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Improvement on frozen mitochondria bioassay: a methodological remark.

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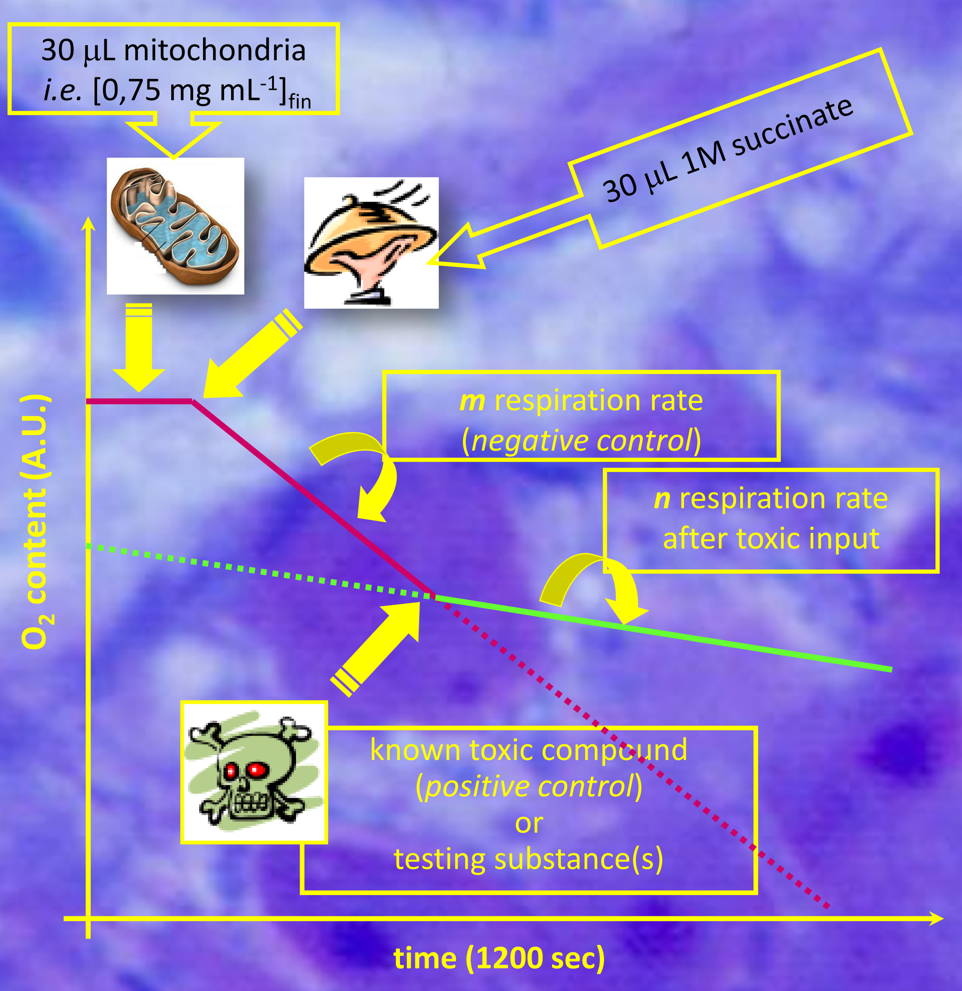
INTRODUCTION

The protocol for the bioassay with the mitochondria of beef heart frozen at -22 °C, (developed by Iero, Manente, Bragadin and Perin, in *Chemosphere*, 52, 2003) requires that the reaction cell is thermostatically controlled at 25 °C. This value was chosen because it is used as a reference for the state environmental standard (*Standard Ambient Temperature and Pressure*, SATP).
The choice is not, therefore, been supported by assessments on the effectiveness of the test at this temperature, but was dictated by the practice of reporting results to a standard temperature value.
Finally, it was decided to make a comparison between the working temperature of 25 °C and the 37 °C one, chosen as close to cattle body temperature (estimated to be 38.6 °C), then "normal" working temperature for mitochondria extracted from heart.



FM22 test highlights

FM22 end point → ΔR = 1 – *n*/*m*

n = *m*, when testing substance(s) don't show acute toxicity for mitochondria

n < *m*, when testing substance(s) show acute toxic/stressful/disrupting action for mitochondria

special effect: n > m, when testing substance(s) show a *uncoupling action **, like FCCP behaviour, classifiable as long term toxic action for mitochondria

- fast, cheap and easy to handle bioassay
- monitoring O_2 consumption as ΔR
- no ATP production
- LC_{50} determination

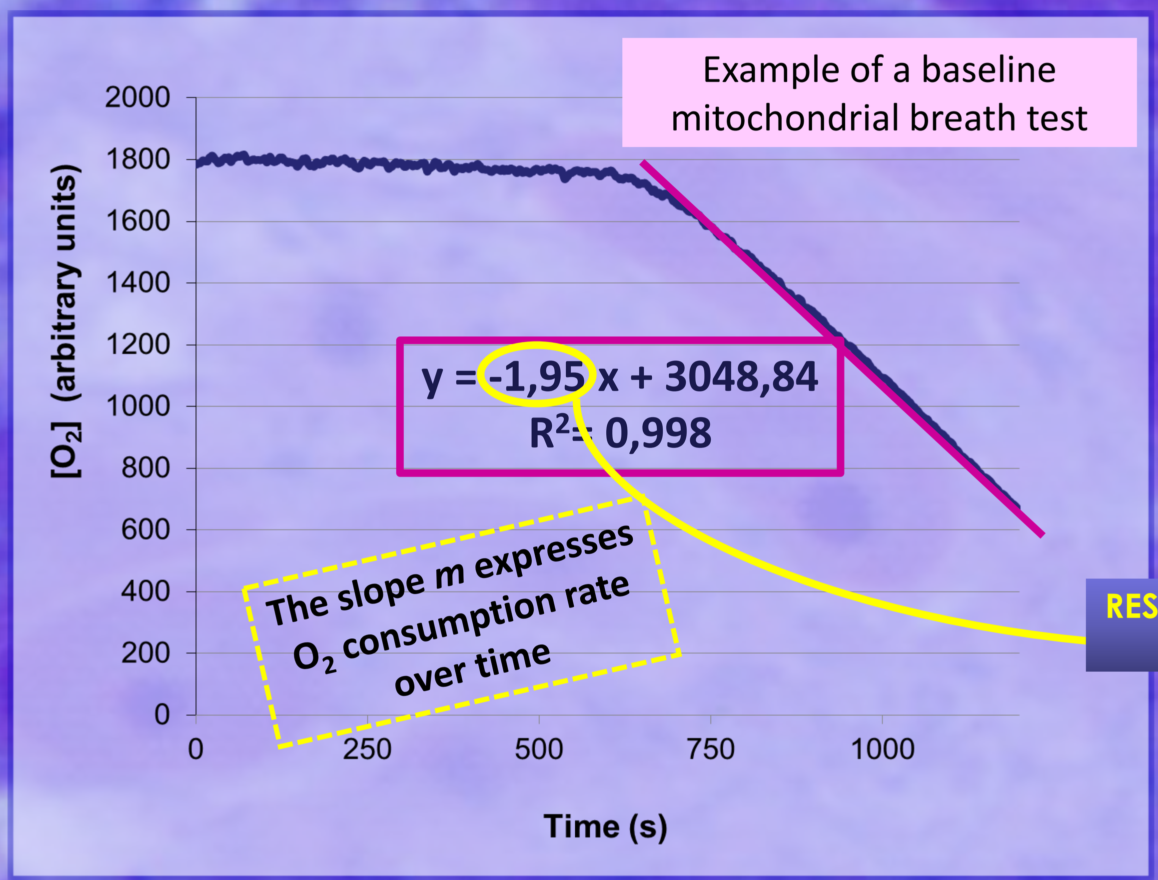
Test set-up:

- ✓ a Clark Oxygen electrode interfaced to a pc
- ✓ a closed water-jacketed (thermostated at 25 °C) 2.5 mL vessel (*reaction chamber*)
- ✓ respiration *medium*: 0,25 M sucrose, 0,1 M TRIS-HCl pH 7,4
- ✓ test run 20 min, loading 1200 data.

Bioassay general steps:

- *beef mitochondria* : prepared (Azzone *et al*., 1979) and stored at -22 °C (standardized method)
- *blank test* : to verify the linear fitting of respiration
- *pure compound toxicity test* : to verify the method sensitivity
- *internal control for each test* : toxicity is quantified comparing the slope (*m* and *n*) *before* and *after* adding the compound(s)
- statistical methodology identified a *break-point* in the linear fitting (linear fitting of O_2 consumption was verified by R^2).

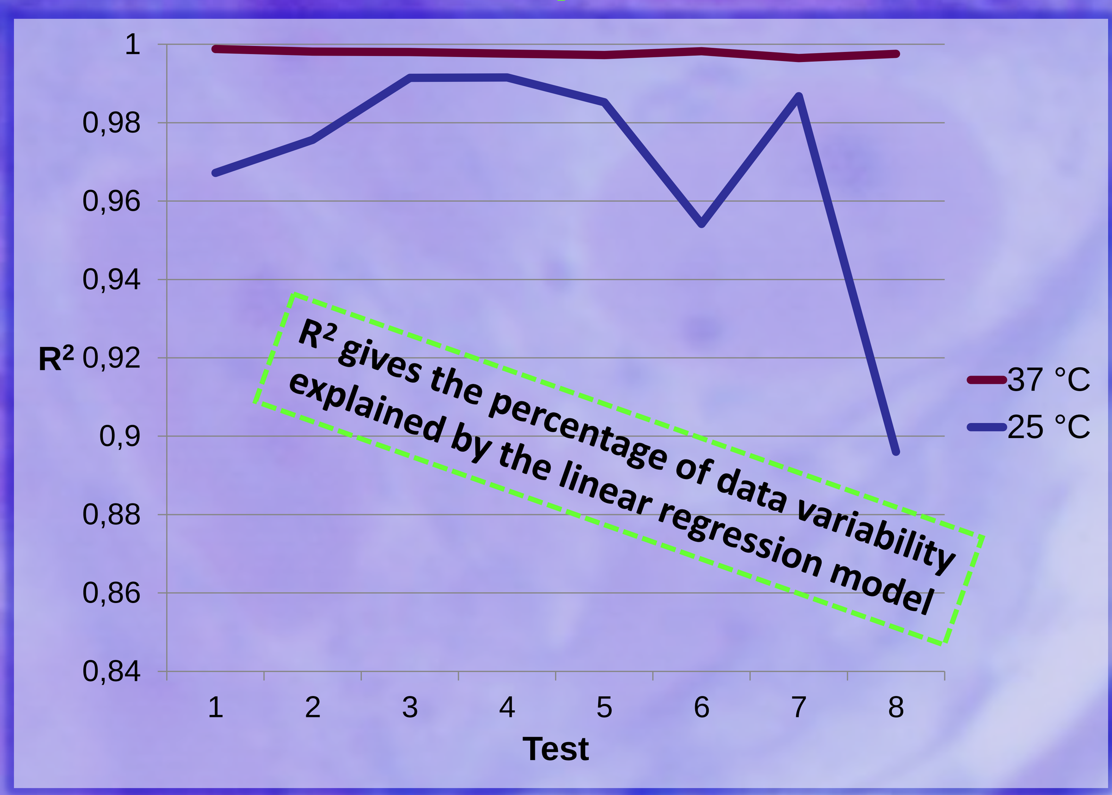
Ist PHASE - Comparison between 25 °C and 37 °C working temperature



test	25 °C *m*	25 °C R^2	37 °C *m*	37 °C R^2
1	-0,30	0,967	-1,90	0,999
2	-0,30	0,976	-2,06	0,998
3	-0,37	0,991	-1,95	0,998
4	-0,32	0,992	-1,93	0,998
5	-0,32	0,985	-1,73	0,997
6	-0,26	0,954	-1,80	0,998
7	-0,42	0,987	-1,93	0,996
8	-0,36	0,896	-2,05	0,998
average	-0,33	0,968	-1,91	0,998

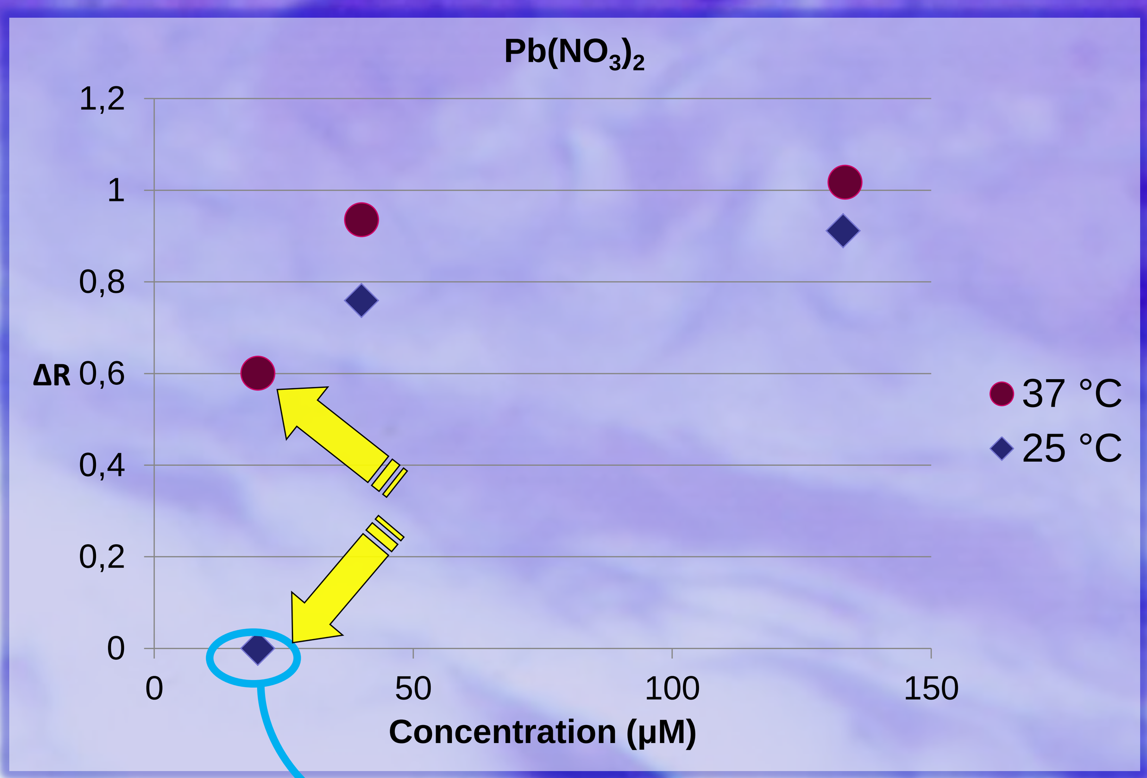
REGRESSION COEFFICIENT

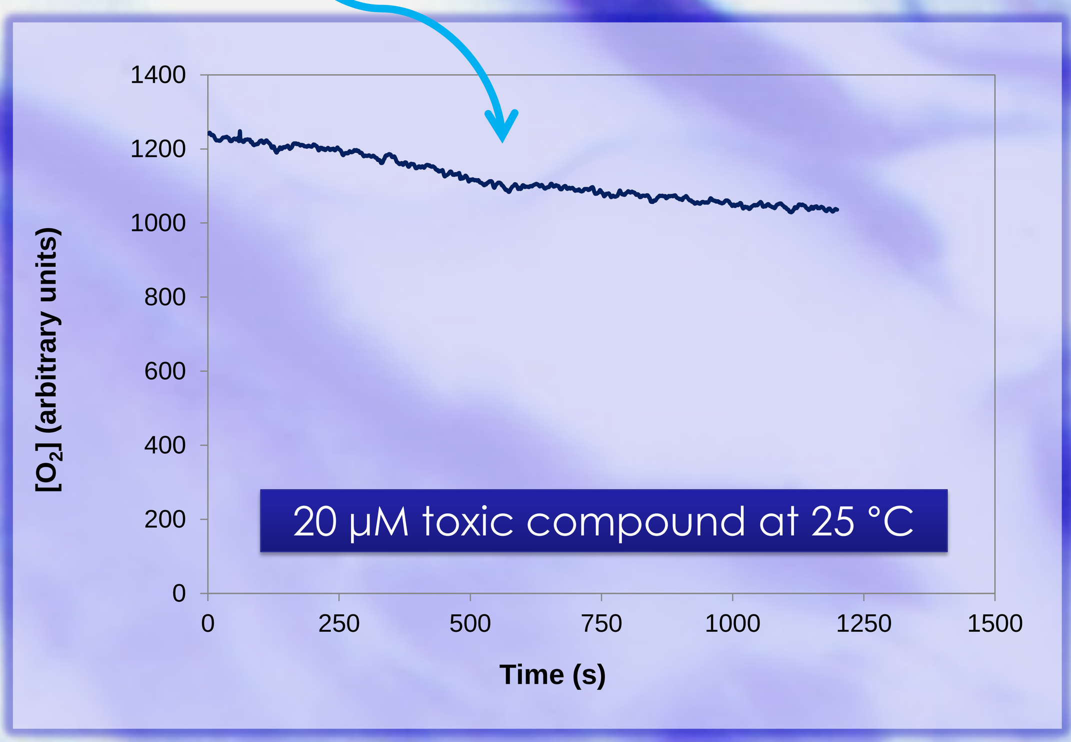
In the first phase, a series of tests for the basal respiration only were carried out in order to compare respiration rates and the quality of the regressions obtained.
Comparing the average value of *m* in both cases, it can be seen that at 37 °C mitochondrial respiration rate is almost 6 times the level recorded at 25 °C: it is reasonable to assume that the enzyme systems responsible for the transport of electrons in the inner mitochondrial membrane evolved to perform their functions optimally at a temperature characteristic of the environment in which they are immersed (a beef heart cell). At temperatures lower than optimum, the activity of these enzymes will decrease and thus the rate at which reactions they promote take place.



Comparison of obtained values highlights that in the case of tests conducted at 25 °C the coefficient of determination has a higher variability and lower values that in the case of tests conducted at 37 °C. In the case of tests conducted at 37 °C, the output signal from the measurement system is more stable and this confirm the hypothesis of mitochondria greater efficiency at this temperature.

IInd PHASE – FM22 toxicity test: comparison between 25 and 37 °C





Toxicity tests were performed at 37 °C; then, known but variable amounts of toxic compound [*e.g.* $Pb(NO_3)_2$ *on the left*] were injected at 480 sec to highlight inhibition trend at this temperature and to compare it with obtained values at 25 °C.
The dependence of respiratory inhibition by the concentration of toxic is detectable at both temperatures. They seem to except the values corresponding at the concentration of 20 μM (ΔR = 0.6 at 37 ° C and ΔR = 0 at 25 ° C), but if we look to the pattern of consumption of O_2 along time for the tests at 25 ° C we can observe as the index of toxicity is equal to zero because, in these cases, the change in slope between the first and the second part of the curve is not so clear for the macro in Excel® to consider it statistically significant.

CONCLUSIONS

✓ **Running the tests at 37 °C, it can therefore calculate the toxic effect of the compound at concentrations lower than those achievable with the test at 25 °C.**

✓ **It can be concluded that the bioassay with frozen beef heart mitochondria is more sensitive and accurate at 37 °C.**

✓ **This reason, together with considerations concerning the rate of respiration higher (due to higher enzyme activity) and increased signal stability, lead to the conclusion that in order to optimize the performance of the test is preferable to control the temperature of the cell reaction at 37 °C.**

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KEYWORDS
Mitochondria
Temperature
Toxicity
Test
Control