Considering the cephalopod

Octopuses, squids, and cuttlefishes all have potential as lab animals. As models emerge, what goes in to keeping them happy and healthy in the lab?

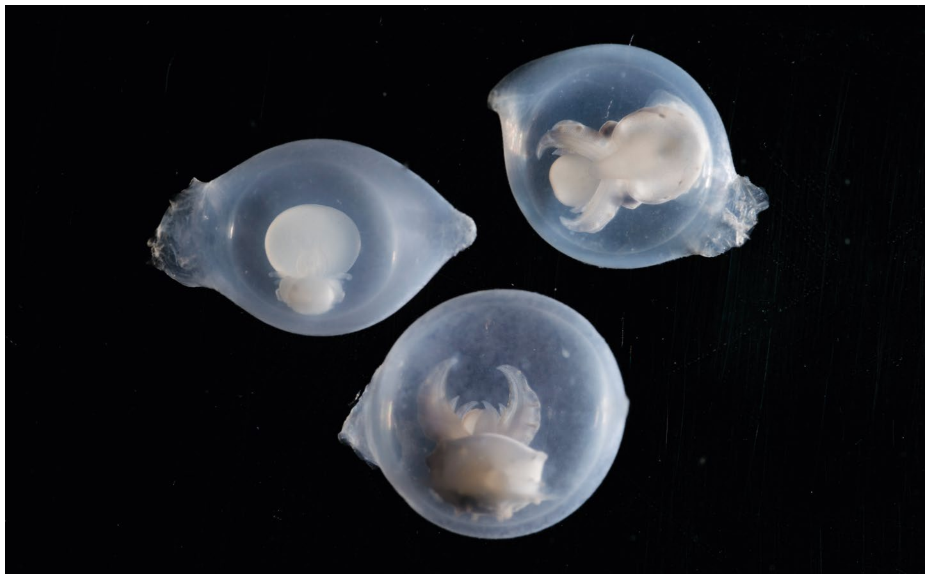
Ellen P. Neff

From the chilly depths of Great Harbor near Woods Hole, Massachusetts, an intake pipe draws sea water, ferries it under Water Street to the Marine Biological Laboratory's (MBL) campus and on to the second floor of the Marine Resource Center building.

Once there, it passes through a gauntlet of pumps and filters and into rows of water tables and variously sized aquarium tanks. The water needs to be cleaned and frequently re-cleaned to keep the sensitive animals that inhabit it happy and healthy. Some will have come from the wild, as far away as Hawaii or Australia while others, if all is going well, will have been bred and born in the lab.

"How we've been successful is just extreme attention to detail," explains Bret Grasse, "We're very careful about everything we do."

Grasse is the Manager of Cephalopod Operations at the MBL. His charges are a fascinating group of invertebrates: striped pajama squid and bobtails, flamboyant and stumpy cuttlefish, the zebra octopus and its two-spot cousin. The goal is to help figure out how to make such cephalopods better lab animals.



Say hello to some emerging models, like these Flamboyant cuttlefish, *Metasepia pfefferi*, in their eggs. Credit: Marine Biological Laboratory/T. Kleindinst

Intriguing invertebrates

How many ways can evolution make a complex brain? "All mammalian brains are really similar, and so we study comparative models like rats and mice to understand the human brain, but it doesn't get at this bigger question: what are the fundamental principles of complex brains?" asks Robyn Crook, a researcher at San Francisco State University.

Wunderpus photogenicus, wonderful & photogenic. Credit: R. Crook

"We need to have something that really has a separate evolutionary history and is kind of fundamentally different," she says. "Cephalopods are perfect for that."

Cephalopods are a unique group, characterized by considerable cephalization—the word 'cephalopod' literally means 'head-foot'. There are about 800 living species that have been evolving for over 500 million years, and the animals fall into one of four main groups: octopuses, squids, cuttlefish, and nautiluses (of which there are just a small number of living representatives). Class Cephalopoda sits in the Phylum Mollusca, but unlike most of their hard-shelled relatives, the cephalopods are, for the most part, soft-bodied invertebrates; only the nautilus species retain an external shell, though cuttlefish find a little structure from the chitinous "pen" found in their mantles. The smallest known cephalopods weigh less than a gram and stretch only a centimeter while the largest, like the aptly named giant squid, can be over 30 feet long and weigh several hundred pounds.

The animals are no strangers to research. Physiologists Alan Hodgkin and Andrew Huxley received the 1963 Nobel Prize in Physiology or Medicine thanks to the long-finned squid and its giant axons, which helped the duo establish the basics of ion channels and how the nervous system conducts electrical currents.

Different cephalopods continued to be studied by researchers who want to better understand their unique abilities[1]. Despite how different they are from mammals and other vertebrates, cephalopods have evolved complex neuoranatomies, sensory systems, and behaviors. They have more alien attributes too, like the ability to regenerate tissue, change the shape and patterns of their skin, and even edit their own RNA.

Roger Hanlon, a scientist at the MBL who has worked with a number of cephalopods over the course of his career, sees in them the potential to complement the more traditional lab animals like rodents, nonhuman primates, and other small invertebrates. "I think it fits in there because it's a different brain, it's a different model," he says. "It's the only example we

The brilliant bobtail *Euprymna scolopes*.
Credit: R. Crook

have in evolution and phylogeny where another animal group has not gone the vertebrate route and has still produced a very large, complex brain and what looks like equally complex behavior." They could make intriguing biomedical models, basic biological models, or even bioengineering models, he suggests.

With so many species to choose from, the field could benefit from converging on a few "models". But what's a researcher to choose? "They all want the perfect model," says Hanlon. The ideal is a small cephalopod with a short life cycle that will reliably produce large and transparent eggs and larvae and that displays complex behavior. "No such species exists, to our knowledge," he says.

There are though some compelling candidates, some of which have long been used in research already. There's the European cuttlefish, *Sepia officinalis*, with hundreds of publications to its name, Hanlon suggests. Or the Hawaiian bobtail squid, *Euprymna scolopes*, well established by Margaret McFall-Ngai at the University of Hawaii as a model of host-microbe symbiosis; Joshua Rosenthal at MBL recently received an NSF EDGE award to further develop the bobtail into the first genetically tractable cephalopod species. There are octopuses to consider too. In 2015, the first cephalopod to have its genome sequenced was the two-spot octopus, *Octopus bimaculoides*[2]. Other cephalopods are likely to follow soon.

"The hope," says Crook, whose own lab works with a variety of octopuses, squids, and cuttlefishes, "is that once we can start getting really good tools for a couple of different species then the field as a whole might be able to converge on those animals." With standard models, standard practices can be established and improved.

The move towards standards is an important consideration given the state of cephalopod regulations. "The regulation issue is at a really interesting point at the moment because almost everywhere outside of the US has vertebrate-like regulations on cephalopod research now," says Crook. "There's a requirement to provide appropriate housing and appropriate analgesia and anesthesia and appropriate euthanasia and all of the things that would be required for vertebrate animals."

In 1986, the United Kingdom included *Octopus vulgaris* as a protected species in the Animals [Scientific Procedures] Act 1986. Canada regulated the use of cephalopods in research in 1991, New Zealand and Australia in 1999 and 2004, respectively, Norway in 2011. The European Directive 2010/63/EU enacted protections as of January 1, 2013.

"The way that the legislation is framed, it just says that it's necessary to minimize pain, suffering, distress and lasting harm," says Daniel Osorio, a vision researcher at the University of Sussex who has been involved with efforts in the European Union to educate and train cephalopod researchers about complying with regulations they hadn't had to consider before. As for other regulated species in Europe, there are severity scales—mild, medium, and severe—to guide assessments[3]. There's no difference in the principle to applying the scale to cephalopods, he says, "but far less is known about them."

"In the US, we're not regulated in any way," says Crook but "we're in an interesting position of being able to provide evidentiary support for regulations that are already in place in other countries."

Live fast, die young—caring for cephalopods

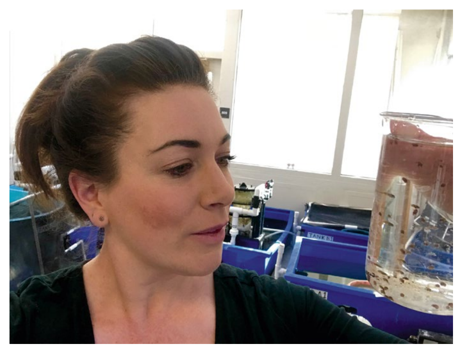
Cephalopods, in general, can be challenging to keep in captivity, in large part due to their basic biology and life histories: most mature, reproduce, and die within six months to a year.

"The good news about cephalopods is they grow like rockets," says Hanlon. The bad news? "They're active carnivores—these are not pellet-eating animals." Though not for a lack of trying—for a time, Hanlon had NIH funding to "turn a cephalopod carnivore into a 'pellet-ivore.'"

"We had all the right juju in the pellets, but getting them to eat the pellets was really difficult because motion is a key stimulus," he recalls. They made pellets that would flutter or spiral through the water in front of the animals in the hopes of prompting their visual attack responses, but to little avail.

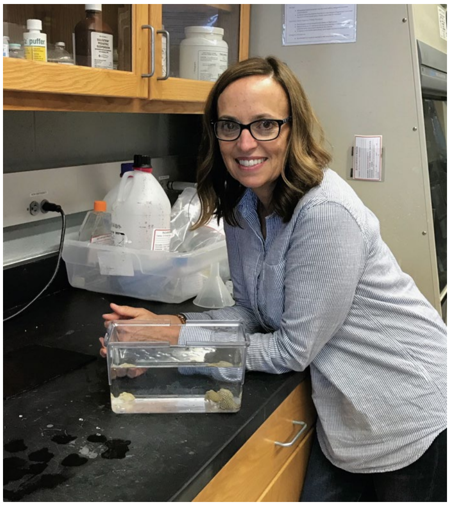
Live food it is—and the animals need a lot of it to keep up with their extremely fast metabolisms, says Grasse. That can get expensive and time-consuming for caretakers who must raise a second set of organisms and follow feeding schedules that involve multiple meals per day. And what goes in, must come out. "They produce on average about three to five times the amount of ammonia of a similar sized fish," he explains, to which they are incredibly sensitive.

Cephalopods have thin skin—literally. In some cases, it can be just a single cell-layer thick. "So any sort of deterioration in the water chemistry or water quality directly affects their biochemistry," says Grasse. If they become too stressed, they'll "ink," releasing a black plume of pigment that can black out their tanks. Proper filtration is therefore key, as is careful attention to other variables like temperature and pH.



Robyn Crook with a beaker of newly hatched Peacock bobtail squid, *Euprymna berryi*.
Credit: R. Crook

On the whole, cephalopods require fairly pristine water quality and have a penchant for live food. But there are species-specific differences that must be considered when establishing different cephalopods in the lab. "Octopuses for example are territorial, so if we keep a bunch of them at a time they need to be in separate tanks and we have to visually isolate them so that they don't see each other and get stressed out by having a near neighbor, which they would never have in the wild," says Crook. "The squids and cuttlefishes are more group tolerant so they tend to do better in small groups than they do separately."



Veterinarian Lisa Abbo in the lab with striped pajama squid, *Sepioloidea lineolata*. Credit: L. Abbo

Environmental enrichment is important too. "We try to give them as natural an environment as possible," Crook says. Sandy substrate for squid, nooks for octopus to hid in. Life in an empty tank is not a life for them.

Working with cephalopods often still involves working with wild animals. "That means there are huge regional differences in the species that are available and there's really no predictability in the background of the animal," says Crook. "You don't know what its prior experience is, you don't know what age it is, you often don't know what sex it is, and so all these things can obviously have huge effects on things like appropriate anesthesia and analgesia."

Lab-bred lines can help account for those variables, and there are a handful of species that can be bred through several generations, like the bobtail squid and European cuttlefish. However, lab populations will eventually start to suffer from inbreeding, and fresh animals must be brought in from wild populations to replenish the genetic pools. "Inbreeding is always a big concern," says Hanlon. "And we know almost nothing about inbreeding depression in cephalopods except that it does occur." There's a need for improved brood stock management and better understanding of gene flow in different cephalopods to keep generations healthy and fit over time.

One goal at the MBL is to improve the process for the candidate species they are working to culture. Cephalopod sexes are separate, and most usually mate just once in their lives. Hatchlings quickly emerge from fertilized eggs, and the cycle begins again.

To catch fertilized eggs as they are released, the cephalopod team at MBL is attempting to swap up some schedules. Like mice, many species of cephalopods are active when humans are usually asleep. For example, as they try to breed their cuttlefish for reliably timed embryos, they are inverting the animals' schedules so that a researcher who needs to collect those embryos can be available—and awake.

The problem of pain

Inevitably though, animals will become sick or injured. When a cephalopod isn't well, it's not always so easy to tell. "Sometimes, the first sign of anything being wrong is that they just die," says MBL's resident veterinarian Lisa Abbo. "That can be really frustrating." One of the most common ailments she sees are skin issues: given their thin skin and the reality that they live in tanks, cephalopods can end up with lesions, which run the risk of becoming infected. "Usually it's something that we can see, although that can be challenging too because the minute I walk up to an animal, they will often change the color or pattern of their skin, sometimes even the texture," she says. Is what she's seeing an injury, or just part of the pattern that particular animal feels like displaying at the moment?

Veterinary care, in Abbo's experience so far, tends to be empirical. Cephalopods weren't part of the curriculum in vet school, so she relies on experience and a network of other veterinarians for advice about tackling their care. A more robust knowledgebase would be welcome. "I think there needs to be more work, learning more about their behaviors, their natural history in the wild, and how we can use that information to be able to assess them," she says.

Assessing, preventing, and treating pain is a particularly important consideration for lab animal caretakers, but cephalopods aren't like other lab animals. "One of the challenges that I've found is that you can't take what you would do in a fish and apply it to a cephalopod," says Abbo. "So how do I assess pain in a cephalopod? How can I treat it? A researcher might want to do a particular surgery—how do I anesthetize it? How do I make sure it's going to recover well?"

Pain is particularly complex. "There's not any solid evidence that cephalopods suffer the emotional component, the distressing aspect of pain that is the major welfare concern," says Crook. The animals do have sensory neurons that respond to injurious stimuli, "but that doesn't necessarily mean that the animal also has the emotional circuits to suffer as a result of that sensation," she explains.

"However, it doesn't hurt to sort of proceed on the assumption that that

A skin lesion to be treated in the European common cuttlefish, *Sepia officinalis*. Credit: L. Abbo

might be possible and then go ahead and do your best to protect the welfare of the animal anyway," she says. That philosophy shapes work in the Crook lab. Over the past few years, they have been working to systematically assess analgesia and anesthesia in different species, in a way that bypasses the difficult underlying question of the animals' pain perception.

In their most recent work published in *Frontiers in Physiology*[4], they used electrodes to test whether two commonly used anesthetics, ether ethanol and magnesium chloride, could prevent the transmission of electrical signals from peripheral injuries to the brains of stumpy cuttlefish and two different species of small octopus. The drugs did just that, both immobilizing the animals and blocking sensory signals. "Anesthesia literally means 'the absence of sensation,'" explains Crook. "If you have an animal that is not sensing noxious stimuli in its periphery, by definition it can't experience pain."

A caveat? The species in the study were tropical. "Metabolism of drugs tends to be

A young two-spot octopus, *Octopus bimaculoides*. Credit: Marine Biological Laboratory/T. Kleindinst really temperature dependent," she says. She's confident that their results are broadly applicable to other tropical cephalopods, but what animals from temperate climes? Over in Woods Hole, Abbo and her colleagues at MBL are currently replicating the study in cold water species and hope to have those results published for the field soon.

Crook's lab will be considering euthanasia in the future too, as well as questions about cephalopod welfare over time, such as whether negative experiences in their early lives influences future outcomes. That question has potential relevance to humans too, in addition to contributing to better understanding of the animals themselves.

Jetting onward

It's an exciting time to be thinking about cephalopods. "All of the sudden we're looking at these animals in a really different way," says Crook. "Really thinking about, how vertebrate-like are they really? Which is a question I've always considered in a neurobiological context but it's interesting to see that now in the broader context of their use as a lab animal."

And efforts like those at the MBL to improve husbandry and develop better tools and approaches for working with the animals are intended to spread the adoption of cephalopods in other interested labs. "What we've been trying to do here at MBL is work with some of the more hearty, more 'user friendly' species" says Grasse. "We really want it to be accessible to a wide variety of studies and scientists."

As the cephalopod star rises, now is a good time to understand their needs better. Although the United States does not regulate the animals, there's a sense that it may only be a matter of time before cephalopods attract more attention. Some institutions are already preparing—Crook notes that she now routinely receives requests from colleagues at other institutions for help with developing their Institutional Animal Care and Use Committee protocols for cephalopods. "Even though it's not legally required, the institution specifically has decided that they would like to have a protocol for those animals in their facility," she says.

"It's my suspicion that we'll probably go that way at some point in the future," says Abbo, though she hopes any formal changes in the US will be enacted thoughtfully and with the input of the researchers and veterinarians who oversee the animals.

In the meantime though, caring for cephalopods will go on. "For us, I feel pretty comfortable that we're doing everything to our ability to make sure that the animals are taken care of," says Abbo. That's an important consideration not just for her, but for the researchers too who will be using the animals to help answer their research questions. A stressed or sick cephalopod won't do the scientific community any favors.

"It goes back to wanting to control for variables," she says. "If you want to get something meaningful from your hard work, well, you better take care of your animals." ❐

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Published online: 12 December 2018
https://doi.org/10.1038/s41684-018-0199-0

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