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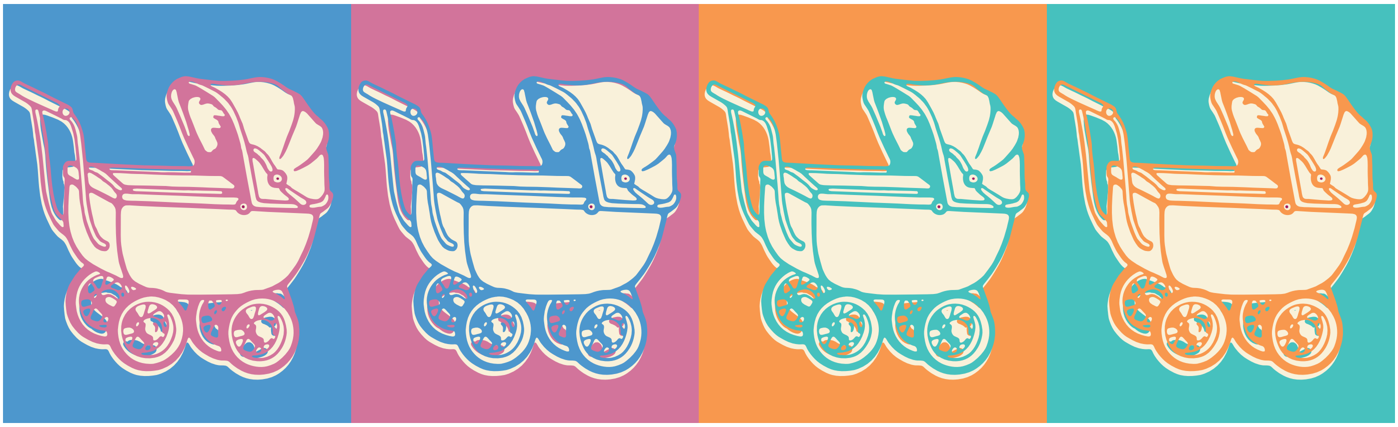

Welcome to the nursery

As they pursue their puzzles of interest, scientists are inventive caretakers of the organisms in their keep, especially the young.

By Vivien Marx

University of Oregon zebrafish research pioneer George Streisinger put marbles at the bottom of fish tanks to keep zebrafish parents from eating their own freshly laid eggs, as they often do, says Washington University School of Medicine developmental biologist Lilianna Solnica-Krezel. The eggs settled into crevices between the marbles and thus avoided becoming a meal.

Researchers care about the welfare of organisms in their keep and are especially attentive to the young, such as seedlings, larvae, pupae, embryos, hatchlings or pups. As they focus on babies and parenting, scientists must be observant and inventive. Solnica-Krezel and her colleagues at Washington University School of Medicine maintain one of the world's largest zebrafish facilities, with 9,000 zebrafish tanks, 1,000 of which make up the nursery. She embraces ideas from the work of Nobel laureates Christiane Nüsslein-Volhard and Eric Wieschaus, who used forward-genetic screens in fruit flies and explored the mutations that showed up to learn about gene function. The approach is, says Solnica-Krezel, "the concept of asking organisms to tell us what's important."

Mindful observations of ants and mice

In many species, parents are watchful caretakers of their young. As scientists watch the watchers, they sometimes see what others miss, as in the following example about collective parenting in ants.

Ant eggs hatch into larvae. Next is metamorphosis in a pupal stage from which an adult emerges. As Rockefeller University scientists and their colleagues point out, pupae were once thought to be "passive members of the colony"[1]. Not true, as observation, measurement and analysis by the team revealed[1,2]. Postdoctoral fellow Orli Snir, in the lab of Rockefeller University researcher Daniel Kronauer, found that a "social fluid" secreted by pupae in the clonal raider ant *Ooceraea biroi* elicits parental care behavior. Adults draw benefit from imbibing this moulting fluid—it's nutritious, and it contains hormones and growth factors that might be shaping development. A metabolite inventory also yielded neuroactive substances that could be regulating behavior, says Kronauer.

Adults not only consume the fluid but also share it with larvae by placing newly hatched larvae on pupae secreting the fluid. It's a balance: if too much of the fluid accumulates, fungal infection can take hold and pupae perish. Snir came to the lab after working on fruit fly epigenetics, says Kronauer, and was more open to noticing things compared to someone "who has watched ants for years and knows the literature in and out," and who might have missed an observation or deemed it trivial. Key, in his view, "was careful behavioral observation combined with a fresh pair of eyes and an open mind."

Developing a research gaze that catches subtleties takes training. Working with mice, staff are trained to see anything out of the ordinary in pups and adults, says Michael Campagna, who directs veterinary services for The Jackson Laboratory (JAX) Mice, Clinical and Research Services (JMCRS), which handles JAX's external activities with the academic and biopharma communities. After the 21 days of gestation, mouse neonates "all start out pretty much pink," he says, with birth followed by another phase of dependency on the mother. Until around day 12, he says of pup life, "it's just nursing and hanging out." Weaning is usually complete in around 3 weeks.

Mouse strains can have different temperaments, says Andrew Schile, a scientific advisor to JMCRS who was interviewed jointly with Campagna. Caretakers get to know the animals and to distinguish normal appearance and behavior and deviations from that. "What is normal for one strain of laboratory mouse might be completely abnormal for another one," says Schile. Some strains are normally heavier than others. A strain might be typically docile, so hyperactivity or anxiety would merit a closer look, he says.

Pupae secrete a nourishing fluid that elicits parental care behavior in the clonal raider ant *Ooceraea biroi*. Ants place larvae on pupae, sharing the social fluid with them.

Protocols for plants and zebrafish

"For successful experiments, all need to pay attention to the precise experimental design and every detail of the proper growth environment, from watering, light condition, temperature, nutrients and relative humidity to photoperiod and plant clock," says Jen Sheen, a plant biologist at Massachusetts General Hospital. One has to control pathogens and insects based on experimental plans, keep accurate records and be observant.

Like a healthy fertilized egg, says Sheen, a healthy seed able to germinate is one that the mother plant has packed with all the nutrients an embryo needs to grow into a healthy seedling. Until the stored nutrients are used up during a seedling's growth and development, its only external needs are water, warm temperature, light and oxygen for converting nutrients into energy.

In the lab, seed desiccation involves drying to a 10% water content. Good storage conditions include cool temperatures and low humidity, around 20%. Storage at −20 degrees maintains longevity, so that seeds can germinate decades later. Records indicate, she says, that lotus seeds have germinated after 1,000 years. With only light and without exogenous sugar, nitrogen and most minerals, *Arabidopsis thaliana* seeds can yield embryos and healthy seedlings in 3 to 4 days. A lack of light leads to elongated stems developing from the seed; the plants are etiolated, there is no leaf expansion, and photosynthesis and root system development are hindered. Corn has large, nutrient-packed seeds that supply an embryo sufficiently to develop into a healthy seedling in light or darkness in about 2 to 3 weeks. But they need water, warm temperature and oxygen.

When experiments involve longer seedling development periods, agar or soil provide exogenous nutrients and physically anchor the root system, says Sheen. Dried seeds are resistant to many environmental stressors, but once germination starts, developing seedlings are vulnerable to factors including cold and hot temperatures, too much or too little water, wind, high salt, pathogens and pollutants.

Edward Rybicki, from the University of Cape Town, and his team work on ways to make protein-based vaccines using plants or plant cells. They grow *Nicotiana benthamiana*, a small Australian desert plant that is well suited to expressing 'foreign' proteins. To pursue this goal, says Rybicki, requires optimizing conditions. He says that his team wants young plants to grow from seed to seedling to mature plant "as quickly as possible, because that's when they're useful."

With zebrafish, which have been a model organism for decades, established protocols are in place, says Krasimir Slanchev, staff scientist at the Max Planck Institute for Biological Intelligence in Martinsried, Germany. He's in the lab of Herwig Baier, who calls Slanchev the lab's "zebrafish rearing expert." The lab has 4,000 zebrafish tanks. Zebrafish are Slanchev's favorite research organism: they are easy to work with, they have high fecundity and the choice of tools for genetic screens, sensors and probes is large. Zebrafish develop outside the mother's body and feed on the yolk up until day 7. They are juveniles for around 2 to 3 months and are then sexually mature.

Both larvae and embryos are transparent. When held in warm agarose, they can be easily imaged, for example to chronicle cartilage or brain development. The zebrafish nervous system can be stimulated and responses recorded as the animals swim about or evade predators. Scientists can also explore basic nervous system functions with gene-editing tools. The Baier lab is part of a large project to build a connectomic zebrafish brain atlas involving stacks of electron micrographs of brain tissue.

PhD student Ashley Parker studies cichlids in the Baier lab at the Max Planck Institute for Biological Intelligence. In the lab, a tumbler (left) simulates the swirling that eggs and hatchlings would experience in the mother's mouth. Parker 3D-prints shells (middle and right) to observe cichlids that lay eggs in abandoned snail shells.

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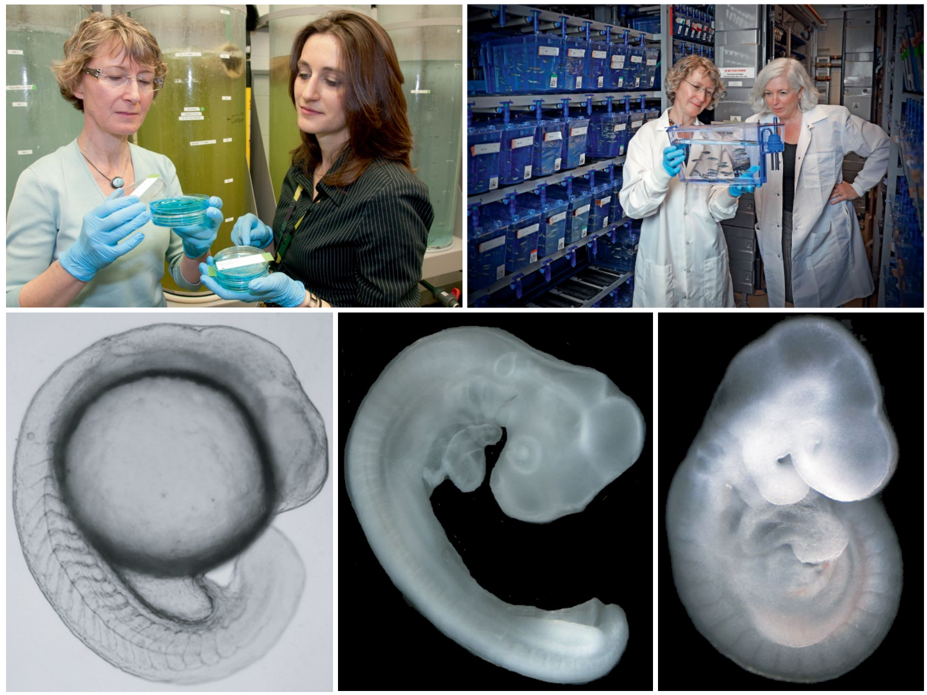

The Washington University School of Medicine has one of the world's largest zebrafish facilities, with 9,000 tanks, 1,000 of which hold the nursery. Top row: developmental biologist Lilianna Solnica-Krezel studies the very early stages of development—gastrulation. Top left, Solnica-Krezel (at left) with colleague Isa Roszko in front of funnels in which rotifers, which are live zebrafish feed, grow. Top right, Solnica-Krezel with colleague Helen McNeill. Bottom row: a cross-species embryo comparison.

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With protocols for maintaining young zebrafish, it's about getting the balance just right, says Slanchev. "The biofilter needs to be okay, the feeding scheme needs to be okay." Beyond the standard protocols, "we have some tricks that we use to increase the survival rate of the babies and also their performance: we feed with live food." These nutritious meals are rotifers that the lab raises on a microalgae. The rotifers are live prey that the zebrafish larvae practice their predatory ways on. Then, they transition to a diet of small crustaceans that are rich in protein and minerals, which boosts growth.

Equipped to breed fish and mice

Practice-based design shapes breeding approaches. Over the years, Washington University School of Medicine scientists have developed creative technology and techniques to support zebrafish breeding. Solnica-Krezel says that 25 robots handle the feeding. In terms of automation, "we were definitely a pioneer," she says. When she started in the field, only a handful of zebrafish labs were active globally. The number has grown to an estimated 1,500 labs that, for example, exchange data through resources such as the Zebrafish Information Network.

As a graduate student, gastrulation fascinated her and she loved genetics. Zebrafish, she realized, were a model for doing both. From the moment of fertilization, zebrafish are accessible to observation and experimentation. Compare human, mouse and zebrafish at the gastrula stage[3] and, she says, "you see how similar they are." Zebrafish embryos develop fast: within 24 hours after fertilization, a zygote develops into an embryo with a typical vertebrate body plan; it has rudiments of organs and a beating heart. "By comparison, mouse and human zygotes will divide once in this time," she says.

When fish arrive at the facility from outside Washington University, they live in quarantine tanks in a separate area. "Only their babies can go downstairs to the fish facility," Solnica-Krezel says, and only after being disinfected to avoid transmitting infection of any kind. In the main facility, eggs are collected in round petri dishes, whereas the quarantine facility uses rectangular ones to minimize the risk of mix-ups. Just as in mouse facilities, she says, quarantine facilities "protect your animals and protect your research." Among the threats are nematodes and fish tuberculosis, caused by a different bacterium than in people, but "it can be also very devastating for fish." For breeding, a tank with two compartments is used. At night, a male and a female are placed separately in each compartment. In the morning, a separator between the compartments is removed and mating takes place. To scale up egg capture from Streisinger's trick with marbles at the tank bottom, zebrafish labs tried out submerged small mouse cages in a tank and replaced the cage bottom with a wire mesh, says Solnica-Krezel. At Washington University, researchers collaborated and tested different versions of tanks made by Tecniplast, which led to the polycarbonate tank currently in use. Eggs drop through a mesh to the tank's second bottom.

With mice, although sometimes one male and one female animal are brought together, says Schile, it's more frequent to set up a trio of two females and one male. Pups born to trio-mated parents tend to weigh a bit more than pups from pair-mated parents. "That could be indicating the benefits of having two females present who can assist each other in the raising of the young and the litters," he says. Caretakers at The Jackson Laboratory all wear personal protective equipment and gloves and are not allowed to wear perfume or aftershave because that irritates the mice. Staff are attentive to sex differences related to development and behavior.

Enriched environments for mice and fish

Mice interact with objects, play, hide and need material to build a nest, says Campagna. Some strains like objects such as cardboard tunnels, he says. They chew up paper to make a nest or use cotton squares called nestlets. Campagna advises the use of the *Guide for the Care and Use of Laboratory Animals*. As the authors note, this edition "strongly affirms the principle that all who care for, use, or produce animals for research, testing, or teaching must assume responsibility for their well-being." *The Principles of Humane Experimental Techniques*, published in 1959, was commissioned by Major Charles Hume, who founded the Universities Federation for Animal Welfare. It was authored by zoologist, psychologist and classical scholar William Russell and microbiologist Rex Burch. The book introduced

Pups usually nurse for 3 weeks. These pups, from mouse strain 004200, are between 3 and 5 weeks old.

ethics principles in experimentation—the 3Rs of replacement, reduction and refinement, which have become widely established and in many countries are the basis for regulations.

Replacement refers to avoiding or replacing animal use, for example with computational tools, when feasible. Reduction is about minimizing the number of animals used. Refinement relates to minimizing factors such as pain, suffering or lasting harm. Some researchers add rehabilitation to encompass caretaking of animals after they leave the lab.

Beyond regulations, ethical practices and established protocols, and with organisms that are less frequently studied, scientists develop ways to help their organisms thrive in their keep. Young organisms receive special consideration. Researchers also aim to respect and honor the roles of indigenous peoples and the locality of organisms and avoid helicopter research—which is when, for example, scientists from the Global North collect samples or do field research in a country of the Global South without involvement or regard for local populations and collaborators. It's a practice that irks her, says Ashley Parker, a PhD student in the Baier lab. She is from Cape Town, South Africa, and studies cichlids.

Cichlids are native to lakes in Africa such as Lake Tanganyika, which stretches between the Democratic Republic of the Congo, Burundi, Tanzania and Zambia. When the lab's collaborator heads to the lake to get fish, he collaborates closely with locals. "This is also something that's very close to my heart, because I also have an issue with the European people going to Africa, using their animals," she says, to benefit only their own research careers. Africa's abundance of resources and biodiversity is sometimes used at the expense of local populations.

Parker and Slanchev, who were interviewed jointly, have friendly battles about their research organisms. "We both think we work on better species," says Parker. For brain studies, zebrafish are superior, she says, but to explore social structure and behavior, she and a few other lab members choose cichlids. The animals are territorial: they know and remember which fish ranks higher. "A fish needs to know its standing," she says, and recall past fights to decide if a new conflict is worth it.

Existing knowledge about cichlids mainly stems from ethologists' observations in the 1970s and 1980s. Parker spent the first part of her PhD designing tanks for breeding cichlids, developing methods such that their life in the lab mimics the natural world as closely as possible. The many cichlid species have numerous breeding strategies. Some lay eggs on surfaces or in pits, others carry their young in their mouth and still others use abandoned snail shells. It's unclear how these small fish manage this, says Parker, but some cichlids take snail shells much larger than their 4-centimeter length and drill the shells into the sand. Only the shell's entrance peeks out, and the female lays eggs in this enclosure. Other species mark territory with mountains of shells.

To learn about what goes on inside in the dark of these shells, she made 3D-printed shells. "I created these shells that basically are cut open in the back," she says. Using infrared cameras, she tracks the mother's activity after laying eggs. "They're going in and out of the shell constantly," she says. To bring oxygen to the hatchlings, the mother fans with her fins around the clock. She sleeps with her head poking into the shell, wakes up, fans vigorously and then "it's this very obvious flop and she goes back to sleep."

In mouth-breeding cichlids, which are better studied than shell dwellers, the mother holds the eggs, and then the hatchlings, in her mouth. She swirls them, likely for buccal cleaning. In this phase, she does not eat. Says Parker, "They starve themselves for 3 weeks carrying these babies and lose so much weight." To mimic this mouth swirling in the lab, the researchers built a tumbler. Gentle water flow moves the embryos. Without it, they will not develop normally. To mimic the mother's movements in the shell, Parker uses a syringe pump attached to a small plastic tube that leads into the 3D-printed shell.

Cichlid species diversity is an "evolutionary paradise," she says, and the species radiation makes these animals a powerful model to work with. For example, one can study them based on behavioral differences.

Attention to cephalopod welfare

"Cephalopods are fantastically strange," says Caroline Albertin, Hibbitt Early Career Fellow at the Marine Biological Laboratory in Woods Hole, Massachusetts. As an embryologist, she focuses on understanding cephalopod embryos and their development. Cephalopods are an understudied taxon with complex central nervous systems, flexible sucker-lined arms, a muscular mantle with three hearts, two gills and an ink sac. "They have so many evolutionary novelties," she says.

Albertin met her first octopus as a graduate student in Cliff Ragsdale's lab at the University of Chicago. A single octopus embryo floated in a basket, which is what they call their nursery in this 250-gallon sea tank. The embryo began move around in its transparent chorion, a soft kind of egg shell. "All of a sudden, it hatched, changed color, inked and swam away," she says. She knew that her career would be about understanding how this remarkable animal is set up during development. Along with Roger Hanlon and other scientists at the MBL, she explores how this organism can help with understanding the unique biology of cephalopods. This work includes sequencing genomes, testing gene function, exploring the extensive RNA editing that goes on in these animals and maintaining them in the lab.

Several high-quality genomes for squid and octopuses exist, "with more on the way, and we've been able to employ CRISPR to knock out gene function in squid," says Albertin. They carefully chose a species to bring into the lab to breed it and be sure that embryos will do well. Aquarists help them develop cephalopod husbandry approaches. The team settled on *Euprymna berryi*, the Japanese hummingbird bobtail squid, originally obtained from Daniel Rokhsar's lab at the Okinawa Institute of Science and Technology. The MBL team is now developing resources for this species, she says.

Cephalopods are very sensitive to water quality, which makes that a primary concern when keeping them in the lab. Regular water changes are a must, as is daily cleaning of

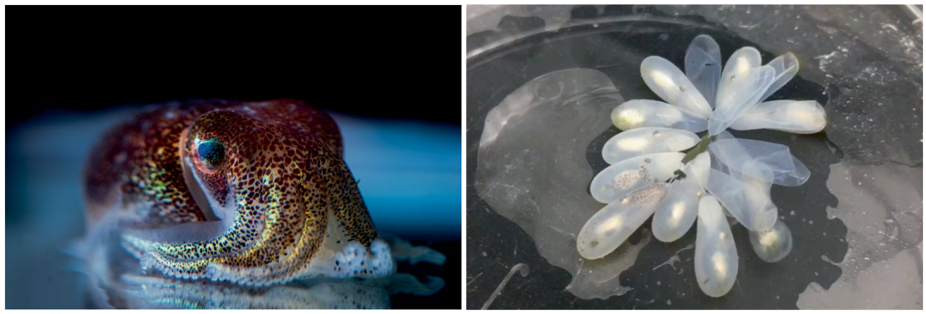
When mouse pups are born, they rely on their mother.

CREDITS: TOP LEFT THE JACKSON LABORATORY; LOWER RIGHT: CALLISTA IMAGES / GETTY embryos once mothers deposit their babies onto substrates. There's a large yolk, which the embryos consume while surrounded by a protective chorion. Cephalopods edit their own RNA at much higher rates than other animals, an aspect Albertin and others at MBL study[4]. There is much to learn from these animals, she says: "We're at an exciting moment where many of these questions can now be studied at a functional level."

For the past ten years, says Fiorito Graziano, who directs the Stazione Zoologica Anton Dohrn in Naples, research use of cephalopods has been governed by strict rules, and they are the sole invertebrates for which this is true. Working with cephalopods, he says, "it's exactly the same like working with primates." This approach is based on precautionary principles about preventing pain, suffering, distress and harm. Of late, he says, discussions and regulations have emerged, for example in the UK, about sentience in cephalopods and crustaceans, which implies these animals may have the neural basis for consciousness.

As Graziano and colleagues explore what makes cephalopods "happy," he says, drawing quotation marks in the air, he and his colleagues place welfare, of both young and adult animals, front and center in their work[5]. Cephalopod gastrulation is entirely unlike human gastrulation, which means "they are totally different from us," says Graziano. Clearly, their brain is complex, and recent work on the chemotactile senses has revealed new facets to learn from these animals[6,7].

Thinking about cephalopod welfare is not new, says Graziano. In the early twentieth century, German zoologist Georg Grimpe developed an overview of the care and use of cephalopods in research and, for example, developed elaborate systems for moving live octopus from Naples to labs through Europe by train. He published best practices and guidelines on cephalopod care in 1928, a document that Graziano and his colleague Giovanna Ponte translated in its entirety for a paper[8].



The Marine Biological Laboratory is developing resources to work with the Japanese hummingbird bobtail squid (left). Right, *Octopus bimaculoides* embryos and hatchlings.

Keeping cephalopods happy is ultimately about mimicking in the lab the best conditions the animal experiences in the wild, says Graziano. This pertains to water quality, food and behavioral needs. The animals are predators and they like to explore objects, which is why enrichment of their environment matters. At the same time, he says, "if you play with the animal, you don't know if playing," such as placing objects in the tank that the animal interacts with, "will interfere with the final outcome of the purpose of this study."

As they explore the behavioral and cognitive abilities of these animals, cephalopod researchers need a keen awareness. "It's always a balance," he says, of maintaining the five freedoms: freedom from hunger and thirst, freedom from discomfort, freedom from pain, injury or disease, freedom to express normal behavior and freedom from fear and distress. A cephalopod might weigh 500 grams and have a 10-centimeter mantle, but their eight arms extend 30 centimeters in any direction, which means a tank cannot be confining, especially because these animals need to hunt. The team in Naples and researchers across Europe have compiled "*mandata minima*" for the European Commission as requirements when using cephalopods in research. Graziano hopes such approaches will also be used elsewhere. To his knowledge, at the US National Institutes of Health, work is now underway to revise the guidelines related to research with cephalopods.

Locality matters when taking care of organisms, says Graziano. The sea is around 150 meters away from where he sits, he says, as he looks out the window. But many researchers eager to study cephalopods are not this close. He and his team share knowledge and best practices with labs wherever they may be. He discourages both helicopter research and using animals without respect for their native environment. The scientists embrace the concept that when learning about animals and using them for research purposes, one needs to optimize their living conditions, says Graziano, because "the animal is always right."

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