from the glycogen particles. Hence, cryoprotectant production is unrestrained during freezing. Signal transduction via PKA has a key role in the reorganization of metabolism in natural cryopreservation, but we are only beginning to understand the possible roles of other signal transduction pathways in mediating responses to freezing. Mitogen-activated protein kinases (MAPKs) mediate a vast number of cellular responses including gene transcription, cell growth and programmed cell death in response to many different extracellular signals. Sub-families of MAPKs exhibit wide involvement in natural cryopreservation and hibernation. For example, MAPK subfamilies (e.g., p38MAPK, ERK and JNK) respond differently in mammalian torpor; each exhibits organ-specific patterns of response. Furthermore, recent results suggest a role for a novel MAPK during extracellular freezing in the marine periwinkle. It is expected that the development of novel "kinomic" methodologies will allow us to advance our understanding of how specific protein kinases contribute to cryopreservation in the natural world. (Conflicts of interest: None declared. Source of funding: None declared.)

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69. To freeze or not to freeze: insects in winter. Janet M. Storey, Carleton University, Ottawa, ON, Canada

Winter survival for many species of insects requires strategies for sustaining viability at temperatures below 0 °C. Typically, this means either freeze tolerance (a high percentage of body water converted to extracellular ice) or freeze avoidance (deep supercooling to maintain the liquid state). Two species of gall insects that overwinter as larvae on goldenrod have been extensively used by our lab and others as models of each strategy - the freeze tolerant gall fly, *Eurosta solidaginis*, and the freeze avoiding gall moth, *Epiblema scudderiana*. Recent studies in our lab have explored a novel aspects of metabolic regulation and cell preservation that underlie natural cold hardiness in these species. Long term winter survival is enhanced by minimizing and prioritizing ATP use and analysis of energy-expensive cell functions such as ion pumping has revealed active suppression of Na^+K^+ATPase and Ca^{2+}ATPase activities mediated by protein kinases. Mitochondrial activity is also suppressed over the winter including reduced activities of enzymes such as cytochrome c oxidase. By contrast, survival of freeze induced anoxia/ischemia by freeze tolerant species appears to be aided by up-regulation of the hypoxia inducible factor, HIF-1, to coordinate responses that provide hypoxia/ischemia protection. Long term viability over the winter is also aided by increased expression of a variety of chaperone proteins (heat shock proteins, glucose-regulated proteins, crystallins) that stabilize protein structure/function in cytoplasmic, endoplasmic reticulum and mitochondrial compartments. Multiple signal transduction cascades are involved on both a seasonal and a temperature responsive basis; these include actions by cAMP-dependent protein kinase (PKA), mitogen-activated protein kinases and several protein phosphatases. Overall, our studies reveal the cold hardy insect species use a suite of dynamic responses to modulate cellular metabolism in response to seasonal, low temperature, and freeze/thaw cues. (Conflicts of interest: None declared. Source of funding: NSERC, Canada)

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Session 10. Cryobiology of aquatic species

70. Survival of pacific oyster, Crassostrea gigas, oocytes in relation to intracellular ice formation. Liliana Salinas-Flores [a], Serean L. Adams [b], David Wharton [a], Matthew Downes [a], Miang H. Lim [a], [a] University of Otago, Dunedin, New Zealand; [b] Cawthron Institute, Nelson, New Zealand

During cryopreservation, cells are exposed to a number of stresses that may compromise post-thaw viability. Intracellular ice formation (IIF) is one such stress and is generally correlated with cell death. In this study, the effect of IIF in Pacific oyster oocytes was assessed using cryo- and transmission electron microscopy (TEM). Gametes were obtained by "stripping". Oocytes, suspended in seawater, were diluted 1:1 with 20% ethylene glycol in Milli-Q water in 10 steps at room temperature. After an additional 10 min, oocytes were cooled to 0 °C, held for 5 min, then cooled to −10 °C at 1 °C min^{-1}, manually seeded and held for a further 5 min. From −10 °C, oocytes were cooled at either 30, 5, or 0.3 °C min^{-1} to −70 °C. Cell darkening ("flashing") was observed between −38 and −52 °C and −36 and −60 °C, for oocytes cooled at 30 and 5 °C min^{-1}, respectively. No evidence of darkening was observed in oocytes cooled at 0.3 °C min^{-1}. A more detailed study was performed using TEM. Oocytes were prepared and cooled to −10 °C as previously described, but frozen using two cooling programmes. Programme-A involved cooling from −10 °C at 0.3 °C min^{-1} to −35 °C, then plunging samples into liquid nitrogen [Tervit et al 2005; Cryobiology 51:142-151]. Programme-B was designed to reduce IIF and involved cooling at 0.1 °C min^{-1} to −60 °C, holding for 30 min then plunging. Transmission electron microscopy revealed that oocytes were at two developmental stages when frozen, prophase I ($53.1 \pm 1.6\%$) and metaphase I ($46.0 \pm 1.7\%$). Irrespective of the stage and cooling programme used, all oocytes contained ice in the cytoplasm. The mean diameter of the ice crystals did not differ significantly between Programme-A and Programme-B ($0.93 \pm 0.02\mu$m and $0.94 \pm 0.01\mu$m, respectively) but the percentage of ice in the cytoplasm did ($24.37 \pm 0.86\%$ and $21.68 \pm 0.78\%$, respectively). Not all prophase I oocytes had ice in the nucleus. Predictably, the percentage with ice in the nucleus was significantly higher in oocytes frozen using Programme-A ($68.51 \pm 4.54\%$) than those frozen using Programme-B ($14.39 \pm 2.51\%$). Interestingly, post-thaw fertilization was significantly higher at the faster cooling rate and higher plunge temperature ($41.96 \pm 5.93\%$ versus $6.24 \pm 0.64\%$). The results indicate that observations of IIF obtained from cryo-microscopy are limited to larger sized ice crystals. Although Programme-B reduces the amount of ice in the cell by allowing more time for dehydration, fertilization is reduced significantly; suggesting that oyster oocytes may be more tolerant to IIF than to solute effects and cell shrinkage. (Conflicts of interest: None declared. Source of funding: The New Zealand Foundation for Research, Science and Technology [CAWX0304]).

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71. Basic studies on the cryopreservation of Japanese whiting (Sillago japonica) embryos. Sk. Mustafizur Rahman, Sullip Kumar Majhi, Toru Suzuki, Carlos Augusto Strüssmann, Rikuo Takai, Tokyo University of Marine Science and Technology, Tokyo, Japan

The purpose of this study was to examine the suitability of cryopreservation protocols for the embryos of Japanese whiting (*Sillago japonica*), a small-sized, prolific marine fish with pelagic eggs that is a good model for cryopreservation studies. Embryos at various developmental stages were exposed to 10, 15, and 20% solutions of six cryoprotectants [propylene glycol (PG), methanol (MeOH), dimethyl sulfoxide (Me_2SO), dimethylformamide (DFA), ethylene glycol (EG) and glycerol (Gly)] in sea water and twelve vitrifying solutions [VS1 (PG 30% and DFA 20%), VS2 (PG 25% and DFA 15%), VS3 (PG 20% and DFA 10%), VS4 (PG 30% and Me_2SO 20%), VS5 (PG 25% and Me_2SO 15%), VS6 (PG 20% and Me_2SO 10%), VS7 (PG 30% and MeOH 20%), VS8 (PG 25% and MeOH 15%), VS9 (PG 20% and MeOH 10%), VS10 (PG 30% and EG 20%), VS11 (PG 25% and EG 15%), VS12 (PG 20% and EG 10%)] for 20 minutes and the hatching rate was determined after returning them to sea water. Hatching rates decreased with increasing cryoprotectant concentrations and toxicity to whiting embryos varied in the order of PG < Me_2SO < DFA < EG < MeOH < Gly. Somites and tail elongation stages were more tolerant to cryoprotectant solutions than gastrula and pre-hatching stages. Embryos (somites) tolerated a five step (5×4 min) better than a four step (4×5 min) impregnation with vitrifying solutions. No significant reduction in hatching rate compared to the control was observed when embryos were treated (five steps) with VS3 and VS6. None of the embryos survived after exposure to VS4, VS7, VS10 and VS11 while the remaining solutions VS1, VS2, VS5, VS8, VS9 and VS12 gave hatching rates between 3.4% and

80.9%. Intra-embryonic ice formation measured by DSC showed that only vitrifying solutions VS6 (−31.1 °C) and VS8 (−32.6 °C) caused a significant decrease in the nucleation temperature as compared to controls (−26.1 °C). The best vitrifying solutions in terms of post-thawing embryo morphology were VS9 for gastrula (4.1%) and pre-hatching (24.83%), VS8 for tail elongation (27.9%) and VS12 for somites (7.7%) stages. The tail elongation stage was more tolerant to chilling injury than other developmental stages but none of the embryos could resume development and hatch. The results of nucleation temperature obtained by DSC analysis and observation of the morphology and clearness of the embryos revealed that impregnation of embryos with vitrifying solutions in the conditions of the present study did not prevent ice formation during cooling and warming. However, the current study revealed that vitrifying solutions composed of PG with MeOH or EG appeared to be promising in terms of tolerance by the embryo and morphology following thawing. (Conflicts of interest: None declared. Source of funding: None declared.)

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72. Cryobiological properties of immature zebrafish oocytes assessed by the ability to be fertilized and to develop to term. Shinsuke Seki, Toshimitsu Kouya, Ryoma Tsuchiya, Delgado M. Valdez Jr., Bo Jin, Naoya Saida, Magosaburo Kasai, Keisuke Edashige. Kochi University, Nankoku, Kochi 783-8502, Japan

We have found that when immature zebrafish oocytes at stage III were matured in 90% L-15 medium (pH 9.0) containing 0.5% BSA and 1 μg/ml 17α, 20β-dihydroxy-4-pregnene-3-one, a high proportion of them could be fertilized and develop to term. As a preliminary study for the cryopreservation of immature zebrafish oocytes, we examined the sensitivity of the oocytes to chilling, cryoprotectant toxicity, osmotic swelling, and osmotic shrinkage. Their viability was examined by their ability to mature, to be fertilized, and to hatch, using the new *in vitro* maturation method. When immature oocytes were exposed to the culture medium at a low temperature (5, 0, or −5 °C) for 30 min, hatching rates resulted from oocytes exposed to 0 and −5 °C (24 and 16%, respectively) were significantly lower than the rate of control (25 °C, 41%) ($P < 0.05$), indicating that immature zebrafish oocytes are sensitive to chilling at 0 °C or below. For cryopreservation, therefore, vitrification would be more suitable than slow-freezing. When immature oocytes were exposed to 8% ethylene glycol or 10% glycerol solutions at 25 °C for 30 min, no oocytes resulted in hatching. On the other hand, when oocytes were exposed to 5% methanol, 10% methanol, 10% propylene glycol or 9.5% Me_2SO solutions, hatched embryos were obtained; the rates being 41%, 31%, 18%, and 6%, respectively. Therefore, methanol and propylene glycol are less toxic to immature zebrafish oocytes than other cryoprotectants tested. To examine the sensitivity to osmotic swelling, immature oocytes were suspended in 40-90% Leibovitz L-15 medium at 25 °C ALT(248) for 60 min. The hatching rate (control; 36% in 90% L-15 edium) decreased significantly when the concentration of L-15 medium was decreased to 60% (21%), and most oocytes did not result in hatching in 40% and 50% L-15 medium (0% and 1%, respectively). Therefore, immature zebrafish oocytes are sensitive to osmotic swelling. To examine the sensitivity to osmotic shrinkage, immature oocytes were suspended in 90% Leibovitz L-15 medium containing 0-0.3 M sucrose at 25 °C for 30 min. The hatching rate (control; 43% in 90% L-15 medium without sucrose) decreased significantly when oocytes were exposed to the medium containing 0.1 M sucrose (13%). No oocytes resulted in hatching after exposure to the medium containing 0.3 M sucrose. Thus, immature zebrafish oocytes are highly sensitive to osmotic shrinkage. Considering the osmotic sensitivities of oocytes, stepwise permeation and stepwise removal of the cryoprotectant would be necessary. For the cryopreservation of immature zebrafish oocytes, vitrification using methanol and propylene glycol as the cryoprotectant with stepwise treatment would be suitable. (Conflicts of interest: None declared. Source of funding: None declared.)

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73. Assessment of the cryoprotectant permeability and cryoprotectant toxicity of aquaporin 3-expressing immature medaka (Oryzias latipes) oocytes. Delgado M. Valdez Jr., Shinsuke Seki, Takao Hara, Naoya Saida, Yu Nishikado, Magosaburo Kasai, Keisuke Edashige, College of Agriculture, Kochi University, Nankoku, Kochi, Japan

Oocytes of teleosts have not been successfully cryopreserved. We have demonstrated that artificial and temporal expression of aquaporin 3 (AQP3) enhances the permeability of immature medaka oocytes to water and cryoprotectants [Valdez et al., Cryobiology, 53: 60-68, 2006]. In the present study, we examined the permeability to various cryoprotectants and the sensitivity to cryoprotectant toxicity of AQP3-expressing medaka immature oocytes in order to determine a suitable cryoprotectant for their successful cryopreservation. Medaka oocytes at the germinal vesicle stage were obtained 2-3 h before the start of the dark period. They were cultured for 6-7 h without treatment or after injection with AQP3 cRNA. The cryoprotectant permeability (P_S) of intact and AQP3-expressing oocytes was determined from their volume change in 90% TCM-199 containing 8% (v/v) ethylene glycol (EG), 10% (v/v) propylene glycol (PG), or 9.5% (v/v) Me_2SO for 1 h at 25 °C or 4 °C. To study the sensitivity of oocytes to cryoprotectant toxicity, intact and AQP3-expressing oocytes were suspended in each cryoprotectant solution for 1 h at 25 °C or 4 °C. Then, the oocytes were washed and cultured in 90% TCM-199 for 10-14 h at 26 °C. Mature oocytes were inseminated, and cleaved oocytes were incubated in Hanks' solution for 14 days at 26 °C. Hatching was regarded as the criterion for term development. At 25 °C, P_s values of AQP3-expressing oocytes (3.0-3.9 $\times 10^{-3}$ cm/min) were 2-3 times higher than those of intact oocytes (1.2-2.0 $\times 10^{-3}$ cm/min). At 4 °C, P_s values of AQP3-expressing oocytes (1.0-1.6 $\times 10^{-3}$ cm/min) were 3-4 times higher than those of intact oocytes (0.3-0.5 $\times 10^{-3}$ cm/min). However, P_s values of both types of oocytes at 4 °C were much lower than those at 25 °C. Although the P_s values of AQP3-expressing oocytes were not markedly different among the three cryoprotectants, PG was the most permeating cryoprotectant to AQP3-expressing oocytes at both temperatures. When oocytes were exposed to various cryoprotectants at 25 °C, 36-37% of intact and AQP3-expressing oocytes exposed to PG hatched, whereas most of the intact and AQP3-expressing oocytes exposed to EG and Me_2SO did not hatch. When oocytes were exposed to cryoprotectants at 4 °C, results were similar to those at 25 °C with both types of oocytes. This suggests that PG is a suitable cryoprotectant for cryopreservation of immature medaka oocytes, but the toxicity of PG is not decreased by lowering the temperature. For the successful cryopreservation of immature medaka oocytes, it would be necessary to increase the permeability of oocytes and to decrease the cryoprotectant toxicity further. (Conflicts of interest: None declared. Source of funding: None declared.)

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74. Cryopreservation of greenshell mussel (Perna canaliculus) oocytes. Serean L. Adams [a], H. Robin Tervit [b], John F. Smith [b], Rodney Roberts [a], Liliana Salinas-Flores [c], Lindsay T. McGowan [b], Steve Webb [a], Steven F. Mullen [d], John K. Critser [d], [a] Cawthron Institute, Nelson, New Zealand; [b] AgResearch, Hamilton, New Zealand; [c] University of Otago, Dunedin, New Zealand; [d] University of Missouri, Columbia, MO, USA

The Greenshell™ mussel (*Perna canaliculus*) is the main aquaculture species in New Zealand. The industry presently on-grows wild sourced spat (juveniles). However, development of hatchery technology for reliable seed supply and selective breeding is now well underway. Cryopreservation is a powerful tool for selective breeding as it enables genetic material from selected stock to be stored and crossed at will. The aim of this study was to develop a method for cryopreserving oocytes of the Greenshell™ mussel. A number of key cryopreservation variables were tested in preliminary experiments based on a successful method we developed for Pacific oyster oocytes [Tervit et al 2005; Cryobiology 51:142-151]. The ability of oocytes to be fertilized post-thawing was used as the criterion for success and then in subsequent experiments, further development to D-stage