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CLASSICS

An early classic study of freeze avoidance in marine fish

John Duman discusses Per Sholander et al.'s classic paper 'Supercooling and osmoregulation in northern fishes', published in the *Journal of Cell and Comparative Physiology* in 1957.

Marine teleost (boney) fishes are thermal conformers (body temperature equals water temperature) and hypo-osmoregulators (their osmotic concentration is lower than that of the seawater). This not only necessitates a considerable energy expenditure on osmoregulation and water balance but also exposes species inhabiting cold ice-laden seas to freezing, as the freezing point of their body fluids is generally –0.5 to –0.7°C while the temperature of normal seawater from these regions in winter is approximately –1.9°C. Consequently, fishes at these temperatures are supercooled by over 1°C, and therefore should freeze, especially if they contact ice that would then easily propagate across the body surface. The question posed by Per Scholander, Leonard van Dam, John W. Kanwisher, Harald T. Hammel and Malcolm S. Gordon (Scholander et al., 1957) was how the large populations of fishes present in polar seas evolved to survive this potentially lethal situation. The expected answer was that these fishes increase the solute concentration of their body fluids so that they become isosmotic, or slightly hyperosmotic, to the water, in the fashion of marine invertebrates. However, this turned out not to be the answer. Through a series of field studies, conducted mainly on fishes collected in waters off the coasts of northern Labrador and southern Baffin Island in eastern Canada, the authors demonstrated that the freezing points of the body fluids of shallow water fishes were sufficiently low to prevent freezing; however, they were unable to identify the responsible antifreeze. In contrast, because of the absence of ice at depth to seed their body fluids, most deep water fishes did not lower their freezing points and remained supercooled.

Before commenting further on this study, it is useful to mention something of the remarkable career of the lead author (Scholander, 1990). While in medical school at Oslo University in Norway, Scholander determined that his real passion was for biological research. Though his basic medical training was quite useful over his career, Scholander was a comparative physiologist in the extreme (Schmidt-Nielsen, 1987). His research included diving physiology in marine mammals, temperature regulation in mammals, facilitated transport of O_2 by hemoglobin, countercurrent exchange in various physiological systems such as swimbladders of fishes and heat exchangers in mammals and birds, transport of water in the xylem of redwood trees, excretion of NaCl by mangroves, measurement of gases long sequestered in glacial ice to identify ancient atmospheres, subzero temperature adaptations (including freezing tolerance) of arctic invertebrates and, of course, freezing resistance in fish. Any interesting and unsolved biological phenomenon, no matter the organism, was fair game. Inspection of most introductory biology textbooks identifies multiple examples of research topics, in both animal and plant physiology sections, that Scholander first identified, investigated and generally solved. He was certainly a major force in the period often mentioned as the golden age of comparative physiology. In addition, he exuded the Viking wanderlust of his ancestors. Over the course of his career his research took him to Greenland, Alaska, Australia, Tierra del Fuego, Panama, etc., in order to study the organisms best adapted to the problem of interest. Even as a medical student, he took part in expeditions to Greenland and Spitsbergen (Svalbard) Island north of Norway. In fact, his PhD was awarded for his work on the lichens of Spitsbergen.

Scholander originally came to the United States in 1939 on a Rockefeller Fellowship with Lawrence Irving at Swarthmore just as World War II was beginning in Europe, initiating a scientific relationship that continued for decades. At the time of his initial work on cold tolerance in fish, Scholander was at the Marine Biological Laboratory at Woods Hole. My personal connection to Scholander, and also to Hammel, occurred when I was a graduate student in the early 1970s at the Scripps Institution of Oceanography at the University of California San Diego, where both had taken faculty positions. I had formal classes from them, both had laboratories in the Physiological Research Laboratory on the same floor as Art DeVries – with whom I was a student – and Scholander was a member of my thesis committee. DeVries had recently identified the now well-known antifreeze glycoprotein as the missing polar fish antifreeze that was the primary subject of the Scholander et al. (Scholander et al., 1957) article being considered here. That article, along with a subsequent publication (Gordon et al., 1964), plus lectures on the cold water fish problem by Scholander and Hammel, and, of course, the DeVries publications, peaked my interest in the fish antifreeze. Especially exciting to me was the combination of science and exotic fieldwork in the Arctic and Antarctic.

The research described in Scholander and colleagues' classic publication (Scholander et al., 1957) resulted mainly from a series of expeditions to the eastern Canadian arctic. The weather was so extreme during the initial winter (April, 1953) trip to Nain in northern Labrador that the group was forced to divert to Frobisher Bay on southern Baffin Island, where after 2 weeks spent chopping holes through more than a meter of ice, only three small sculpins (probably *Myoxocephalus scorpius*) were collected. A blood plasma sample was obtained from the largest fish, a small sample of eye fluid from the smallest and the

Classics is an occasional column, featuring historic publications from the literature. These articles, written by modern experts in the field, discuss each classic paper's impact on the field of biology and their own work.

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Fig. 1. The camp at Hebron Fjord in summer 1954. Scholander in foreground. Photo credit: Malcolm Gordon.

medium sized fish was eaten by a husky dog before it could be sampled. Such is fieldwork during winter in the arctic. The authors reported: 'Freezing point determinations made in a tent 20 miles from the base showed that these fish avoid freezing by simply becoming isotonic with the seawater'. The second part of this statement, regarding isotonicity, turns out to be incorrect, not because the measurements were improperly done but because the interpretation of the freezing point was incorrect, for reasons I will discuss later.

Because of the problems associated with the initial trip, a second expedition was mounted during the summer of 1954, to Hebron Fjord in northern Labrador (Fig. 1). A summer trip was thought to be suitable because previous oceanographic studies showed that deep water in the fjord remained at –1.7°C all year (Iselin, 1932). Consequently, fish collected at depth are exposed to potentially freezing conditions even in summer. During this second trip, numerous fish of multiple species were collected from cold (–1.73°C) deep water and warmer (4-7°C) shallow near-shore water. To their surprise, freezing points measured in plasma and eye fluid in the fish, both deep and shallow water species, were –0.75 to –0.96°C, indicating that (1) the deep fish spend their entire lives supercooled by about 1°C, and consequently are in danger of freezing, and (2) the winter depression of freezing points in shallow fishes is seasonal (Fig. 2). To determine whether this is possible, they conducted experiments where fishes were held in a trough at –1.0 to –1.7°C. Because this was a field situation, and therefore they lacked the ability to control temperature by standard means, they 'controlled temperature by freezing out ice from the relatively brackish surface water', much in the way that adding salt in the presence of ice lowers the temperature in a home ice cream maker. When fishes were placed into the ice trough at –1.7°C, the fish, both shallow and deep water, froze. The critical point here is that not only were the fish at –1.7°C but also they were in contact with ice. In contrast, if the fish were placed in a net and lowered to –1.7°C water at 100 m in the fjord, most remained unfrozen. When those individuals that survived at depth were placed into the ice trough at –1°C, they froze and died.

The following winter, back at Woods Hole where more controlled temperatures were available, the freezing studies were continued using local killifish, *Fundulus heteroclitus*, and other species. They determined that the fish survived when supercooled to –2°C, or even –3°C, in the absence of ice; however, addition of ice at these same temperatures or at –1.5°C resulted in freezing. An additional experiment was conducted in which fish skin was stretched over the opening of a tube, the tube being filled with isotonic saline, and the apparatus placed into supercooled seawater. Ice touching the skin resulted in seeding across the skin and freezing of the interior solution. So, the problem of survival of the deep water fish in Labrador supercooled by 1°C was solved. In the absence of ice to seed the fish, they remain supercooled by this amount without freezing. However, this did not explain the situation of shallow water species that survive with ice in the environment for much of the year.

The initial results on the two sculpins collected off Baffin Island in winter 1953 had indicated that this species lowered their freezing point (–1.5 to –1.6°C), becoming approximately isosmotic to the seawater in winter, and thus avoided freezing in the same fashion as marine invertebrates. To further investigate this possibility, they went back to Hebron Fjord in winter (March) of 1955, collected fjord cod (*Gadus ogac*) and sculpin (*M. scorpius*) by jigging through the ice and determined their plasma freezing points. This confirmed the earlier results, demonstrating that these shallow water fishes lowered the freezing point of their plasma in winter (approximately –1.5°C) relative to summer (approximately –0.8°C) (Fig. 2). However, while these values are close to the measured temperature of the seawater in winter (–1.73°C), the fish still appeared to be slightly supercooled, and therefore would possibly freeze if they contacted ice, a likely scenario in these waters. Also, the responsible antifreeze remained unknown.

At this point it is time to look ahead to the answer to how these fishes inhabiting ice-laden seawater remain unfrozen, and suggest why such innovative and successful researchers missed the final answer to their puzzle. The freezing/melting point is a colligative property of water, generally dependent only on the solute concentration. If an aqueous sample, such as normal fish plasma, is sealed in a glass capillary tube, placed into a temperature-controlled bath so that it can be observed with a microscope, frozen, and the temperature then slowly raised until only a small crystal remains unmelted, this temperature constitutes the melting/freezing point of the sample. If the temperature is raised by just 0.01°C, the ice crystal melts and disappears (the melting point), and if the temperature is lowered a similar amount the crystal grows (the freezing point). Therefore, the freezing and melting points of water are identical, and the terms are generally used interchangeably. However, as DeVries later showed (DeVries and Wohlschlag, 1969; DeVries, 1971), in the presence of the protein or glycoprotein antifreeze from polar fish there is a difference of as much 1.7°C between the melting temperature (the colligative melting point) and the temperature at which the

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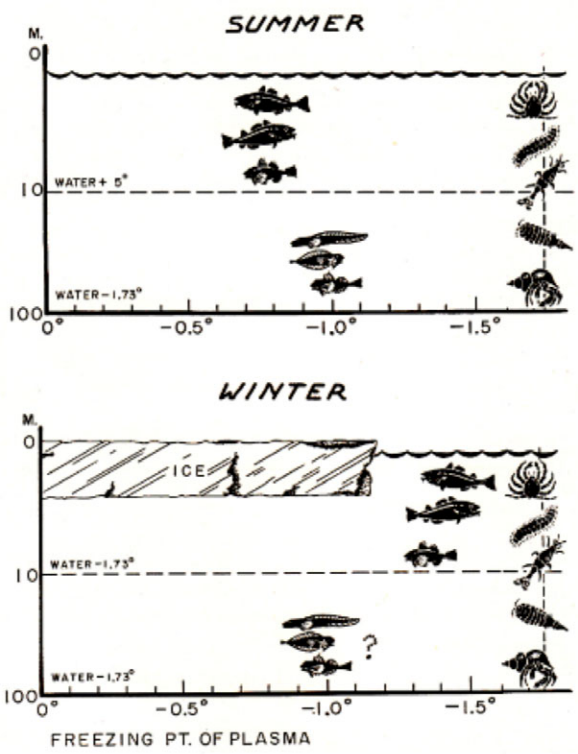

Fig. 2. Freezing points of plasma of shallow and deep water fishes from Hebron Fjord in summer (top) and winter (bottom). Original figure.

crystal will grow (the hysteretic freezing point), reflecting the ability of the protein antifreezes to inhibit the growth of ice. DeVries termed this difference thermal hysteresis, and it is now used to identify the presence of these antifreeze proteins (DeVries, 1986). This unique situation was, of course, unknown to Scholander and colleagues. The 'freezing point' they reported was actually an average of the temperature where the last small crystal remained (melting point) and the temperature where 'the crystal edges sharpen up and new spicules may begin to form' (the incipient freezing point). They stated that while their measurements conformed closely to handbook values for salt solutions, 'in fish plasma, especially that of winter fishes, the melting and freezing temperatures were less sharp and were often as far apart as 0.1°C'. In fact, Hammel later told me that the differences in melting and freezing temperatures of the fish plasma were often much greater. However, given the field conditions under which the determinations were made, this problem was understandable, as temperatures were raised and lowered in a crude fashion by adding ice and brine to the bath.

While the freezing points of the shallow water fish decreased in winter to a temperature that apparently protected them from freezing, the nature of the antifreeze remained unknown. Na^+ and Cl^- ions typically contribute ~80–90% of the solute making up the osmotic concentration of the extracellular fluids of animals. Therefore, it was logical to hypothesize that the shallow fishes might simply increase these ions in winter, becoming isosmotic in the fashion of marine invertebrates. However, while Scholander et al. demonstrated that the Na^+ and Cl^- concentrations did increase slightly in winter relative to summer, the ions were not responsible for the much lower winter freezing points (Fig. 3). Neither were the non-protein nitrogen levels in the sculpin, presumably measured because they knew that elasmobranchs possess high urea and trimethyl amine oxide concentrations that, along with Na^+ and Cl^-, are the primary constituents that render sharks and rays isosmotic with seawater. Consequently, they were unable to identify the antifreeze in the arctic teleosts.

A second study on the teleost freezing resistance topic was published a few years later that included two of the authors (including Scholander) of the 1957 paper (Gordon et al., 1962). Cod (*G. ogac*) and sculpin (*M. scorpius*) from Hebron Fjord were again studied, along with tomcod (*Microgadus tomcod*) from nearshore shallow water off New Brunswick, Canada. The primary purpose was to identify the antifreeze, but in spite of an extensive search that included numerous low molecular mass solute candidates, they were again unsuccessful. However, non-protein nitrogen levels in the serum of *G. ogac* were high, and the authors suspected that the missing antifreeze was a component of this fraction. In fact, they were half correct. These cod have an antifreeze glycoprotein (Van Voories et al., 1978),

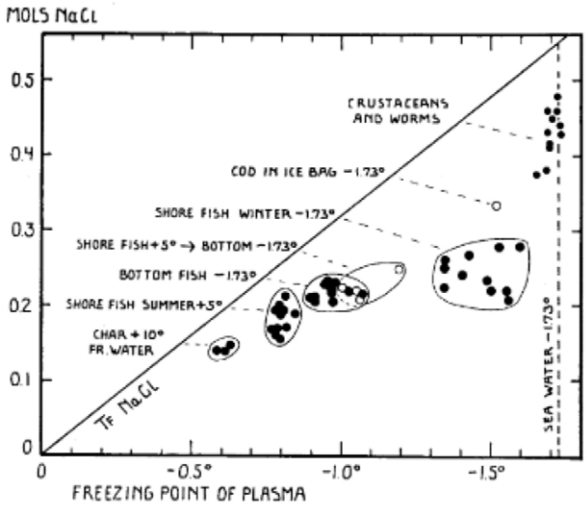

Fig. 3. Freezing points versus chlorides in fishes and invertebrates from Hebron Fjord. Note that as the freezing points decrease, the percentage due to chlorides decreases, indicating the increasing importance of the missing antifreeze. Original figure.

but this protein is not precipitated by the treatments used to remove protein for the non-protein nitrogen measurement. Therefore, the non-protein nitrogen fraction did contain the unknown antifreeze, but it actually was a protein.

Why were these exceptional physiologists unable to identify the elusive antifreeze as a protein? Because they 'knew' that a protein couldn't be the antifreeze. Only low molecular mass solutes, such as inorganic ions or small organic solutes such as glycerol, should be responsible for the depressed winter freezing points of the fishes. After all, the molal freezing point depression constant for water is 1.86, meaning a solute concentration of ~0.48 Osmol is required to depress the freezing point of fish plasma from a summer value of –0.80°C to the winter value of –1.70°C (Δ0.9°C). Consequently, based on colligative properties, proteins could not possibly be the antifreeze, because even if the antifreeze was a small protein of 10 kDa, an impossibly high concentration of 4300 g l^{-1} would be required. However, as DeVries initially demonstrated in his classic studies over a decade later, the antifreeze proteins and glycoproteins do not function via colligative properties (DeVries, 1971).

One reason to read, or reread, this article is to gain insight into the thought processes, even when incorrect, of these extremely capable scientists. This is especially obvious because of the editorial policies of the day that permitted their writing to elaborate on the logic and intuition that went into the planning and interpretation of the research, even permitting inclusion of some fairly anecdotal points that add greatly to the narrative. Although the studies were not successful in identifying the actual antifreeze responsible for the depressed winter freezing points of arctic fishes, we should not be overly critical of this shortcoming. While hindsight is often perfect, rarely is that the case with first research efforts attempting to answer difficult questions, especially when the answers are counter to widely accepted scientific theory. Although their work came up short after a long and exhaustive search, Scholander et al. did show that the fish prevented freezing by using antifreeze that must be highly unusual. Therefore, the studies described in this publication set the stage for the insightful and innovative work of DeVries that in turn, lead us to today (Duman and Olsen, 1993; Zongchao and Davies, 2002; Griffith and Yaish, 2004; DeVries, 2005; Raymond et al., 2008; Duman et al., 2010) where the many varied antifreeze proteins that have been identified in numerous species of animals, plants, fungi, bacteria, etc., continue to be studied by multiple laboratories around the world.

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References

DeVries, A. L. (1971). Glycoproteins as biological antifreeze agents in antarctic fishes. *Science* **172**, 1152-1155.
DeVries, A. L. (1986). Antifreeze glycopeptides and peptides: interactions with ice and water. *Met. Enzymol.* **127**, 293-303.
DeVries, A. L. (2005). Ice, antifreeze proteins and antifreeze genes in polar fishes. In *Life In The Cold* (ed. B. M. Barnes and H. V. Carey), pp. 307-316. Institute of Arctic Biology, University of Alaska, Fairbanks.
DeVries, A. L. and Wohlschlag, D. E. (1969). Freezing resistance in some antarctic fishes. *Science* **163**, 1073-1075.
Duman, J. G. and Olsen, T. M. (1993). Thermal hysteresis activity in bacteria, fungi and primitive plants. *Cryobiology* **30**, 322-328.
Duman, J. G., Walters, K. R., Sformo, T., Carrasco, M. A., Nickell, P. and Barnes, B. M. (2010). Antifreeze and ice nucleator proteins. In *Low Temperature Biology of Insects* (ed. D. Denlinger and R. E. Lee), pp. 59-90. Cambridge, UK: Cambridge University Press.
Gordon, M. S., Amdur, B. H. and Scholander, P. F. (1962). Freezing resistance in some northern fishes. *Biol. Bull.* **122**, 52-62.
Griffith, M. and Yaish, M. W. (2004). Antifreeze proteins in overwintering plants: a tale of two activities. *Trends Plant Sci.* **9**, 399-405.
Iselin, C. O'D. (1932). A report on the coastal waters of Labrador, based on explorations of the 'Chance' during the summer of 1926. *Proc. Am. Acad. Arts Sci.* **66**, 1-37.
Raymond, J. A., Christner, B. C. and Schuster, S. C. (2008). A bacterial ice-binding protein from the Vostok ice core. *Extremophiles* **12**, 713-717.
Van Voories, W. V., Raymond, J. A. and DeVries, A. L. (1978). Glycoproteins as biological antifreeze agents in the cod, *Gadus ogac* (Richardson). *Physiol. Zool.* **51**, 347-353.
Scholander, P. F. (1990). *Enjoying a Life in Science: an Autobiography of P. F. Scholander*, 226 pp. Fairbanks: University of Alaska Press.
Scholander, P. F., van Dam, L., Kanwisher, J. W., Hammel, H. T. and Gordon, M. S. (1957). Supercooling and osmoregulation in northern fishes. *J. Cell Comp. Physiol.* **49**, 5-24.
Schmidt-Nielsen, K. (1987). Per Scholander 1905-1980. In *Biographical Memoirs*, Vol. 56, pp. 386-413. Washington, DC: National Academy of Sciences.
Zongchao, J. and Davies, P. L. (2002). Antifreeze proteins: an unusual receptor–ligand interaction. *Trends Biochem. Sci.* **27**, 101-106.