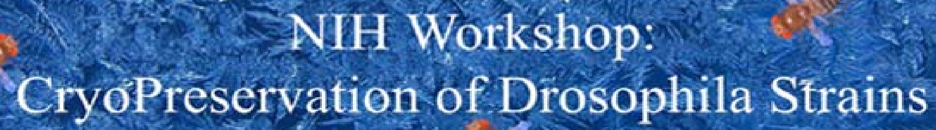

Report

Workshop held on Wednesday, July 13, 2016
Orlando World Center Marriott, Orlando, FL

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1 - Executive Summary

Drosophila melanogaster is widely used in the biomedical research community to study development, model human diseases and undertake high-throughput drug discovery. However, reliable and cost-effective approaches for long-term preservation of *Drosophila* stocks are lacking. To address this gap, the Office of Research Infrastructure Programs in the Division of Program Coordination, Planning and Strategic Initiatives and the National Institute of Neurological Diseases and Stroke co-sponsored a workshop titled "Cryopreservation of *Drosophila* Strains" in Orlando, Florida on July 13, 2016. The objective of this workshop was to evaluate the potential and practicality of developing efficient preservation methods for long-term storage of *Drosophila* stocks. Among the 40 participants, which included 12 speakers, were fly experts, stock center personnel and cryobiologists from universities, private companies, and government agencies of the United States, Canada, Europe and Japan, including the NIH and the USDA.

The workshop was divided into three sessions with presentations followed by discussion. In Session 1, two keynote speakers discussed the status and challenges of *Drosophila* cryopreservation and laid out several potential strategies for long-term preservation of *Drosophila* strains. Session 2 focused on emerging and novel technologies potentially applicable to cryopreservation of *Drosophila* strains. This session was further divided into three panels. In Panel 1, three talks focused on genetic and dietary approaches. The speakers discussed what can be learned from insects in nature to improve freeze tolerance and temperature sensitivity to model and optimize cryopreservation and extend cold-storage in *Drosophila*. Panel 2 focused on cryopreservation via sperm, embryos and larvae. Speakers discussed *in vitro* fertilization protocols with cryopreserved sperm, cryopreservation protocols of mouse and fish ovaries applicable to *Drosophila* cryopreservation, and approaches to cryopreserve larvae. Panel 3 concerned robotics and instrument development in vertebrates, humans and insects potentially of interest for optimizing cryopreservation protocols in *Drosophila*. Speakers discussed techniques to anhydrously preserve vertebrate germinal vesicles, vitrification methods in human infertility treatment that overcome chilling sensitivity, microfluidics methods that could optimize high-throughput cryopreservation protocols, and the development of effective and economic automation methods for cryopreservation of insect embryos. The last session, Session 3, was reserved for open discussion and recommendations. Participants discussed the potential value of long-term stock preservation, protocol standardization, strategies better suited for strain preservation in labs rather than stock centers, improvement of existing storage methods, and development of novel approaches. Finally, participants made several recommendations to the NIH that delineated research priorities for long-term preservation of *Drosophila* strains through multidisciplinary approaches.

2 - Background: *Drosophila* Resources and the Need for Cryopreservation

Drosophila melanogaster is one of the leading biomedical models for understanding the basic biology of animal systems. The genetic, genomic, cellular and developmental biology lessons acquired from studies of *D. melanogaster* and its relatives often directly impact on our understanding of the biology of evolutionarily distant metazoans, notably humans and the other major vertebrate models (e.g., mouse, zebrafish) as well as on closer relatives, including other invertebrate biomedical models (such as *C. elegans*) and arthropods of medical or agricultural importance. The importance of *Drosophila* research is reflected in the recognition of many *Drosophila* researchers for major scientific prizes and membership in national and international scientific academies. The impact of *Drosophila* is also reflected in the symbols and names of many genes of humans and other species – named based on the orthology to the pioneer genes of the family that were first discovered in *Drosophila melanogaster*. *Drosophila* research in the –omic era continues to play a leading role in understanding biological pathways and networks, genome variation and evolution, and increasingly as a test-bed for translational research.

The importance of *Drosophila* as a research tool relies on several characteristics: ready culturability, a small genome size, high fecundity, short generation time, and the ease in recovering mutations. The genome is nearly entirely sequenced, very well annotated and readily transformed. Its favored use in many labs around the world for more than 100 years has led to the development of a large suite of elegant tools which allow easy analyses of gene structure and function. These tools exist in the form of fly lines (stocks) that carry chromosomal aberrations, useful mutations and/or transgenic constructs. The number of these stocks is increasing at a rapid rate, especially as new technologies like CRISPR can and are being applied. Currently there are >150K stocks held in the Centers at Bloomington, Vienna and Kyoto and this number is growing. Unfortunately, there is no easily applied method of long term storage of *Drosophila* and living cultures must be maintained. Thus, it is this burgeoning of stocks that in part has brought about this meeting to discuss the potential to bring cryopreservation technology to the *Drosophila* system. It must be noted that methods have been published (Steponkus et al. 1990 *Nature* 345: 170-172; Mazur et al. 1992 *Science* 258: 1932-1935), but they are labor-intensive and have variable success rates, and thus are impractical for application to many stocks. A further issue resides in the collections of stocks held in individual laboratories. Keeping even a modest number of lines is a time consuming and added expense to running a laboratory working on *Drosophila*. Fly stocks need to be "turned over" on a schedule of about every 2 weeks when they are maintained in an optimal temperature range of 22 °C to 25 °C. While cryopreservation may not be practical for individual labs, some method of increasing the interval between stock changes could be quite useful to many investigators and reduce costs significantly.

3 - Summary of Presentations and Discussion

3.1 - Overview of Challenges & Cryopreservation

Ideally, a protocol as simple as those used on *C. elegans* would be widely used in individual labs and stock centers. Barring that possibility, if the current published technique could be made more reliable with a consistent recovery of >50% of the preserved animals and shown to work on multiple genotypes not just wild type flies, it would find some applicability.

Cryopreservation would be useful for maintaining those stocks that are in low demand, hard to create or of important historical significance. Moreover, keeping stocks as living cultures means that undetected second site mutations can accumulate, and any mobile genetic elements in the stock line can and will undergo changes in position. If it is important to maintain the genomic sequence in stasis, then cryopreservation would seem an excellent solution. However, it is unlikely that any regularly ordered stocks (*e.g*., as tools for genetic mapping and expression analyses) would be frozen.

Based on the current literature and the natural history of other insects that can resist low temperature, there are a few potential strategies for cryopreservation:

1. Use a strategy based on cryopreservation of vertebrate embryos – *i.e.*, cryopreservation by ultra-rapid cooling in media containing high concentrations of cryoprotectant agents (often referred to as vitrification). As noted, this protocol has been developed but it is technically challenging and would need significant improvement. Alternatively, there are strategies used in pest species (screwworm and house fly) that might be modified and applied.

2. Use a strategy based on inducing diapause. Diapause is a period of suspended suspension of development that can occur at the embryonic, larval, pupal, or adult stage, depending on the species. *Chymomyza*, a drosophilid fly, undergo diapause in the fall when temperatures drop. Can *Drosophila* be induced to undergo a diapause-like state? It is done in nature by increasing proline concentration. Maybe changes in things such as diet in *Drosophila* would work.

3. Use a strategy based on changing food composition. This is not a freezing protocol but rather a technique to lower sugar and increase lipids to enhance fly lifespan. Such a technique could be useful to individual labs that do not desire or cannot adopt cryopreservation.

4. Use a strategy based on expressing transgenes. This would involve the introduction of constructs that express anti-freeze proteins or aquaporins. A major drawback is the need to introduce the transgene in all lines that need to be frozen.

In summary, if a protocol can be developed, it needs to be easy to use, extremely reliable and not require a large labor investment. The following sections present current state-of-the-art approaches for long term maintenance of organisms and organs and potential technology areas that could be developed to affect cryopreservation or life cycle extension in *Drosophila*.

3.1.1 – The Challenges of *Drosophila* Cryopreservation and the Potential of Aqua-Glyceroporins
Hugo Bellen, Baylor College of Medicine

Dr. Hugo Bellen discussed the challenges of *Drosophila* cryopreservation and presented the potential of using aqua-glyceroporins to address these challenges. Dr. Bellen noted that cryopreservation of flies is a long sought-after way to reduce the manual effort that is required for maintenance of many *Drosophila* stocks. Cryopreservation of flies will save stocks that are not kept at stock centers owing to their low demand or stocks that are not easy to generate. However, current protocols for the preservation of *Drosophila*, such as vitrification of embryos using ethylene glycol, are not easy to implement and do not offer a very efficient way to recover frozen embryos/larvae. In the last decade, attempts have been made to adapt strategies employed by naturally freeze tolerant insects to freeze *Drosophila*. While these studies have not yielded a successful protocol available to all fly researchers, they have been instructive in unraveling the mechanisms that are employed by these freeze tolerant animals. Study of *Chymomyza*, a drosophilid family member which has been shown to survive periods of deep freeze, suggest that diapause and metabolic changes in larvae are critical for their survival under extremely cold conditions. Other studies on freeze tolerant insects have shown that osmotic regulation and accumulation of glycerol using aquaporins are key adaptations in insects that survive harsh winters. Dr. Bellen stated that his lab had commenced an effort to use aquaporins (HC-3) from Cope's gray tree frog (*Hyla chrysoscelis*) combined with dietary changes to allow *Drosophila* larvae and embryos to accumulate glycerol within their cells. They have generated *Drosophila* lines that over-express the HC-3 gene and accumulate glycerol when fed a high glycerol diet. While these strains are not currently freeze-tolerant, improving the expression of the HC-3 gene and streamlining the freezing protocol might yield a facile way to successfully cryopreserve Drosophila larvae.

3.1.2 – Applied Cryobiology in Managed Insect Systems
Joseph Rinehart, USDA-ARS

Dr. Joseph Rinehart discussed the management of insect systems using applied cryobiology. Dr. Rinehart noted that whether in an agricultural, industrial, or laboratory setting, facilities that mass rear insects can be greatly aided by the implementation of cold storage technologies. Due to their ectothermic physiology, relatively simple cold storage protocols can often be employed to increase the longevity of an insect. "Shelf life" can be further increased by coupling cold storage with fluctuating thermal regimes or by manipulating other micro-environmental parameters such as humidity or oxygen concentrations. For many insects, especially those from temperate climates, the overwintering physiology associated with diapause can be hijacked to further increase longevity; however, this process can be complicated by the need to understand the diapause initiation process, which not only limits the life stage that can be stored, but can be altered or even absent in certain strains. Finally, cryopreservation offers the ability to store important germplasm indefinitely, but involves a complicated process that may appear daunting to end users, thereby limiting implementation. Importantly, rather than picking a specific strategy, managed insect systems are often best served by developing a suite of cold storage technologies that can be used together to accommodate various needs. Dr. Rinehart further described his group's experience with other insect species. He suggested that the *Drosophila*

community would be best served by developing all three cold storage strategies (cold storage, diapause, and cryopreservation), including research that focuses on underlying mechanisms to facilitate further protocol refinement as well as serving as a foundation for a systematic analysis of strain to strain variances. Finally, Dr. Rinehart recommended that resources should be dedicated to technology transfer, including improving the ease and accessibility of storage protocols as well as ensuring the quality of post-storage insects.

3.2 - Comparative, Dietary & Modeling Approaches for Cryopreservation

3.2.1 – Overview

Cryopreservation of *Drosophila melanogaster* has benefits to the community. These include protection of stocks against genetic drift, reduction of space needed for storage, decreased costs of maintenance, and prevention of loss or contamination. While a successful method has been described for embryo cryopreservation (Mazur et al., 1992), it has not been adopted. Several reasons contribute to the lack of use of embryo cryopreservation, such as the difficulty in executing these protocols and the limited survival of animals (including robust, wild-type stocks) upon retrieval.

Session 2 of the workshop had two themes. The first theme considered strategies used by wild flies to survive challenging environments in their natural habitats, environments that include periods of extreme low temperatures. The second theme focused on the use of mathematical modeling to inform experimental design for cryopreservation. Collectively, session 2 presentations provided recommendations that included (1) development of alternative strategies that extend lifespan and reduce generational time, thereby incorporating some of the benefits of cryopreservation and (2) development of computer-assisted learning to optimize development of cryopreservation protocols.

3.2.2 – Insect Freeze Tolerance: Lessons from Nature

Brent Sinclair, University of Western Ontario

Many insects survive extreme environmental conditions that include freezing temperatures. This suggests these animals have mechanisms permitting adaptation that could be exploited to improve survival at low temperatures.

D. melanogaster is a chill-susceptible species killed at above-freezing temperatures that do not cause ice formation. In contrast, several species of drosophilids are freeze tolerant. In these species, embryos do not survive freezing, but some adults and many larvae survive. Freeze tolerant species use many mechanisms to survive low and freezing temperatures, natural mechanisms that maintain fluids in a liquid state to prevent the generation of ice crystals. Natural freeze tolerance includes alterations in the production of antifreeze proteins, carbohydrates, or amino acids, all changes that could induce osmotic dehydration. Strikingly, freeze tolerance has evolved independently multiple times, suggesting that these mechanisms represent physiologically relevant and important survival strategies. Understanding natural strategies of freeze tolerance will provide used information that has the potential to convert of *D. melanogaster* from a cold sensitive to a freeze tolerant organism.

Cold tolerance of larvae and adults is plastic. Acclimation to the cold improves cold homeostasis. Acclimation is achieved through changes in temperature, diet, gut microbes, photoperiod, and short-term hardening treatments that improve tolerance. Notably, cold tolerance is improved by some species of yeast (*Lachancea kluyveri*), but not by the *S. cerevisiae* usually used in *Drosophila* diet. Dr. Sinclair discussed data published by Vlad Koštál (Czech Academy of Sciences) from two preconditioning methods in *D. melanogaster*. One included feeding animals with food containing high concentrations of proline or glycerol. The second involved cold acclimation, conferred using a daily thermal period of temperature ramp up and down that simulates artificial autumn, known as the fluctuating

thermal regime. Notably, Dr. Sinclair reported the best survival of animals subjected to both regimes, with 50% survival of animals frozen at -5ºC for short periods (3 hours). Dr. Sinclair noted that using only methods that promotes intracellular osmotic dehydration (feeding proline or glycerol) is challenging, as the fly gut acts as a barrier for absorption. This limitation is particularly true for glycerol, wherein concentrations as high as 4 M glycerol are needed in the hemolymph to survive low temperatures. The combination regime provided improved survival, but this was limited to short times. As such, this method is not suitable for long-term cryopreservation at liquid nitrogen temperatures.

Dr. Sinclair has begun studies to define the mechanism of induced cold tolerance. To this end, gene expression changes associated with cold acclimation were defined, with ~1000 genes responding. Pathways identified include those involved with proline and glutathione metabolism, iron balance, and the cytoskeleton. Further, Dr. Sinclair noted that there is also an interaction with the gut microbiome, an area that needs further investigation. Strikingly, data obtained to date demonstrate that altering the microenvironment promotes adaptation and should be considered as a method to reduce generation times in flies. Dr. Sinclair predicted that natural methods of increasing acclimation might provide short-term solutions with some of the benefits of cryopreservation.

3.2.3 – A Temperature-Dependent Shift in Dietary Preference Alters the Viable Temperature Range of *Drosophila*
Marko Brankatschk, Max Planck Institute

D. melanogaster survives year-round in a variety of habitats that experience wide ranges of temperature fluctuations. Dr. Brankatschk considered how diet might influence adaptation. Notably, he tested the effects of two diets, each balanced in the proportions of calories represented in protein, lipid and carbohydrate. One diet was a standard yeast diet and the second was a plant diet comprised of cornmeal, malt, and sunflower oil. Differences between yeast food (YF, based on Baker's yeast, *Saccharomyces cerevisiae*) and plant food (PF) include the shorter, more saturated lipids and fungal sterols in yeast, compared with the longer, more unsaturated lipids and phyto-sterols in plants. Dr. Brankatschk showed data supporting that the PF diet improves survival at low temperatures.

Temperature affects food preference in females. These striking observations indicate that survival at different temperatures depends upon a behavioral adaptation. YF is preferred at 25-30ºC, while PF is preferred at temperatures below 15ºC. Notably, YF improves development and survival at high temperatures, while PF improving development and survival at low temperatures. Further, at low temperatures, plant fed flies remain mobile and are capable of geotaxis, whereas yeast fed flies are immobile. Tests of survival at subzero degrees showed that plant fed flies can survive freezing in water for 24 hours and are fertile after a few days. Plant-fed flies also have a longer lifespan than yeast-fed flies, due to changes in the insulin signaling pathway. Lifespan extension occurs both at 25°C and at cooler temperatures. Strikingly, plant fed flies can live for 6 months at 12°C. These data show that diet has a substantial impact on flies raised at low temperatures (12°C).

The mechanism of how low temperature affects plant-fed flies was investigated. Although insulin signaling is critical for high temperature survival of yeast-fed flies, manipulation of insulin signaling did not affect larval survival at low temperature. However, differences in YF and PF do affect membrane lipid composition, which parallels differences in the makeup of lipids within the dietary source. Further, the biophysical properties of membranes were studied using a laurdan dye assay. These studies showed that membrane

fluidity was unchanged in liposomes isolated from yeast- or plant-fed flies at high temperatures (20-30°C), but increased only in liposomes isolated from plant-fed flies raised at 12°C. These data suggest that feeding flies on plants promotes survival at low temperatures through changes in membrane lipid composition that improves fluidity.

In the wild, temperatures fluctuate daily. To determine whether diet impacted development and cold tolerance in a natural setting, flies fed on the two diets were placed outside in Dresden, Germany from September to January, where daily temperatures ranged from a daytime high of 23°C /12°C to a nighttime low of -1°C /-9°C. Better pupation was observed for plant- versus yeast-fed larvae. When nighttime temperatures dropped to -4°C, no yeast-fed larvae pupated and no adults emerged. In contrast, plant-fed larvae continued to develop and adults emerged. These data suggest that feeding on PF, but not YF, allows flies to survive outdoor conditions up to midwinter. Further, natural seasonal adaptation of flies might reflect dietary changes that prepare flies for survival in changing environmental conditions.

3.2.4 – Cryopreservation Modeling Enables Rational Protocol Design and Develops Informed Hypotheses: The Past, Present and Future of Modeling Cryopreservation in Drosophila
James Benson, Northern Illinois University

Vitrification is a cryopreservation procedure that prevents ice crystal formation both within and outside of cells. Executed optimally, vitrification (glass formation) improves material survival after long-term storage following freezing. Multiple factors impact vitrification, conferring challenges to optimization of experimental procedures. Dr. Benson noted that mathematical modeling is useful in defining parameters relevant to damage mechanisms and providing information to guide experimental optimization.

Ice formation during freezing is harmful to material survival. During freezing, salt concentrations increase and promote ice formation. In studies of *Drosophila*, no ice is present at 22°C, ice and liquid exist at -10°C, and only ice exists at -40°C. Ice-free cryopreservation or vitrification can be achieved experimentally using cryopreservation agents (CPAs) such as glycerol that reduce salt concentrations. Yet, optimal CPA concentrations are often cytotoxic. Additionally, material survival depends upon the time period during which freezing is achieved. Notably, both slow and fast freezing times are associated with injury. Together, current observations emphasize the need to identify the best CPA concentrations and time points for robust cryopreservation.

Cryobiology is embodied by challenges of mass transport and heat dissipation. Dr. Benson described how mathematical modeling of physical properties of transport improves the understanding of water and solute exchange. Further, he noted that computational methods were used to optimize procedures for freezing adherent endothelial cells. Dr. Benson was optimistic that mathematical models can be developed for stage 14-16 embryos *D. melanogaster* because there is so much information about the organism. The goal is to understand how damage accumulates, then use models to test the parameters. Good models can lead to good protocols.

3.2.5 – Round Table Discussion

The discussion began with a question about cryoprotective agents. Speakers noted that different substrates are used due to technical issues (rate of movement) and the outcome (better glass formation). The best CPAs have the highest rates of cellular entry. Cocktails of

CPAs have less toxicity for cells. Next, a participant asked whether proline was a CPA. Speakers noted that proline does not appear to be a vitrifier (a chemical that readily forms a glass at low concentrations and temperatures), but its mechanism of action is unclear. Protection occurs from feeding flies 15 mM proline during rearing. Dr. Toner stated that proline might contribute to collagen production, providing a connection to extracellular matrix formation. As noted in Dr. Sinclair's talk, the extracellular matrix, especially of the gut, appears important in cold tolerance in other insects.

The discussion then turned to effects of diet on longevity. Questions were raised about the wide scale application of the PF diet. It was noted that a paper was published in the early 1990s that examined cholesterol additives to food; these studies reported results similar to those found for PF. Dr. Brankatschk noted that the causative agent in PF is not known. One participant asked whether diet impacts the cold tolerance of eggs. Although extensive studies have not been completed, it appears that late stage embryos die before they freeze. The discussion returned to the PF diet. Use of this diet was noted to represent a method to decrease transfer of stocks, which might be very helpful to the community. Members of the Bloomington stock center noted that maintenance of stock vials for prolonged times is often accompanied with food drying out and mold/ fungal contamination.

In a follow up email, Dr. Brankatschk provided additional information. He noted that fungicide is need to kill the yeast that transfers with flies from the YF. With the addition of fungicides (and low food pH perhaps due to propionic acid), the bacterial isolates show no temperature differences. Dr. Brankataschk shared that the amount of agar in PF needs to be tested further, with his current protocols using a 50% reduction. In addition, Dr. Brankatschk shared that circulating temperature improves stock keeping protocols. He uses fluctuations between 12°C and 18°C, which requires transfers to be done every 4 months in a 25-mL volume of food. Special stoppers are used to prevent mites. A commercial source is used, since transfers occur infrequently. With this strategy, mites are not a problem.

Discussion turned to Dr. Benson about whether membrane fluidity can be modeled, especially as Dr. Brankatschk noted diet influences this parameter. Dr. Benson noted that membrane fluidity gets batched into temperature studies because of the underlying assumption that the critical aspect of cryopreservation is exchange and permeability.

3.3 - Cryopreservation of Sperm, Embryos, and Larvae

3.3.1 – Overview

The two established cryopreservation protocols – and all successful insect cryopreservation programs – focus on cryopreservation of embryos. However, genetic material of other organisms is cryopreserved in a range of life stages. For example, sperm is cryopreserved both in species with external fertilization (such as economically-important marine invertebrates), as well as in those for which *in vitro* fertilization is necessary, including zebrafish and humans. Cell lines (including those derived from insects) are also regularly cryopreserved. As discussed above, many *Diptera* have freeze tolerant larvae, raising the question of whether a focus on embryos is going to be most productive. In this session, the speakers explored several alternative targets for cryopreservation and the technical challenges that need to be overcome to facilitate these approaches.

3.3.2 - Developing *Drosophila* IVF protocols for Preservation of *Drosophila* Stocks
Jianjun Sun, University of Connecticut

Sperm of many species are routinely cryopreserved, but for species like *Drosophila* with internal fertilization, artificial insemination or *in vitro* fertilization (IVF) is necessary to produce offspring. There is an IVF protocol for a sawfly (*Hymenoptera*). Dr. Sun presented information about ongoing experiments to develop IVF in *Drosophila melanogaster*. Dr. Sun and his group have identified an octopamine-based method to induce ovulation (necessary for producing oocytes for fertilization) and a piezo-actuated intracytoplasmic sperm injection technique (based on that used for other species), which allows them to inject sperm heads directly into the oocytes. Although in its early stages, this method could facilitate functional long-term storage of genetic material via well-established sperm cryopreservation methods.

In discussion/clarifications, Dr. Sun pointed out that he has not yet had the injection system operating for long enough to determine the viability of embryos post IVF. The positioning of these tail-less sperm is important during the injections, and it is important to note that this IVF works even though the sperm are not motile, because only the nucleus is injected. One potential problem with a sperm cryopreservation approach is that only half of the genome is preserved (although this should be less of a problem for inbred strains); also, mitochondrial genomes are not preserved if only sperm are cryopreserved.

3.3.3 - How to Preserve Genetic Materials When It Is Difficult to Cryopreserve Their Embryos
Shinsuke Seki, Akita University, Japan

Classic cryopreservation of embryos (including the Mazur/Steponkus method for *Drosophila*) relies on cryoprotectant loading, extremely rapid cooling to favor vitrification over intracellular ice formation (IIF), and rapid rewarming to also preclude IIF. Dr. Seki and colleagues (including Peter Mazur) showed that survival of cryopreservation by mouse embryos is more sensitive to the rewarming rate than either cryoprotectant concentration or cooling rate. Using extremely fast rewarming rates can offset a 50% decrease in cryoprotectant concentration in mouse embryos, implying that optimization of embryo cryopreservation (possibly including *Drosophila*) should begin with a standardized and maximized rewarming rate. However, not all embryos are amenable to cryopreservation due to their large size. Dr. Seki presented results from a model fish (medaka). Medaka whole

testes including spermatogonial stem cells were cryopreserved and the cells dissociated from cryopreserved testicular cells were transplanted into juvenile, immune-compatible fish. The resulting recipients produced both functional sperm and eggs, based on sex of recipient. If Dr. Seki try to cryopreserve *Drosophila* strains, he indicated he would try to vitrify embryos with extreme rapid warming. If it is not possible, the germline stem cells should be cryopreserved.

In the next talk (see Section 3.3.4), Dr. Gubb pointed out that there is a protocol for transplanting *Drosophila* larval gonads (Brüschweiller & Gehring 1973 *Experentia* 29: 134-135), which suggests that this approach could be feasible. Dr. Seki thought that cryopreserving whole testes should be possible for *Drosophila.*

3.3.4 - Genetic and Physiological Approaches to Minimizing Freezing Damage: Ice-Nucleation Dynamics in Cryoprotected First Instar Larvae

David Gubb, CNRS, France

Cryopreservation protocols for embryos, while well-suited to relatively slow-dividing mammalian embryos, are a challenge for *Drosophila*, in part because of the acute temperature sensitivity during embryonic development. By contrast, early instar larvae are surprisingly robust, can potentially be handled in liquid culture, and have substantial capacity to repair damage during development. Newly-hatched first-instar larvae are approximately the same size as embryos, although cryoprotectant loading is difficult; however, it is possible to get larvae to at least feed on cryoprotectants. Some protocols yield a small amount (~11%) survival after cryopreservation in liquid nitrogen, but reproducibility is low. First-instar larvae appear to be killed by freezing at -25 to -30°C; this freezing temperature could be increased either by feeding them on a medium containing *Pseudomonas syringae* as an ice nucleator, or in transgenic flies expressing the *P. syringae* ice nucleator gene. Dr. Gubb will deposit these transgenic lines in the Bloomington Stock Center. Although these are preliminary data, there is good reason to consider newly-hatched larvae as a potential alternative target for cryopreservation.

Dr. Gubb clarified that they have not yet fully explored the influence of the freezing medium on survival, that they have not used dimethyl sulfoxide, and that ice nucleating protein is naturally-occurring in *P. syringiae*, which uses it to induce frost damage on leaves. He believes that the ice formation in the larvae is intracellular, and that dehydration could improve survival (the larvae seem to tolerate it). He suggested that exposure to N_2 gas arrests embryonic development; this could be used to facilitate staging of embryos. It was pointed out that the insect gut is highly selective and feeding is unlikely to be successful at delivering cryoprotectants to cells. James Benson suggested that it might be possible to load embryos with cryoprotectants and then cryopreserve newly-hatched (and cryoprotectant-loaded) larvae.

3.4 - Robotics and Instrument Development

3.4.1 - Overview

Even if a robust preservation protocol is developed, high-throughput can be limited by both storage capacity and handling efficiency. Alternatives to cryopreservation, such as anhydrous storage, are well-used for materials preservation, and possible for germplasm, but not yet viable for living organisms. Handling efficiency can be improved by optimizing the cryopreservation protocol to improve viability of preserved material, or by automating the handling. The latter approach can greatly increase viability as well as throughput as both the developmental stage and the treatment prior to cryopreservation must be very precisely timed.

3.4.2 - Anhydrous Preservation Methods for Genome Resource Banking

Gloria Elliott, University of North Carolina at Charlotte

In insects, as in most systems, the predominant means of germplasm storage is cryopreservation. However, anhydrous preservation, which involves water removal without low temperature storage, has shown promise in other models. Dr. Elliott presented data on their successes with feline germinal vesicles. Using permeabilization and low-power microwaves, her group has been able to initiate isothermal vitrification by removing water from dilute sugar solutions to a high viscous state without the use of low temperatures. Once they achieved a point of dehydration that balanced cell survival with storage longevity, they could store germinal vesicles at both 4°C and room temperature for up to 4 weeks. At that time point, samples were observed to have 30% degeneration and DNA breaks. They are currently using similar techniques in sperm with similar success.

Discussions involved whether similar results could be achieved for *Drosophila*. Dr. Elliott did have several examples of organisms (anhydrobionts) that regularly conduct similar drying processes naturally, including the bdelloid rotifer (*Philodina roseola)*, brine shrimp (*Artemia franciscana)*, and water bears (*Tardigrada richtursius).* Another example that was mentioned was the case of the African sleeping midge (*Polypedilum vanderplanki*) that can also undergo anhydrobiosis. However, significant differences are evident between these naturally occurring examples and *Drosophila*. Dr. Elliott concluded by suggesting that in conjunction with advances in *Drosophila* IVF, dry preservation may be a viable alternative.

3.4.3 - Similarities in Historical Development and the Approach to Improvement of Vitrification Methods for Human Infertility Treatment and the Preservation of *Drosophila* Strains

Steven F. Mullen, Cook Regentec

Dr. Mullen's presentation centered on similarities and differences of *Drosophila* cryopreservation efforts and those in other organisms. Regardless of the model, the key parameters of a successful cryopreservation method include cryoprotectant selection, how cryoprotectants enter the cells, careful selection of the sample holding device, the rate of temperature change, and how cryoprotectants are removed from cells after storage. All of these parameters require optimization while some have proven equally problematic in both insect and mammalian models. For instance, the chill sensitivity of the cells is a common

thread, which can be somewhat mitigated by adjusting the rate of temperature change. Selection of a sample holding device is also critical because, among other things, it will directly affect chilling and warming rates of the samples. While the Steponkus et al. publication in 1990 and the Mazur et al. publication in 1992 addressed some of these issues, many more remain unresolved. In short, there is ample room for further improvement. Better cryoprotectant cocktails will need to be developed to reduce toxicity and to improve permeabilization. A better understanding of chill injury could improve the overall process. Microfluidics could lead to high throughput systems, and a mechanical system could be employed to minimize error and to improve exact timing.

3.4.4 - The Power of Microfluidics to Move Cells and Solutes

Mehmet Toner, Harvard Medical School

Using microfluidics to move cells and solutes on chips should be considered a valuable tool as *Drosophila* cryopreservation technologies move forward. Benefits of microfluidics include microscale precision, multiplexing/parallelization, and the ability to automate/scale-up. For instance, microfluidics can create a precise gradient of solutes, be used to precisely treat individual cells, and can control reaction times, all of which could greatly improve several aspects of the cryopreservation process. Microfluidic processes can be used to manipulate cells, oocytes, and embryos as well. Microfluidically controlled temperature gradients have been used to control gene expression in developing *Drosophila* embryos. In non-insect systems, it has been used for cryoprotectant loading of oocytes, and to control the microenvironment experienced by a developing embryo. Individual embryos can be trapped, moved, or treated in a precise manner as dictated by the protocol. Finally, microfluidics lends itself to scaling-up by large volume processing, automation, and development of multiple parallel processes. Microfluidics could be used for the preservation of *Drosophila* germplasm similar to its use in improving human reproductive medicine.

3.4.5 - Technology and Cost Benefits of Automation of Arthropod Embryo Cryopreservation

Arun Rajamohan, USDA-ARS

A major factor contributing to the low implementation rates of insect embryonic cryopreservation protocols is their perceived complexity. While some of the complexities of the Mazur/Steponkus method were simplified in the Leopold method, insect cryopreservation protocols have still not been widely implemented. To make the protocol accessable to screwworm mass-rearing facilities, a robotic automation system was developed. This system has been successfully used by non-cryobiologists to repeatedly preserve large numbers of embryos. Additionally, embryos processed by robotics resulted in improved hatching rates after cryopreservation, likely due to the reduction in human error. These robotic systems were produced in a cost-effective manner. Using open-source microcontrollers and other off-the-shelf components, a system can be assembled for under $250. The system can be run in a cost-effective manner as well, costing about $14.70 per “run” of 1000 embryos. Due to the open-source nature of the current system, it could easily be adapted to different insect strains and species.

Other aspects of embryonic cryopreservation are less amenable to automation, such as developmental stage selection. For all insects, one must select the stage at which yolk is minimized but barriers to permeability (especially cuticle) are not well developed. In the

case of *Drosophila melanogaster*, embryonic stage 15/16 still contains a problematic amount of yolk, while stages 17/18 have too much development. Hence, embryos at stage 16/17 transition are optimal for cryopreservation. At present, this must be determined by trained laboratory technicians every time cryopreservation is conducted. This will be further complicated by the fact that strain-to-strain differences will undoubtedly alter the timing of the optimal cryopreservation stage. Another issue is that staging is rarely uniform throughout any given sample of embryos. Dr. Rajamohan feels that getting 80% of the embryos to the correct stage will result in a 40% cryopreservation success rate.

4 - Summary of Discussion Session

4.1 - Need for Preservation

Stock centers are currently at capacity, leading to regular culls of stocks. Although the current cost of maintaining a stock is low, there are objective risks at all levels, ranging from disasters destroying the building (and the stocks) to the long-term (and well-documented) accumulation of mutation. Finally, the lack of capacity means that entire resources are sometimes culled upon retirement, which means that we lose unique genotype combinations, particularly multigenic strains.

Cryopreservation is thus particularly appealing for long-term preservation of important genotypes (e.g. drivers) without mutation accumulation, or for the preservation of unique, but low-use, resources for future use. Effective cryopreservation could also lead to new research opportunities, for example, mutation accumulation.

There is a possibility that many simple mutations will not require cryopreservation, since the advent of CRISPR means that mutations stored *in silico* as sequence information can be relatively easily regenerated (at least, more easily than the current cryopreservation/recovery practices would allow). Thus, given the need for strain-by-strain optimization as described for the screwworm by Dr. Rinehart, the initial cryopreservation problem can be made more tractable by identifying a smaller number of high-value lines to prioritize for optimization.

In other cases, it appears that some of the pressure for cryopreservation is driven by a need to enhance short-to-medium-term storage, for example, to allow PIs to buffer the effort of maintaining fly stocks when shifting labs, between funding, or during personnel changes. In these situations, some sort of ‘stock life extension’ that reduces the turnover time could be effective. Already many lab keep backup lines at 18°C to reduce flipping time, but several speakers demonstrated the capacity of phenotypic plasticity in response to a variety of cues to extend lifespan, increase cold tolerance, and/or decrease development rate.

4.2 - Choice of Cryopreservation Strategy/Target

There was much enthusiasm about dehydration as a storage method. The advantages of room temperature storage are very compelling. The method described by Dr. Elliott was for germplasm, not whole cells; however, there has been substantial work on anhydrous preservation at least of blood cells. Anhydrobiosis has been studied in many systems (reviewed by Watanabe 1986 *Appl. Entomol. Zool.*), with the most promising insect model being the African sleeping chironomid, *Polypedilum vanderplankii* (Hinton 1960 *Nature* 188: 336-337; Kikawada et al. 2007 *PNAS* 104: 11585-11590). Anhydrobiotic storage of *P. vanderplankii* cells may be possible (Watanabe et al. 2016 *Cryobiology* 73: 93-98), but it should be noted that it has not been possible to get even sister species of *P. vanderplankii* to survive dehydration. Any induction of anhydrobiosis will likely require genetic manipulation of transporters, cytoskeleton, and intracellular cytoprotective proteins such as Heat Shock Protein and Late Embryogenesis Abundant proteins, since these are the elements (alongside high intracellular trehalose concentrations) that appear to be common to anhydrobiotic organisms.

Dr. Benson suggested loading embryos with cryoprotectants and then cryopreserving newly hatched larvae. It is unclear whether the embryo will develop with very high cryoprotectant loads, or whether it will metabolize the cryoprotectants.

4.3 - Potential Problems/Concerns

Researchers need to be careful about what they are preserving. Some important phenotypes have epigenetic origins, which will likely be lost with most of the proposed methods, so there may be some lines that simply need to be maintained using current methods. Similarly, preserving sperm or germinal vesicles will not preserve the mitochondrial genome.

Many participants raised concerns about the amount of effort required for any given method, ranging from moving stocks to the difficulty of (*e.g.*) implanting cryopreserved ovaries. It must be recognized that no current method looks like it will be close to the simplicity seen in methods developed for *C. elegans*. Even optimized cryopreservation methods are labor intensive during both preservation and recovery. As noted above, in some circumstances, cryopreservation will not suit specific labs or facilities.

A big problem with not flipping flies regularly (as is proposed with stock life extension) is the accumulation of mold and mites. Dr. Brankatschk stated that they have not had problems with mold in the rooftop stocks in Germany (note that those experiments also have uncontrolled low, fluctuating temperatures). Little appears to be known about the mites and molds, and the methods for controlling them (including the chemicals we use as preservatives and preventatives) have changed little in more than half a century.

Concern was raised about cycling temperatures being bad for incubators. Many fluctuating temperature regimes simply cycle vials in and out of a cold room manually (at a stock center scale, this could be achieved, for example, with conveyors). The perception that incubators cannot cycle the temperature is largely false, although it can place more strain on the compressors. Drs. Sinclair, Rinehart, and Hahn all have incubators on temperature cycles, and they have been going strong for years to decades. Sinclair's experience is that Sanyo incubators handle this better than the Percivals used in many fly labs.

4.4 - Other Thoughts

Development of standardized protocols could provide an opportunity to standardize other aspects of fly research, for example food recipes, which could improve reproducibility among labs in some experiments.

5 – Recommendations

5.1 - Determine a Minimum Set of ~500 'Essential' Drosophila melanogaster Strains, and Optimize the Mazur/Steponkus Embryo Cryopreservation Technique to Cryopreserve at Least 5000 Embryos of Each of These Strains.

Insect embryo cryopreservation is not a 'one size fits all'; often, several protocols must be developed to cover a range of genotypes. Although there are tens of thousands of *Drosophila melanogaster* strains in stock centers, the increasing utility of the CRSIPR/Cas9 system is such that soon it may be possible to 'rebuild' specific mutations from sequence information. However, the backbone of *Drosophila* genetics is a broad set of driver and reporter genes and the chromosomal inversions and markers that underlie almost all studies. Thus, these core tools are the highest priority for immediate long-term cryopreservation to guard against catastrophic loss of one or more stock centers, or of the genetic lines themselves. We suggest that the *Drosophila* community identify and prioritize these lines, and that existing methods be optimized to safeguard these core tools.

Embryonic cryopreservation is a technically challenging process, a problem which is exasperated by the fact that any large-scale *Drosophila* cryopreservation program must be readily accessible to a wide variety of end-users, many of whom are not cryobiologists. Additionally, any specific step, including selection of cryoprotectants or their equilibration protocol, can have substantial downstream implications. Therefore, sublethal and other unintended consequences of cryoprotectant selection should be properly vetted prior to wide-scale implementation.

During protocol development, we recommend that resources be devoted to investigating the sublethal effects of different cryoprotectants, including but not necessarily limited to reductions in fitness, DNA damage, and trans-generational effects. Additionally, we recommend that protocol development should address accessibility of the cryopreservation process. Key components of accessibility would be technological solutions to the key challenge of embryo staging prior to cryopreservation, robotic solutions for consistent sample processing, and investigating how microfluidics can be used during key steps such as cryoprotectant loading.

5.2 - Develop Stock Life Extension Protocols to Reduce Stock Care Requirements for Medium-Term Maintenance in Labs

Many research laboratories do not specifically require cryopreservation of strains for the decades-to-centuries timeframes for which cryopreservation is best suited, but instead need to reduce the input necessary to maintain medium-sized stock collections for months-to-years. For example, labs may maintain occasionally-used driver lines as an alternative to ordering them afresh from a stock center every year, generate a set of constructs but not have capacity to characterize them all quickly, or need to reduce maintenance effort during personnel changes, when between grants, or when shifting institutions. In these situations, increasing the interval between maintenance to >3 months would be sufficient.

Drosophila melanogaster is phenotypically plastic; temperature, diet, gut microbiota, and photoperiod all affect development time and adult longevity, and – in combination – could potentially be optimized to yield protocols that dramatically reduce the requirements for maintenance. Concurrently, we need to address the major challenges in keeping stocks for long periods (food drying, mold, mites) to ensure that the cultures can remain viable in the less-than-ideal conditions of a low-maintenance incubator.

We recommend supporting research specifically targeted at extending lifespan and increasing the maintenance interval for *Drosophila* stocks, with a goal of reducing maintenance requirements to twice per year for most strains. We further recommend supporting research that critically assesses current culturing protocols, specifically exploring methods, fungicides, diet compositions, and acaricides, that may reduce the prevalence of pests in *Drosophila* culture.

5.3 - Explore Non-Traditional Preservation Approaches

Embryo cryopreservation is well-established in other insects and has been developed for *Drosophila*. Nevertheless, there are other approaches to long-term preservation of *Drosophila* stocks and genetic resources, including (but not limited to) dehydration and cryopreservation of implantable gonad tissues (ovaries/testes), sperm, and first instar larvae. Some of these may be substantially easier in terms of storage conditions (*e.g.* anhydrobiotic tissue can be kept at room temperature), success (cryopreservation of ovaries or sperm may have higher survival) or convenience (a larval method might be more robust to variation in developmental stage).

We recommend taking a broad approach to supporting the basic and applied research necessary to evaluate and develop *Drosophila* preservation approaches other than embryo cryopreservation.

6 – Acknowledgements

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Appendix 1: Workshop Agenda

Cryopreservation of *Drosophila* Strains
Wednesday, July 13, 2016
Conference Room: Denver room at the Convention Center
Orlando World Center Marriott, 8701 World Center Drive
Orlando, FL 32821, USA, 1-888-789-3090

Purpose and Overview of the Meeting: The goal of this workshop is to evaluate the potential and practicality of using cryopreservation to maintain *Drosophila* stocks. This workshop will offer a forum for stakeholders to review the needs, current status, limitations or bottlenecks, novel techniques and future directions of cryopreservation in *Drosophila*. The workshop will be divided into three sessions with short talks from experts and participant discussion.

8:00 - 8:15 **Registration**

8:15 - 8:20 **Introduction and Welcome**
Sige Zou (ORIP, NIH)

8:20 - 9:15 **Session 1**: **Overview of Challenges to Cryopreservation in *Drosophila* and Other Insects**
Session Chair: Thomas Kaufman (Indiana University, USA)

8:20-8:40 The Challenges of *Drosophila* Cryopreservation and the Potential of Aqua-Glyceroporins
Hugo Bellen (Baylor College of Medicine, USA)
8:40-9:00 Applied Cryobiology in Managed Insect Systems
Joseph Rinehart (USDA-ARS, USA)
9:00-9:15 Q&A

9:15 – 1:00 **Session 2: Emerging and Novel Technologies Applicable to Cryopreservation in Drosophila**

9:15-10:15 **Panel 1 Genetic and Dietary Approaches for Cryopreservation**
Panel Chair: Pam Geyer (University of Iowa, USA)

9:15-9:30 Insect Freeze Tolerance: Lessons from Nature
Brent Sinclair (University of Western Ontario, Canada)
9:30-9:45 A Temperature-Dependent Shift in Dietary Preference Alters the Viable Temperature Range of Drosophila
Marko Brankatschk (Max Planck Institute, Germany)
9:45-10:00 Cryopreservation Modeling Enables Rational Protocol Design and Develops Informed Hypotheses: The Past, Present, and Future of Modeling Cryopreservation in Drosophila
James Benson (Northern Illinois University, USA)
10:00-10:15 Q&A

10:15 - 10:30 Break[1]

10:30-11:30 **Panel 2 Cryopreservation via Sperm, Embryos and Larvae**
Panel Chair: Brent Sinclair (Western University, Canada)

10:30-10:45 Developing Drosophila IVF Protocols for Preservation of Drosophila Stocks
Jianjun Sun (University of Connecticut, USA)
10:45-11:00 How to Preserve Genetic Materials when it is Difficult to Cryopreserve their Embryos
Shinsuke Seki (Akita University, Japan)
11:00-11:15 Genetic and physiological approaches to minimizing freezing damage: ice-nucleation dynamics in cryoprotected first instar larvae
David Gubb (CNRS, France)
11:15-11:30 Q&A

11:30 - 12:30 Lunch Break[1]

12:30 - 1:45 **Panel 3 Robotics and Instrument Development**
Panel Chair: Joseph Rinehart (USDA-ARS, USA)

12:30-12:45 Anhydrous Preservation Methods for Genome Resource Banking
Gloria Elliott (University of North Carolina at Charlotte, USA)
12:45-1:00 Similarities in Historical Development and the Approach to Improvement of Vitrification Methods for Human Infertility Treatment and the Preservation of Drosophila Strains
Steven F. Mullen (Cook Regentec, USA)
1:00-1:15 The power of Microfluidics to Move Cells and Solutes
Mehmet Toner (Harvard Medical School, USA)
1:15-1:30 Technology and Cost Benefits of Automation of Arthropod Embryo Cryopreservation
Arun Rajamohan (USDA-ARS, USA)
1:30-1:45 Q&A

1:45 - 2:30 **Session 3: Questions/Open Discussion/Recommendations**
Session Chairs: Mehmet Toner (Harvard Medical School, USA) and Toshiyuki Takano-Shimizu (Kyoto Stock Center, Japan)

1:45-1:50 Summary of Cryopreservation Technologies
Mehmet Toner and Toshiyuki Takano-Shimizu
1:50-2:30 Open Discussion and Recommendations

[1]Lunch, and refreshments for breaks will not be provided by Federal Government funds. Each attendee is responsible for covering them at their own expense.

Scientific Advisory Board: Pamela Geyer (University of Iowa), Thom Kaufman (Indiana University), Joseph Rinehart (USDA), Brent Sinclair

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NIH Organizing Committee: Michael Bender (NIGMS), Miguel Contreras (ORIP), Desiree von Kollmar (ORIP), Daniel Miller (NINDS), Sige Zou (ORIP).

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Appendix 2: List of Workshop Participants

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