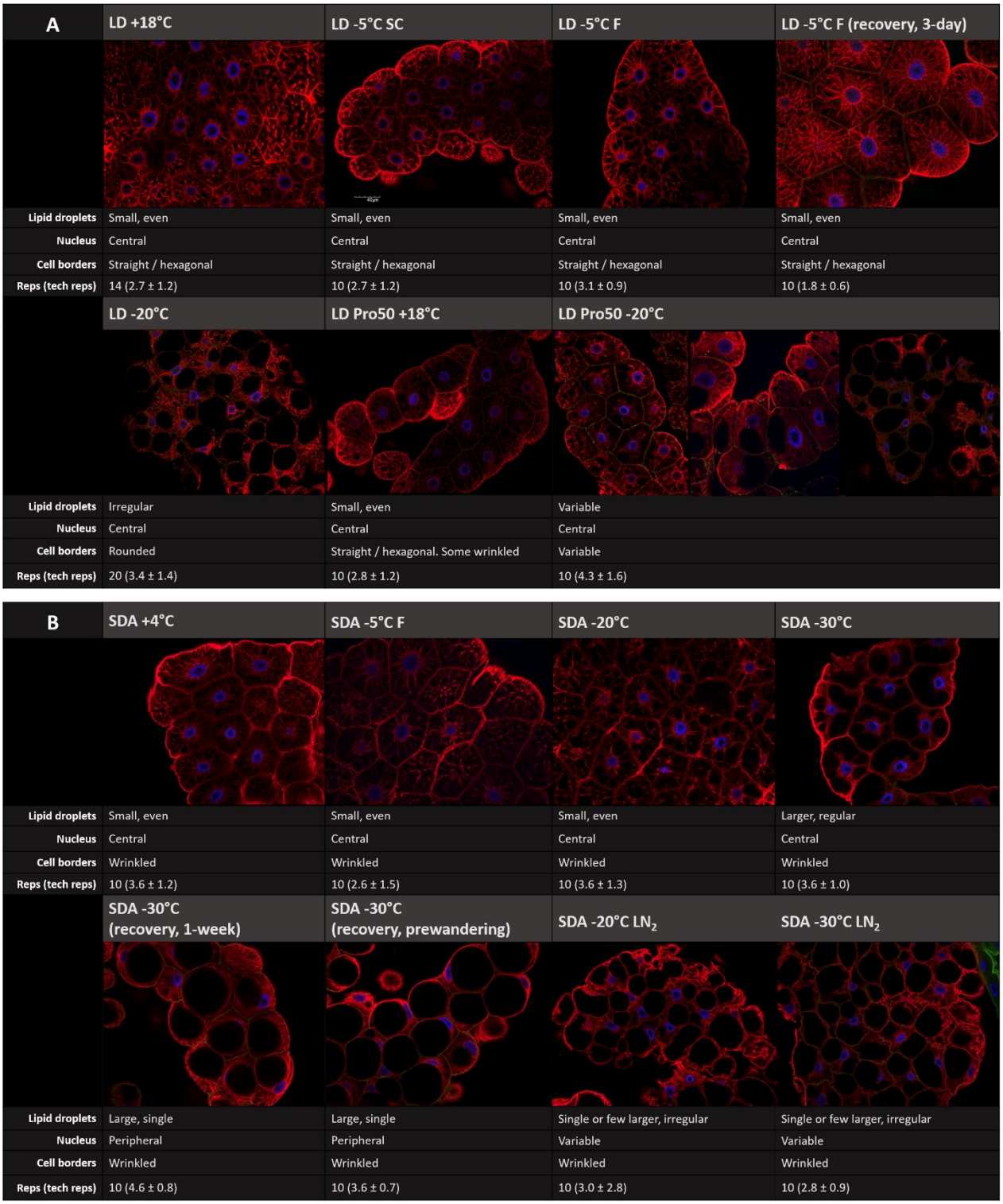

Figure S5. Representative fat body mid-section images for *Chymomyza costata* larvae of all freeze tolerance variants and treatments, indicating the extent of lipid droplet coalescence, cell border morphology, and α-tubulin radial structure (confocal microscopy).

A: LD - long-day reared, active variant, B: SDA - diapausing, cold-acclimated variant. LD Pro50 – LD larvae supplemented with 50 mg proline per g diet, SC: larvae supercooled to -5°C without freezing, F: larvae frozen at -5°C.

Larvae were cooled gradually and held at target temperatures for 1 hour before rewarming (details in Figure S2). Tissues were dissected, fixed, and stained immediately after cold treatments. Larvae exposed to LN_2 were first pre-frozen to either -20°C or -30°C. Prewandering SDA larvae had recovered for approximately 3 weeks. Red: α-tubulin (anti-α-tubulin antibody), green: F-actin (phalloidin, generally not visible), blue: nuclei (DAPI). Biological replication (number of larvae) and technical replication (mean ± s.e.m. fat body tissues imaged per larva) are indicated below each image.

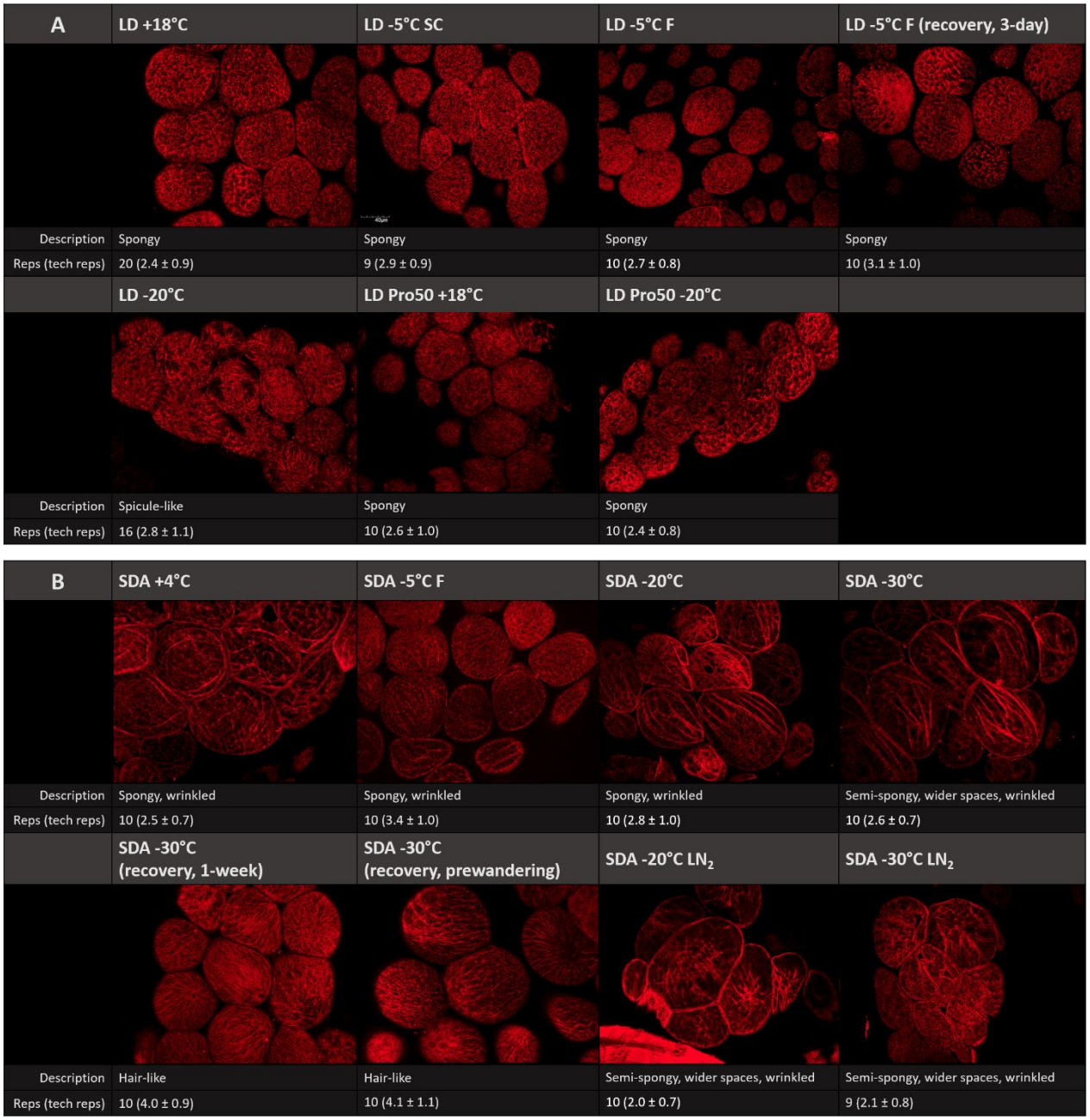

Figure S6. Representative *Chymomyza costata* larval fat body surface images for all freeze tolerance variants and treatments, indicating cell border morphology and α-tubulin radial structure (stained by anti-α-tubulin antibody, confocal microscopy).

A: LD - long-day reared, active variant, B: SDA - diapausing, cold-acclimated variant. LD Pro50 – LD larvae supplemented with 50 mg proline per g diet, SC: larvae supercooled to -5°C without freezing, F: larvae frozen at -5°C. Larvae were cooled gradually and held at target temperatures for 1 hour before rewarming (details in Figure S2). Tissues were dissected, fixed, and stained immediately after cold treatments. Larvae exposed to LN_2 were first pre-frozen to either -20°C or -30°C. Prewandering SDA larvae had recovered for approximately 3 weeks. Biological replication (number of larvae) and technical replication (mean ± s.e.m. fat body tissues imaged per larva) are indicated below each image.

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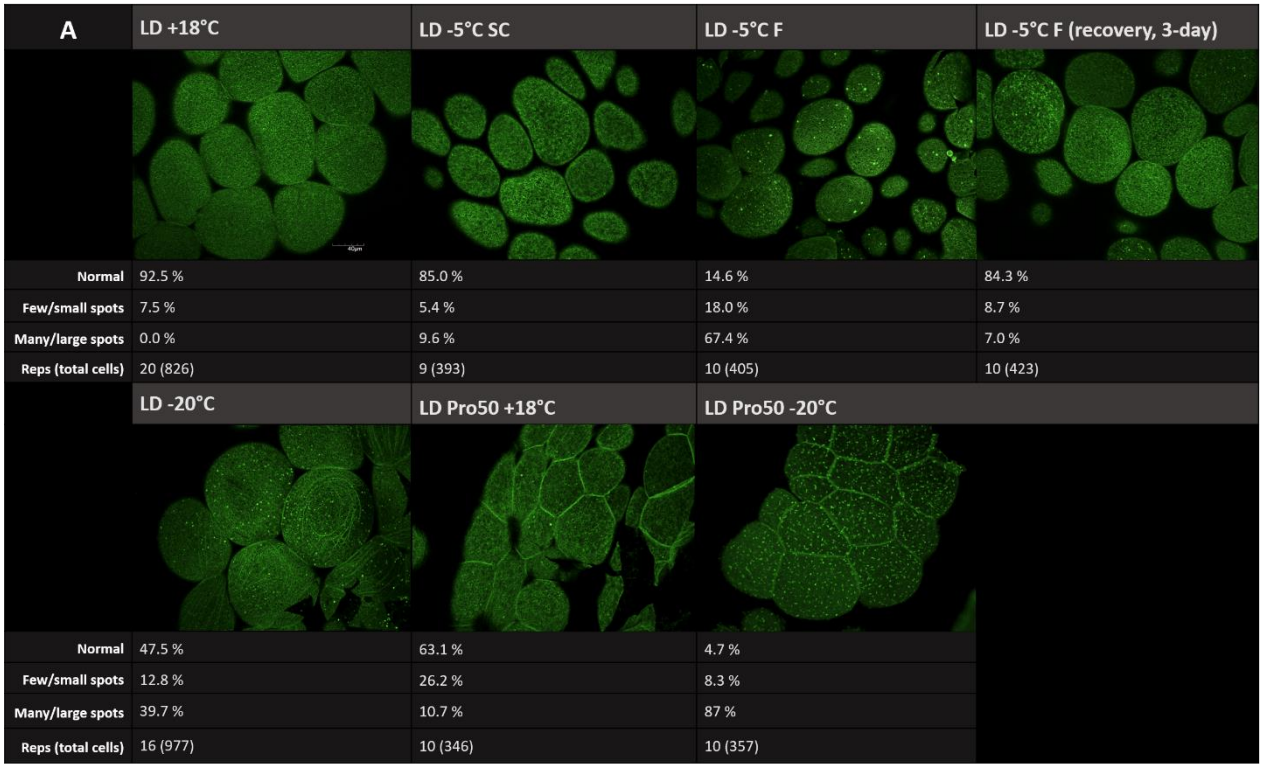

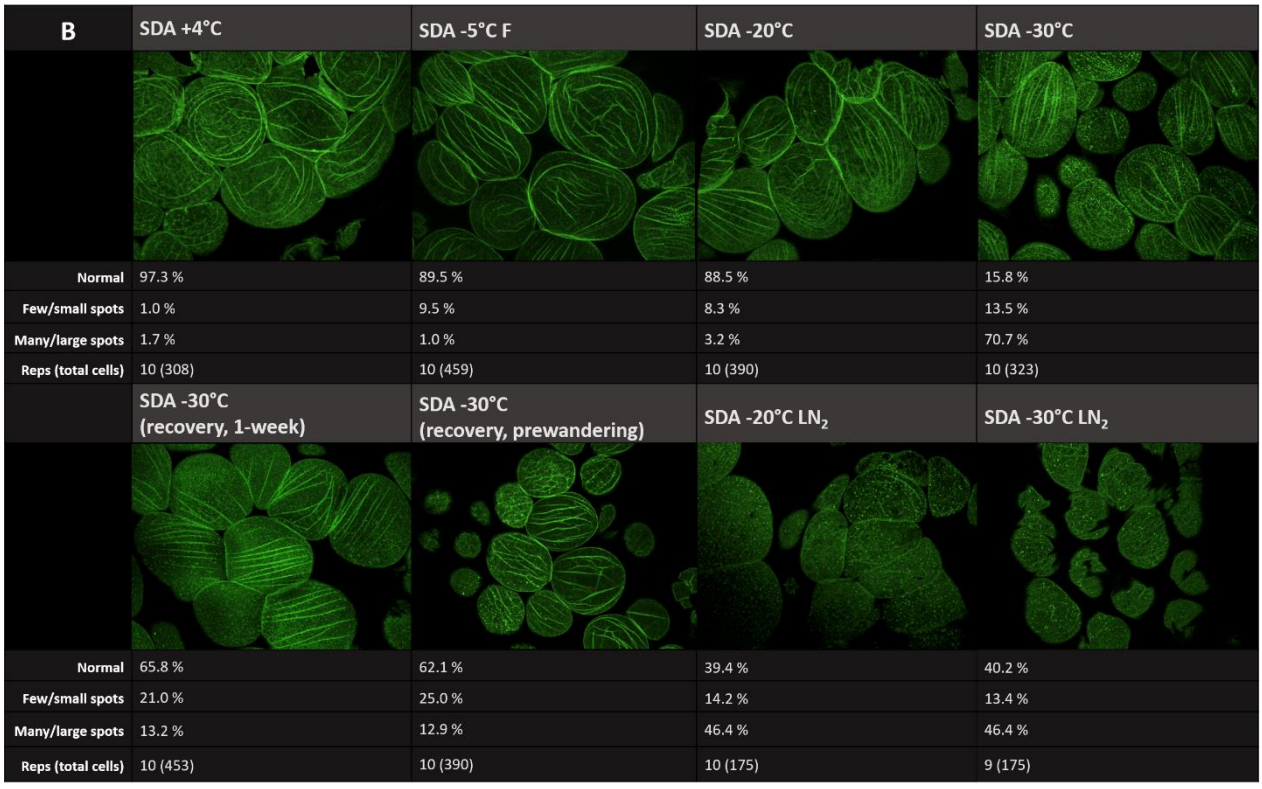

Figure S7. Representative *Chymomyza costata* larval fat body surface images for all freeze tolerance variants and treatments, indicating cell border and F-actin morphology (stained by phalloidin, confocal microscopy).

A: LD - long-day reared, active variant, B: SDA - diapausing, cold-acclimated variant. LD Pro50 – LD larvae supplemented with 50 mg proline per g diet, SC: larvae supercooled to -5°C without freezing, F: larvae frozen at -5°C. Larvae were cooled gradually and held at target temperatures for 1 hour before rewarming (details in Figure S2). Tissues were dissected, fixed, and stained immediately after cold treatments. Larvae exposed to LN_2 were first pre-frozen to either -20°C or -30°C. Prewandering SDA larvae had recovered for approximately 3 weeks. Biological replication (number of larvae) and technical replication (total number of fat body cells assessed across all biological replicates) are indicated below each image.

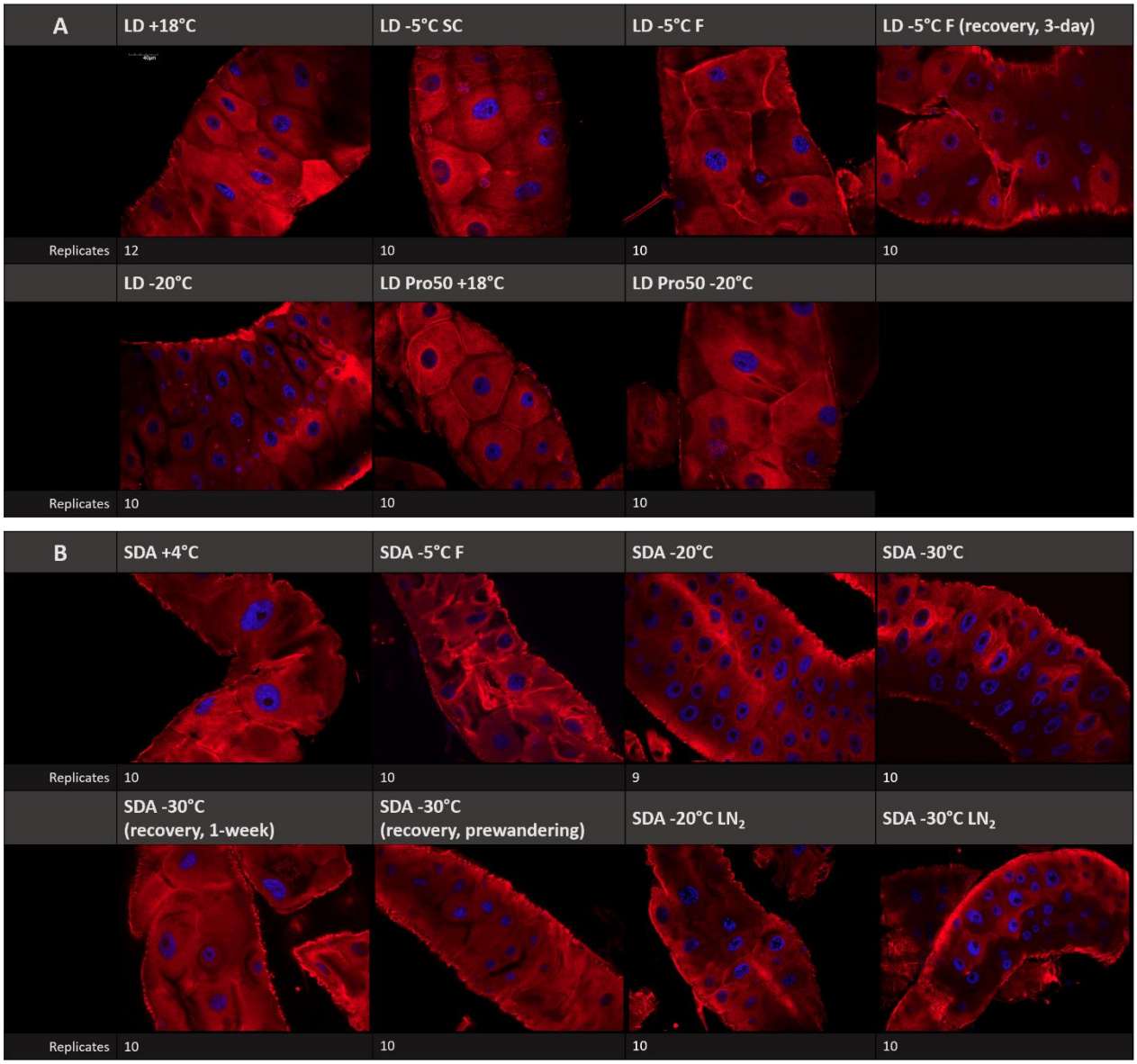

Figure S8. Representative *Chymomyza costata* larval anterior midgut epithelia for all freeze tolerance variants and treatments (confocal microscopy).

A: LD - long-day reared, active variant, B: SDA - diapausing, cold-acclimated variant. LD Pro50 – LD larvae supplemented with 50 mg proline per g diet, SC: larvae supercooled to -5°C without freezing, F: larvae frozen at -5°C. Larvae were cooled gradually and held at target temperatures for 1 hour before rewarming (details in Figure S2). Tissues were dissected, fixed, and stained immediately after cold treatments. Larvae exposed to LN_2 were first pre-frozen to either -20°C or -30°C. Prewandering SDA larvae had recovered for approximately 3 weeks. Red: α-tubulin (anti-α-tubulin antibody), blue: nuclei (DAPI). The biological replication (number of larvae) is indicated below each image.

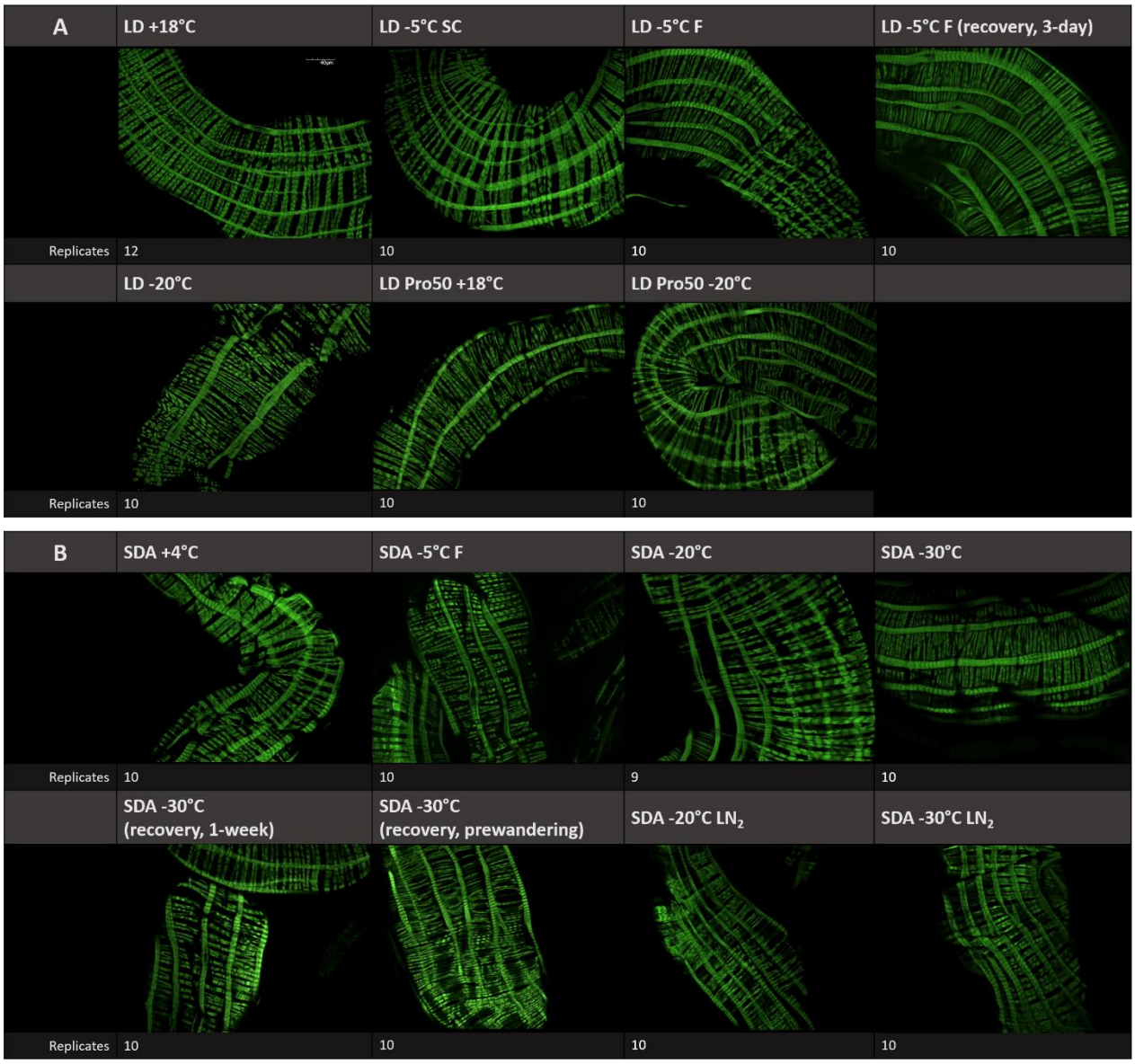

Figure S9. Representative *Chymomyza costata* larval anterior midgut muscle for all freeze tolerance variants and treatments (visualized by F-actin staining with phalloidin, confocal microscopy).

A: LD - long-day reared, active variant, B: SDA - diapausing, cold-acclimated variant. LD Pro50 – LD larvae supplemented with 50 mg proline per g diet, SC: larvae supercooled to -5°C without freezing, F: larvae frozen at -5°C. Larvae were cooled gradually and held at target temperatures for 1 hour before rewarming (details in Figure S2). Tissues were dissected, fixed, and stained immediately after cold treatments. Larvae exposed to LN_2 were first pre-frozen to either -20°C or -30°C. Prewandering SDA larvae had recovered for approximately 3 weeks. Biological replication (number of larvae) is indicated below each image.

Figure S10. Representative Malpighian tubules from *Chymomyza costata* larvae of all freeze tolerance variants and treatments (confocal microscopy).

A: LD - long-day reared, active variant, B: SDA - diapausing, cold-acclimated variant. LD Pro50 – LD larvae supplemented with 50 mg proline per g diet, SC: larvae supercooled to -5°C without freezing, F: larvae frozen at -5°C. Larvae were cooled gradually and held at target temperatures for 1 hour before rewarming (details in Figure S2). Tissues were dissected, fixed, and stained immediately after cold treatments. Larvae exposed to LN_2 were first pre-frozen to either -20°C or -30°C. Prewandering SDA larvae had recovered for approximately 3 weeks. Red: α-tubulin (anti-α-tubulin antibody), green: F-actin (phalloidin), blue: nuclei (DAPI). Biological replication (number of larvae) and technical replication (mean ± s.e.m. Malpighian tubules imaged per larva) are indicated below each image.

Table S1. Cold exposure regimes for malt fly larvae of various freeze tolerance states. LD: long-day (non-diapause, weakly freeze tolerant), LD Pro50: LD with dietary supplementation of 50 ± 5 mg proline per g of diet (non-diapause, moderately freeze tolerant), SDA: short-day acclimated (diapausing, extremely freeze tolerant), LN_2: liquid nitrogen, F: freeze treatment at -5°C (larvae in tubes were overlain with an ice crystal to ensure inoculation and freezing, verified by an exotherm), SC: supercooled (larvae cooled to -5ºC did not freeze as verified by a lack of exotherm). Recovered larvae were returned to rearing conditions at +18°C immediately after cold exposure and assessed after 3 days (LD), 1 week (SDA), or upon reaching the prewandering stage (SDA, approximately 3 weeks). Details of acclimations and cold exposure regimes are provided in Supplementary Material Figs. S1 and S2. Survival details from Rozsypal et al. (2018).

Treatment	Exposure temperature (°C)	Survival to adulthood (%)
LD +18	+18	91
LD -5 SC	-5 (unfrozen)	72*
LD -5 F	-5 (frozen)	39
LD -5 F (recovery, 3-day)	-5 (frozen)	39
LD -20	-20	0
LD Pro50 +18	+18	75
LD Pro50 -20	-20	34
SDA +4	+4	86
SDA -5 F	-5 (frozen)	76-86
SDA -20	-20	76-86
SDA -30	-30	76
SDA -30 (recovery, 1-week)	-30	76
SDA -30 (recovery, prewandering)	-30	76
SDA -20 LN_2	-196 (pre-frozen to -20°C)	0
SDA -30 LN_2	-196 (pre-frozen to -30°C)	39

*Determined in the present study