SMITHSONIAN Zoogoer

Frozen Assets

Frozen sperm, eggs, and other tissues are truly cool tools for scientists trying to preserve biodiversity—at the National Zoo and in the wild.

by Caroline Treadway

Mary Crauderueff literally has one of the coolest jobs in the world. She's an archivist. But Crauderueff isn't sorting papers. Fresh out of school with a master's degree in library science, she's just landed a dream job as the archivist for the Smithsonian's collection of frozen biological samples.

Many departments at the Smithsonian store huge amounts of frozen material, from milk and soil to mosquitoes and blood. It all needs to be organized. At the National Zoo's reproductive science laboratories, rows of squat, metal freezers guard the secrets to survival for some of the world's most endangered species. These tanks store sperm, eggs, and reproductive tissue in liquid nitrogen at nearly minus 200° Celsius. Such cold temperatures can suspend life within a cell for 20 to 30 years, or even longer.

Frozen material, though, is useful only if you know what you have. "If you want to make dinner, and you have unlabeled stuff in your freezer, you don't really know what you have," Crauderueff explained. Her job, she said, is "like cleaning out the freezer, starting fresh and saying, 'I know the peas are labeled and they're good, so I'm going to keep them.' Organization is really key in terms of coming back to it in 50 years and saying, 'OK, we know exactly what's in each vial and each container, so we can use it.'"

The Smithsonian is not quite at that point yet. "Strangely enough we don't really know exactly how many frozen specimens we have within the whole Smithsonian Institution," said Pierre Comizzoli, a National Zoo scientist who specializes in cryopreservation, or freezing, of eggs, mostly from mammals. Collected over the past 30 years, the Zoo's bank of frozen material is one of its most valuable assets. Scientists use the material for artificial insemination, in vitro fertilization, and basic research on wild, endangered animals. But the frozen samples will be much more useful once they've been counted and entered into a database.

Hard Lessons

In 1978, Louise Brown, the first human baby conceived via in vitro fertilization, was born. National Zoo scientists weren't far behind in exploring new reproductive techniques. Eager to apply these technological breakthroughs to endangered animals, David Wildt and his then student JoGayle Howard plunged headfirst into

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Zoo scientist Pierre Comizzoli dissects ovaries from a black-footed ferret and treats them with cryoprotectant before freezing them in liquid nitrogen. *(Mehgan Murphy/NZP)*

the uncharted territory of wild animal reproductive science. (Wildt later headed the Zoo's Center for Species Survival and Department of Reproductive Sciences. Howard became a theriogenologist, or scientist who studies animal reproduction, for the Zoo.)

"We were a bit ahead of our time, and a bit naïve because we thought the practical application of these techniques would be a lot easier than it actually was," Wildt said.

Armed with data from human fertility clinics and the commercial cattle industry, Howard and Wildt engineered artificial insemination techniques for cheetahs using frozen sperm. But when their attempts failed, the two scientists realized that cheetahs, and their reproductive systems, were nothing like cows or humans.

"We thought, 'Oh, we'll just use these high-tech techniques and produce all these endangered species,'" said Howard. "What we learned, the hard way, is we didn't know the basic biology of these species."

Initial failures forced the scientists to start from scratch with each species they wanted to breed, learning the reproductive biology and developing tailored breeding protocols. "There's this conventional wisdom that all mammals reproduce in the same general way, and that couldn't be further from the truth," said Wildt. "The mechanisms for reproduction are as diverse as the species themselves."

Female giant pandas, for example, come into heat only once a year, for 24 to 72 hours. Bats can store sperm for weeks, even months. Nonmammals vary widely too. Some sharks can store sperm for two years. Other animals, such as whiptail lizards, don't even need a male to reproduce.

Once Howard and Wildt understood a species' basic reproductive biology, they honed the techniques for freezing sperm. Each species' sperm has a widely different cold sensitivity. For example, panda sperm freezes well, but cheetah sperm doesn't.

"Each freezing protocol needs to be adapted for a specific species," said cryopreservation expert Comizzoli. "You can't freeze the sperm of a wild pig like you freeze the sperm of an elephant or freeze the sperm of a cat."

According to Comizzoli, some sperm die in the freezing process, so it's important to start with a healthy sample. That's not as easy as it sounds. Many wild and endangered animals don't have high numbers of healthy sperm to begin with due to their reduced genetic diversity.

Zoo scientist JoGayle Howard (seated) and intern Natalka Melnycky assess the quality of black-footed ferret sperm. They view the sample on a screen hooked up to a microscope.(*Mehgan Murphy/NZP*)

High-Tech Triumph

Years of research eventually paid off for Wildt and Howard. Using artificial insemination techniques and frozen sperm, they've helped cheetahs, giant pandas, and black-footed ferrets reproduce.

Black-footed ferrets *(Mustela nigripes)* were a dramatic success story. They were considered extinct until 1981, when scientists found a small pocket of survivors in Wyoming. By 1985, there were only 18 left.

In cooperation with U.S. Fish and Wildlife Service, Howard used blackfooted ferret semen, frozen for more than ten years, to help save the species, which once populated the Great Plains. Through reintroduction programs, approximately 800 black-footed ferrets now live wild in the western grasslands of the United States and Canada.

Given the small starting population, inbreeding (which can cause disease, infertility, and high infant mortality rates) was a major concern among scientists. "If you start with 18, inbreeding will happen over time," said Howard. "You just want to make it as gradual as possible."

To minimize inbreeding, the Zoo's Jon Ballou, a population biologist and researchscientist, engineered a "computer dating" program. The software uses algorithms to pair mates as distantly related as possible. Ballou's computer program is now used by Species Survival Plan programs and zoos worldwide to ensure genetic diversity in breeding programs.

"The only way we could increase gene diversity was to either find a brand-new animal from the wild that's unrelated— and they've looked for over 20 years and there are no new wild ferrets—or use frozen semen," Howard said.

"With frozen semen, you're capturing the gene diversity of that animal in time. It's frozen with that sample," Howard said. "What if a whole species disappears, like the scimitar-horned oryx, which has gone extinct [in the wild] in my career here? Well, we have semen frozen from that animal 20 years ago." That frozen semen can help zoos boost genetic diversity among the captive oryx population.

Equal Time for Eggs

Reproductive techniques with frozen semen solve another big problem for Zoo scientists: moving animals. Moving is stressful, for both animals and caretakers. Once they arrive at their new home, animals have to be quarantined to ensure they don't bring in disease, introduced to a new environment, and paired with a new mate, whom they might not even like. It doesn't take a theriogenologist to figure out it's much easier to ship frozen elephant sperm than to ship an elephant!

The same goes for wild animals. Artificial insemination technology using frozen sperm allows scientists to collect samples from the wild and bring them back to reproductive labs in the U.S. without moving a single animal. Scientists can use this frozen wild sperm to introduce new genes in zoo populations, strengthening the gene pool. At the same time, frozen semen from zoo animals can bolster genetic diversity in wild populations by bringing in fresh genes. National Zoo experts like Wildt and Howard view zoo animals as an insurance policy against habitat destruction and inbreeding, which can devastate wild populations.

But sperm is only half of the genetic equation. To have full control over genetic diversity, Zoo scientists realized they needed to learn how to freeze eggs too. “Everyone was saying, ‘What about the female? You’re not banking the gamete of the female,’” Howard said. “Even in human medicine, freezing eggs is hard. That’s when Pierre [Comizzoli] came on board.”

Comizzoli, who specializes in freezing eggs and embryos, joined the National Zoo’s team of reproductive scientists eight years ago. According to Comizzoli, cryopreserving both male and female genomes is critical for ensuring genetic diversity in wild animal populations, now and in the future.

Problem is, it’s much harder to freeze eggs than sperm. Eggs are larger, contain more water and fat, and have many more delicate cellular structures that don’t freeze well. There are also far fewer of them. A single semen sample contains millions of sperm, so even if only 50 percent survive the freezing process, millions of sperm are still able to fertilize an egg. But scientists can only extract a few eggs from females at a time. Each is precious and very fragile.

Comizzoli has also recently found that freezing whole slices of ovarian tissue can yield many more eggs, in various stages of development. They can be used to study optimal freezing and thawing procedures.

Ovarian tissue contains many early-stage eggs that can be cultured and matured in the lab for in vitro fertilization. This new technology will eventually allow scientists to create genetically desirable, healthy embryos from a variety of rare species.

Cryobiology helped Zoo scientists breed black-footed ferrets, once thought to be extinct. (*Mehgan Murphy/NZP*)

Human-Animal Links

“We are facing the same problems as in human reproductive medicine,” said Comizzoli. He recently joined forces with scientist Teresa Woodruff’s Oncofertility Consortium, which is funded by a grant from the National Institutes of Health. Consortium scientists work to discover the best techniques for freezing ovarian tissue in both humans and animals. Doing so can preserve the fertility of young cancer patients who want to have children in the future. Cancer treatments can irreversibly harm a woman's ovaries, compromising her fertility. Before treatment, a patient can opt to have part of her ovaries removed and frozen.

When a female patient recovers, the cryopreserved ovarian tissue can be thawed and grafted back into her reproductive tract. It can also be mixed with sperm in vitro to create an embryo, which can be transferred to the mother. According to Comizzoli, more than 30 women have given birth to healthy babies this way.

"That's really exciting," Comizzoli said, "because the animal models we use are obviously different from humans, but what we are learning can potentially be applied to improve techniques in humans and inversely in rare animal species. We are the National Zoo, and obviously we are interested in wildlife conservation, biodiversity, but I think it's really important to keep a link with what's going on in human medicine. If we can contribute, it's even better. That's kind of the cutting edge we are talking about."

Big Job

In a perfect world, the Smithsonian's vast bank of cryopreserved material would be completely organized and accessible so scientists could use frozen samples for daily research. Each sample would be labeled with specific information: what the sample is, who collected it, when it was collected, and how it was frozen. All this information would be stored in a computer database that scientists could access to improve their techniques, share knowledge, and keep up with colleagues' research. But every good archive needs a good archivist, and that's where Crauderueff comes in.

"If you have researchers who go into the field, do an awesome study, and leave their files in a box, unmarked, then there's no record of what's in there and it's completely useless," said Crauderueff, who's excited to bring the various arms of the Smithsonian together in a new way.

"Normally an archivist works with papers and maybe photographs or reel-toreel film. I like feeling like I'm part of something bigger," she continued. "It's all toward the preservation of species, investigating and learning. And in the long term making sure there's still biodiversity in 50 years."

—Smithsonian Zoogoer *intern* CAROLINE TREADWAY *is a graduate journalism student at Boston University.*

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