



Disruptions of mitochondrial integrity and function in response to cold in the larvae of *Chymomyza costata*

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Introduction:

Some insects can survive freezing of body fluids and some are exceptional by surviving even in liquid nitrogen (LN₂). Understanding the underlying mechanisms of this **extreme freeze tolerance** may help to develop new techniques of cryopreservation of biological material (tissues, organs, whole organisms).

The drosophilid fly, *Chymomyza costata*, is one of two insect species for which survival in liquid nitrogen has been proven. We found earlier that the extreme freeze tolerance in this species is based on entry to diapause (developmental arrest, metabolic suppression), cold acclimation, accumulation of L-proline (up to 0.5 M) and slow freezing followed by vitrification of unfrozen solutions.

Here we asked whether the sensitivity of mitochondria to freezing stress may be the cause of mortality in non-acclimated (non-diapausing) larvae of *C. costata*.

Materials & Methods:

Larvae of *C. costata* of two contrasting phenotypes were compared:

- LD:** non-diapausing, active, warm-acclimated long days, 18°C, **no survival in LN₂**
- SDA:** diapausing, dormant, cold-acclimated short days, 4°C, **50% survival in LN₂**

The larvae were frozen to -30°C and their **fat body** and **hindgut tissues** were dissected for analysis of:

- Morphology and integrity** – TEM
Chemical fixation, semi & ultra sections; Image analysis - ImageJ software
- Quantity** – Confocal microscopy
MitoTracker™ Green staining, nDNA vs. mtDNA
- Functionality** – Citrate synthase activity;
O₂ consumption (PreSens SDR)

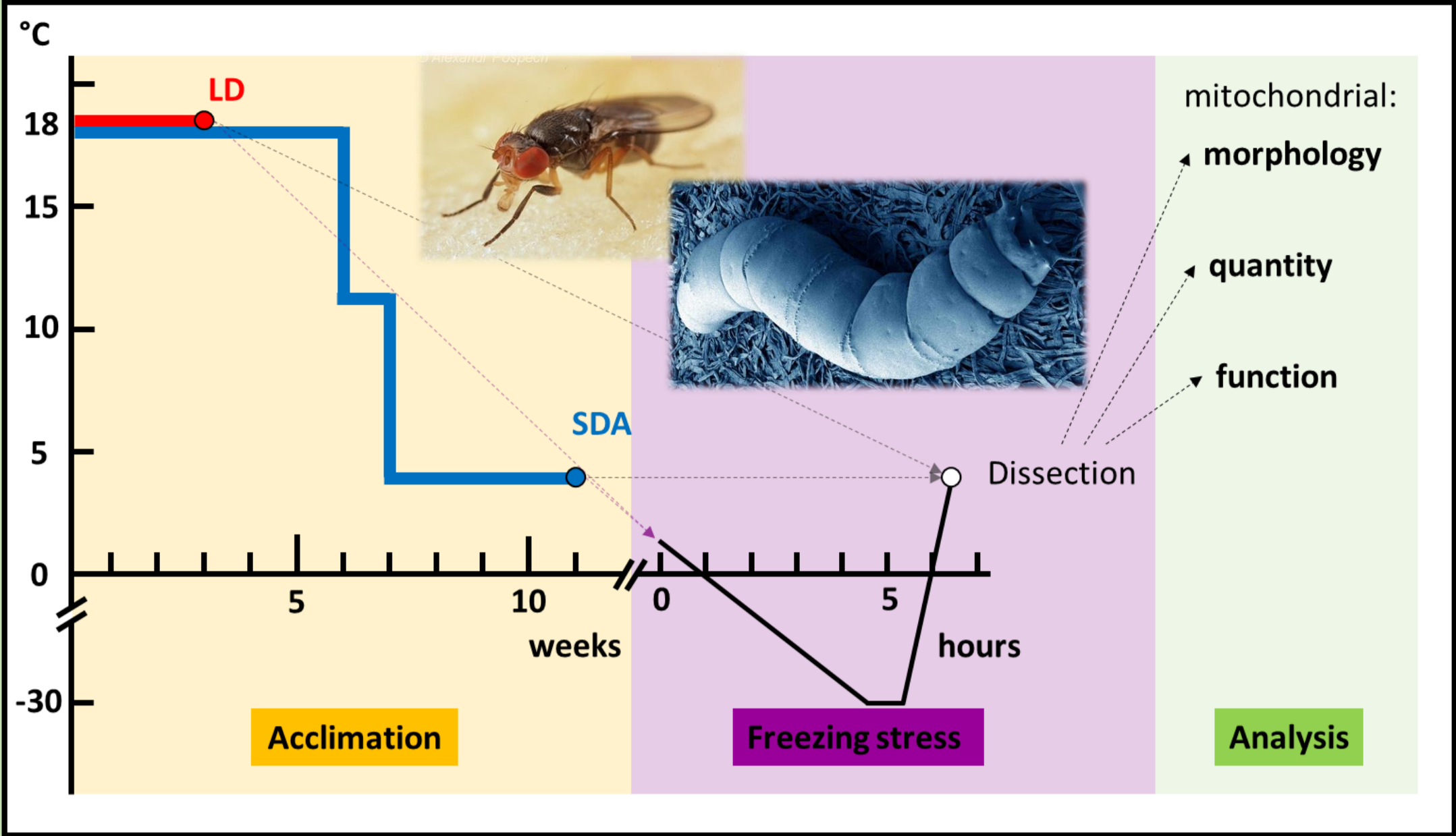
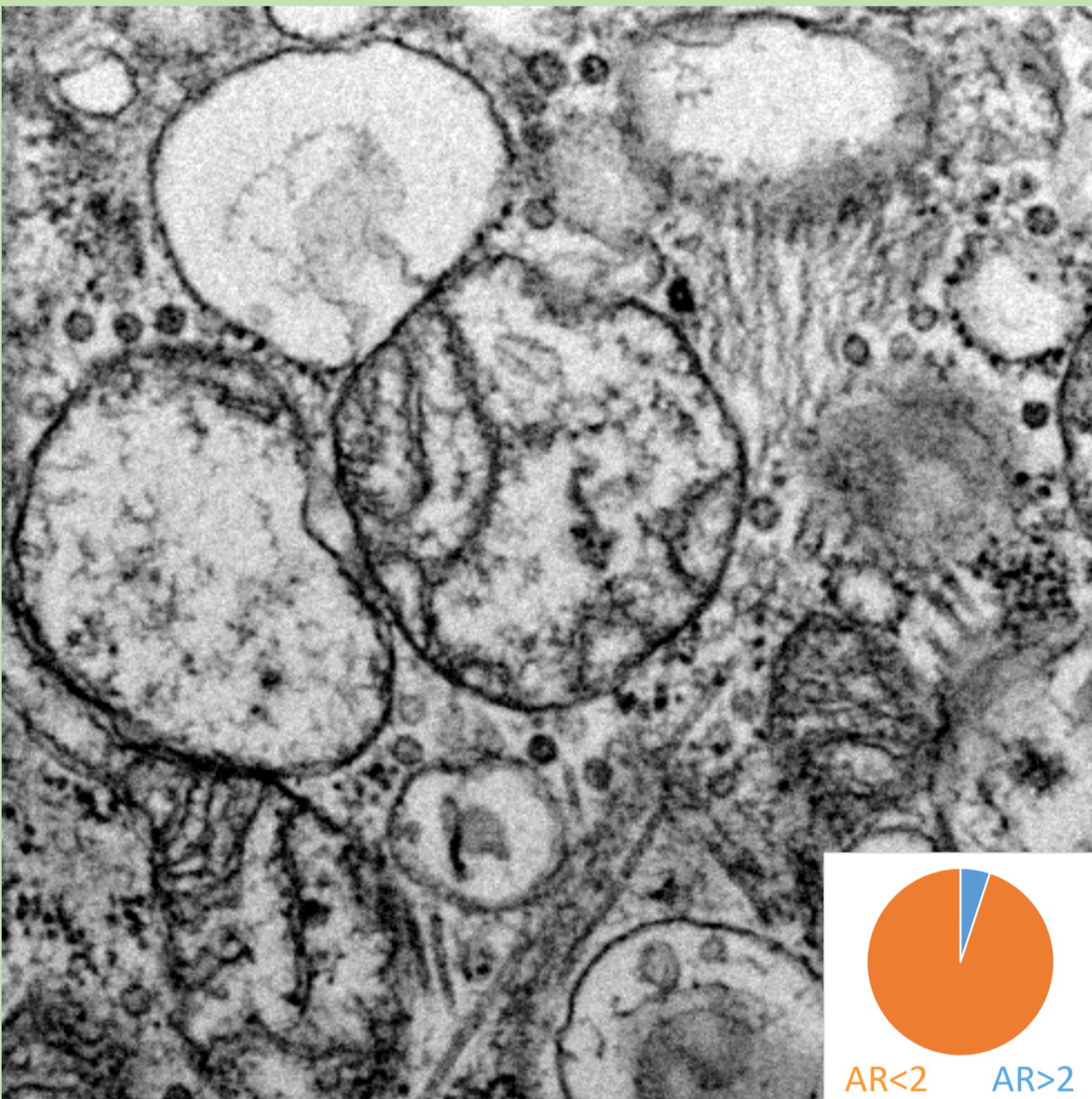
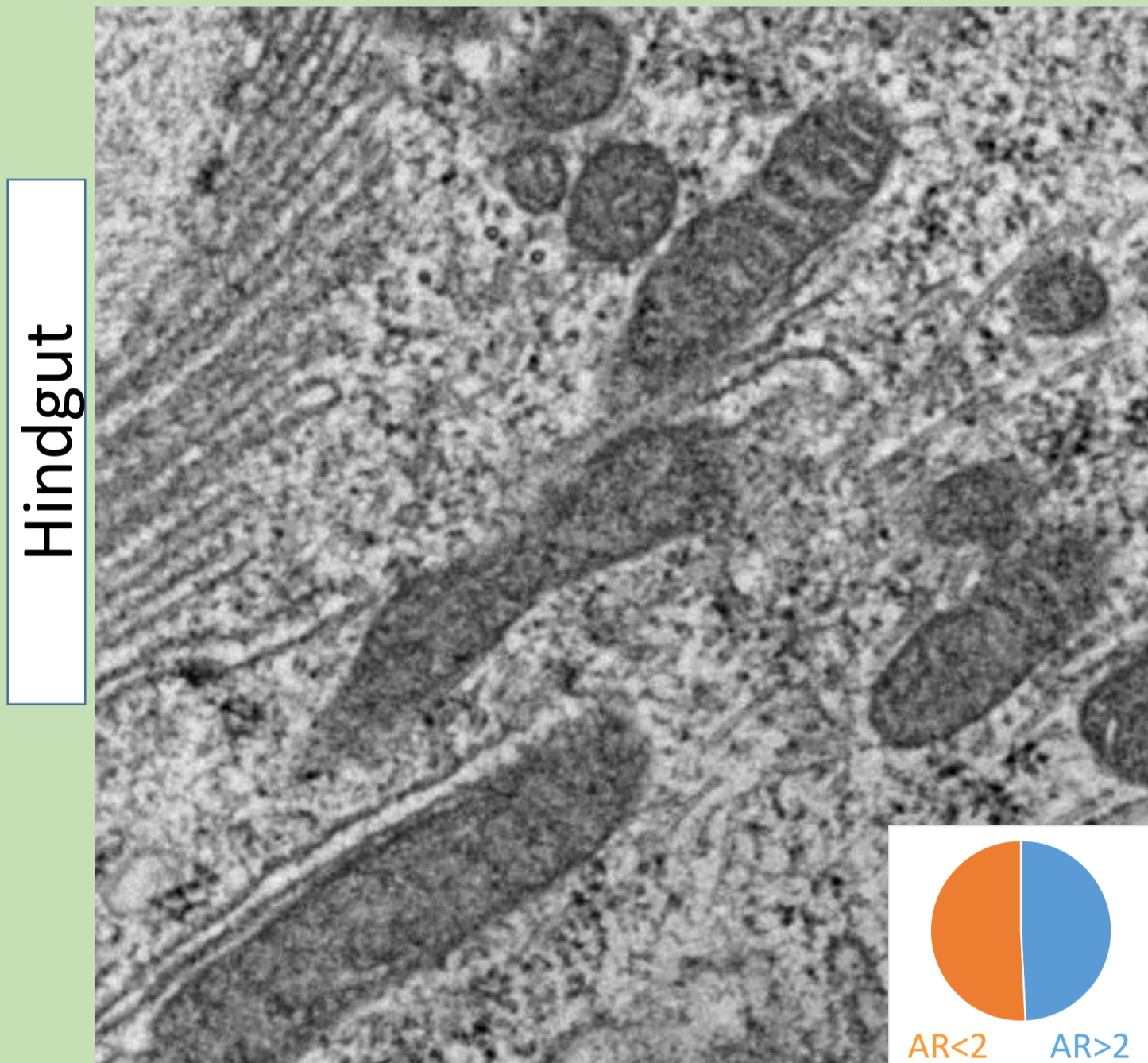
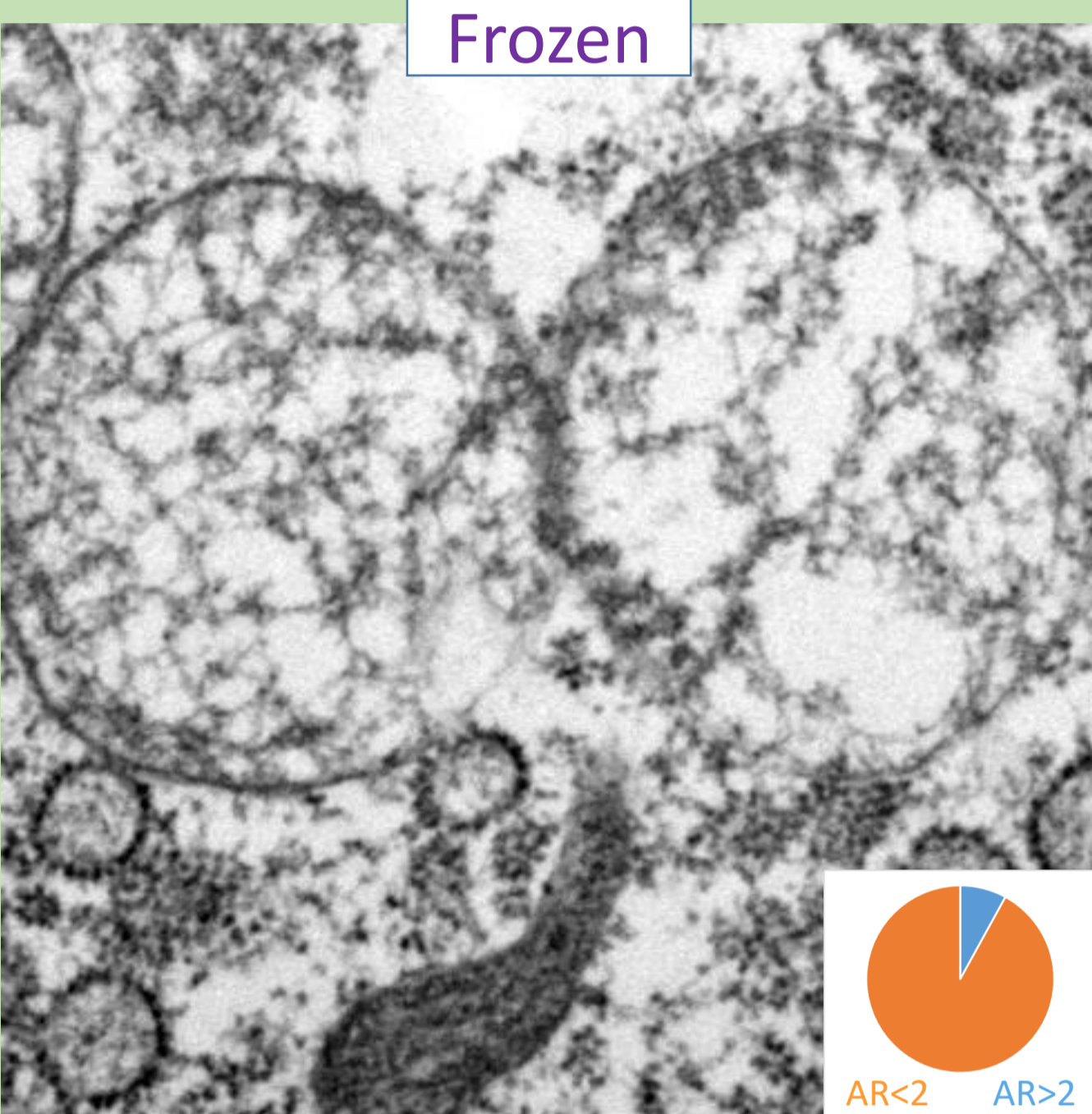
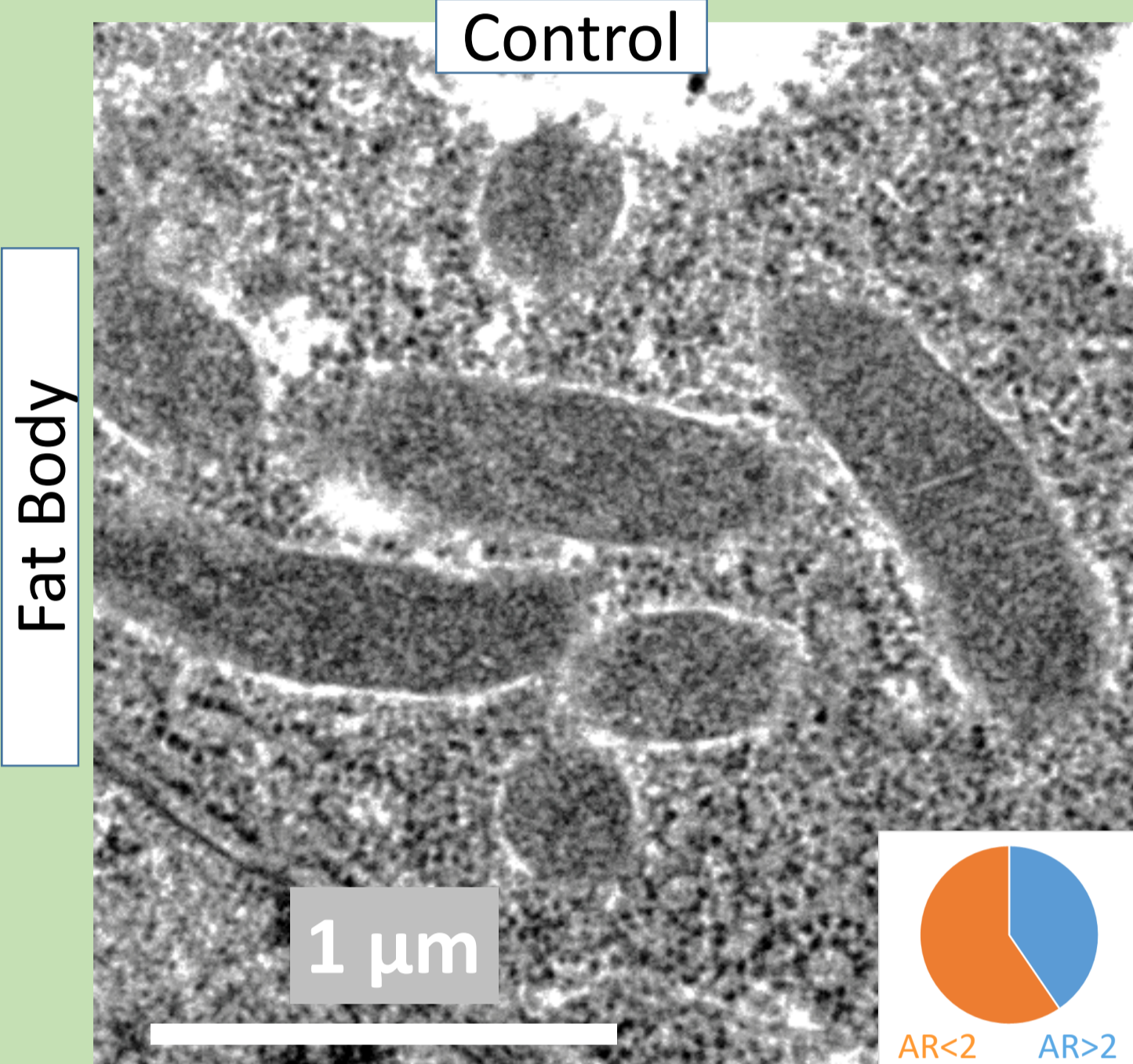


Fig. 1: Experimental design
Larvae were acclimated in order to produce two phenotypes, LD and SDA with contrasting freeze tolerance at -30°C (LD, no survival; SDA, 90% survival). Larvae of both phenotypes were exposed to freezing stress (-30°C) and their mitochondria were analyzed. Controls were not exposed to freezing stress.

Results: 1) Mt morphology and integrity:

LD (no survival at -30°C or in LN₂)



SDA (90% or 50% survival at -30°C or in LN₂)

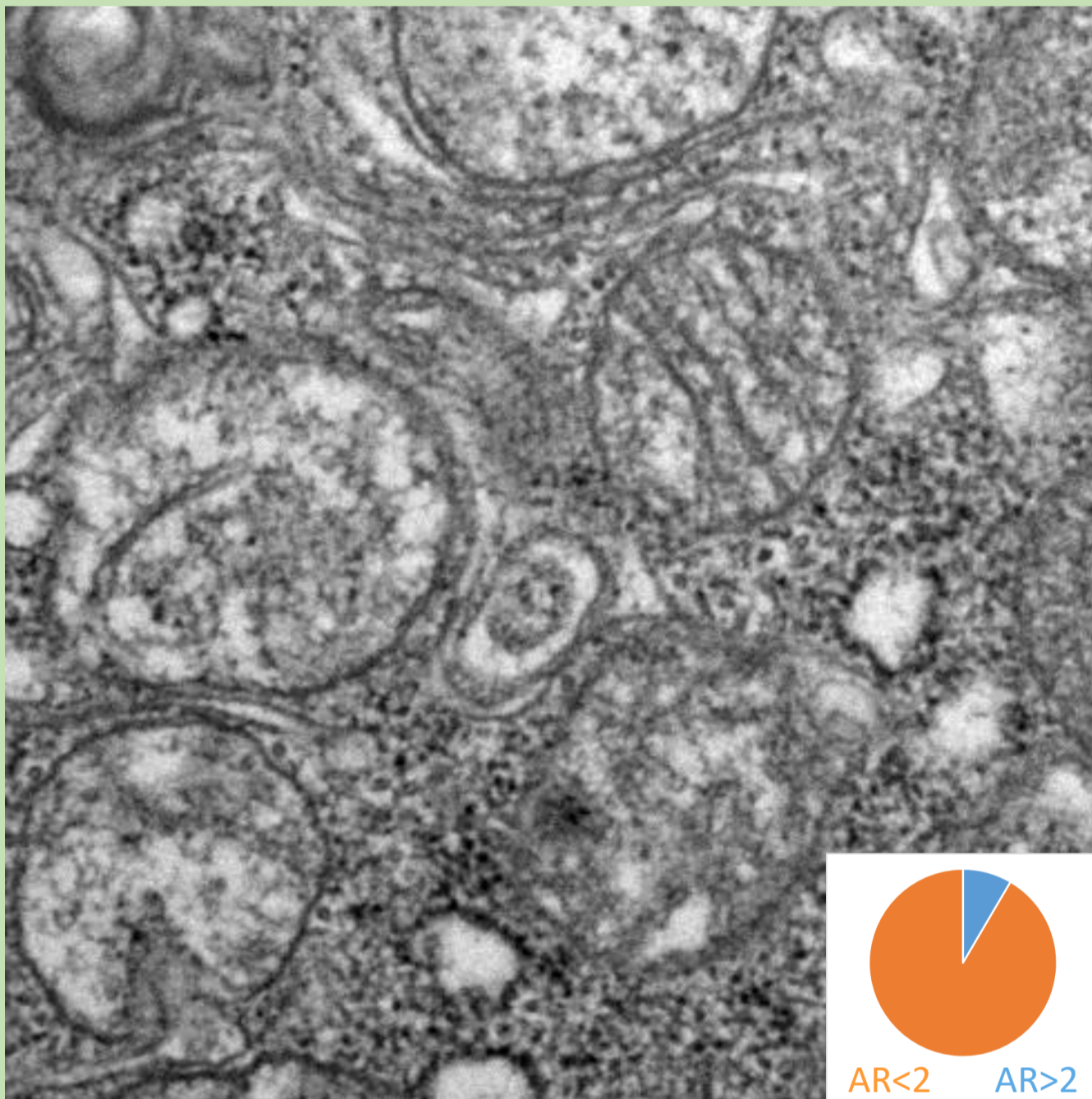
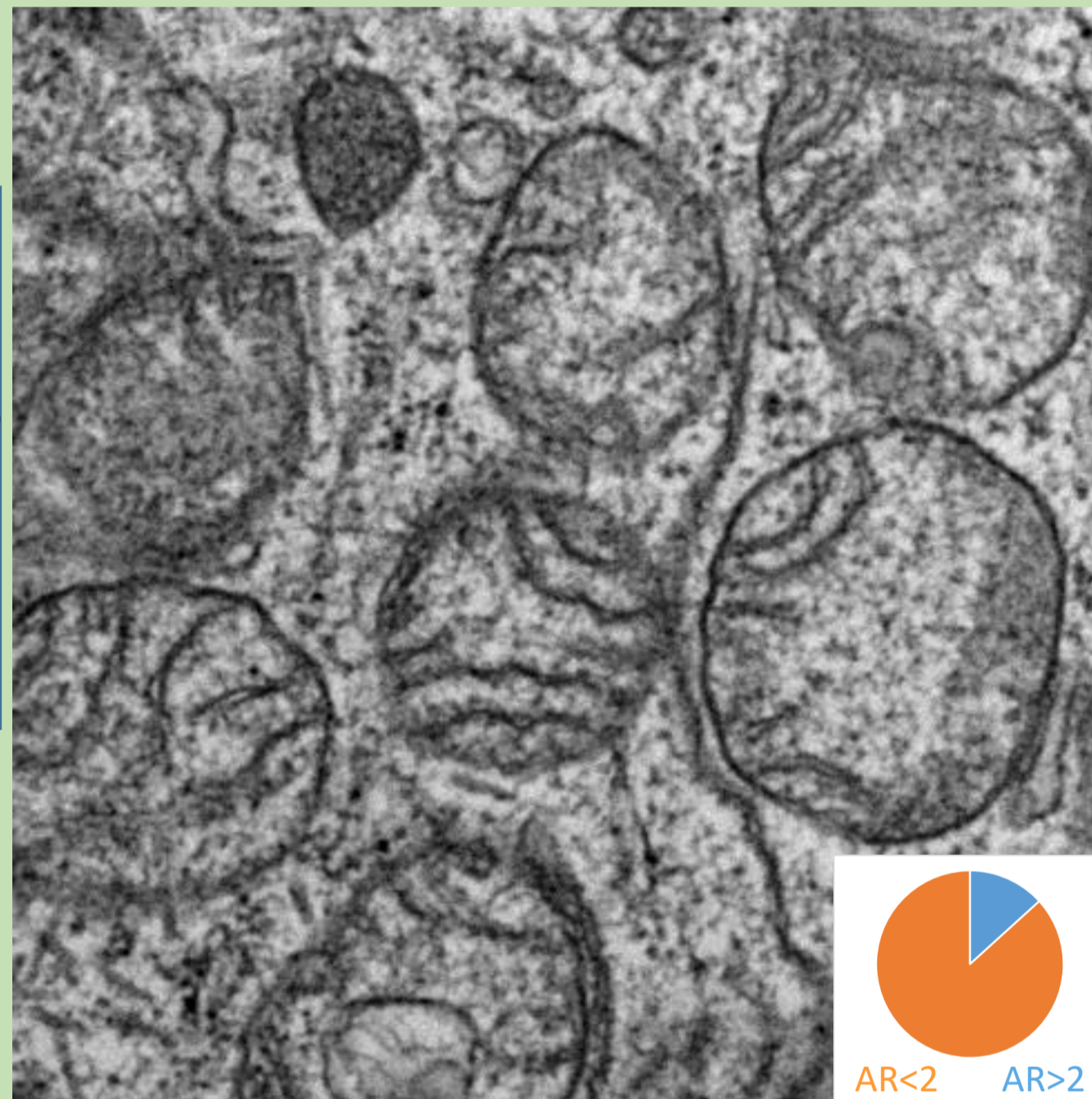
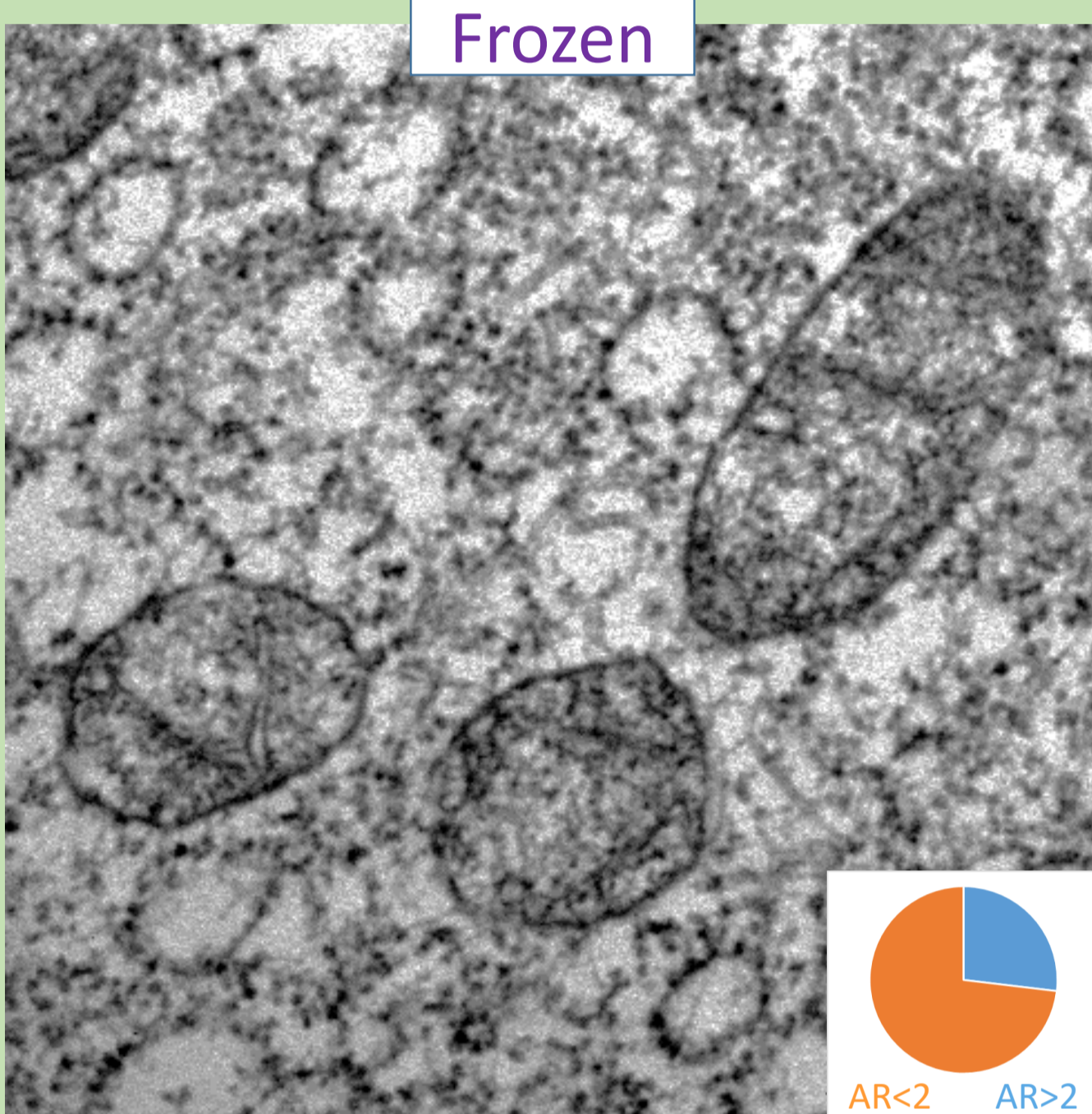
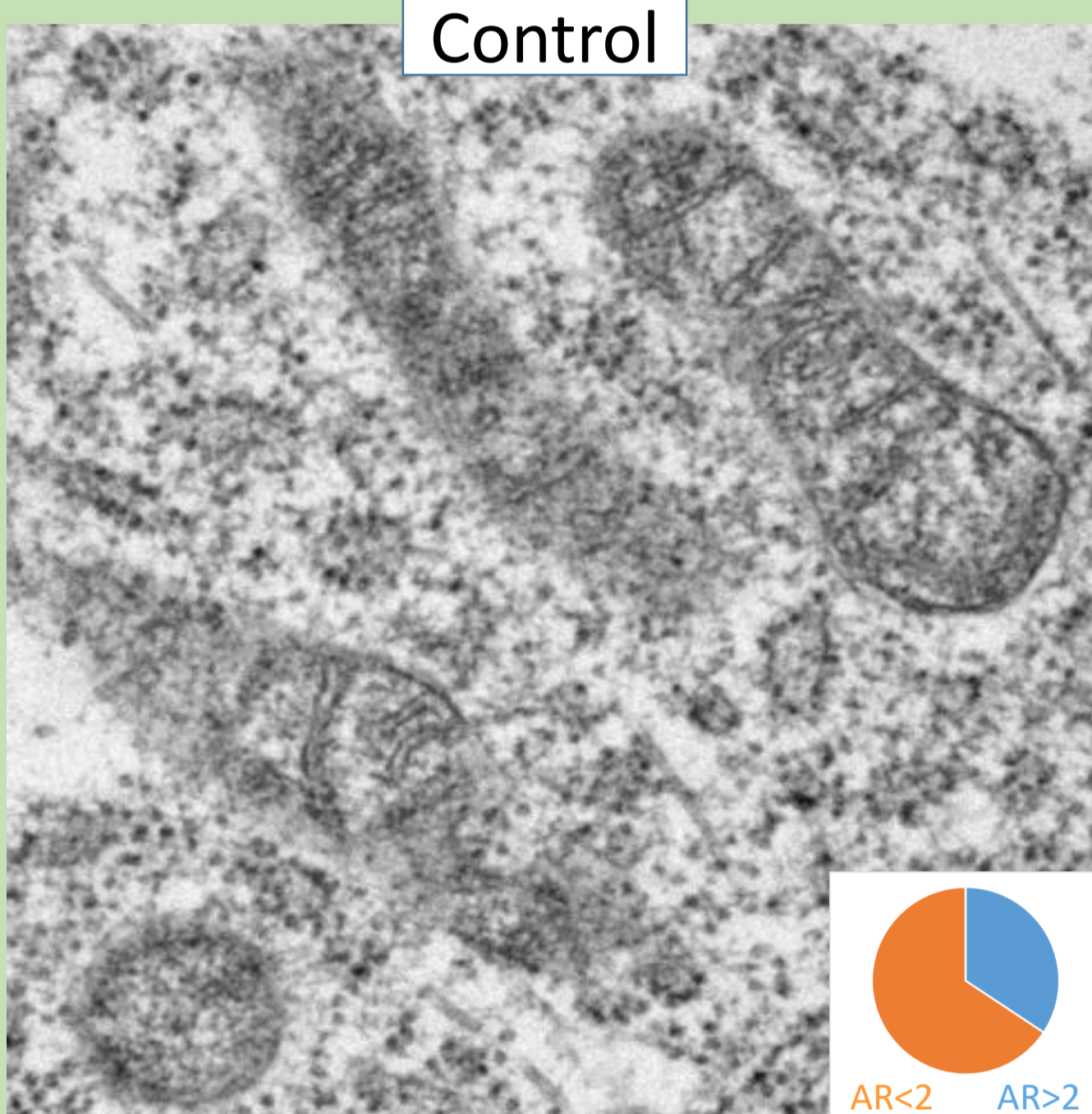


Fig. 2: Representative TEM micrographs of fat body and hindgut tissues of *C. costata* larvae. Mitochondria of control **LD** larvae are of typical rod shape with well developed internal structure (dense cristae) and clearly visible outer membrane while **SDA** mitochondria show tendency to rounding (especially in hindgut) and losing internal organization (sparse cristae). **LD** mitochondria are obviously destroyed by freezing to -30°C (they swell, lose internal organization, and some of them burst) while **SDA** mitochondria show no or little change upon freezing to -30°C. Pie diagrams represent mitochondrail shape expressed as Aspect Ratio (AR, ratio of major vs. minor axis of fitted ellipse). Scale bar is 1 µm for all micrographs.

2) Mt quantity:

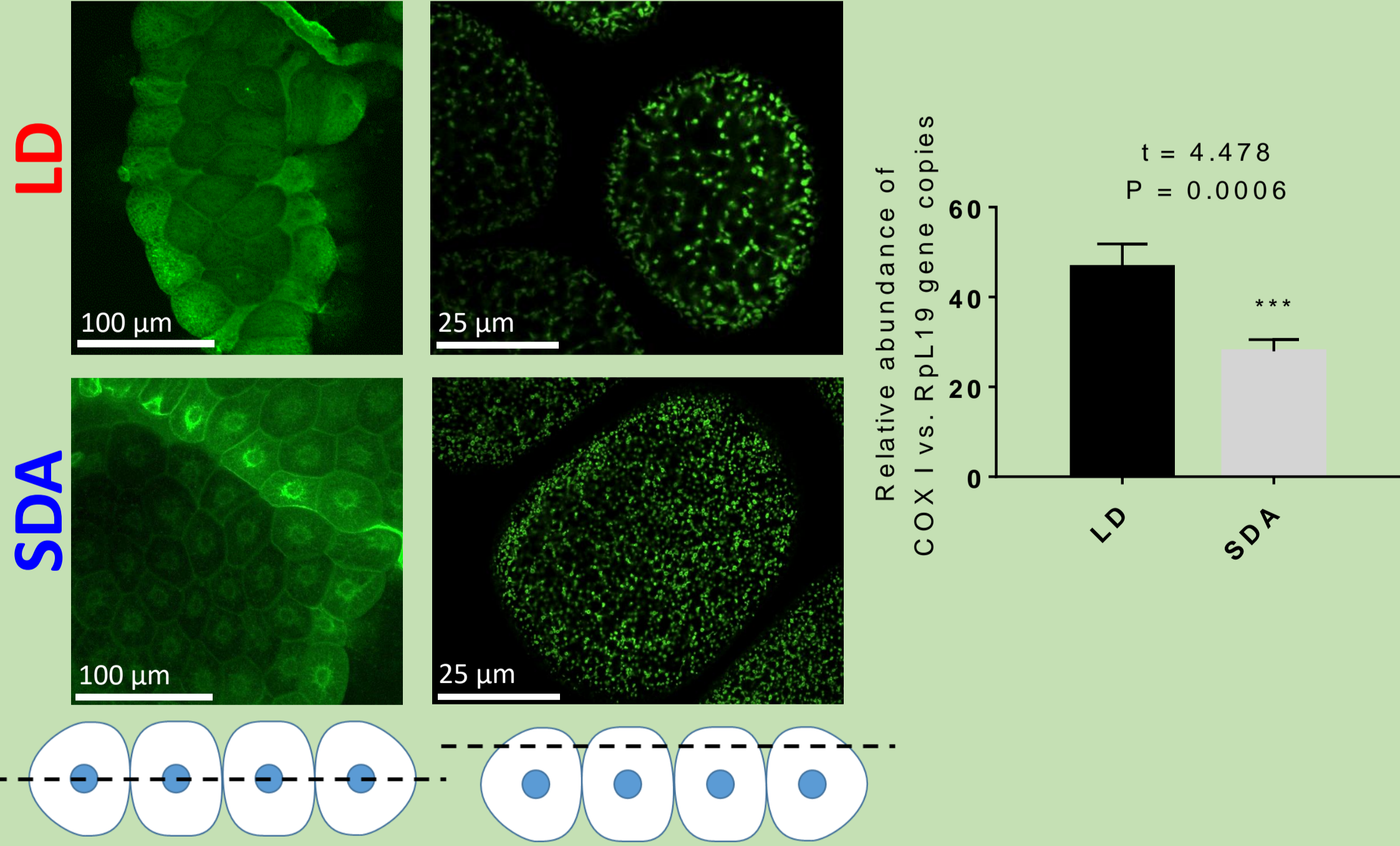


Fig. 3: Mt staining by MitoTracker Green shows that Mt are unequally distributed inside cells and also withing fat bodytissue. Mt counts apparently do not differ between **LD** and **SDA**.

Analysis of relative abundance of Mt (*cox I*) / nuclear (*RpL19*) DNA indicates that Mt counts are approximately 2-fold lower in **SDA** than in **LD** fat body tissue.

3) Mt functionality:

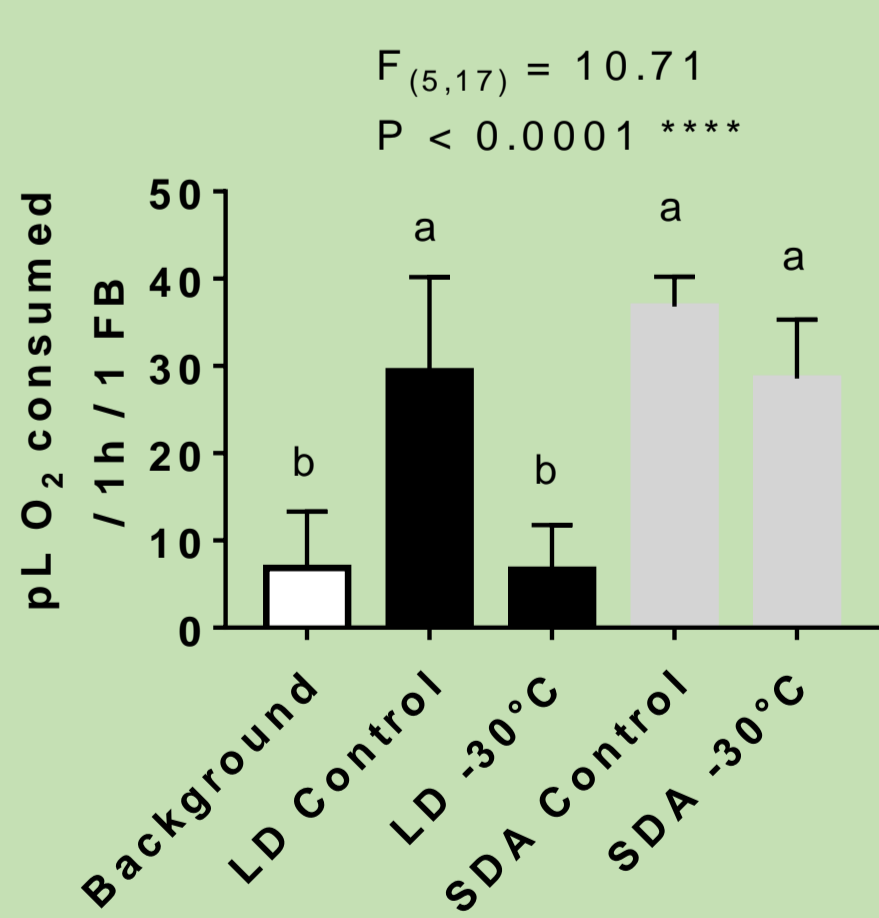
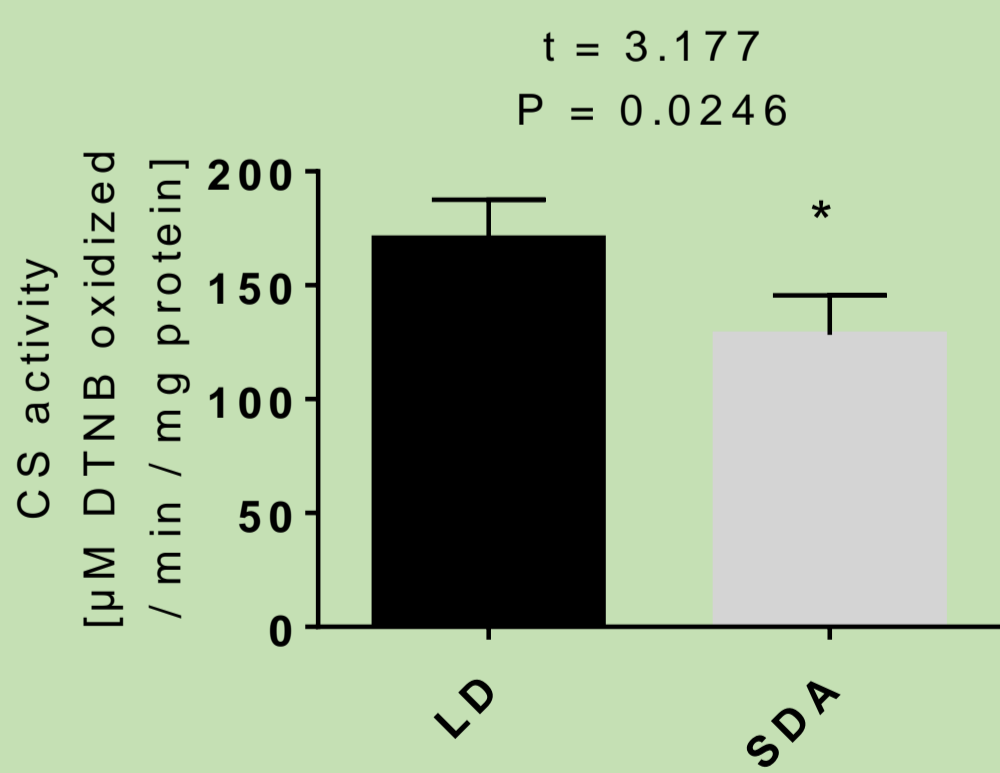


Fig. 4: Citrate synthase activity is approximately 1.5-fold lower in **SDA** than in **LD** fat body tissue.

The **O2 consumptions** do not differ between control **LD** and **SDA** tissues. **LD** tissue shows absolute loss of respiratory activity upon freezing, while **SDA** tissue maintains full respiratory activity after freezing.

Summary:

- Non-diapausing larvae (**LD**) have rod-like shaped mitochondria with well developed internal organization. Freezing to -30°C causes massive mitochondrial destruction – loss of internal organization, swelling, ruptures. Fat bodies dissected from frozen **LD** larvae do not show any sign of respiration capacity.
- Mitochondria in diapausing and cold acclimated larvae (**SDA**) are apparently different from those in **LD** larvae (especially in hindgut) - they are more circular, showing less densely packed internal structure. The counts of **SDA** mitochondria are likely reduced (approximately 2-fold) but show similar respiration rate at 23°C as **LD** mitochondria. Most importantly, the **SDA** mitochondria also do lose neither morphology nor functionality upon freezing.
- Our ongoing experiments focus on the role of **L-proline** in preservation of Mt integrity and functionality upon freezing stress. We feed LD larvae proline-augmented diet and also incubate tissues dissected from LD larvae in proline-rich medium. We ask whether treatments with proline show better survival after freezing stress.

Acknowledgement: This study was supported by Czech Science Foundation (GACR, grant no. 16-06374S to V.K.)