


**COMMENTARY**

# How hot are single cells?

 Robert S. Balaban 

All energy conversion processes of the cell generate heat, as these processes are invariably inefficient. This is true for the generation of ATP via glycolysis or oxidative phosphorylation, as well as work functions. Thus, heat generation is an inescapable aspect of cellular physiology. The evolution of endothermy maintaining body temperature of 37–39°C in mammals has been proposed to allow for environmental niche expansion due to enhanced thermoregulatory capacity (Crompton et al., 1978; McNab, 1978; Ruben, 1995) and peak sustained performance (Bennett and Ruben, 1979; Ruben, 1995). An increase in basal, or resting, metabolic rate in endotherms is thought to be generated by the high mitochondrial content and metabolic demand of internal organs. The cause of this high resting-metabolic rate remains a source of controversy, but processes such as high ion permeability of plasma and mitochondrial membranes and high protein and membrane turnover have been proposed to underlie this phenomenon (Ruben, 1995). Importantly, the maintenance of homeothermic endothermy can be enhanced by dynamic “insulation” from the environment provided by feathers and fur, as well as more static structures such as subcutaneous fat and large body mass that greatly decreases the resting heat loss to the environment (Boldrinia et al., 2018). Oyama et al., in the current issue of *Journal of General Physiology*, present well-designed experiments measuring the surface temperature of different isolated cells during alterations in the energy metabolism to examine topology of heat production in single mammalian cells.

As discussed above, heat generation is a basic property of an endotherm cell. However, isolated single cells, without any insulation factors or summation of adjacent cells (Baffou et al., 2014), cannot maintain a high cellular temperature, which is why we heat perfusion chambers for single-cell studies. However, with the bath at physiological temperature and the cell in close thermal contact with the bath/chamber, does a temperature gradient exist between cell and bath as well as between cellular organelles? If dynamic temperature gradients exist between organelles, this could represent a cellular regulatory mechanism that has been overlooked (Lane, 2018).

Over the years there have been many attempts at measuring cellular and intracellular temperature using different physical approaches with many groups interpreting this data to represent

a large temperature gradient in isolated cells (Zohar et al., 1998; Zeeb et al., 2004; Suzuki et al., 2007; Gota et al., 2009; Okabe et al., 2012). Chretien et al., (2018) interpreted localized temperature sensitive fluorescent probe data to support the notion of a large temperature gradient between the mitochondria and cytosol with the mitochondria matrix approaching 50°C. The magnitude of this gradient was a very surprising result (Lane, 2018).

A brief comment on the potential of cellular mitochondria matrix reaching 50°C, as these insights also apply to the general temperature gradient issues inside single cells. Isolated mitochondria become uncoupled at temperatures >45°C (Zukiene et al., 2010; Anderson and Gillooly, 2018) questioning the viability of mitochondria at 50°C. NADH fluorescence decreases with increasing temperature. 50°C would almost eliminate both free and bound NADH fluorescence, extrapolating the data from Zelent et al. (2007). However, NADH fluorescence from mitochondria far exceeds the cytosolic emission (Avi-Dor et al., 1962; Estabrook, 1962), enabling mitochondrial NADH fluorescence to image mitochondrial structure in muscle cells (Eng et al., 1989; Rothstein et al., 2005; Lewellyn et al., 2008). Consistent with these NADH fluorescence amplitude measures, the mitochondrial NADH lifetime in intact cells is similar to isolated mitochondria in the nanosecond range (Wakita et al., 1995; Blacker et al., 2014), implying similar temperature environment. Thus, the temperature dependence of oxidative phosphorylation and the behavior of the intrinsic chromophore, NADH, do not support the notion of “hot” mitochondria. Finally, mitochondrial NADH fluorescence in the heart has been shown to be insensitive to workload (Heineman and Balaban, 1993), again inconsistent with a change in matrix temperature with increases in oxidative phosphorylation. NADH fluorescence is sensitive to many parameters besides temperature, including binding and enzyme activity, but the results are not consistent with a dynamic matrix temperature approaching 50°C.

Clearly recording an intracellular organelle at this high a temperature is unexpected and can also be challenged when considering the basic thermal conductivity and power generation of cellular organelles (Baffou et al., 2014; Lane, 2018). Physical modeling predicting a gradient more than  $10^{-5}$  K cannot

---

National Health, Lung, and Blood Institute, National Institutes of Health, Bethesda, MD.

Correspondence to Robert S. Balaban: [balaban@nhlbi.nih.gov](mailto:balaban@nhlbi.nih.gov).

© 2020 Balaban. This article is distributed under the terms of an Attribution–Noncommercial–Share Alike–No Mirror Sites license for the first six months after the publication date (see <http://www.rupress.org/terms/>). After six months it is available under a Creative Commons License (Attribution–Noncommercial–Share Alike 4.0 International license, as described at <https://creativecommons.org/licenses/by-nc-sa/4.0/>).

exist in a single cell (Baffou et al., 2014). This so-called “ $10^{-5}$  gap” (Lane, 2018; Suzuki and Plakhotnik, 2020) between theory and the interpretation of intracellular temperature measures—found using many approaches—has generated considerable debate (Baffou et al., 2014; Takei et al., 2014; Baffou et al., 2015; Suzuki et al., 2015; Lane, 2018; Suzuki and Plakhotnik, 2020). Potential explanations for this discrepancy have included the size of heat source, the homogeneity of cellular thermal conductivity and “nonthermal” alterations in temperature probe performance (i.e., artifacts). On the last point, intracellular probes, especially in the protein- and membrane-rich organelle environments, are likely sensitive to more than temperature alone. Indeed, a common summary of most of these discussions on the  $10^{-5}$  gap is that “...the experimental results should also be critically considered, and new methods of temperature measurement more accurate and less susceptible to artifacts should be developed” (Suzuki and Plakhotnik, 2020).

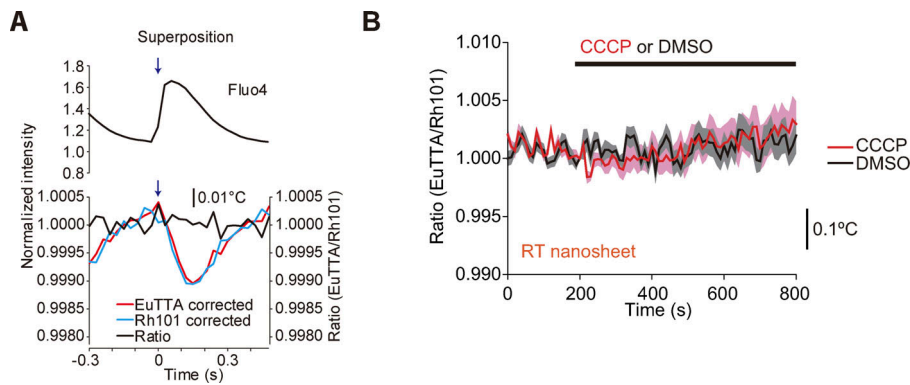
Toward this goal, the article from Oyama et al. (2020) in this issue of the *Journal of General Physiology* provides a new method and procedures to monitor the surface temperature of isolated cells avoiding many of the pitfalls of intracellular measurements. These authors used a ratiometric approach on cells grown directly on a fluorescent thermometer nanosheet. The nanosheet was a poly(methyl methacrylate) membrane embedded with a temperature-sensitive fluorescent probe (EuTTA) and temperature-insensitive probe (rhodamine 101 [Rh101]) for ratiometric compensation of optical artifacts. Growing different cell types, including cardiac myocytes, HeLa cells, brown fat cells, and neurons, on this membrane permitted the dynamic imaging of the cellular surface temperature under a wide variety of conditions. The authors employ a careful technical approach in attempting to measure temperature gradients on the order of a fraction of a degree. The technical problems they encountered are illuminating with regard to the difficulty of these measures, even on the surface of the cell. The most surprising result, from a technical perspective, was the observation that EuTTA fluorescence in the gel was sensitive to not only temperature but also small changes in the cell–gel interface. This “non-temperature” sensitivity was demonstrated with dead cells on the gel or contracting cardiomyocytes that altered EuTTA fluorescence independent of temperature. The nature of the surface contacts interfering with gel-based EuTTA fluorescence is yet ill-defined, but likely due to an optical interference at the cell–gel interface. The authors, to their credit, detected this error and developed a ratiometric method with a temperature-insensitive fluorescent probe (Rh101), with fluorescent properties close to EuTTA but spectrally distinct, to compensate for both primary and secondary filter effects (Kubista et al., 1994). This compensation system using an inert reference compound has been used in prior biological fluorescence studies (Koretsky et al., 1987; Resch-Genger and Ameloot, 2008; Lee et al., 2009) and was apparently effective in this application. This was best demonstrated in contracting cardiomyocytes where EuTTA fluorescence was changing with each beat (Fig. 1 A), but this was due to a gross optical artifact affecting Rh101 emission to the same extent. The ratio of EuTTA and Rh101 emissions demonstrated no specific change in EuTTA or associated

change in temperature with stimulated contraction and associated cellular work. These unexpected artifacts, such as cells binding to the gel or contracting, underlines the difficulty of performing these highly sensitive experiments all while looking for minute changes in temperature. The authors must be commended for identifying these artifacts and providing a reasonable correction getting closer to the physical measure desired. This is not always the case for single-cell fluorescent experiments in the literature where optical artifacts have impacted many interpretations over the years. With a properly corrected system, Oyama et al. (2020) undertook an impressive set of studies on a wide variety of cell types and protocols previously shown to apparently generate a localized temperature gradient effects inside cells. In agreement with the  $10^{-5}$  gap calculations, these authors found no evidence for a large increase in external temperature associated with alterations that should greatly stimulate energy conversion processes in a variety of cells. This is a very important “negative” result in that implies any temperature gradient is confined to intracellular compartments.

From my own experience, the most convincing demonstration of the lack of correlation of surface temperature with metabolic rate was the authors’ cardiac myocyte experiment with CCCP uncoupler (reproduced in Fig. 1 B) a condition that should induce maximum mitochondrial heat production. Similar results were found in brown fat cells. Importantly, cardiomyocytes have many subsarcolemmal mitochondria located just under the plasma membrane (Glancy et al., 2017), positioning these potential heat sources close to the temperature-sensitive membrane. As seen in Fig. 1 B, no significant change in the EuTTA/Rh101 fluorescence was detected in cardiac myocytes treated with CCCP, consistent with theoretical calculations even in this highly energetic cell. Similar results were obtained on all the cell types studied with different methods of stimulating cellular energy metabolism. Again, this is an important contribution to this controversial field and clearly limits the possibility of cellular temperature gradients to localized intracellular sites.

The authors point out that many intracellular fluorescent temperature probes have non-temperature influences on their fluorescent emission much like the effects that they corrected for with EuTTA. They specifically point out these nonthermal issues with thermo yellow but nevertheless used this probe in these studies as a point of comparison with their extracellular measures. It must be noted that this new extracellular temperature measurement cannot be used to rule out the existence of intracellular thermal gradients, with the caveat of subsarcolemma mitochondria in cardiomyocytes would be very close to the temperature sensitive gel. The fact that multiple methods apparently detect intracellular gradients cause the authors to speculate that local temperature gradients do exist in the cell with “high probability” despite the  $10^{-5}$  gap.

With the clear demonstration by Oyama et al. (2020) that essentially no thermal gradient exists from the cell to the extracellular space, how could intracellular organelle temperature gradients exist? One possibility is that the heat source is highly localized in mitochondria or in ATP using ATPase activity such as SECA or myosin. However, given the maximum flux per



**Figure 1. Surface temperature measures from beating cardiomyocytes. (A)** Paced cardiomyocytes. Superimposed data of Fluo4 (top), and EuTTA (red), Rh101 (blue), and EuTTA/Rh101 (black; bottom). Data obtained by 10 stimuli were superimposed. A calibration bar of 0.01°C. Arrows indicate time of stimulation (data from Fig. 4 D in Oyama et al. (2020)). **(B)** Measurement for CCCP-induced thermogenesis in rat neonatal cardiomyocytes. Time course of changes in the EuTTA/Rh101 ratio of ratiometric thermometer (RT) nanosheets with cardiomyocytes upon application of 10  $\mu$ M CCCP (red) or 0.1% DMSO (black). Number of cells, 18 ( $n = 3$ ) for both CCCP and DMSO. The horizontal black line indicates the period during which CCCP or DMSO was present. Data from Fig. S11 B in Oyama et al. (2020).

cytochrome chain and most ATPase proteins, it is unlikely that only a few localized reactions could meet the energy metabolism needs of the cell. Histochemistry studies also show a relatively diffuse distribution of these enzymes in their organelles.

Another possibility is limited thermal conductance due to membranes and proteins which would “trap” heat near intracellular organelles. However, thermal conductivity of protein and lipids are lower by only a factor of 2 to 4, they do not approach the  $10^{-5}$  gap (Leitner, 2008; Nakano et al., 2010; Baffou et al., 2014; Bastos et al., 2019; Suzuki and Plakhotnik, 2020). That is, in vitro measures of thermal conductivity of these biological structures do not support a local “containment” of a heat source in the cell. Indeed, if the bilayers and proteins provided large barriers ( $10^5$  times greater than water) for heat dissipation, then common fluorescence imaging methods such as confocal microscopy, which deposits large amounts of heat into optically absorbent organelles, would be problematical. Indeed, Schönle and Hell (1998) demonstrated the importance of radial heat dissipation in high resolution cellular optical microscopy to reduce the local thermal load. With these theoretical concerns, it would be ideal to measure heat propagation across a single cell to determine if “excluded” regions exist, assuming that heat insulation is reciprocal. An approach to do this was recently presented by Yang et al. (2011) in plants using the intrinsic fluorescence from chlorophyll as a temperature indicator and an external localized heat source. As the subcellular thermal conductivity of the living cells is still one of the assumptions in the  $10^{-5}$  gap theoretical calculation, this type of study might prove useful in mammalian cells to evaluate the existence of thermal barriers within the cell.

The work by Oyama et al. (2020) clearly demonstrates that the surface temperature of isolated mammalian cells is not affected by the rate of energy metabolism in the cell. Thus, if temperature gradients exist, they must be restricted to small intracellular events. I look forward to new approaches of imaging temperature gradients within cells, as the  $10^{-5}$  gap between some measures and theory remains. If intracellular temperature gradients do exist, it would lead to a very new biophysical picture of the cell, as well as potential cellular regulatory networks.

## Acknowledgments

Eduardo Ríos served as editor.

The author thanks Paul Jobsis, Han Wen, Jay Knutson, and Dillon Chung for useful discussions concerning this commentary.

The author declares no competing financial interests.

## References

- Anderson, D.M., and J.F. Gillooly. 2018. Comparing the temperature dependence of mitochondrial respiration among vertebrates. *Evol. Ecol. Res.* 19:659–668.
- Avi-Dor, Y., J.M. Olson, M.D. Doherty, and N.O. Kaplan. 1962. Fluorescence of pyridine nucleotides in mitochondria. *J. Biol. Chem.* 237:2377–2383.
- Baffou, G., H. Rigneault, D. Marguet, and L. Jullien. 2014. A critique of methods for temperature imaging in single cells. *Nat. Methods.* 11: 899–901. <https://doi.org/10.1038/nmeth.3073>
- Baffou, G., H. Rigneault, D. Marguet, and L. Jullien. 2015. Reply to: “Validating subcellular thermal changes revealed by fluorescent thermosensors” and “The 10(5) gap issue between calculation and measurement in single-cell thermometry”. *Nat. Methods.* 12:803. <https://doi.org/10.1038/nmeth.3552>
- Bastos, A.R.N., C.D.S. Brites, P.A. Rojas-Gutierrez, C. DeWolf, R.A.S. Ferreira, J.A. Capobianco, and L.D. Carlos. 2019. Thermal Properties of Lipid Bilayers Determined Using Upconversion Nanothermometry. *Adv. Funct. Mater.* 29. 1905474. <https://doi.org/10.1002/adfm.201905474>
- Bennett, A.F., and J.A. Ruben. 1979. Endothermy and activity in vertebrates. *Science.* 206:649–654. <https://doi.org/10.1126/science.493968>
- Blacker, T.S., Z.F. Mann, J.E. Gale, M. Ziegler, A.J. Bain, G. Szabadkai, and M.R. Duchan. 2014. Separating NADH and NADPH fluorescence in live cells and tissues using FLIM. *Nat. Commun.* 5:3936. <https://doi.org/10.1038/ncomms4936>
- Boldrinia, J.L., M.P. Viana, S.F. dos Reis, and B. Henning. 2018. A mathematical model for thermoregulation in endotherms including heat transport by blood flow and thermal feedback control mechanisms: changes in coat, metabolic rate, blood fluxes, ventilation and sweating rates. *Lett. Biomath.* 5:129–173. <https://doi.org/10.30707/LIB5.1Boldrinia>
- Chretien, D., P. Benit, H.H. Ha, S. Keipert, R. El-Khoury, Y.T. Chang, M. Jastroch, H.T. Jacobs, P. Rustin, and M. Rak. 2018. Mitochondria are physiologically maintained at close to 50 degrees C. *PLoS Biol.* 16: e2003992.
- Crompton, A.W., C.R. Taylor, and J.A. Jagger. 1978. Evolution of homeothermy in mammals. *Nature.* 272:333–336. <https://doi.org/10.1038/272333a0>
- Eng, J., R.M. Lynch, and R.S. Balaban. 1989. Nicotinamide adenine dinucleotide fluorescence spectroscopy and imaging of isolated cardiac myocytes. *Biophys. J.* 55:621–630. [https://doi.org/10.1016/S0006-3495\(89\)82859-0](https://doi.org/10.1016/S0006-3495(89)82859-0)
- Estabrook, R.W.. 1962. Fluorometric measurement of reduced pyridine nucleotide in cellular and subcellular particles. *Anal. Biochem.* 4:231–245. [https://doi.org/10.1016/0003-2697\(62\)90006-4](https://doi.org/10.1016/0003-2697(62)90006-4)
- Glancy, B., L.M. Hartnell, C.A. Combs, A. Femnou, J. Sun, E. Murphy, S. Subramaniam, and R.S. Balaban. 2017. Power Grid Protection of the

- Muscle Mitochondrial Reticulum. *Cell Rep.* 19:487–496. <https://doi.org/10.1016/j.celrep.2017.03.063>
- Gota, C., K. Okabe, T. Funatsu, Y. Harada, and S. Uchiyama. 2009. Hydrophilic fluorescent nanogel thermometer for intracellular thermometry. *J. Am. Chem. Soc.* 131:2766–2767. <https://doi.org/10.1021/ja807714j>
- Heineman, F.W., and R.S. Balaban. 1993. Effects of afterload and heart rate on NAD(P)H redox state in the isolated rabbit heart. *Am. J. Physiol.* 264: H433–H440.
- Koretsky, A.P., L.A. Katz, and R.S. Balaban. 1987. Determination of pyridine nucleotide fluorescence from the perfused heart using an internal standard. *Am. J. Physiol.* 253:H856–H862.
- Kubista, M., R. Sjöback, S. Eriksson, and B. Albinsson. 1994. Experimental correction for the inner-filter effect in fluorescence spectra. *Analyst (Lond.)*. 119:417–419. <https://doi.org/10.1039/AN9941900417>
- Lane, N.. 2018. Hot mitochondria? *PLoS Biol.* 16. e2005113. <https://doi.org/10.1371/journal.pbio.2005113>
- Lee, Y.-E.K., R. Smith, and R. Kopelman. 2009. Nanoparticle PEBBLE sensors in live cells and in vivo. *Annu. Rev. Anal. Chem. (Palo Alto, Calif.)*. 2:57–76. <https://doi.org/10.1146/annurev.anchem.1.031207.112823>
- Leitner, D.M.. 2008. Energy flow in proteins. *Annu. Rev. Phys. Chem.* 59: 233–259. <https://doi.org/10.1146/annurev.physchem.59.032607.093606>
- Llewellyn, M.E., R.P. Barretto, S.L. Delp, and M.J. Schnitzer. 2008. Minimally invasive high-speed imaging of sarcomere contractile dynamics in mice and humans. *Nature*. 454:784–788. <https://doi.org/10.1038/nature07104>
- McNab, B.K.. 1978. The evolution of endothermy in the phylogeny of mammals. *Am. Nat.* 112:1–21. <https://doi.org/10.1086/283249>
- Nakano, T., G. Kikugawa, and T. Ohara. 2010. A molecular dynamics study on heat conduction characteristics in DPPC lipid bilayer. *J. Chem. Phys.* 133. 154705. <https://doi.org/10.1063/1.3481650>
- Okabe, K., N. Inada, C. Gota, Y. Harada, T. Funatsu, and S. Uchiyama. 2012. Intracellular temperature mapping with a fluorescent polymeric thermometer and fluorescence lifetime imaging microscopy. *Nat. Commun.* 3:705. <https://doi.org/10.1038/ncomms1714>
- Oyama, K., M. Gotoh, Y. Hosaka, T.G. Oyama, A. Kubonoya, Y. Suzuki, T. Arai, S. Tsukamoto, Y. Kawamura, H. Itoh, et al. 2020. Single-cell temperature mapping with fluorescent thermometer nanosheets. *J. Gen. Physiol.* 152. <https://doi.org/10.1085/jgp.201912469>
- Resch-Genger, U., and M. Ameloot, editors. 2008. Standardization and quality assurance in fluorescence measurements I: techniques. Springer, New York. 496 pp. <https://doi.org/10.1007/978-3-540-75207-3>
- Rothstein, E.C., S. Carroll, C.A. Combs, P.D. Jobsis, and R.S. Balaban. 2005. Skeletal muscle NAD(P)H two-photon fluorescence microscopy in vivo: topology and optical inner filters. *Biophys. J.* 88:2165–2176. <https://doi.org/10.1529/biophysj.104.053165>
- Ruben, J.. 1995. The evolution of endothermy in mammals and birds: from physiology to fossils. *Annu. Rev. Physiol.* 57:69–95. <https://doi.org/10.1146/annurev.ph.57.030195.000441>
- Schönle, A., and S.W. Hell. 1998. Heating by absorption in the focus of an objective lens. *Opt. Lett.* 23:325–327. <https://doi.org/10.1364/OL.23.000325>
- Suzuki, M., and T. Plakhotnik. 2020. The challenge of intracellular temperature. *Biophys. Rev.* 12:593–600. <https://doi.org/10.1007/s12551-020-00683-8>
- Suzuki, M., V. Tseeb, K. Oyama, and S. Ishiwata. 2007. Microscopic detection of thermogenesis in a single HeLa cell. *Biophys. J.* 92:L46–L48. <https://doi.org/10.1529/biophysj.106.098673>
- Suzuki, M., V. Zeeb, S. Arai, K. Oyama, and S. Ishiwata. 2015. The 10(5) gap issue between calculation and measurement in single-cell thermometry. *Nat. Methods*. 12:802–803. <https://doi.org/10.1038/nmeth.3551>
- Takei, Y., S. Arai, A. Murata, M. Takabayashi, K. Oyama, S. Ishiwata, S. Takeoka, and M. Suzuki. 2014. A nanoparticle-based ratiometric and self-calibrated fluorescent thermometer for single living cells. *ACS Nano*. 8:198–206. <https://doi.org/10.1021/nn405456e>
- Wakita, M., G. Nishimura, and M. Tamura. 1995. Some characteristics of the fluorescence lifetime of reduced pyridine nucleotides in isolated mitochondria, isolated hepatocytes, and perfused rat liver in situ. *J. Biochem.* 118:1151–1160. <https://doi.org/10.1093/oxfordjournals.jbchem.a125001>
- Yang, C.-Y., C.-S. Liao, Y.-Y. Tzeng, and S.-W. Chu. 2011. Visualization of Heat Propagation in Biological Tissues with Two-Photon Fluorescence Microscopy. SPIE, San Francisco. <https://doi.org/10.1117/12.875026>
- Zeeb, V., M. Suzuki, and S. Ishiwata. 2004. A novel method of thermal activation and temperature measurement in the microscopic region around single living cells. *J. Neurosci. Methods*. 139:69–77. <https://doi.org/10.1016/j.jneumeth.2004.04.010>
- Zelent, B., T. Troxler, and J.M. Vanderkooi. 2007. Temperature dependence for fluorescence of  $\beta$ -NADH in glycerol/water solution and in trehalose/sucrose glass. *J. Fluoresc.* 17:37–42. <https://doi.org/10.1007/s10895-006-0146-0>
- Zohar, O., M. Ikeda, H. Shinagawa, H. Inoue, H. Nakamura, D. Elbaum, D.L. Alkon, and T. Yoshioka. 1998. Thermal imaging of receptor-activated heat production in single cells. *Biophys. J.* 74:82–89. [https://doi.org/10.1016/S0006-3495\(98\)77769-0](https://doi.org/10.1016/S0006-3495(98)77769-0)
- Zukiene, R., Z. Nauciene, J. Ciapaite, and V. Mildaziene. 2010. Acute temperature resistance threshold in heart mitochondria: Febrile temperature activates function but exceeding it collapses the membrane barrier. *Int. J. Hyperthermia*. 26:56–66. <https://doi.org/10.3109/02656730903262140>