Investigating cryoinjury using simulations and experiments: 2. TF-1 cells during graded freezing (interrupted slow cooling without hold time)

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Abstract

Cryopreservation plays a key role in the long-term storage of native and engineered cells and tissues for research and clinical applications. The survival of cells and tissues after freezing and thawing depends on the ability of the cells to withstand a variety of stresses imposed by the cryopreservation protocol. A better understanding of the nature and kinetics of cellular responses to temperature-induced conditions is required to minimize cryoinjury. An interrupted freezing procedure that allows dissection of cryoinjury was used to investigate the progressive damage that occurs to cells during cryopreservation using slow cooling. Simulations of cellular osmotic responses were used to provide interpretation linking states of the cell with events during the freezing procedure. Simulations of graded freezing (interrupted slow cooling without hold time) were correlated with cell recovery results of TF-1 cells. Calculated intracellular supercooling and osmolality, were used as indicators of the probability of cryoinjury due to intracellular ice formation and solution effects, providing direct links of cellular conditions to events in the freezing process. Using simulations, this study demonstrated that both intracellular supercooling and osmolality are necessary to explain graded freezing results.

Introduction

For simulations to be used in designing cryopreservation protocols, it is necessary to compare simulations with empirical data to further understanding of the relationship between calculated parameters and related experimental outcomes. Graded freezing (interrupted slow cooling without hold time) provides insights into the two types of freezing injury which can affect cell recovery: solution effects and intracellular ice formation [14]. McGann used this freezing procedure to explore the two major types of freezing injury by determining the subzero temperature ranges where different sources of cryoinjury occur [15]. The graded freezing procedure involves slow cooling (1°C/min for many nucleated mammalian cells) to various subzero temperatures and then either thawing directly or rapid cooling by plunging into liquid nitrogen and later thawing (see schematic - Figure 1). With this procedure it is possible to separate injury sustained during the slow cooling phase to the subzero experimental temperatures, from that sustained upon rapid cooling to storage temperatures. Various cooling rates can also be used to explore the effect of time during cooling on cell recovery.

Mazur previously used simulations to explore the effects of solutions, osmotics and temperatures on cellular systems [11], and reported that the water permeability and its temperature dependence are important parameters necessary to predict changes in the volume of intracellular water with temperature during cooling. Furthermore, that study reported that predictions of the probability of intracellular ice formation can be made based on the amount of intracellular water and the temperature of the cell. Subsequently, Mazur indicated that cooling rates greater than 1°C/min would result in a supercooled cytoplasm in yeast [11,13]. Supercooling is the degree to which a solution is cooled below its thermodynamic freezing point and was used as an indicator of the potential for intracellular ice formation [14]. Ebertz also

reported the use of supercooling as an indicator for intracellular ice formation for simulations on corneal endothelial, epithelial and stromal cells [4]. It has been shown previously that intracellular ice formation is associated with between -5 and -15 degrees of intracellular supercooling, when extracellular ice is present [12]. Diller further examined the probability of intracellular ice formation based on the synergistic interaction of cooling rates and supercooling [2,3]. During cooling, the cell approaches osmotic equilibrium across the plasma membrane either by osmotic dehydration or the formation of ice. Therefore, at low cooling rates, cells dehydrate, resulting in low supercooling. At high cooling rates, there is insufficient time for dehydration, so supercooling increases, making intracellular freezing more likely [2]. Karlsson et al. developed a mechanistic model, including water transport across the cell membrane, ice nucleation, and crystal growth, for predicting the kinetics of intracellular ice formation [6].

Previously, we have demonstrated that intracellular supercooling can be used as the sole indicator for cryoinjury in two-step freezing (rapid interrupted cooling with a hold time) [20]. However, based on Mazur et al.'s 'two-factor hypothesis', solution effects injury must also be considered to determine the optimal cooling rate for cryopreserving a cell type using slow cooling [14]. Karlsson et al. have also shown the need for an effective model of solution effects injury [7]. During slow cooling, cell injury is related to prolonged exposure to the high solute concentrations resulting from extracellular ice formation [14]. This study explores the use of intracellular parameters to distinguish between the two major types of injury sustained during the different phases of the cooling protocol: supercooling, as an indicator of intracellular freezing; and intracellular osmolality, as an indicator of solution effects injury.

The objective of this study was to perform simulations of interrupted slow cooling freezing experiments using parameters for TF-1 cells previously reported [19] (Table 1), and to

compare the cell survival outcomes with calculations of intracellular supercooling and intracellular osmolality, as cells were cooled slowly to various subzero temperatures before rapid cooling.

Simulation specifications

The specifications for our simulations of cellular responses at low temperatures have previously been described in detail [20]. Briefly, there are four main elements to the simulations: 1) the change in the composition of the extracellular solution as ice forms at low temperatures; 2) cellular osmotic responses to changes in composition of the extracellular solution and the resulting change in the composition of the intracellular solution; 3) the temperature dependence of the cellular osmotic permeability parameters; and 4) changes in sample temperature as a function of time.

Measured temperature profiles in the experimental samples were used in the simulations. Since the cells are initially in osmotic equilibrium with the isotonic extracellular solution, no osmotic changes occur before ice nucleation. The assumptions in our simulations are that a) the simulation begins on ice nucleation at the freezing point of the extracellular solution and b) the extracellular solution remains in equilibrium with extracellular ice. The simulations used measured osmotic parameters of TF-1 cells [19] and solution parameters [5] (listed in Table 1c).

Experimental materials and methods

TF-1 cell freezing experiments

Descriptions of TF-1 cell culture and freezing experiments have been previously reported in detail [19]. Briefly, TF-1 cells (ATCC, Manassas, Virginia) were cultured according to ATCC

guidelines. Samples of 0.2 mL cell suspension in serum-free RPMI, were transferred to glass tubes and cooled in a 0 °C wet ice bath for 5 min. Control samples were removed and either warmed in a 37 °C water bath or plunged into liquid nitrogen. Experimental samples were transferred into a methanol bath preset at -3 °C and allowed to equilibrate for 5 min prior to ice nucleation in each tube with cold forceps. After ice was nucleated at -3 °C, samples were held for 3 minutes to allow for the dissipation of the nucleation heat. The bath was then cooled at 0.9 °C/min, and the temperature monitored using a Type T thermocouple. At each experimental temperature (-3, -6, -9, -12, -15, -20, -30, and -40 °C), one set of duplicate samples was thawed directly in a 37 °C water bath and another set was plunged into liquid nitrogen (250 °C/min). Plunge samples were kept in liquid nitrogen for a minimum of one hour prior to being thawed in a 37 °C water bath. Cell membrane integrity was used as an indicator of cell damage during freezing, as it has been shown that the membrane is a primary site of freezing-thawing injury [1]. It has also been shown that there is a correlation between intracellular freezing and membrane damage for cells in suspension [1,12].

Results and discussion

Graded freezing simulations

Figure 2 shows the measured temperature profiles used in the simulations (profiles shown to -40 °C). After ice nucleation at -3 °C, the sample temperature increased to the freezing point of the solution (-0.6 °C). Figure 3 shows calculated cell volumes as a function of temperature for the corresponding cooling profiles shown in Figure 2. As expected for linear cooling, simulations show that cell volumes during the various experimental temperature profiles only

differ during the plunge step. Specifically, the calculated volume for the -3 °C experimental temperature profile differs considerably from the other experimental temperatures.

Figure 4 shows the calculated supercooling in TF-1 cells cooled at 1°C/min to various subzero temperatures before rapid cooling. For all cooling profiles, there was a small increase in supercooling after ice nucleation, followed by a decrease as the cell dehydrates during the 3 minute hold. TF-1 cells maintained equilibrium (i.e. no increase in supercooling) during slow cooling, indicating that 1 °C/min was sufficiently low enough to allow the cells to remain in osmotic equilibrium during cooling to all the experimental temperatures. During the plunge step, there was an additional increase in supercooling as a result of rapid cooling, with the pattern similar for all experimental temperatures.

Figure 5 shows the calculated intracellular osmolality during cooling at 1°C/min to various temperatures before rapid cooling. After extracellular ice nucleation, there was an increase in intracellular osmolality as the cell dehydrated in response to the increased extracellular osmolality to the experimental temperatures. During the plunge step, there was a lower increase in intracellular osmolality during the subsequent rapid cooling step. The calculations for intracellular osmolality do not account for intracellular ice or precipitate formation.

Maximum intracellular supercooling and osmolality during cooling

It has been suggested previously that most damage resulting from exposure to low temperatures occurs at temperatures above -40 °C [10]. The highest degree of supercooling was calculated between ice nucleation and -40 °C and was reached at the end of the cooling profiles (i.e. supercooling reached in the plunge step by the time the cells are cooled to -40 °C). The pattern of supercooling was consistent for all experimental temperatures. In Figure 4, the arrow

indicates where the maximum supercooling was determined for the -3 °C experimental temperature, as an example. The maximum supercooling for each experimental temperature profile was separated into the maximum supercooling which occurred down to the experimental temperature (direct thaw) and the maximum supercooling which occurred during the plunge step (plunge). The results were then summarized and plotted as a function of experimental temperature (Figure 6). The maximum supercooling calculated for the direct thaw group was low (~2 °C) and since it occurred during the ice nucleation step, it was equal for all experimental temperature profiles. This indicates that there should be no damage due to intracellular ice formation for TF-1 cells cooled slowly to -40 °C. However, high maximum supercooling was calculated for the plunge group at high subzero experimental temperatures, decreasing at lower experimental temperatures. This indicates that there should be significant damage due to intracellular ice formation for TF-1 cells plunged from at least the higher experimental temperatures. This analysis demonstrates that for TF-1 cells cooled at 1 °C/min intracellular ice formation plays a role in freezing injury only during the plunge step.

Similar to the pattern for intracellular supercooling, the highest intracellular osmolality was reached at the end of the cooling profiles (i.e. osmolality reached in the plunge step by the time the cells are cooled to -40 °C). The arrows in Figure 5 indicate where the maximum osmolality was determined for direct thaw and plunge for the -20 °C experimental temperature, as an example. The maximum osmolality for each experimental temperature profile was separated into the maximum osmolality which occurred down to the experimental temperature (direct thaw) and the maximum osmolality which occurred during the plunge step (plunge). The results were then summarized and plotted as a function of experimental temperature (Figure 6). The maximum osmolality calculated for the direct thaw group increased with decreasing

experimental temperature, where cells are exposed to the increasing osmolalities for longer durations. The maximum osmolality calculated for the plunge group also increased with decreasing experimental temperature.

Comparison of theoretical and experimental results

The maximum intracellular supercooling and osmolality calculated throughout the cooling profiles were used to interpret loss of membrane integrity after graded freezing experiments. Figure 7 shows previously published membrane integrity as a function of experimental temperature [19] with the results divided into 2 sections.

Direct thaw

In Section 1 (-3 to -6 °C), there is minimal loss of membrane integrity for the direct thaw samples; while in Section 2, there is a decline in membrane integrity for the direct thaw samples with decreasing experimental temperature. For directly thawed samples in Section 1, the corresponding section in Figure 6 shows low maximum supercooling and also in Figure 6 shows low maximum intracellular osmolality. TF-1 cells can therefore tolerate exposure during slow cooling to a calculated 3 Osm/kg. Lovelock reported that exposure to, and dilution from, 0.8 M salt concentration causes sensitivity to thermal and mechanical stresses and from 3 M salt concentration causes hemolysis of human red blood cells [9]. The experimental data for TF-1 cells demonstrated that there was a progressive decrease in membrane integrity in Section 2 (below -6 °C), as the calculated intracellular osmolality increased with a 50 % loss in membrane integrity at ~6.6 Osm/kg. Since the calculated supercooling in the direct thaw samples remained low at all experimental temperatures, this loss of membrane integrity can be attributed to high intracellular osmolality (yellow area in Figure 7).

Plunge

Experimental results from the plunge-thaw samples can also be interpreted using the calculated maximum supercooling and osmolality in the plunge step. In Section 1 of experimental temperatures (-3 to -6 °C), high maximum supercooling (Figure 6) and lower increase in osmolality in the plunge step (Figure 6) corresponded to approximately 75 % loss of membrane integrity (Figure 7). The damage in this section can therefore be attributed to intracellular ice formation (blue area in Figure 7). In figure 7 Section 2 of experimental temperatures (-6 to -30 °C) for the plunge step, shows a trend of reduced membrane integrity, with decreasing maximum supercooling (Figure 6) and increasing maximum intracellular osmolality (Figure 6) (green area in Figure 7). The two indicators of injury are oppositely dependent on the experimental temperature, resulting in an apparent maximum in recovery for the plunge samples. This maximum has been previously reported [15-18,22-26].

Figure 8 also shows membrane integrity data further analyzed by calculating the plunge-thaw cell recovery as a percentage of the direct-thaw cell recovery, showing the additional damage incurred during the plunge step. This supports the view that recovery after the plunge step is only partially limited by damage incurred during the direct thaw step and that there is additional damage to the cells during the plunge step. Lastly, there is no range of experimental temperatures in this study that minimizes the likelihood of intracellular ice formation and solution effects injury sufficiently to achieve high cell recovery using this graded freezing protocol for TF-1 cells.

Impact of including intracellular protein

Solution properties of the cytoplasm are complex and have yet to be elucidated for nucleated cells. We have previously discussed the inclusion of a protein as a component of the cell cytoplasm in simulations [20], assuming that the protein in TF-1 cells has thermodynamic

properties similar to those of hemoglobin, which are well documented. The inclusion of protein as an intracellular component had little effect on the calculated maximum supercooling (Figure 9a) or maximum intracellular osmolality (Figure 9b) for direct thaw or plunge profiles. As expected, including protein in the cytoplasm is not expected to change the calculated intracellular osmolality during slow cooling because the cell is in equilibrium with the extracellular solution, where the osmolality is determined by the temperature. However, the presence of protein did have an effect on the maximum salt (KCl) concentration for the direct thaw cooling profiles (Figure 10). This demonstrates that while the inclusion of protein may describe the solution thermodynamics of the cytoplasm more accurately, it does not substantially affect simulation results for graded freezing.

Contrasting graded freezing and two-step freezing simulations

A previous study has shown that decreased membrane integrity due to intracellular ice formation in a two-step freezing procedure (rapid cooling interrupted with a hold time) could be predicted using intracellular supercooling as an indicator of intracellular ice formation for TF-1 cells following two-step freezing [20]. In this study, we also calculated the intracellular osmolality as a function of hold temperature for two-step freezing of TF-1 cells to explore the potential contributions of high intracellular osmolality to decreased membrane integrity. Calculations of maximum intracellular osmolality for the first step were comparable to graded freezing direct thaw calculations. However, the time spent exposed to these conditions is significantly longer using graded freezing and thus contributes to a decrease in membrane integrity. Calculations of maximum intracellular osmolality for the second step were also comparable to graded freezing plunge thaw calculations. However, similarly the time spent

exposed to these conditions is low for both two-step and graded freezing and therefore, damage is primarily associated with high intracellular supercooling. Calculated intracellular supercooling alone could be used to explain membrane integrity results for two-step freezing (rapid cooling interrupted with hold time); however, calculated intracellular supercooling alone was not sufficient to explain graded freezing (interrupted slow cooling without hold time) results and calculated intracellular osmolality was required.

Conclusions

Empirical approaches have been used to develop most cryopreservation procedures currently in use. However, more difficult to cryopreserve cells and complex cellular structures require application of theoretical understanding of cellular osmotic responses to low temperatures [4,8,21]. This study demonstrated the use of intracellular supercooling and osmolality as indicators of intracellular ice formation and solution effects injury, respectively. For direct thaw samples, high intracellular osmolality alone correlated with decreased membrane integrity with lower subzero experimental temperatures. For samples cooled rapidly from -3 and -6 °C, intracellular supercooling alone was correlated with decreased membrane integrity. However for samples cooled rapidly from lower subzero temperatures, a combination of high intracellular osmolality and supercooling were correlated with decreased membrane integrity which is what one would expect with damage associated with slow cooling.

The impact of including intracellular protein as an intracellular component in simulations minimally affected the calculated maximum supercooling and osmolality during interrupted slow cooling. However, the inclusion of protein in simulations may be important for predicting cellular responses using other cooling profiles or cryoprotectants. Although it was beyond the

scope of this study, it should be noted that there may be an effect of nucleation heat with sample volumes larger than the 0.2 mL volumes used in this study, especially at lower cooling rates based on the ability of the cooling system to dissipate the heat produced from nucleation.

Based on the findings of this study and our previous study on two-step freezing [20], we have shown that supercooling alone could be used to explain membrane integrity results for two-step freezing (rapid cooling interrupted with hold time); however, supercooling alone was not sufficient to explain graded freezing (interrupted slow cooling without hold time) results. Therefore, we suggest using both intracellular supercooling and osmolality as indicators of injury for simulations of slow cooling cryopreservation protocols. Simulation results from this study can be used to understand why standard slow cooling cryopreservation protocols require a permeating cryoprotectant, such as Me_2SO, to avoid solution effects damage. Furthermore, the results could be used to support how solution effects can be avoided without the use of cryoprotectants by using two-step freezing.

This study combined the effects of intracellular supercooling and osmolality as indicators of potential damage due to intracellular ice formation and solution effects, respectively. Furthermore, this study demonstrates the usefulness of simulations, combined with interrupted freezing in attempting to determine the optimal cryopreservation protocol for a particular cell type. Specifically, interrupted freezing provides useful insight into the mechanisms of damage conferred by slow cooling over a range of subzero temperatures [15]. It allows for the manipulation of different variables of the cryopreservation protocol, cooling rates, plunge temperatures, cryoprotectants, and storage temperatures, so that a comparison between them can be made. Ultimately, both procedures allow for further understanding of cryoinjury and thus the potential for an optimum protocol to be determined.

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Figure captions

Figure 1. A schematic of graded freezing (interrupted slow cooling without hold time) [19]. Direct-thaw paths are indicated by dashed lines and the plunge-thaw paths are indicated with solid lines. Ice nucleation is indicated. Experimental temperatures are circled.

Figure 2. Simulation input temperature as a function of time, measured post-nucleation during cooling at 1 °C/min to various subzero experimental temperatures (-3°C to -30°C), prior to rapid cooling (250°C/min). The experimental temperatures are indicated on the graph. The figure insert shows the entire measured temperature profile including prior to the nucleation step for one set of experimental conditions.

Figure 3. Calculated relative cell volume as a function of temperature for TF-1 cells cooled according to the temperature profiles in Fig 2.

Figure 4. Calculated intracellular supercooling as a function of temperature for TF-1 cells cooled according to the temperature profiles in Fig 2. "Max" indicates where the maximum supercooling occurs during the cooling profile (for simulations down to -40 °C, where simulations were stopped) for a particular experimental temperature.

Figure 5. Calculated intracellular osmolality as a function of temperature for TF-1 cells cooled according to the temperature profiles in Fig 2. "Max" indicates where the maximum intracellular osmolality occurs during the direct thaw and plunge steps of the cooling profile (for simulations down to -40 °C, where simulations were stopped) for a particular experimental temperature.

Figure 6. Maximum intracellular supercooling (blue) and intracellular osmolality (red) during direct thaw (dotted line) and plunge (solid line) as a function of experimental temperature for TF-1 cells. Vertical dashed line is for reference in Figure 7.

Figure 7. Membrane integrity for TF-1 cells (±SEM; normalized to controls) in serum-free RPMI following graded freezing [19] indicating sections where loss of membrane integrity is a result of supercooling (SC; blue area), osmolality (OSM; yellow area), or both (green area), for the graded freezing procedure.

Figure 8. Membrane integrity for TF-1 cells (±SEM; normalized to controls) in serum-free RPMI following graded freezing and includes a plunge-thaw curve normalized with (i.e. as a percentage of) the direct-thaw data (blue long dashes).

Figure 9. Cell model with (blue) and without (black) intracellular protein. (a) Maximum intracellular supercooling and (b) maximum intracellular osmolality during direct thaw (dotted line) and the plunge step (solid line) as a function of experimental temperature for TF-1 cells.

Figure 10. Maximum intracellular KCl concentration. Cell model with (blue) and without (black) intracellular protein during direct thaw as a function of experimental temperature for TF-1 cells.

Table 1. Parameters used in simulations: (a) isotonic solution composition, (b) solution parameters, and (c) osmotic parameters for TF-1 cells

a) Isotonic solution composition [20]

	Extracellular	Intracellular
NaCl	0.170 molal	0.010 molal
KCl	0.005 molal	0.133 molal
Protein	0	0.004 molal
Total Osmolality	0.300 Osm/kg	0.300 Osm/kg

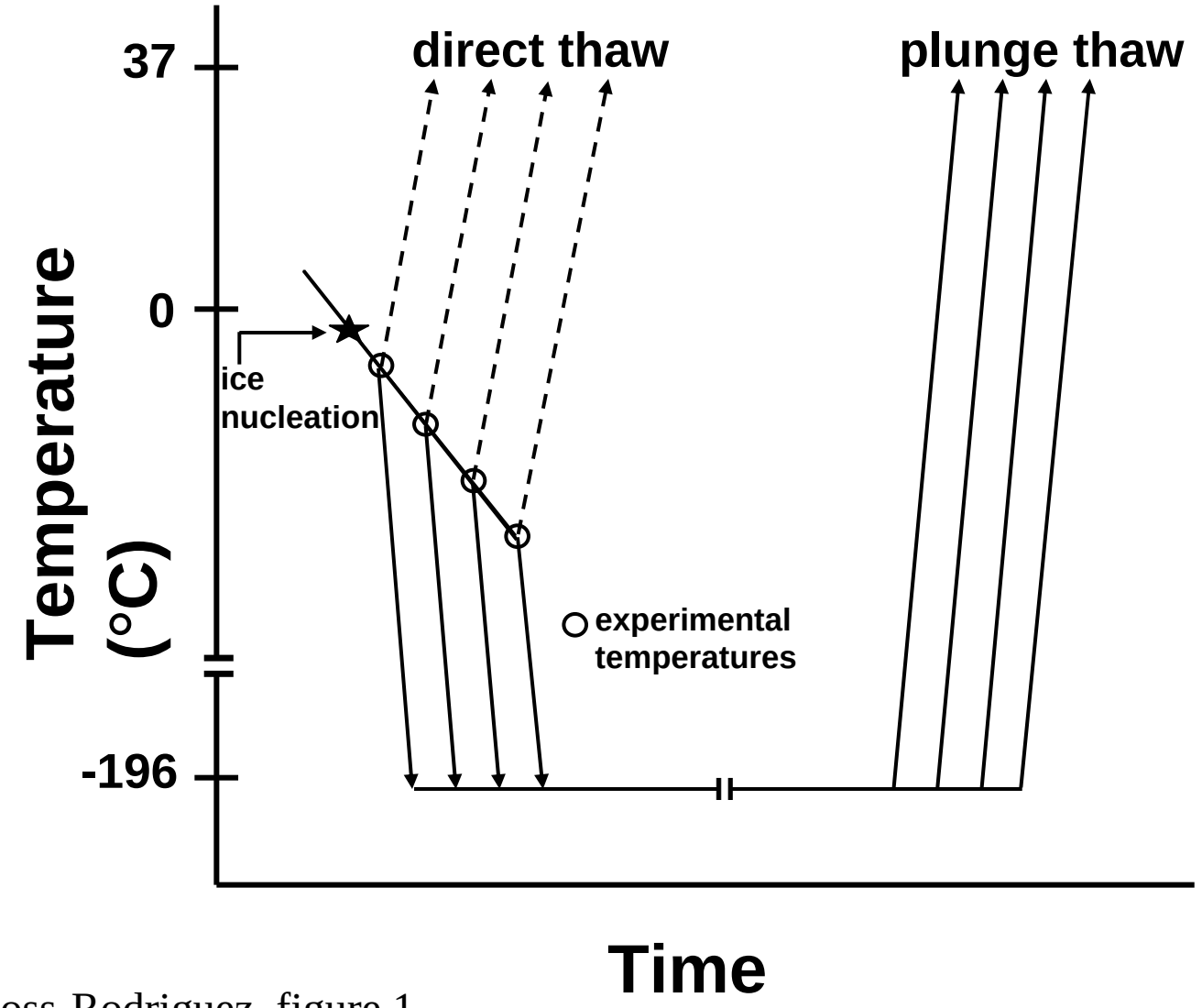
b) Solution parameters [5]

	B (mol/kg solvent)$^{-1}$	C (mol/kg solvent)$^{-2}$	K
NaCl	0.02986	0	1.702
KCl	0	0	1.742
Protein†	49.3	3.07×10^4	1

c) Osmotic parameters [19]

Isotonic volume, V_o	916 μm^3
Inactive fraction, b	0.361
E_a (Activation Energy for L_p)	14.2 kcal/mol
k (Pre-exponential factor for L_p)	1.33×10^{10} $\mu m^3/\mu m^2$/min/atm

†based on values reported for hemoglobin
L_p is the hydraulic conductivity
B is the second osmotic virial coefficient
C is the third osmotic virial coefficient
K is the dissociation constant for electrolytes



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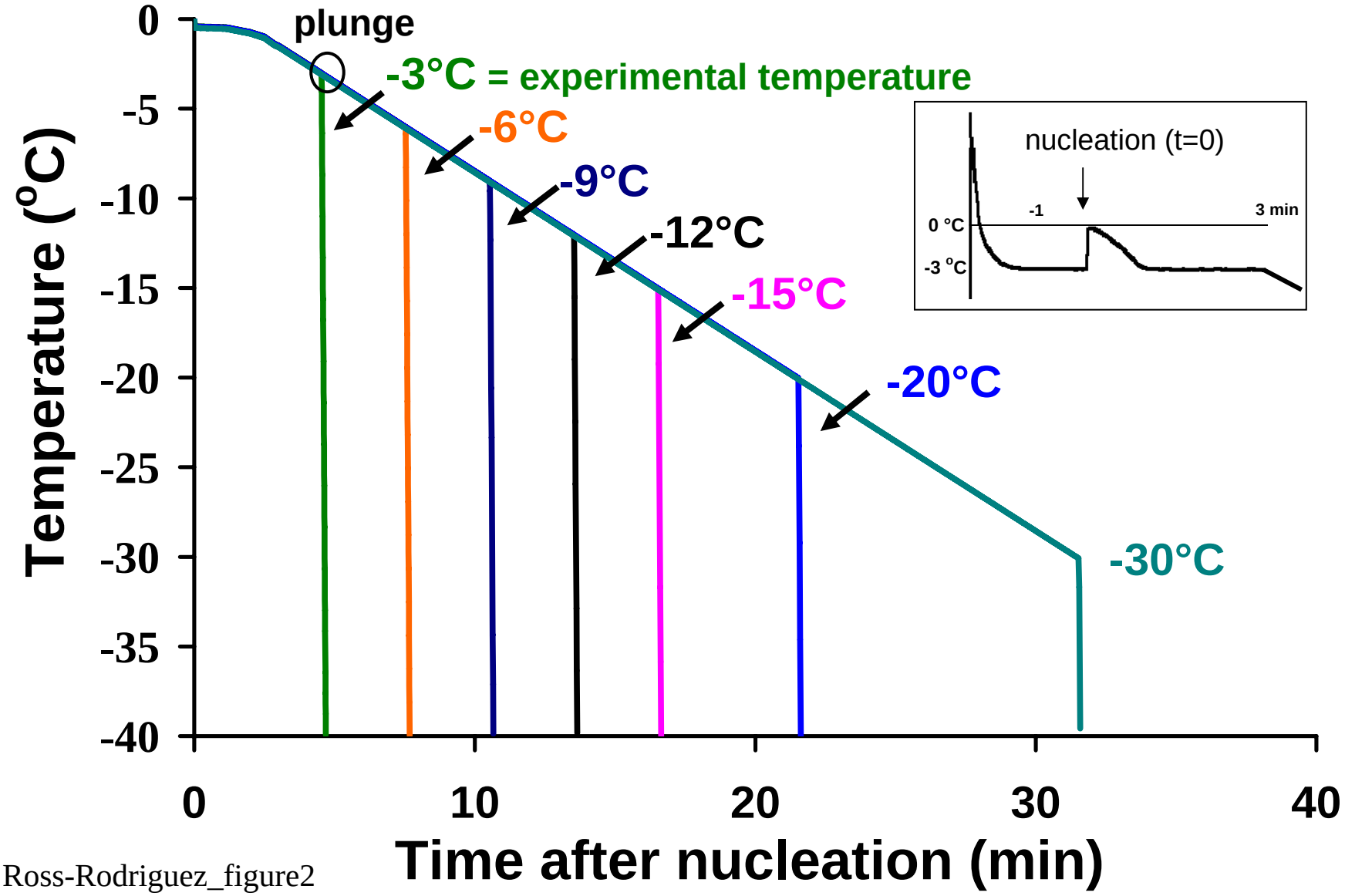

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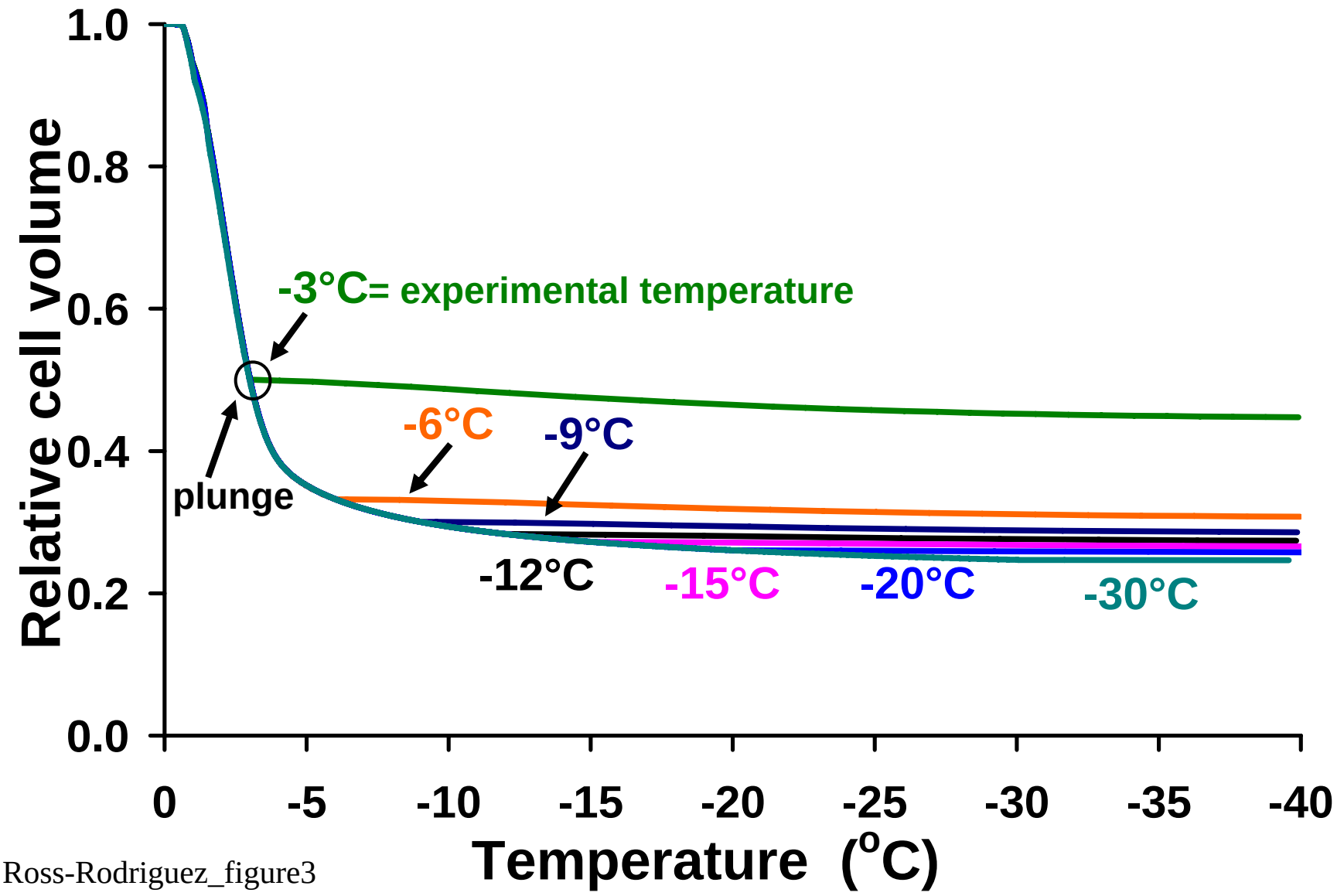

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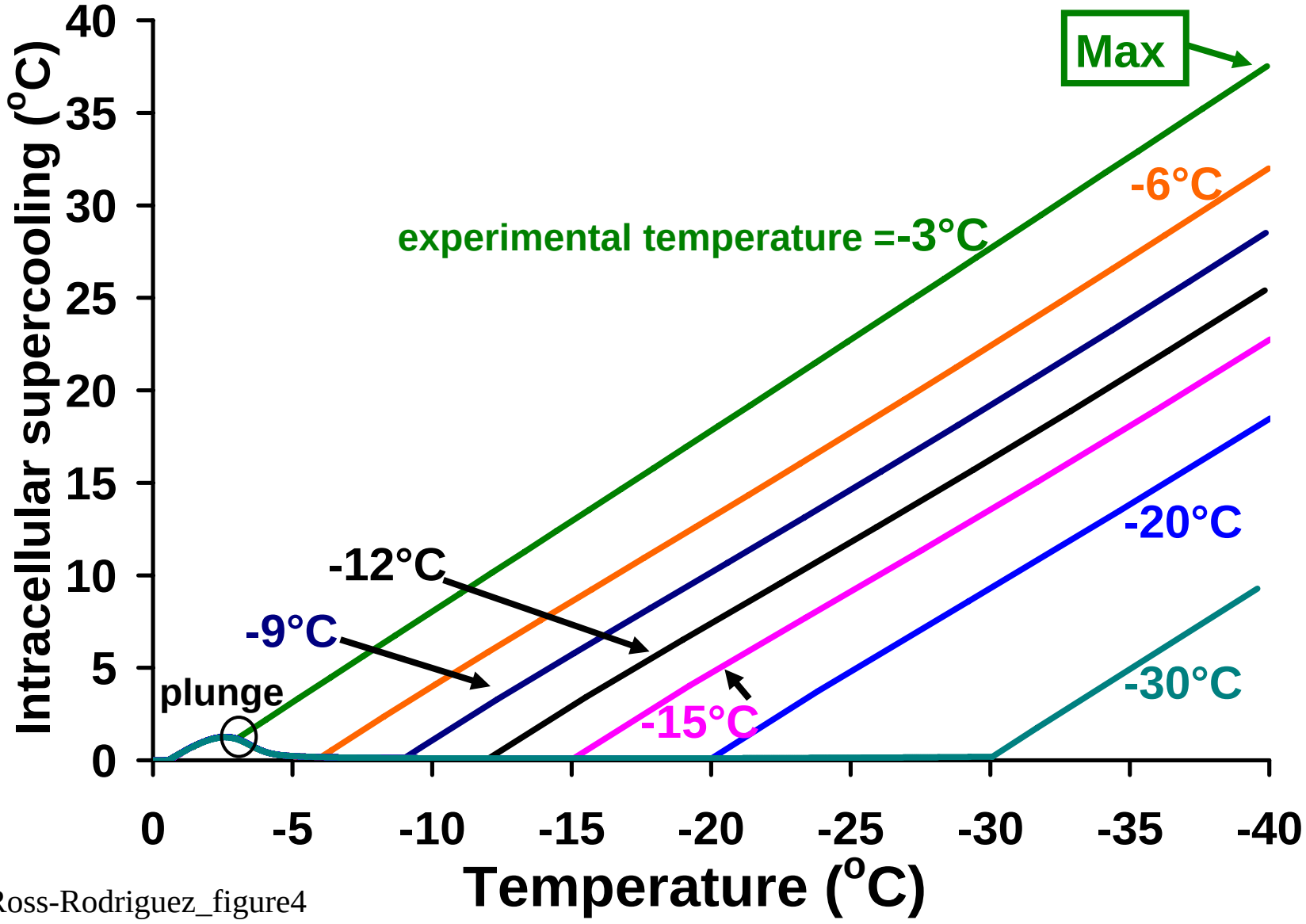

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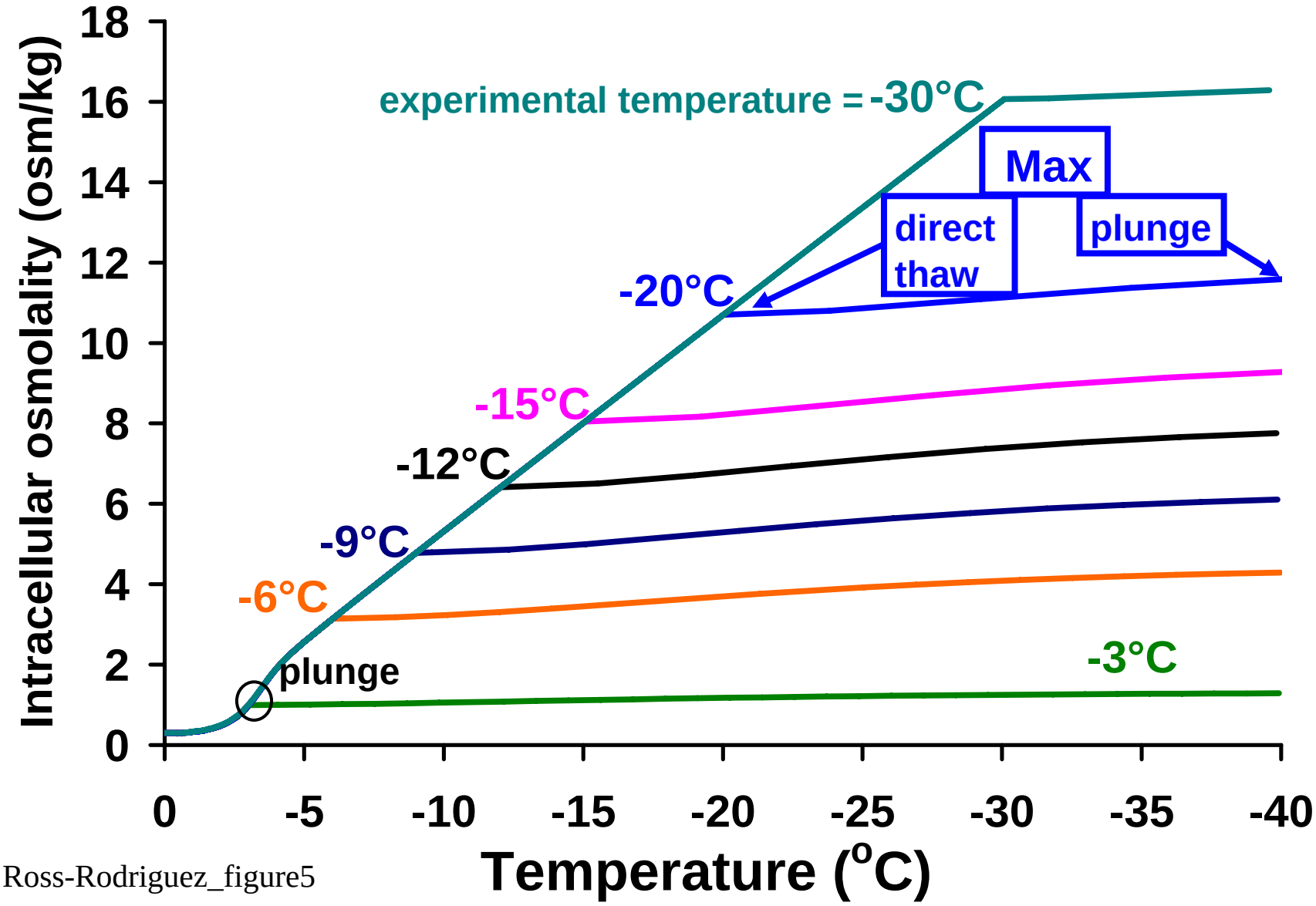

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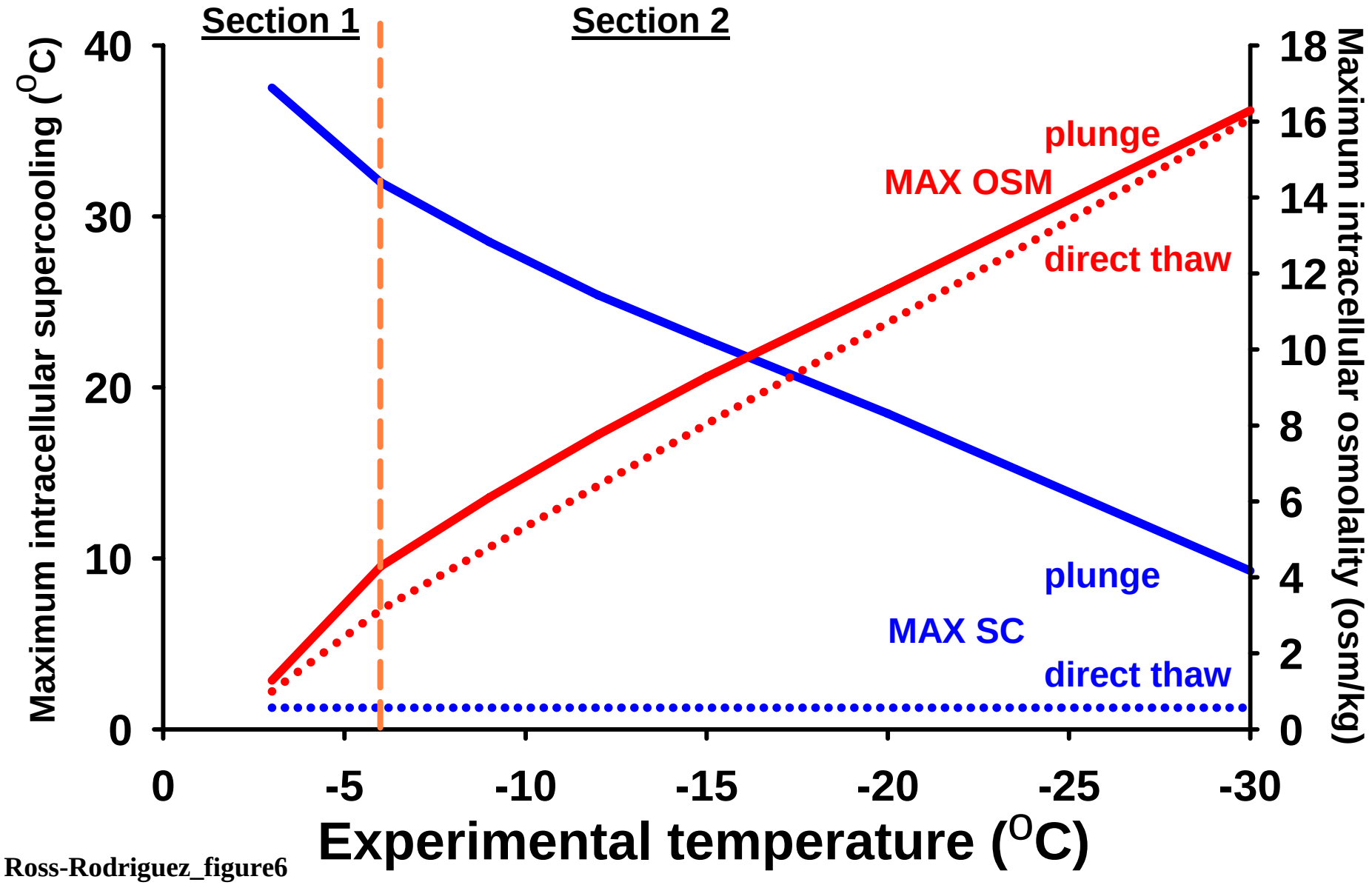

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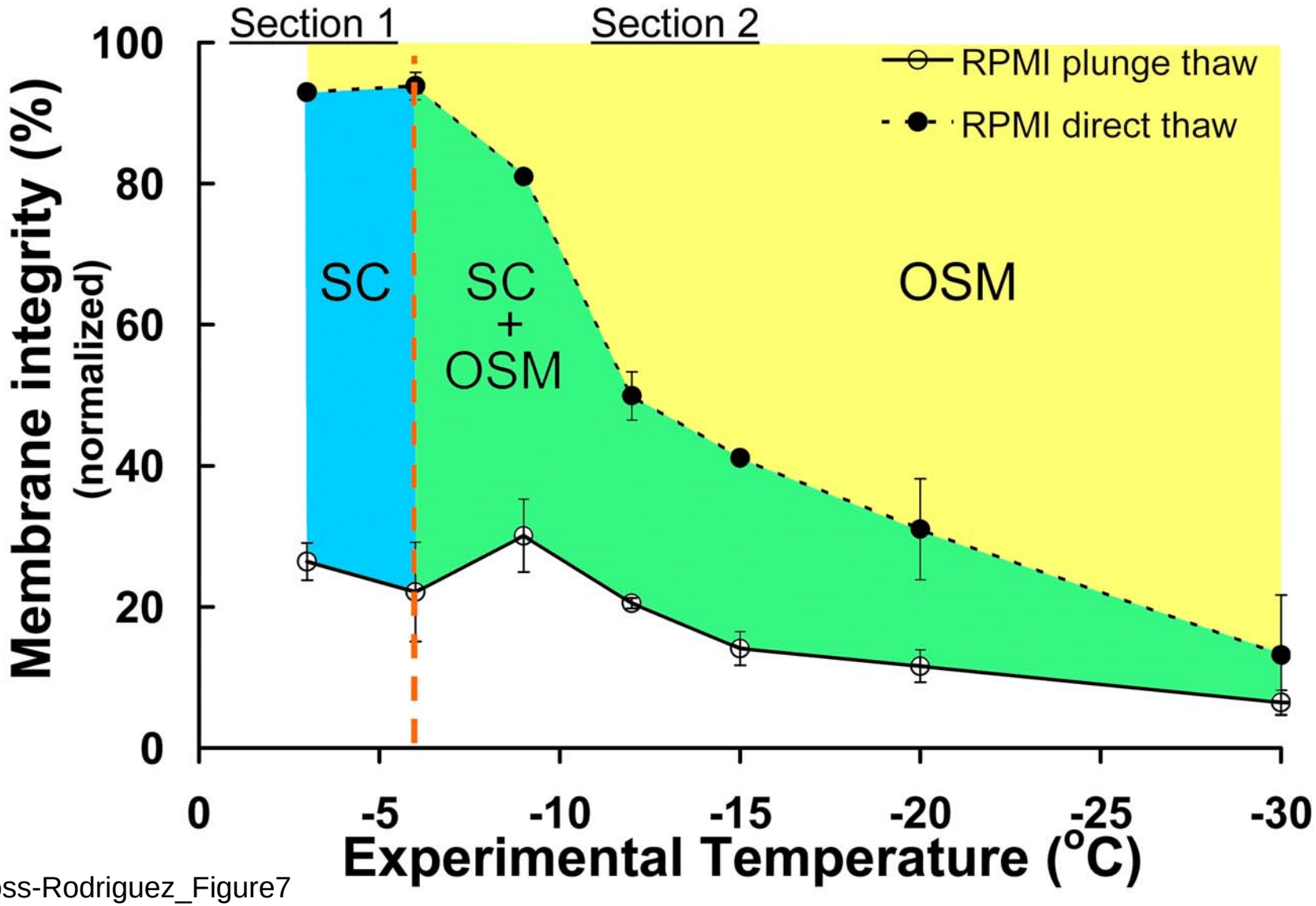

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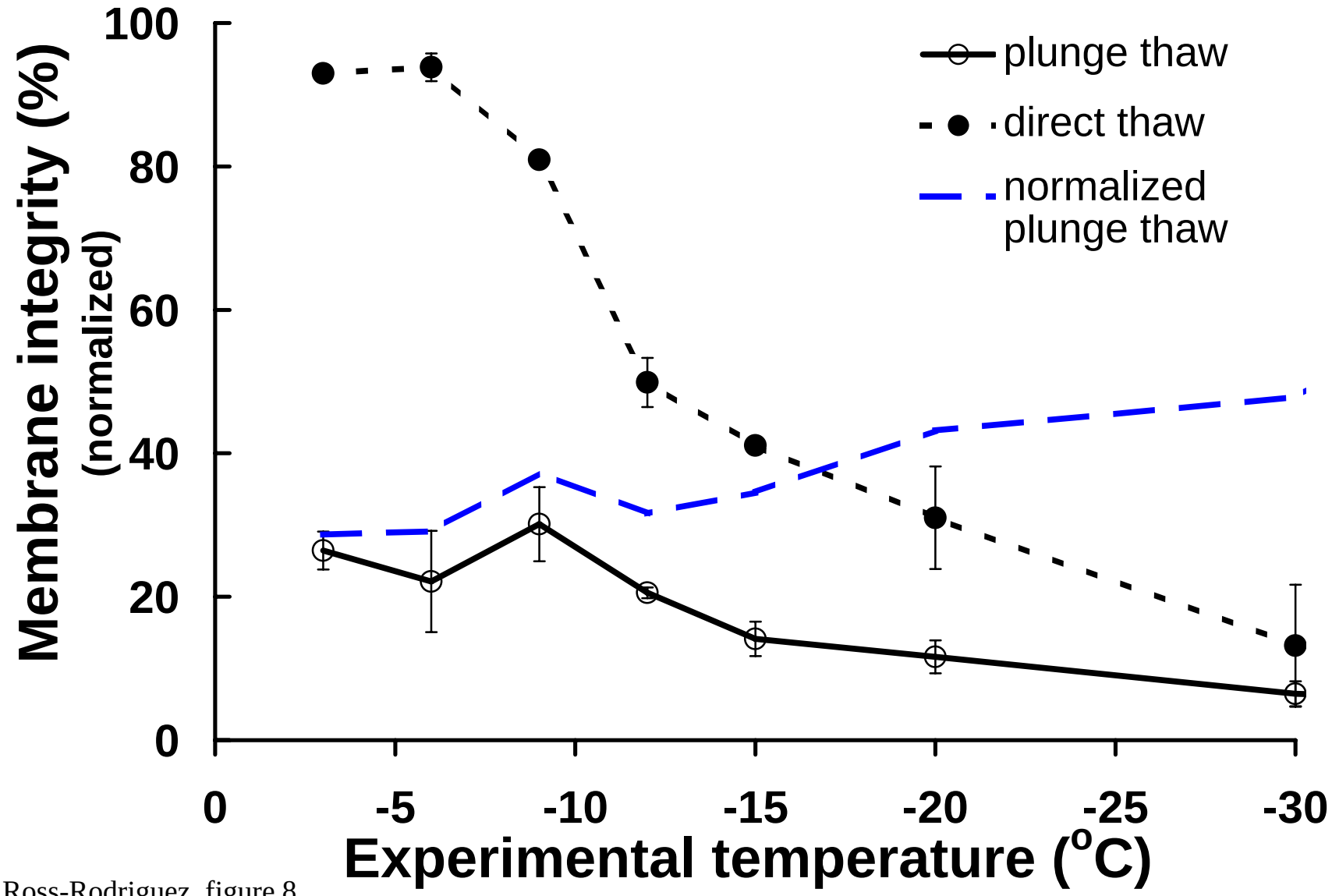
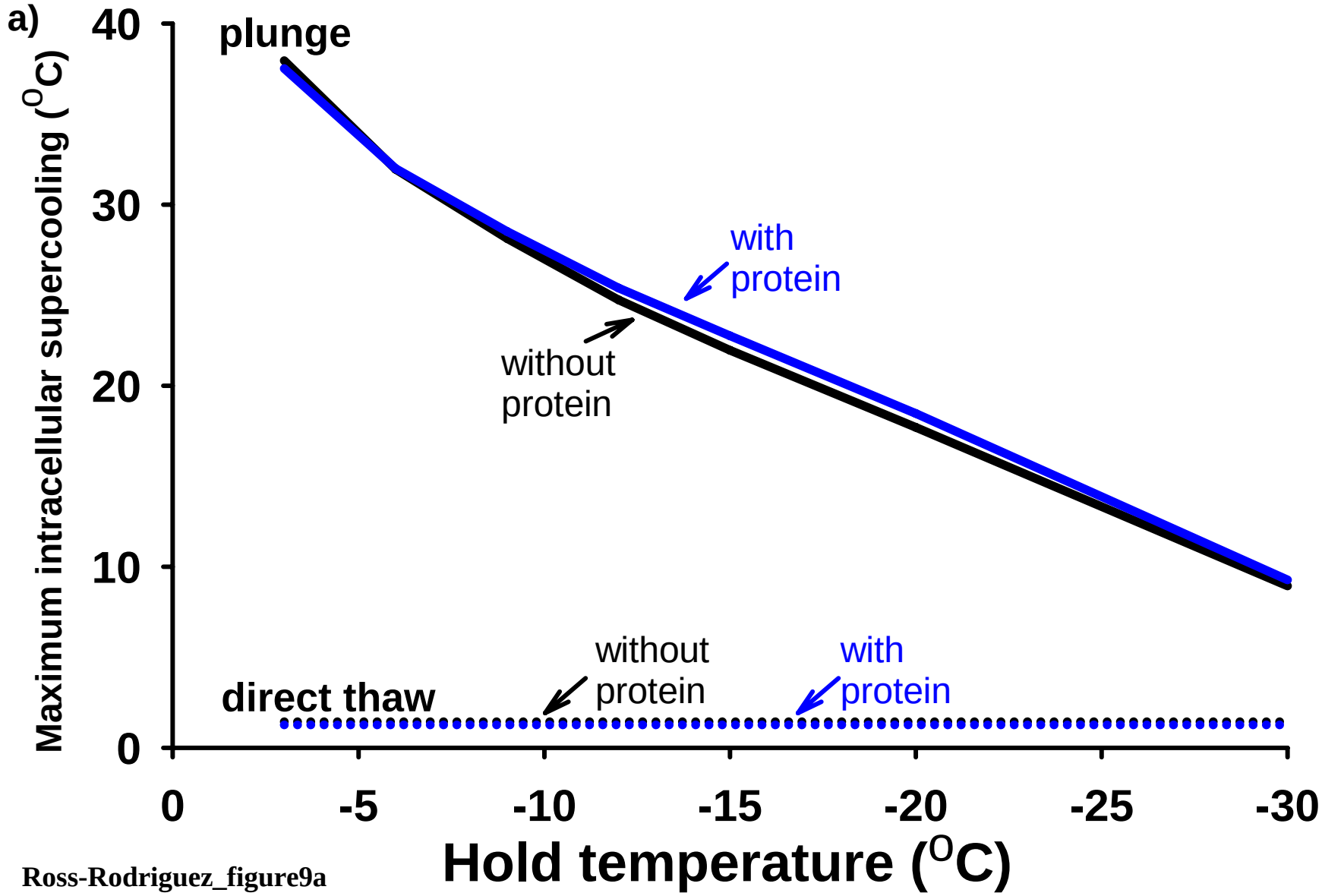
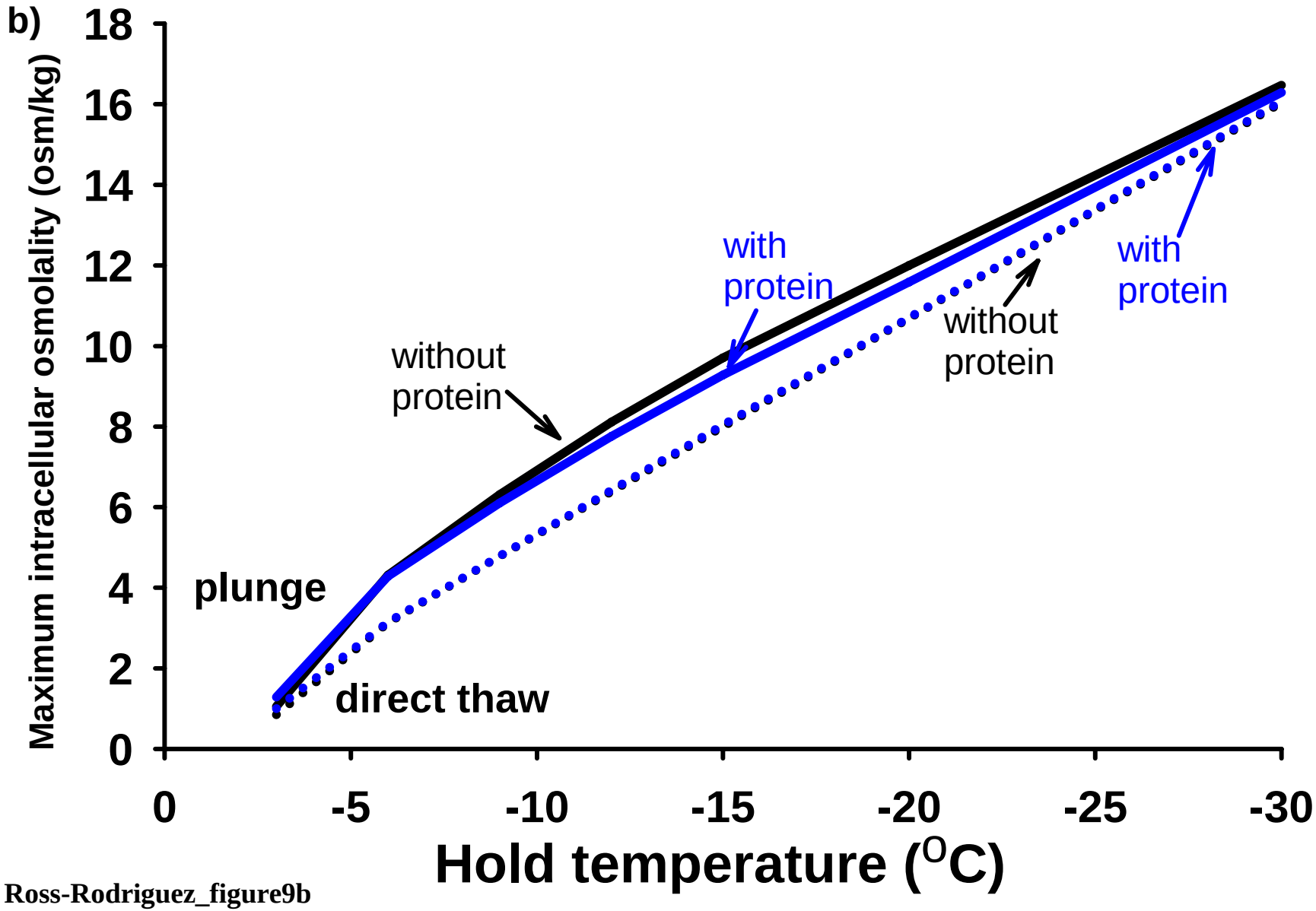


Ross-Rodriguez_figure 8



Ross-Rodriguez_figure9a



Ross-Rodriguez_figure9b