Cryopreservation in České Budějovice, Czech Republic

Comfortably Cold

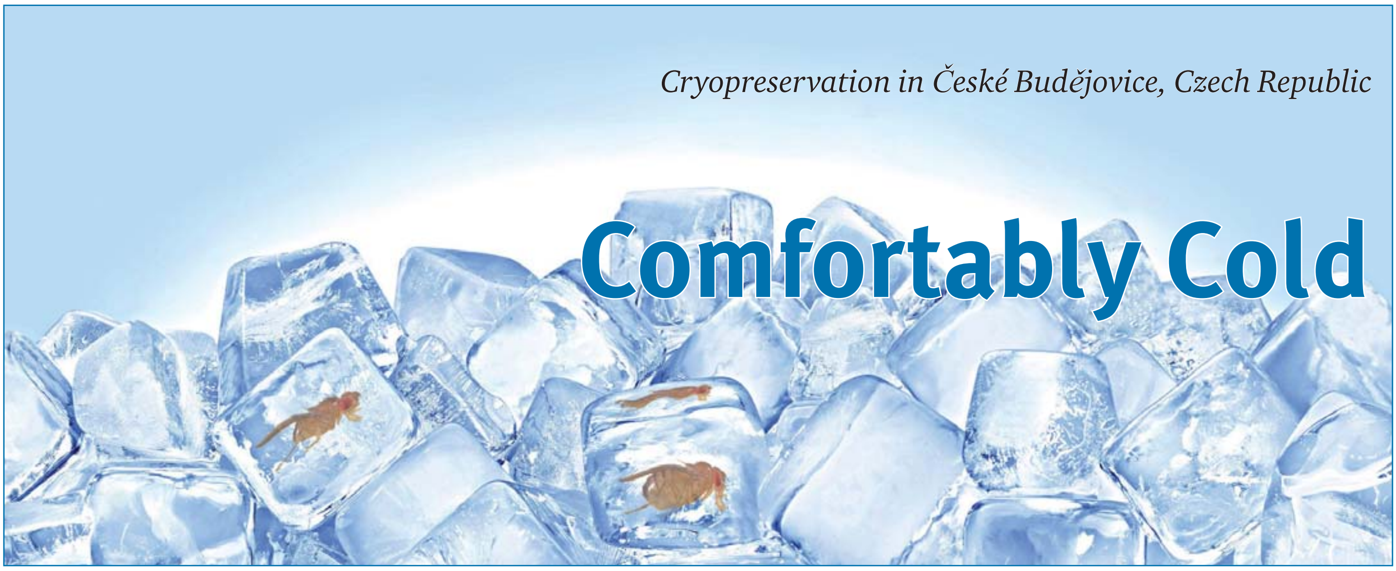

Photo (ice cubes): Fotolia/aris sanjaya

Jack Frost is not every organism's best friend. However, Vladimir Kostal and colleagues have recently shown that simple manipulations are enough to make warmth-loving insects like *Drosophila* tolerate sub-zero temperatures.

Exposing insects to the freezing cold has a long history. Already, back in the 18th century, French scientist René Antoine Ferchault de Réaumur observed that some insects survive winter in a frozen state (*Mémoires pour servir à l'histoire des insectes*). Since then, many biologists have been intrigued with the problems facing insects inhabiting cold environments. One such biologist is Vladimir Kostal.

Inspiration from UK, Japan and the US

Working between the Institute of Entomology, Biology Centre Academy of Sciences of the Czech Republic (BC ASCR), and the Faculty of Science, University of South Bohemia, Czech Republic, Kostal's research interests are focussed on insect biology. Currently, he leads a research group that is trying to understand "how insects cope with a seasonal world" including the mechanisms of insect diapause (the developmental delay due to recurrent periods of adverse weather conditions), cryopreservation and cold storage. In total, he tells me, he has been "studying insect overwintering, and the physiological principles of cold tolerance, for about 20 years". Along with Martin Holmstrup (Aarhus University, Denmark) and David Reanult (University of Rennes, France), he is one of only a handful of principle investigators based in Europe working in the field.

Although Vladimir has always been based in the Czech Republic, over the years he has also completed a number of international stays at overseas Universities. These stays include 15 months at the Horticulture Research International in Wellsbourne, England between 1992 and 1995, 31 months at Hokkaido University in Japan between 1997 and 2003, and three months at the Ohio State University, USA in 2008.

A foolish idea

The paper, now published in *PNAS* (109(9):3270-4), was the result of a simple idea that Vladimir and colleagues had come up with back in 2007, suggesting "Let's try to mimic the knowledge obtained by studying freeze-tolerant insects and apply it artificially to non-tolerant ones." The "trick" he told me "was to try transferring only the algorithms, the basic principles, without trying to transfer a whole set of many details ... Sounds quite foolish, but it worked".

Vladimir had previously studied the larvae of an extremely freeze-tolerant drosophilid fly called *Chymomyza costata*, which are capable of surviving even after submergence in liquid nitrogen (-196°C). In contrast to *C. costata*, fruit flies, *Drosophila melanogaster*, are of tropical origin and have weak innate tolerance to even mild chilling. This is illustrated by the halting of immature development in the fruit fly at temperatures below 10°C, the occurrence of chill injury at 6°C and the death of all developmental stages below -5°C after only a few hours.

Learning from a cold-loving fly

Through studying the *C. costata* larvae, Vladimir "revealed some of the basic mechanisms of freeze-tolerance". The freeze-tolerance was shown to be based on two main principles. Firstly, the entering of larvae into diapause (an interruption of development in response to unfavourable environmental conditions) and, secondly, the accumulation of high concentrations of the free amino acid L-proline, a known cryoprotectant, during the acclimatisation process.

Shutting down development

In 2007, Vladimir began researching whether he could take the lessons he learned from the *C. costata* studies, and apply the two main principles of inducing diapause and proline accumulation in the larvae to see whether he could achieve freeze-tolerance in a normally non-freeze tolerant organism, the common fruit fly.

Vladimir Kostal (right) puts his *Drosophila* research on ice, quite literally.

To achieve diapause and cold-acclimatisation Vladimir and co. exposed *Drosophila* larvae to above-lethal temperatures, under a specific "fluctuating thermal regime". This involved transferring fully-grown larvae 'accustomed' to 15°C, rather than the usual 25°C, for 19 days to an environment where the temperature oscillated between 6°C (12 hours) and 11°C (12 hours) for a period of three days, under constant darkness. As a result of this cold-acclimatisation, most larvae entered a dormant state.

They then sought to investigate whether this dormancy allowed the fly larvae to tolerate freezing to temperatures of -5°C. Thus, the flies were gradually frozen to -5°C in the presence of surrounding ice and then gradually melted. They showed that 6.3% of the larvae that had been exposed to the fluctuating thermal regime showed signs of cellular activity and one of the 284 larvae tested could continue developing to the adult stage. In contrast, larvae, which had been kept at a constant temperature of 25°C or 15°C, showed absolutely no freeze-tolerance.

Diet modification

Since cold acclimatising alone was seen to have limited effect only on the freeze tolerance of the fruit fly larvae, the authors went on to test whether the effect of augmenting the diet of these larvae with high levels of known cryoprotectants, proline, glycerol and trehalose could improve freeze-tolerance in conjunction with cold-acclimatisation.

Ultimately, only proline could induce freeze tolerance and that's because only proline could be accumulated to high concentrations within the larvae, whereas accumulation of glycerol and trehalose was limited even when amounts in the diet were increased to very high levels. This was due to the haemostatic control of concentrations of trehalose and glycerol but not of proline. In addition, Kostal *et al.* showed that proline could penetrate deep into tissues, in order to provide cryoprotection.

Also, in the absence of cold-acclimatisation, proline accumulation was seen to improve freeze-tolerance to some degree at the cellular level but the survivors had little ability to continue developing, with only around 2% metamorphosing into adults. In contrast, cold-acclimatised larvae, fed a high-proline diet, showed much higher survival at the cellular level and 9.1% metamorphosed into adults. In addition, the survivors were able to produce viable offspring.

A surprise success

Overall, the results showed that cold-acclimatisation of fly larvae under a specific fluctuating thermal regime accompanied by a proline-rich diet can successfully induce freeze-tolerance in the usually warmth-loving *Drosophila melanogaster*. Exactly as Vladimir had originally hypothesised. However, the success of the study was still "a real surprise" according to Vladimir, "Not only for us but for other colleagues in the field as well. When we found that the freeze-tolerance is transferable to a chill-susceptible species, most of us were quite sceptic because the adaptation for freeze-tolerance is known to be rather rare in nature and it is based on highly complex mechanisms."

Important encouragement

Despite the clear success of Vladimir's research, he describes his research funding as "so, so" and remarks that he is reliant "mainly on national resources (Czech grant agencies) but the amount of money allocated to basic science decreased during the last few years".

Vladimir states, "I think that the main importance of our experiment is that it brings encouragement." He goes on to explain that the work, which they have done here and are continuing to do, going "more deeply into the mechanisms" behind freeze-tolerance induction and extending "closer to more practical applications", will hopefully "contribute to the development of a technique for long-term storage of numerous *D. melanogaster* lineages and mutant strains. Such technique could replace tedious, expensive and risky, continuous rearing practices used in big *Drosophila* centres, such as Binghampton (USA), Vienna (Austria) and thousands of research laboratories all over the world". Since *Drosophila* is widely used in the study of genetic disease and developmental defects, this is clearly of great importance.

Practical applications

Kostal also hopes that, in the long term, "perhaps a similar approach can also be tested for other insects or for other biological materials like tissues and cells", which may benefit many research laboratories, cell banks and tissue banks. In addition, comprehension of cold tolerance has practical applications in understanding the overwinter survival of pest species in agriculture and forestry, and may potentially lead to the development of strategies to overcome and prevent overgrowth of pest populations.

So, what started out as a simple and foolish idea has turned into a brilliant discovery with far-reaching consequences.

NICOLA HUNT

Lab Times

Founded 2006. Issue 3, 2012
Lab Times is published bimonthly

ISSN: 1864-2381

Publisher: *LJ*-Verlag Herfort und Sailer
Kai Herfort, Ralf Neumann

Office: Alte Straße 1, 79249 Merzhausen, Germany
Tel.: +49 (0)761-286869; Fax: +49 (0)761-35738

Management: Kai Herfort, Tel: +49 (0)761-286869

Editors:
Ralf Neumann (Editor-in-chief), Kathleen Gransalke, Kai Herfort, Winfried Koeppelle, Harald Zähringer
Tel.: +49 (0)761-2925884, *editors@lab-times.org*

Reporters:
Latika Bhonsle, Jeremy Garwood, Karin Hollricher, Irena Hreljac, Madhuvanthi Kannan, Steven Buckingham, Alejandra Manjarrez, Rosemarie Marchan, Ralf Schreck

Graphics, Design and Production:
Ulrich Sillmann (Art Director), Kathleen Gransalke, Kai Herfort, Winfried Koeppelle, Ralf Neumann, Harald Zähringer

Cover Photo: iStockphoto

Sales:
Advertising Manager: Bernd Beutel
Top-Ad Bernd Beutel, Schlossergäßchen 10, 69469 Weinheim, Germany
Tel: +49 (0)6201-29092-0
Fax: +49 (0)6201-29092-20
info@top-ad-online.de

Recruitment: Ulrich Sillmann,
Tel: +49 (0)761-2925881, *jobs@lab-times.org*

Printed at: Stürtz GmbH,
Alfred-Nobel-Straße 33, 97080 Würzburg, Germany

Web: *www.lab-times.org*
Webmaster: Carsten Rees
Tel.: +49 (0)761-1563461, *webmaster@lab-times.org*

Prices & Subscription rates:
- price per issue: €4.90
- research institutes/units: free of charge
- annual subscriptions for companies and personal subscribers: €27

Subscribe at *http://www.labtimes.org/kontakt/sub.html*, or mail to: *subscriptions@lab-times.org*

Bank Accounts:
- Volksbank Freiburg
KTO: 319 0 315; BLZ :680 900 00;
BIC: GENODE61FR1;
IBAN: DE24 6809 0000 0003 1903 15
- Basler Kantonalbank
KTO: 16 428.471.36;
BIC: BKBBCHBB;
IBAN: CH06 0077 0016 0428 4713 6