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Multigenerational laboratory culture of pelagic ctenophores and CRISPR–Cas9 genome editing in the lobate *Mnemiopsis leidyi*

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Despite long-standing experimental interest in ctenophores due to their unique biology, ecological influence and evolutionary status, previous work has largely been constrained by the periodic seasonal availability of wild-caught animals and difficulty in reliably closing the life cycle. To address this problem, we have developed straightforward protocols that can be easily implemented to establish long-term multigenerational cultures for biological experimentation in the laboratory. In this protocol, we describe the continuous culture of the Atlantic lobate ctenophore *Mnemiopsis leidyi*. A rapid 3-week egg-to-egg generation time makes *Mnemiopsis* suitable for a wide range of experimental genetic, cellular, embryological, physiological, developmental, ecological and evolutionary studies. We provide recommendations for general husbandry to close the life cycle of *Mnemiopsis* in the laboratory, including feeding requirements, light-induced spawning, collection of embryos and rearing of juveniles to adults. These protocols have been successfully applied to maintain long-term multigenerational cultures of several species of pelagic ctenophores, and can be utilized by laboratories lacking easy access to the ocean. We also provide protocols for targeted genome editing via microinjection with CRISPR–Cas9 that can be completed within ~2 weeks, including single-guide RNA synthesis, early embryo microinjection, phenotype assessment and sequence validation of genome edits. These protocols provide a foundation for using *Mnemiopsis* as a model organism for functional genomic analyses in ctenophores.

Introduction

The growing interest in ctenophores[1–3], a monophyletic group of globally distributed gelatinous marine predators[4–9], primarily stems from recent phylogenomic analyses highlighting their relationship with other animals[1,10–20]. Along with the many unique traits ctenophores possess, such as their iconic ciliary ctene rows[4,21–23], sticky tentacular colloblast cells[4,24–27], aboral apical organ[4,28–30] and biradial rotational symmetry, ctenophores also retain a remarkable suite of modified features found in other animals, for example, a nervous system that lacks many canonical bilaterian neurotransmitters[15,31,32], highly derived mitochondria[33] and mitochondrial genome[34–36], muscle cells despite the absence of canonical muscle specification genes and a defined mesoderm[14,15,37–41], and the presence of a functional through-gut[42]. Most ctenophores exhibit bioluminescence[43,44] and extensive regenerative capacity[45–47], and at least a few ctenophore species are known to exhibit a unique form of juvenile reproduction termed dissogeny[48–50] (but see ref. [51]). These features, along with the early divergence of Ctenophora from the metazoan stem lineage, have made ctenophores a key group for investigating character trait evolution during early metazoan diversification.

A number of studies utilizing periodically abundant seasonal coastal species from the genera *Beroe*, *Bolinopsis*, *Pleurobrachia* and *Mnemiopsis* have been foundational in illuminating remarkable aspects of ctenophore development. For example, studies of early embryological stages[4,52–56] were among the first to recognize that ctenophore embryos undergo a unique phylum-specific cleavage program, hatching as free-swimming juveniles termed cydippids. More recently, molecular phylogenetic analyses suggest that the range of extant body plan diversity among ctenophores stems from an ancestral cydippid-like morphology[19,57,58]. Work with *Beroe* has highlighted gametogenesis and fertilization[59–65]. Perturbation experiments in *Bolinopsis* have identified the requirement of the ctenophore-specific protein, CTENO64, for proper comb plate organization and locomotory function[66], while analyses

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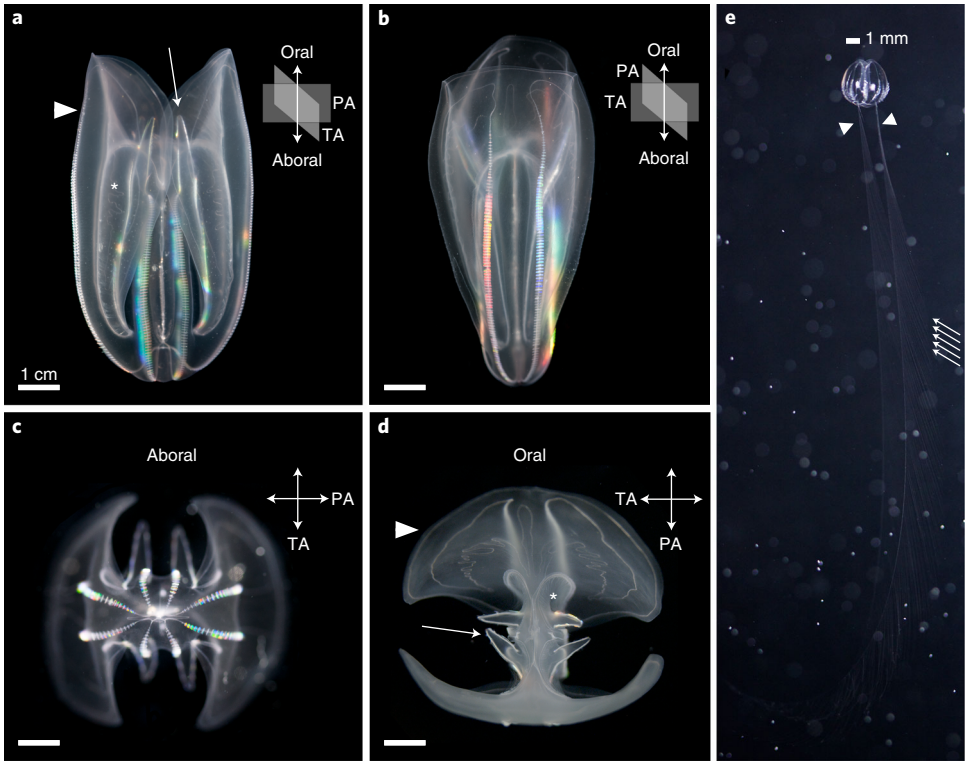


Fig. 1 | Body plan of *Mnemiopsis leidyi* with major axes labeled. a, Lateral view of an adult showing the pharyngeal axial plane. **b**, Same individual as in **a**, viewing the tentacular axial plane. **c**, Adult viewed from the aboral pole. The ctene rows converge at the aboral organ. **d**, Oral view, showing the mouth. **e**, 6 dph juvenile with extended tentacles (arrowheads) and branching subtended tentilla (small arrows). The tentilla are covered with sticky colloblast cells used for prey capture. Marine rotifers, *Brachionus plicatilis*, are visible as small dots in the image. The lobes, marked by arrowheads in **a** and **d**, extend orally. The ciliated medial surfaces of the lobes assist in prey capture and transport to the mouth. The auricles, marked by arrowheads in **a** and **d**, bear giant cilia used to shape water flow between the oral lobes, assisting in both prey capture and swimming. Asterisks in **a** and **d** indicate the position of the food grooves in the adult. Each food groove extends along the body wall, aborally from the mouth, underneath the lobes and is lined with both sticky tentilla for prey capture and the giant cilia used to shape water flow through the tentilla. PA, pharyngeal axial plane; TA, tentacular axial plane.

of gene expression patterns in *Pleurobrachia* adults have provided insights regarding axial patterning[67–71].

Experimental manipulations of *Mnemiopsis* embryos have been particularly useful for examining the establishment of cell fates, axial symmetry, inductive signaling, cleavage clock and organizer activity during ctenophore development[72–84]. Analyses of embryonic gene expression patterns and their perturbation have provided insights regarding the expression and function of transcription factors and signaling pathways controlling cell fate specification and axial patterning during *Mnemiopsis* development[85–96].

Mnemiopsis has also been used as an important ecological model owing to its well-documented invasion capacity[97–101]. A wide range of ecological and physiological experiments have been performed with *Mnemiopsis* in both its native and invasive habitat ranges[99,102–112]. In addition, *Mnemiopsis* is a model for studies of the unique ciliary mechanics associated with pelagic ctenophore swimming behavior[22,113–115].

There are currently no standardized methods for long-term multigenerational maintenance of ctenophores. The principal challenges to reliably closing the life cycle of these fragile animals in a laboratory setting have been replicating a pelagic habitat without strong shear forces and, most importantly, providing a suitable and constant planktonic food source. Here we describe multigenerational laboratory culturing of the Atlantic lobate ctenophore *Mnemiopsis leidyi* (Fig. 1). The goal of the methods detailed below is to highlight the necessary resources and advances in procedures required for culture conditions to close the life cycle of *Mnemiopsis* in the laboratory in order to facilitate a wide range of biological studies including behavioral, physiological, anatomical, cellular, embryological, molecular genetics and functional genomics. These methods were developed to

improve adult fecundity for controlled and consistent daily spawning to produce high-quality embryos that can be used for a range of experimental manipulations. Our methods maximize juvenile growth rates and have been optimized for the maintenance of long-term, healthy transgenerational laboratory strains, expand upon prior short-term protocols[50,116,117], and can be implemented without direct access to natural seawater or coastal populations of *Mnemiopsis*. Overall, our recommendations for optimizing ctenophore husbandry include housing conditions, detailed feeding requirements and improved feeding regimes, the use of light-induced entrainment queues for reliable control over spawning, the collection of embryos, and the rearing of juveniles to adults that are amenable for a variety of experimental designs. We also include detailed recommendations for performing CRISPR–Cas9-mediated genome editing via microinjection of *Mnemiopsis* embryos.

Advantages of laboratory culture of *Mnemiopsis*

Most ctenophores are oceanic and/or deep-water species that are particularly fragile and often have environmental requirements that are extremely difficult to replicate under laboratory conditions[118,119]. However, coastal species such as *Mnemiopsis* are relatively resilient and seasonally available in large numbers. Native habitats of *Mnemiopsis* are found across a wide range of tropical, subtropical and temperate coastal Atlantic environments[120–122]. They are self-fertile hermaphrodites capable of producing large numbers of embryos daily[116,117,123]. Embryogenesis is rapid, with hatching occurring within 18–24 h of oocyte fertilization. Juvenile *Mnemiopsis* cydippids can be easily reared to sexual maturity in small aquaria and egg-to-egg generation time is as short as 3 weeks[75,116] (Figs. 2–4). *Mnemiopsis* embryos, juveniles and adults are transparent, making observation and imaging of *in vivo* tissue and organ function relatively easy[42,96] (Figs. 1, 3 and 4).

A range of experimental methods have been developed for *M. leidyi*, including cell lineage fate mapping during embryogenesis[79], determination of gene expression patterns[90,92,95,124,125], generating primary cell cultures[41,126], and the examination of gene function using morpholino oligonucleotides (MO) and CRISPR–Cas9-mediated genome editing[66,96,127]. Genomic resources available for *Mnemiopsis* include an assembled reference genome[14,128,129] as well as transcriptomic data[14,84,130–132]. These resources will continue to provide insights into unique aspects of the ctenophore genome, for example, lineage-specific gene expansions associated with neurotransmitters and extracellular matrix (ECM) proteins[15,133–135], as well as highlighting an absence of many genes typically found in other animal lineages such as mesodermal specification genes[14], determinants of neural fate and neurotransmitter biosynthesis[15], HOX genes[136], key components of the miRNA biogenesis pathway[137] and components associated with the regulation of innate immune system regulation[138].

Applications

We describe standardized protocols for effective and reliable long-term multigenerational lab cultures of *Mnemiopsis*. These protocols are intended to reduce or eliminate the need for wild-caught animals and thus allow laboratories, far from the ocean, to perform detailed biological analyses on these remarkable animals. Our protocols permit more nuanced observations of ctenophore biology than previously possible and make routine mutational analyses via genome editing approaches practical in ctenophores. Although we focus on *Mnemiopsis* in this report, these protocols have been used to successfully maintain a number of other pelagic ctenophore species (Table 1).

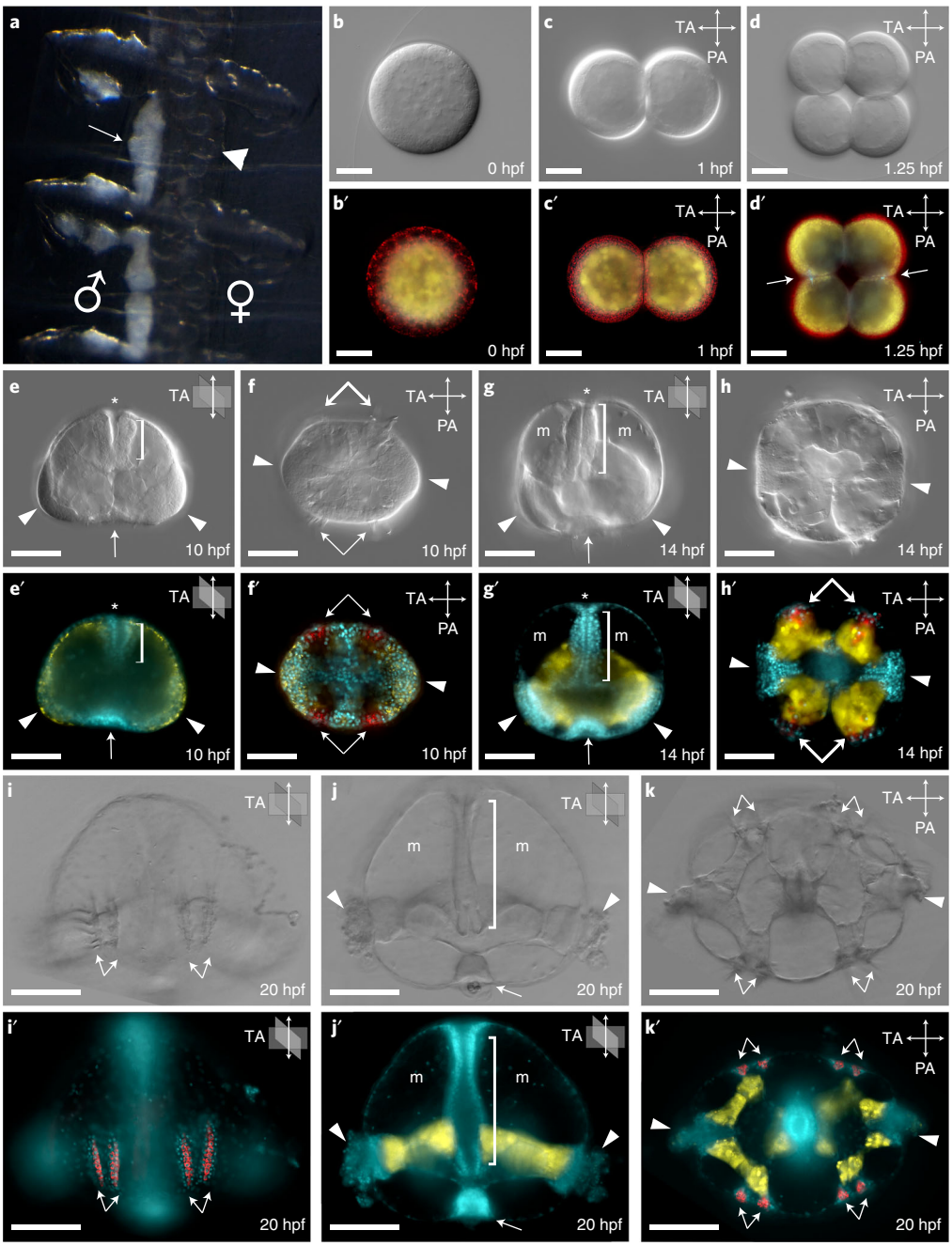
Comparative morphological studies continue to provide critical insight into animal evolution by inferring both the origin of and change to character traits in a phylogenetic context[139–141]. Detailed studies required to understand how the unique attributes of ctenophores may contribute to their fascinating biology also require access to healthy populations of individuals that are representative of normal development. A clear advantage of rearing ctenophores in the lab is the ability to observe morphological characteristics that are otherwise difficult to document in the wild or in fixed material[42]. Importantly, access to the complete ontogeny of *Mnemiopsis* requires the ability to reliably

◀ **Fig. 2 | Equipment used for housing, feeding, spawning and general tank maintenance. a**, Custom 60-cm pseudokreisel. A ball valve and flow meter, marked with an arrow, are used to regulate the velocity of seawater entering the pseudokreisel. **b**, Custom ~300 L planktonkreisel. Three ports used for automated delivery of rotifers are drilled into the top plexiglass panel (Fig. 3d). **c**, Active filtration is performed in a sump tank located below the planktonkreisel. The sump tank combines a biofiltration chamber and a venturi-driven protein skimmer marked with an arrowhead. The flow rate through the planktonkreisel is controlled via a gate valve, marked with an arrow, downstream of the main pump at bottom. **d**, An in-line chiller is used to maintain planktonkreisel water temperature at 20 °C. **e**, Live larval zebrafish for feedings can be maintained for short periods of time in soda-lime glass bowls. **f**, 10 L and 1.5 L tanks are used for spawning individual mature *Mnemiopsis* and for raising juveniles to maturity. On the left are individuals from F43 in a 10 L glass tank. To the right are three smaller 1.5 L plastic tanks containing F44 individuals, a single F44 individual isolated for spawning indicated by S, and on the right F45 individuals that have been transferred from the spawning tank. **g**, Shallow soda-lime glass bowls are also used for spawning as they can be easily screened under a dissection microscope to collect individual embryos for experimental manipulations. Rapid and efficient entrainment of *Mnemiopsis* to a user-defined spawning cycle requires exposure of animals to wide-spectrum lighting containing ultraviolet wavelengths. **h**, 1.5 L tank with length × width × height measurements in millimeters. Arrows indicate additional small holes that have been drilled into the fitted plastic cover to facilitate passive gas exchange. **i**, Individual juvenile *Mnemiopsis* can be raised for several days post-hatching in 400 mL plastic beakers placed in sealed plastic bins that function as humidity chambers to prevent rapid changes in salinity due to evaporation. **j**, Plastic 7.5 mL and 1.5 mL transfer pipettes are cut to increase tip diameter for use in both general maintenance and moving individual juvenile *Mnemiopsis*. **k**, A vacuum hose is used to perform periodic water changes and siphon accumulated organic wastes from the bottom of the planktonkreisel.

close the life cycle to generate long-term laboratory cultures with year-round embryo availability, thus facilitating the production of inbred and discrete genetic lines that are valuable for a range of experimental analyses that rely heavily on laboratory-maintained model systems including, but not limited to, genetic, cellular, embryological, physiological, developmental, ecological and evolutionary studies.

Limitations

A primary limitation remaining for the laboratory culture of ctenophores is the lack of an artificial diet. While we provide details for the standardization of food quality sufficient for long-term reproductive success with live rotifer cultures (Box 1 and Fig. 5), we suggest periodic feeding of live larval fish to promote rapid growth rates and maximize fecundity of adult ctenophores. However, obtaining, housing and feeding vertebrates can be problematic owing to regulatory



constraints associated with vertebrates. We and previous studies[108,109] have managed to achieve continuous egg production on non-vertebrate diets. If fish larvae are unavailable, we suggest supplementing a staple rotifer diet with copepods and/or mysid shrimp (Table 1)[142].

Alternative methods

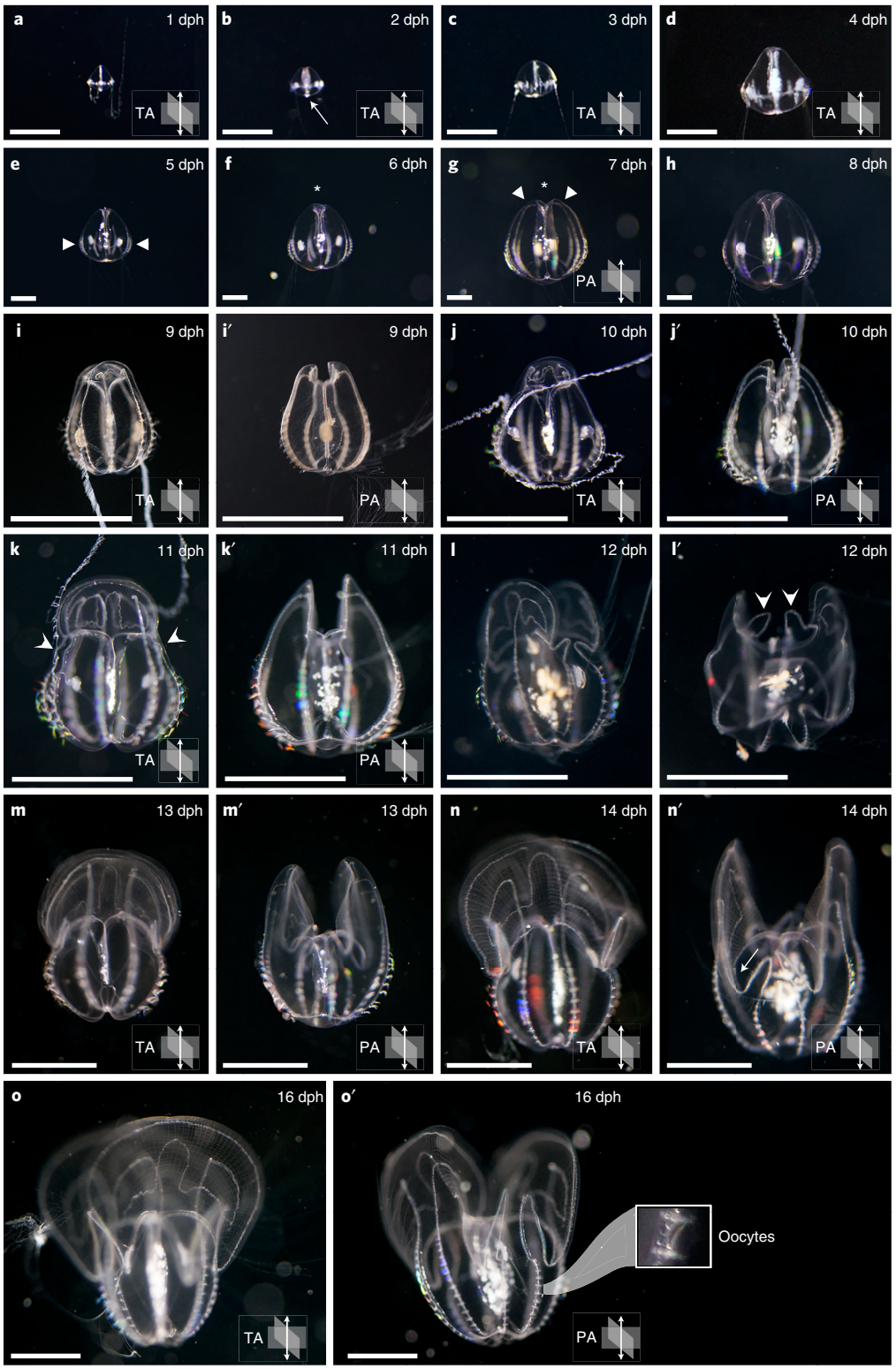
Oceanic ctenophores are particularly fragile and difficult to maintain in a healthy condition for extended periods of time owing to typical water flow and shear forces present in aquaria, including the slow circular currents produced in pseudokreisels. A 'double tube'-based design can be used as an alternative to support extremely delicate ctenophores and other fragile pelagic species. This design reduces water movement and shear forces substantially by encouraging gentle diffusion of seawater between an inner acrylic tube containing animals that is sleeved within a larger diameter outer acrylic tube. The 'double tube' design reduces tank horizontal footprint while increasing tank vertical space and replaces a typical hard bottom with a mesh screen that serves as the diffusion interface between inner and outer tubes[142]. We find long-term maintenance of pseudokreisel tanks easier for coastal ctenophores, such as *Mnemiopsis*, that are more tolerant of environmental perturbations. Importantly, pseudokreisel designs maximize horizontal surface area (Fig. 2b), providing rapid and easy access to animals.

Procedure overview

Our protocol consists of five main stages. In Steps 1–4, we present procedures optimized for the long-term laboratory maintenance of *Mnemiopsis* cultures, including housing and feeding requirements. With the procedures presented in Box 1 we describe the setup, maintenance and harvesting of rotifers from small-scale cultures we use for the staple diet fed to our laboratory *Mnemiopsis* strain. In Step 5A and B, we describe our methods for controlled spawning of adult *Mnemiopsis* via light entrainment. Methods optimized for obtaining embryos for immediate experimentation (i.e., CRISPR–Cas9 via microinjection) are detailed in Step 5A, and methods optimized for general propagation of new and/or existing laboratory strains are detailed in Step 5B. In Steps 6–8, we present

◀ **Fig. 3 | *Mnemiopsis leidyi* embryogenesis. a**, Lateral view of male and female gametes flanking a meridional endodermal canal underneath ctene plates. Left arrow marks the location of a male gonopore. Male gonopores occur between each ctene plate along each ctene row. Right arrowhead marks the location of a female gonopore. Female gonopores occur between each ctene plate along each ctene row. **b**–**k** in vivo DIC images are matched with (**b′**–**k′**) in vivo fluorescent vital dye images. Red MitoTracker fluorescence marks mitochondrial distribution, yellow LysoTracker fluorescence marks lysosomal acidic vacuole distribution and blue Hoechst fluorescence marks nucleic acid distribution. **b**, Newly released 0 hpf embryo. Eggs are ~150–200 μm in diameter, with a dense centrolecithal yolk core and a thin outer layer of cytoplasm. **b′**, Mitochondria are excluded from the yolk-rich egg core. **c**,**c′**, First cleavage occurs ~1 h post-fertilization (hpf) and establishes the pharyngeal axial plane (PA). **d**,**d′**, Second cleavage at ~1.25 hpf is orthogonal and establishes the tentacular axial plane (TA). Arrows in **d′** denote the position of the nuclei and can be used to differentiate PA from TA among the four equal-sized blastomeres at the four-cell stage. **e**,**e′**, Lateral view, oral up, 10 hpf. The establishment of different tissues are clearly visible. The asterisk at the oral pole marks the developing pharynx. The bracket identifies the position of the pharynx. The arrow at the aboral pole marks the position of the cellular thickening associated with the developing aboral apical organ. The arrowheads mark the position of the cellular thickenings associated with the developing tentacle bulbs. **f**,**f′**, Aboral view 10 hpf. The arrows indicate the position of the mitochondria rich ctene rows associated with each quadrant. Each ctene row is organized into a series of reiterating plates along the oral-aboral axis. The two arrowheads denote the position of the developing tentacle bulbs. **g**,**g′**, Lateral view, oral up, 14 hpf. Developing organs are clearly visible. The asterisk at the oral pole and bracket marks the elongated pharynx that connects with the endoderm from the developing gastrovascular cavity forming the central infundibulum of the gut. The mesogleal space, marked by m, is beginning to swell with ECM. The arrow at the aboral pole marks the position of the developing aboral apical organ. The arrowheads mark the position of invaginated ectodermal cells associated with medial internal endodermal-derived cells to form the developing tentacle bulbs. **h**,**h′**, Aboral view 14 hpf. The arrows indicate the position of the mitochondria rich ctene rows associated with each quadrant. The organization of the gastrovascular cavity in each quadrant is clearly visible along with the development of the initial branches of the endodermal canal system that contact the developing tentacle bulbs marked by arrowheads. **i**,**i′**, Lateral surface view, oral up, 20 hpf juvenile cydippid. Each quadrant contains two rows of mitochondria rich ctene rows marked by arrows. **j**,**j′**, Lateral medial view, oral up, 20 hpf juvenile cydippid. Bracket marks the pharynx. The mesogleal space, marked by m, is filled with ECM. Remaining yolk is sequestered in the gut. Arrow marks the position of the aboral apical organ. Arrowheads mark the position of the tentacles and tentacle bulb. **k**,**k′**, Aboral view 20 hpf juvenile cydippid. Arrows mark the position of the ctene rows in each quadrant. Arrowheads denote the position of the tentacles and tentacle bulbs. Centrally, the junction of the pharynx with the infundibulum can be seen. The endodermal canals are tightly associated with the medial inner cells of the tentacle bulbs and inner cells of the ctene rows. Scale bars, 50 μm (**b**–**d′**) and 100 μm (**e**–**k′**). PA, pharyngeal axial plane; TA, tentacular axial plane.

procedures optimized for the rapid growth and development of juvenile *Mnemiopsis* to sexual maturity. These methods can be used to close the *Mnemiopsis* life cycle in as few as 14 d. Finally, our protocol provides procedures and recommendations for the preparation and implementation of CRISPR–Cas9 genome editing experiments (Steps 9–23) via early embryo microinjection (Steps 24–51), and also includes procedures useful for the live imaging of embryonic and juvenile *Mnemiopsis* to assist with characterization of phenotypes with fluorescent vital dyes (Steps 52–66).



Experimental design

The purpose of this protocol is to support long-term culturing and maintenance of *Mnemiopsis* for generating reliable and continuous laboratory stocks of animals that can be used for biological experimentation (for example, daily embryo collections for microinjections). Below we provide detailed specifics of our culturing system. The successful husbandry of *Mnemiopsis* and other ctenophores for the regular production of high-quality gametes requires (1) providing suitable housing to mitigate against shear forces induced by irregular water flow and tissue damage from contact with hard surfaces and, most importantly, (2) the constant availability of high-quality food sources such as live rotifers. As there are no standard methods beyond ours for long-term multigenerational maintenance of ctenophores, we encourage researchers to modify our protocols where appropriate to make the best use of available equipment beyond that listed herein. However, we stress that the recommendations provided below have proven invaluable for maintaining healthy, stable, multigenerational stocks of breeding ctenophores (>F50) that have been used for a wide variety of biological experiments.

Water quality

As a coastal species, *Mnemiopsis* is often found in environments subject to variable salinity, turbidity and temperature over short time scales[143]. For captive cultures, either filtered natural seawater or artificial seawater (ASW; Instant Ocean) can be used. There are several major advantages to using ASW: a dramatic reduction in chance contamination and parasite transmission, the ability to precisely control multiple water quality parameters simultaneously, and, importantly, the distance of the laboratory from a natural seawater source is irrelevant. We typically prepare large volumes of ASW in a mixing cistern by initially removing chlorine and heavy metals from treated public municipal freshwater sources using Prime (Seachem Laboratories). Sea salts are then added to reach the desired salinity range of 23–31 ppt (1.017–1.023 sg) for *Mnemiopsis*. Completely mixed ASW is then transferred to a holding cistern, ready to be used in culture tanks.

Housing

Proper housing for pelagic organisms such as ctenophores is critical (Fig. 2 and Supplementary Figs. 1 and 2). Ctenophores are susceptible to tissue damage from repeated physical contact with tank walls, vigorous currents and aeration. At Monterey Bay Aquarium, a combination of 12 L, 72 L and 150 L pseudokreisels are utilized for culturing *Mnemiopsis* depending on their relative size and number (e.g., smaller pseudokreisel tanks are appropriate for small and/or few animals) (Fig. 2a and

◀ **Fig. 4 | Stages of post-hatching development in *Mnemiopsis leidyi*.** In all panels, oral is up. **a**, *Mnemiopsis* cydippids begin feeding with extended tentacles within an hour of hatching and are ~0.2–0.3 mm in size at 1 dph. The infundibulum contacts the innermost face of each tentacle sheath, ctene row and inner face of the apical organ. **b**, At 2 dph, juveniles are morphologically similar but twice as large. An aboral canal visibly emerges from the infundibulum and bifurcates into two short anal canals terminating with anal pores, marked by the arrow, that flank the apical organ. **c**, At 3 dph, endodermal protrusions appear on the oral side of the infundibulum flanking the pharynx in the tentacular plane and correspond to the developing paragastric canals. **d**, At 4 dph, juvenile cydippids are ~1–2 mm in size. The paragastric canals extend the length of the pharynx, and gastrovascular protrusions that will become the radial and adradial canals appear. **e**, At 5 dph, the distance between the tentacle bulb and the apical organ has increased and more plates have been added to the ctene rows, marked by arrowheads, as the meridional canals extend orally along the oral–aboral axis[157]. **f**, At 6 dph, the stomodeal opening, marked by the asterisk, has begun to widen and thicken. **g**, At 7 dph, the meridional canals, marked by arrowheads, have migrated orally. The stomodeal opening is marked by the asterisk. Aborally, the radial and adradial endodermal canals associated with the infundibulum have formed, and the two anal canals have become distinct from the aboral canal. **h**, At 8 dph, the meridional canals have converged around the stomodaeum just before the outgrowth of the oral lobes. **i**,**j**,**k**,**l**,**m**,**n** and **o** are lateral views in the tentacular axial plane. **i′**, **j′**, **k′**, **l′**, **m′**, **n′** and **o′** are matched lateral views in the pharyngeal axial plane. **i**,**i′**, At 9 dph, transformation of juvenile cydippids towards adult morphology is visible as the oral lobes begin to rapidly enlarge. Oral lobe development is pioneered by the growth, integration and merging of the meridional canals. Patterning of the oral lobe endodermal canal system continues through 12 dph (**i**–**l′**). **k**, At 11 dph, the auricles initially appear as protuberances, marked by arrowheads, flanking the developing oral lobes. **l**,**l′**, At 12 dph, the developing auricles rapidly acquire their distinct finger-like morphology. In **l′**, the arrowheads indicate the developing auricles. **m**,**m′**, At 13 dph, the juvenile cydippid body plan has been extensively remodeled and closely resembles the adult lobate form. The characteristic, tentilla-lined, adult feeding grooves situated between the auricles and lobes have also begun to form and are clearly visible by 14 dph (**n**,**n′**). In **n′**, the arrow indicates the position of a developing food groove. **o**,**o′**, Juveniles reach sexual maturity as early as 14–16 dph. Inset in **o′** is a closeup of mature oocytes. Scale bars, 1 mm (**a**–**h**) and 5 mm (**i**–**o′**). PA, pharyngeal axial plane; TA, tentacular axial plane.

Table 1 | Comparison of critical husbandry characteristics for several successfully cultured pelagic ctenophore species

Husbandry	*Mnemiopsis leidyi*[a]	*Pleurobrachia bachei*[b]	*Hormiphora californensis*[b,173]	*Bolinopsis infundibulum*[b]	*Bolinopsis vitrea*[b]
Temperature (°C)	20–25	10–13	12	10–13	20–25
Salinity (ppt)	23–36	34–36	34–36	34–36	30–36
Size of fertilized egg (µm)	150	35	75	200	?
Hours to hatch	FL: 18–22; MA: 18–24	24–48	12–24	48–72	~24
Size at hatch (µm)	250–300	100	150	300–400	–
First food item	R	C	C	R, C	R
Onset of feeding post hatch	Immediate	Immediate	Immediate	Immediate	Immediate
Juvenile and adult diet	C, F, M, R	C, F, M, R	C, F, M, R	C, F, M, R	C, F, M, R
Age at maturity	16 d	30 d	46 d	> 30 d	–
Spawning cues	FL: 3.5 h dark + light; MA: 8 h dark + light	Overnight dark + 2 h light	Overnight dark + 2 h light	Overnight dark + 2 h light	–
Spawning frequency	Daily	Daily	Daily	Weekly	–
Median life span	~2 years	>1 year	>1 year	>2 years	>1 year

Median life spans are derived from our observations in culture. C, calanoid copepod nauplii; F, fish hatchlings; M, mysids; R, rotifers; FL, Florida; MA, Massachusetts. [a]Using this protocol[41,42,44,96,126,138]. [b]From unpublished culture protocols (M.B., T.K., W.P., W.E.B).

Supplementary Figs. 1 and 2)[144]. Pseudokreisel volumetric turnover flow rates are measured using an in-line flow meter and range from 0.1–0.3 liters per minute (Lpm) (30 cm pseudokreisel) to 0.75–1.5 Lpm (60 cm pseudokreisel) and 7.5–15 Lpm (150 cm pseudokreisel). The circular current flow is produced from a point source with a velocity of ~5–10 cm/s regulated by either a ball valve or a gate valve upstream of the in-line flow meter (Fig. 2a). At the University of Miami, a slightly different pseudokreisel design with angled bottoms, no substrate and very low flow rates[145,146], producing a slow rotating current to keep ctenophores suspended in the water column while minimizing contact with tank walls, is used for larger ~300 L tanks to support long-term cultures (Fig. 2b; planktonkreisel developed with Midwater Systems). These long-term culture tanks are split into two chambers: a narrow rear standpipe area that maintains a constant tank volume irrespective of water flow rate and a front main chamber that houses the ctenophores. Water is exchanged between the two sections via diffusion through 200 µm nylon mesh (Nitex) screen cutouts. The slow circular current in the main chamber is achieved with a 'spray' bar fashioned from PVC piping with small holes positioned just below the water surface. The spray bar directs water flow perpendicular to the Nitex screens that divide the main front chamber from the rear chamber. Filtration, aeration, waste removal and spray bar flow rate are accomplished via a sump-style wet-dry trickle biofilter, a venturi-driven protein skimmer and an adjustable gate valve (Fig. 2c). Efficient protein skimming is critical for reducing the organic waste load resulting from continuous feeding. Debris that settles to the bottom of culture tanks is removed periodically during partial water changes by vacuum (Fig. 2k).

In the wild, *Mnemiopsis* can tolerate a wide temperature range, from <1 °C to 32 °C (refs.[99,123]). We maintain our large long-term pseudokreisel tank temperatures at 20 °C via an in-line seawater chiller (Fig. 2d). Cool temperatures help maintain satisfactory water quality and reduce physiological stress. Each long-term 300 L pseudokreisel tank supports ~20–30 mature adults. A healthy adult, when entrained to a user-defined light cycle (see section below), is capable of producing hundreds to thousands of embryos on a nearly daily basis. While *Mnemiopsis* can tolerate short-term overcrowding, negative effects on growth rates and gamete production are both rapid and severe. To maximize adult growth rates, reliable spawning and long-term viability it is critical to avoid overcrowding. Our best results are achieved with long-term cultures of no more than one adult animal per 10 L of seawater.

Feeding

Ctenophores are highly effective predators[102,119]. Captive cultures of ctenophores are provided with a constant food supply as egg production is enhanced by the continuous availability of a high-quality food source[42,108,147,148]. This is particularly important for maintaining rapid growth rates, reliable

Box 1 | *Brachionus plicatilis* culture maintenance and use ● Timing duration of culture

Small-scale *Brachionus plicatilis* rotifer cultures are maintained in 15 L plastic buckets at RT at a density of ~100–200 rotifers/mL (Fig. 5a) and fed a concentrated algal paste diet (either RG Complete, Nanno 3600, or Rotifer Diet sourced from Reed Mariculture), as described in the steps below. We recommend maintaining a minimum of two rotifer cultures as a backup in case a rotifer population crashes. The number of individual rotifer cultures can be easily scaled up as needed to support the desired number of adult ctenophores in culture. Each 15 L plastic bucket should carry a minimum of ~100,000–200,000 rotifers/L to ensure a sufficient rotifer density for maintaining ctenophores (Fig. 5b). Our ~300 L pseudokreisel housing ≤30 adult *Mnemiopsis* is supplied with rotifers from two 15 L buckets via automated dosing every 20 min from a timer-controlled panel of four peristaltic pumps calibrated to deliver ~6 L of rotifer culture every 24 h (Fig. 5c).

The advantage of maintaining several small-scale rotifer cultures include: (i) limiting the potential for rapid ammonification of concentrated waste products that can collapse an entire rotifer population, (ii) limiting the spread of disease through the entire rotifer population, (iii) limiting the growth of undesirable protists/ciliates through the entire rotifer population, (iv) relatively easy adjustments to feeding rates across multiple ctenophore cultures and (v) minimal additional labor investment to maintain several small cultures. The disadvantages of maintaining small scale rotifer cultures include: (i) rapid rotifer population growth rates need to be closely monitored to prevent overpopulation, and (ii) rotifer culture buckets used for automated dosing to ctenophore tanks require daily seawater replacement.

Procedure

1 Assemble a 15 L plastic bucket system according to manufacturer's guidelines. Systems are available commercially for the conversion of general-purpose 15 L plastic buckets that include air pump, airline tubing and air diffuser (e.g., Reed Mariculture, Compact Culture System).
2 To each bucket, add 15 L of ASW, salinity 20–31 ppt (1.017–1.023 sg), pretreated for removal of chloramines and heavy metals (e.g., Prime water conditioner, Seachem Laboratories).
3 Inoculate buckets with *Brachionus plicatilis* rotifer starter culture (~1 million rotifers, Reed Mariculture).
4 Add 1 mL of concentrated algal mix twice a day.
▲ **CRITICAL STEP** Seawater in culture buckets should be slightly green in color, indicating sufficient algae availability for continuous feeding by rotifers. Rotifer culture productivity is primarily controlled via feeding rate. To increase rotifer populations slowly increase the volume of available algae.
5 Monitor rotifer cultures visually each day for viability, population growth and the presence of undesired protist/ciliate populations.
▲ **CRITICAL STEP** Assess rotifer health by performing counts via light microscopy on 1 mL aliquots from each rotifer culture bucket twice a week.
6 Change the seawater in each rotifer culture bucket once a week to remove algae debris, nitrogenous wastes, undesired protists/ciliates and dead rotifers. To do so, first remove air diffusers and allow rotifer cultures to settle undisturbed for ~30 min, then pour rotifer cultures through a 40 μm sieve.
▲ **CRITICAL STEP** Leave the bottom few centimeters of seawater and settled debris in the bucket. Rinse the rotifer sieve containing concentrated rotifers with clean seawater.
7 Release rinsed, concentrated rotifers into a small container of ASW while cleaning and reassembling the 15 L buckets.
8 Clean rotifer culture buckets with fresh water, wipe bucket surfaces clean of adhering waste and refill with ASW, salinity 23–31 ppt (1.017–1.023 sg), pretreated for removal of chloramines and heavy metals.
9 Distribute concentrated rotifers into the reassembled and clean 15 L buckets. Typically, rotifer counts will transiently drop for 1–2 d post-cleaning before rebounding.
10 To maintain healthy rotifer cultures, harvest no more than ~20–30% volume (3–4.5 L) from each 15 L bucket each day. Replace harvest volume with clean seawater once a day.
▲ **CRITICAL STEP** When manually harvesting rotifer cultures, be sure to use dedicated containers, such as 1 L plastic beakers. This will help to prevent cross-contamination of undesirable protists/ciliates between rotifer cultures that can cause rotifer population collapse.
11 To establish a path for automated transfer from rotifer cultures to ctenophore cultures, attach an appropriate length of silicone tubing leading from the rotifer culture to the inlet side of a peristaltic doser pump.
12 Attach an appropriate length of silicone tubing to the outlet side of a peristaltic doser pump that leads to the ctenophore culture tank.
13 Repeat Steps 11 and 12 as needed for multiple rotifer and/or ctenophore tanks (Fig. 5c,d). Multiple rotifer cultures can be used to dose a single ctenophore tank through the use of a multichannel peristaltic doser pump.
▲ **CRITICAL STEP** Maintain optimal automated doser delivery of rotifers to ctenophore tanks by periodically replacing the silicone tubing feed lines, and flush any in-line check valves.
▲ **CRITICAL STEP** Doser pumps can be programed as desired. For example, 20–30% volume from a single rotifer culture can be delivered to a ctenophore tank every 24 h. Programming is manufacturer dependent.
▲ **CRITICAL STEP** Rotifer culture buckets used for automated dosing to ctenophore tanks should be regularly checked for correct peristaltic pump timing and volume transfer.

entrainment to light cues for efficient control of spawning and the continuous production of high-quality gametes useful for experimental manipulations.

There are currently no artificial diets suitable for long-term ctenophore maintenance. *Artemia* are insufficient for the long-term maintenance of *Mnemiopsis* and other pelagic ctenophore species. Live marine rotifers, *Brachionus plicatilis* (L-type, Reed Mariculture), are our staple food for long-term maintenance of both juvenile and adult *Mnemiopsis* (Fig. 5). Our rotifer husbandry procedures are based on recommendations from rotifer supply and equipment manufacturer (Reed Mariculture) and are highlighted in Box 1. Regulated doses of *Brachionus* are transferred directly to long-term ctenophore cultures in regularly spaced ~20 min intervals via peristaltic pumps calibrated to deliver 6 L per 300 L tank over a 24-h period (Fig. 5c). Extended periods of robust gamete production are achieved by supplementing rotifers with once- or twice-daily feedings of fish larvae. We have had excellent success using freshwater zebrafish larvae (*Danio rerio*) that can be easily sourced from stock

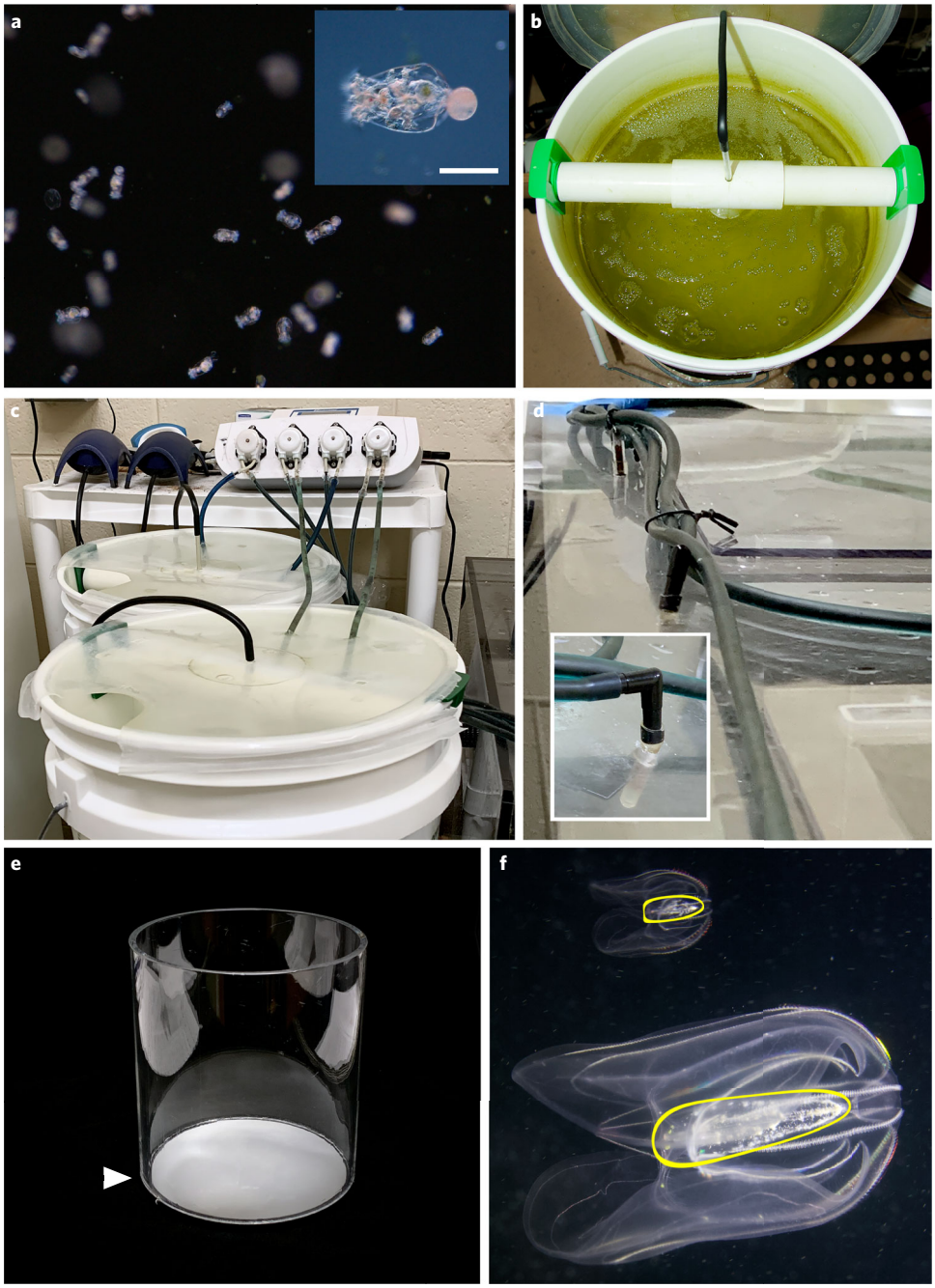


Fig. 5 | Equipment used for maintaining *Brachionus plicatilis* rotifer cultures. a, Live *Brachionus plicatilis*. High-magnification inset at upper right is a dorsal view of an egg-bearing individual, anterior oriented left. Scale bar, 100 µm. **b**, *Brachionus plicatilis* are raised in 15 L buckets. **c**, Programmable peristaltic dosing pumps are used to deliver rotifers from the 15 L rotifer buckets at 20-min intervals. **d**, Rotifer culture is delivered to ctenophores via tubing that connects peristaltic pump outflow to ports drilled into the top panel of the planktonkreisel tank (see inset). **e**, A 40 µm screen is used to sieve rotifer cultures weekly to eliminate waste products and control populations of undesired protists/ciliates. The arrowhead marks the 40 µm screen attached to the bottom of a 10 cm diameter section of acrylic tubing. **f**, Adult *Mnemiopsis* feeding in a planktonkreisel. Yellow ovals demarcate the pharynx. The oral end is oriented to the left. Individual animals are visually screened frequently to ensure the pharynx is ~15–40% occupied with food as an indicator of both the sufficient availability of planktonic food and relative individual health. Note that this qualitative assessment can be made on individuals varying substantially in size and housed within the same tank.

centers (e.g., UM Zebrafish Facility) or commercial aquarium stores (Fig. 2e). Freshwater fish larvae should be briefly washed in ASW and target fed to ctenophores with a pipette (Supplementary Video 1). Larval marine fishes can be added directly to ctenophore culture tanks without the need for

targeted feeding. We have had success feeding ctenophore cultures with a variety of larval marine fishes, including *Coryphaena hippurus* (mahi-mahi), *Rachycentron canadum* (Cobia) and *Atherinops affinis* (topsmelt silverside, 6–15 mm). Cultured copepods and mysid shrimp are also suitable regular prey items for pelagic ctenophores. While others have been able to sustain egg production with cultured copepods and wild mysids[49,75,108,109,116], our best results for maintaining long-term multigenerational cultures competent for daily high-quality spawning are achieved by providing a continuous supply of *Brachionus* rotifers supplemented with fish larvae.

It is critical to avoid overfeeding, as ctenophores will regurgitate the entire contents of their pharynx if overwhelmed with food[42]. The transparency of ctenophores allows for rapid assessment of feeding rates. Approximately 15–40% of the pharynx being filled with food (i.e., 15–40% pharyngeal occupancy) is a good indicator for confirming that individuals have continuous access to food in the water column and for assessing whether the feeding rate is adequate for daily spawning (Fig. 5f). It should be noted that rapid increases in feeding rates can produce spikes in ammonia and nitrite levels that can subsequently lead to tissue damage. In our experience, oral lobe morphology provides a good indicator of relative water quality. Elevated ammonia levels typically result in extensive damage to lobe tissue (lobes that appear 'ragged'). When total ammonia reaches 0.37 ppm, high mortality can result. In most cases, periodic water changes, debris removal and cleaning of tank surfaces will mitigate rapid changes in water quality associated with heavy feeding. Under optimal water quality and feeding conditions, *Mnemiopsis* will rapidly regenerate damaged tissues.

Parasites

A variety of epibiont and endobiont parasites, including hyperiid crustaceans, polychaetes, trematodes, nematodes, cnidarians and protists (including ciliates and amoebas), are known to be associated with *Mnemiopsis* and other ctenophores[149–155]. For example, the ciliate *Trichodina ctenophorii* can often be observed attached to the ctenes of wild-caught *Mnemiopsis*[153]. These ciliates and most other epibionts will typically detach with a few washes of clean ASW and generally do not persist in culture. However, as with any closed-loop aquarium system, buildup of protists, ciliates and bacteria occurs over time. A heavy protist/ciliate infestation can result in ctenophores with ragged lobe morphology and may require tank disassembly and bleaching to effectively eradicate. Biweekly water changes of 5–10% of total system volume, continuous biological filtration and venturi-driven protein skimming are highly effective in keeping most parasite populations in check.

Before introduction into long-term culture tanks, wild-caught ctenophores should be screened for the presence of potential endobiont parasites. Endobiont infections typically appear as opaque lesions, or inclusions, within the mesogleal layer. A common, and particularly detrimental, parasite of *Mnemiopsis* is the sea anemone *Edwardsiella lineata*[154,156]. When present, these parasitic anemones are typically visible embedded within the mesoglea, often adjacent to the pharynx and endodermal canals. Thorough screening of wild-caught ctenophores for *Edwardsiella* infections is necessary before founding long-term cultures. Heavy infestations of these parasites will generally kill the host ctenophore. A culture system inadvertently inoculated with *Edwardsiella* requires disassembly and bleaching to ensure that free-living stages of the parasitic anemone are destroyed.

Spawning

Entrainment to user-defined light cycle

In their natural environment along the North American Atlantic coastline, *Mnemiopsis* spawn at night with variation in the timing of gamete release along a north–south cline. Northern populations of *Mnemiopsis* from Woods Hole, Massachusetts require 6–9 h of darkness before gamete release[78,157]. Southern populations along the Florida coast require 3–5 h of darkness before gamete release[49]. Manipulating light exposure can be easily implemented as an effective tool for controlling the mass release of mature gametes. As seen in Table 1, spawning cues vary among ctenophores. *Mnemiopsis* are self-fertile hermaphrodites; thus, only a single individual is required to obtain viable embryos. Both male and female gonads are located along each ctene row (e.g., Fig. 3a). To increase the production of high-quality gametes for obtaining single-cell embryos for microinjection, adult *Mnemiopsis* are fed fish larvae 24 h before spawning and then fed a fish meal again before being placed in the dark. Spawning of *Mnemiopsis* can be easily entrained to artificial light cues to precisely synchronize and control spawning. Rapid and efficient light cycle entrainment is achieved by exposing *Mnemiopsis* adults to wide-spectrum lighting that also contains short-wavelength ultraviolet light. Our lab-reared southern Florida *Mnemiopsis* reliably spawn in soda-glass bowls (Carolina

Biological Supply) or small plastic tanks (Pioneer Plastics) 3.5–4 h after placing animals in the dark (Fig. 2g,h). Healthy, well-fed, light-entrained animals can be spawned daily.

Animals can be entrained to a custom light cycle spawning regime by shifting the 'lights-off' time in increments of 4 h. The typical 12-h light period can be simply shifted ≤4 h/d until the desired light on/off period is reached. For example, for daytime spawning of *Mnemiopsis*, after ~4 of light exposure, adults are selected and placed in the dark for the spawning cue time specified in Table 1 to stimulate gamete release. After spawning, animals are returned to their long-term culture tank. Thus, by shifting the lighting on/off period of the long-term culture, isolation of individual animals in the dark is a simple method for controlling the time of gamete release. Animals collected from the wild should be started on their natural light cycle with progressive on/off ≤4 h/d shifts until the desired on/off period convenient for experimentation is achieved.

Multiple timed spawning events can be staggered within the same day from animals on the same basic 12-h on/off lighting regime. For example, a morning and afternoon spawning session with our laboratory strain of *Mnemiopsis* maintained on a light on/off period of 1:00/13:00 can be easily achieved as follows. Select individual adults with mature gametes, and place in large soda-glass bowls at 0800 in a dark cabinet or room. These animals will spawn at ~12:00. The remaining animals in the long-term culture tank will transition to light off at 13:00, and individuals with mature gametes can be isolated to large soda-glass bowls for spawning at ~17:00. Thus, experiments requiring precisely synchronized spawns and/or embryos at a specific time can be accommodated without difficulty.

Spawning

After ~3.5 h of dark exposure, animals are introduced to light and screened under a dissecting microscope for mature gametes in soda-glass bowls. In gravid animals, mature sperm and oocytes are visible at the gonopores between ctene plates flanking the underlying endodermal canal[158] (Fig. 3a). Mature sperm often give the ctene rows a gray/white appearance. Approximately 1 h before gamete release, mature sperm compacts between the ctene rows on the male side. During gamete release, the concentrated sperm appears iridescent and can be seen streaming out of the male gonopores. *Mnemiopsis* are broadcast spawners that produce large quantities of sperm. When mature oocytes are presented with too many sperm, high rates of early embryonic failure due to polyspermy can occur. To mitigate against polyspermy and reduce the concentration of sperm in spawning bowls, *Mnemiopsis* are washed two to three times during the initial release of sperm with clean ASW. Typically, mature sperm release initiates at the aboral end of the animal and begins ~10–15 min before mature oocyte release. The mature oocytes are squeezed through the female gonopores opposite to the male gonopores and are fertilized almost immediately by motile sperm in the surrounding seawater. Within a few minutes of oocyte release, egg jelly swells to fill the space between the egg and the expanded outer vitelline membrane.

Eggs are typically competent for fertilization within the first ~30 min of release. In contrast, *Mnemiopsis* sperm remain highly active for several hours after release and sperm swimming activity can persist for as long as 24 h. Egg fertilization rates drop as the outer vitelline membrane expands and the egg jelly swells. To ensure high fertilization rates, we allow shed eggs to remain in spawning bowls for ~10 min after oocyte release before collection. An entire spawning event generally spans 60–90 min from initial sperm release through final egg release. Importantly, we have not observed abnormal phenotypes in progeny derived from successive self-fertilization spawns in our laboratory culture (currently F52, with all generations derived from successive single animal self-fertilizations). Development of crossing schemes will be necessary in the future to facilitate crosses between genetically distinct strains, for example to maintain strains derived in the laboratory via transgenesis or to maintain genetic variation from naturally occurring populations of biological interest.

Embryogenesis and juvenile staging

Embryogenesis

Mnemiopsis embryos undergo a stereotypical, ctenophore-specific cleavage program[73,79,83]. Fluorescent labeling with vital dyes provides a nondestructive means to monitor the development of live embryos and track tissue and organ morphogenesis (as detailed in Fig. 3 (ref. [96])). At room temperature (RT, ~20 °C), gastrulation occurs from 3 to 6 h post-fertilization (hpf), during which aborally located ectodermal micromeres migrate via epiboly to the oral side of the embryo and invaginate to form the blastopore[79,83]. Cellular thickenings visible by 10 hpf on either side of the tentacular axis will develop into the tentacle bulbs and tentacles (Fig. 3e–e′). One of the first

ectodermal structures to appear are the four pairs of ciliary ctene rows, with one pair per quadrant located towards the aboral end of the embryo[83] (Fig. 3f–f′). Each individual ctene row is composed of ectodermally derived polster cells that produce giant motile cilia that fuse into paddle-like structures used to propel the animal through the water column[22]. Distinct organs including the apical organ, pharynx, tentacles and tentacle bulbs are visible in the developing embryo by 14 hpf (Fig. 3g). Five embryonic cell lineages contribute to the development of both the tentacle and tentacle bulb[79,159]. Ectodermal cells associated with the two lateral thickenings invaginate and, along with medial internal endodermally derived cells, form the tentacle bulb. The tentacle core muscles, derived from endoderm, are rooted within the tentacle bulb. In contrast, colloblast cells and other epithelial cell fates associated with the tentacle and tentacle bulb are derived from ectoderm[79,84,96,159]. At this stage, the 'middle' mesogleal space also begins to expand with ECM deposition and the embryo begins to increase dramatically in size. Aborally, the emergence of the first lithocytes is also beginning. As lithocytes differentiate, they are transported along balancer cilia to form the statocyst of the apical organ[30,96,160]. Juvenile cydippids hatch ~18–22 hpf (Fig. 3i–k′).

Juvenile staging

Juvenile cydippids are raised at RT in 10 L glass (Carolina Biological Supply) or 1.5 L plastic rearing tanks (Pioneer Plastics), from hatching to sexual maturity in ASW saturated with rotifers (Fig. 2f,h) before being transferred to larger pseudokreisel or planktonkreisel tanks (Fig. 2a,b). Cydippid stage tentacles are exposed to the environment (e.g., Figs. 1e and 4a–l′) and are thus particularly prone to damage. When transferring juveniles, we trim plastic transfer pipettes to enlarge the openings for unrestricted entry of cydippids (Fig. 2j). It is critical to allow cydippids to retract their tentacles as they are gently suctioned into the transfer pipette. For experiments that require tracking unique individuals, we rear newly hatched individual cydippids in plastic 1.5 L rearing tanks (Pioneer Plastics) or 400 mL Tri-Pour beakers (VWR) saturated with rotifers for the first 7 d post-hatching (dph; Figs. 2h,i and 4a–g).

Rearing tanks/containers do not require an active current, aeration or filtration (Fig. 2f). Juvenile *Mnemiopsis* cydippids are resilient to rapid changes in water quality, and a still water environment enhances their ability to ensnare prey (Supplementary Video 2). Approximately once a week, or when the surfaces of rearing tanks become fouled, juvenile cydippid ctenophores are gently transferred, with trimmed pipettes, to clean rearing tanks with fresh ASW saturated with rotifers. A rearing tank saturated with rotifers can support hundreds of juveniles for the first 1–4 dph. However, to reduce mortality and attain maximal cydippid growth rates, we raise no more than ~50 juveniles in a 10 L rearing tank. Under optimal conditions in which food is not limiting, cydippid stage *Mnemiopsis* will approximately double in size each day for the first 11 d; however, there can be substantial variation in size and growth rate among individuals from the same spawning event.

Cultured *Mnemiopsis* are reproductively competent within ~14–16 dph under ideal growth conditions (see post-hatching details in Fig. 4). In culture, juveniles typically swim through the water column, mouth facing forward. During active feeding, juveniles swim in a looping pattern as they deploy their tentacles and tentilla[28,161] (Supplementary Video 3). At ~8 dph, some juveniles are capable of dissogeny and can produce viable gametes for a short period of time[49] (but see ref. [51]). In culture, the progeny of early reproduction events are readily apparent as an additional class of 'new' smaller juveniles in rearing tanks. The extensible, free-hanging, juvenile tentacles used for prey capture remain during the transition to the adult lobate form (Fig. 4i–l′). Between 13 dph and 16 dph, several morphogenetic patterning events lead to the final adult body plan, including the development of the tentilla-lined adult feeding grooves (Fig. 4m–o′). As the feeding grooves develop, the proximal base of each tentacle bifurcates as it emerges from the tentacle sheath and the tentacles begin to travel along the outer ridge of the newly formed adult feeding grooves. As the feeding grooves continue to develop, tentilla emerge at regular closely spaced intervals along the tentacle-lined outer ridge. Complete resorption of the free-hanging juvenile tentacles takes place over several days. Under optimal growth conditions in the laboratory, food availability exceeds natural conditions (high relative concentration and extended duration). Under conditions of high feed concentration paired with continuous availability, cultured *Mnemiopsis* are reproductively competent at 14–16 dph and are 12–20 mm in size (Fig. 4o–o′).

Microinjection

Preparation and microinjection of embryos

Microinjection (Fig. 6) of small molecules into *Mnemiopsis* embryos has been used to deliver lipophilic dyes to analyze cell fates associated with early embryonic cell lineages[79,82–84], mRNA to track

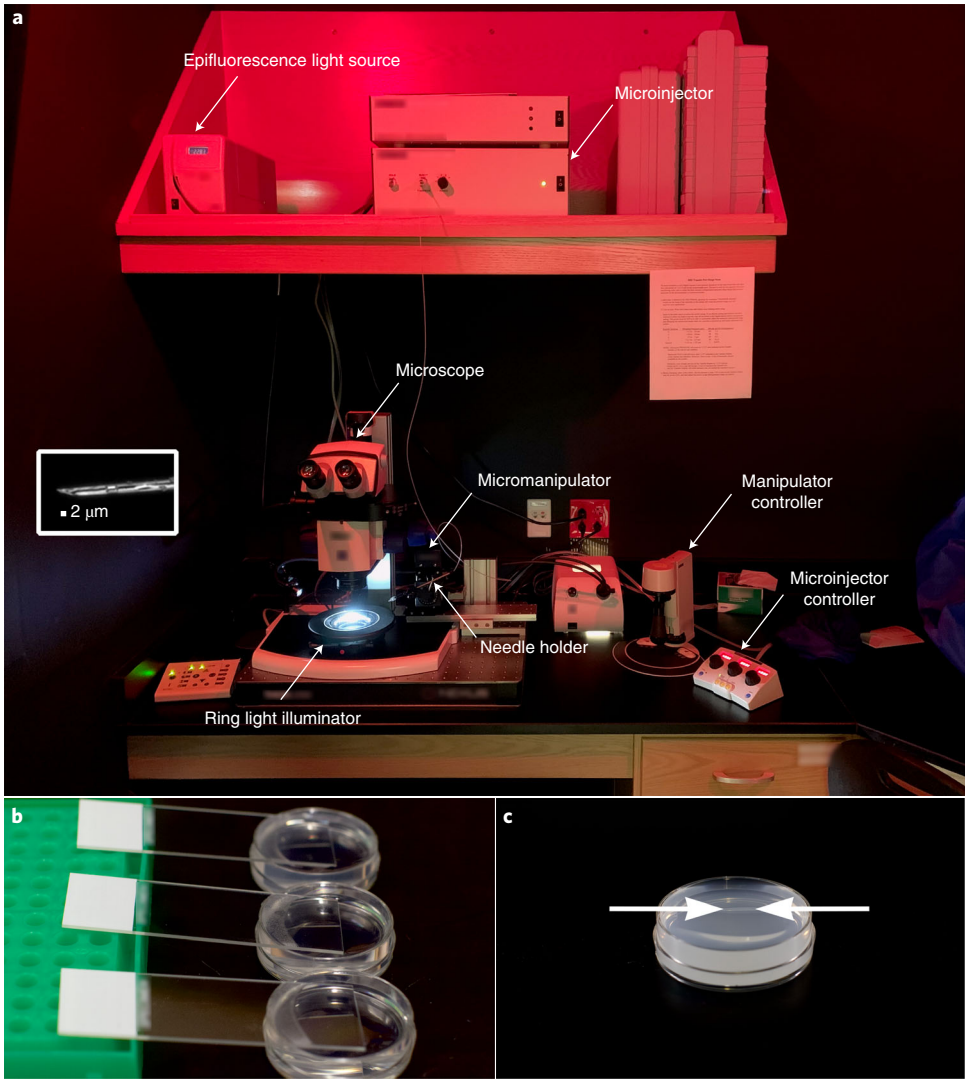


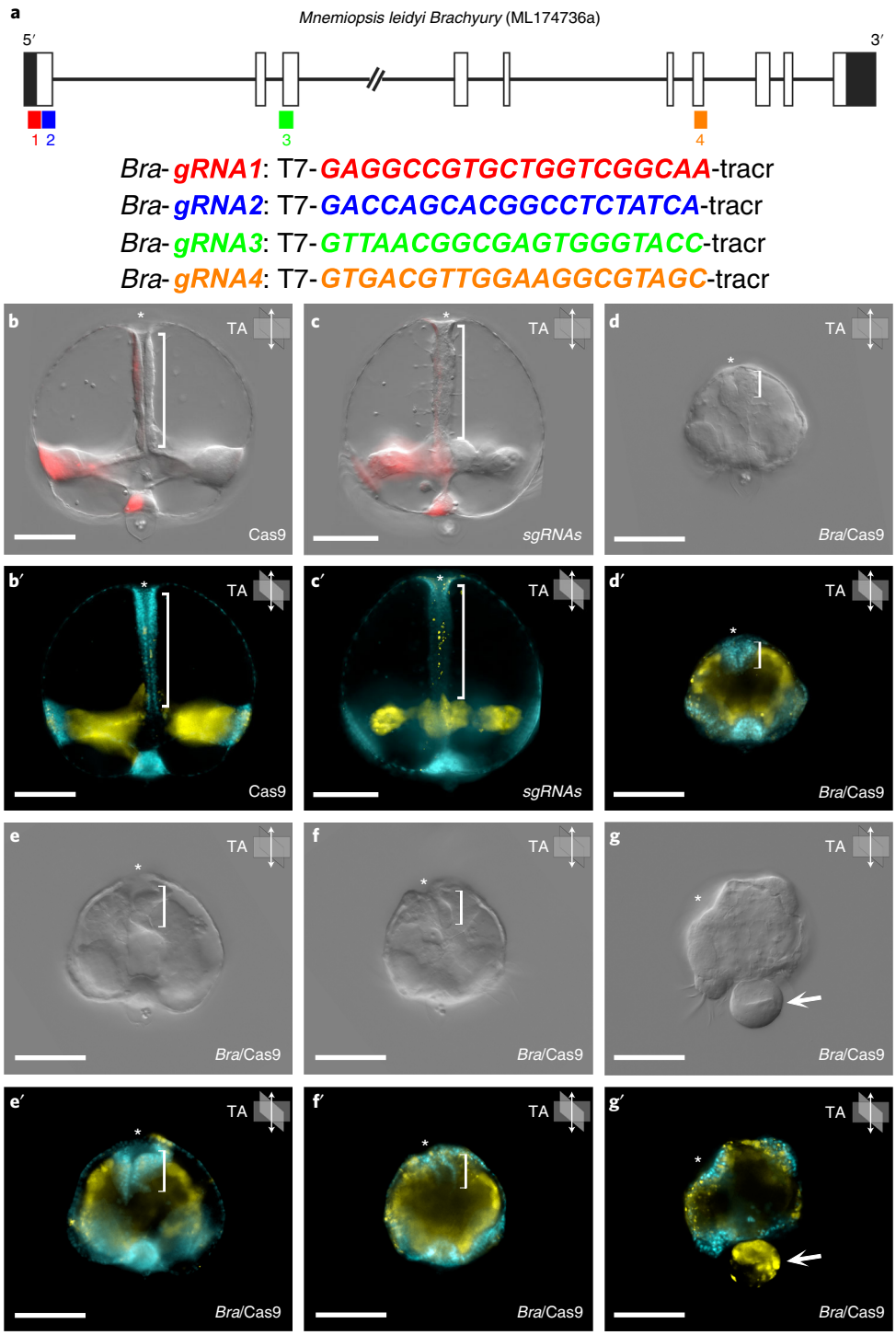
Fig. 6 | Microinjection. a, The microinjection station here is arranged for a right-handed operator. A large-working-distance stereoscopic dissecting epifluorescence microscope is paired with ring light illumination from below and a micromanipulator fitted with a needle holder. The micromanipulator is operated via an inverted joystick for dynamic fine-scale *XYZ*-plane adjustment of needle positioning. Pressurized gas is fed to the needle holder via a microinjector with a controller for fine-scale adjustments to delivery gas pressure. Fluorescent molecules can be monitored via an epifluorescence light source and appropriate detection filters mounted in the microscope light path. Inset on the left is a closeup of a beveled microinjection needle. Scale bar is 2 µm and is representative of the approximate inner diameter opening of the beveled needle tip. **b**, Standard glass microscope slides positioned at a 25–35° angle are used to form an impression in the cooling agarose. **c**, When the microscope slide is removed, the impression creates a steep backstop in the agarose, the 'egg trough' (arrows), against which fertilized and prepared eggs are arranged immediately before microinjection.

subcellular localization of specific proteins[162], MO to knock down gene expression during embryogenesis[66,127], and CRISPR–Cas9 components to abrogate the function of transcription factors during embryogenesis[96]. Here we provide a detailed protocol for microinjection of recombinant Cas9 protein coupled with single-guide RNAs (sgRNAs). This microinjection protocol can be used to deliver a diverse range of small molecules into *Mnemiopsis* embryos.

Using a dissecting microscope, fertilized eggs with expanded outer vitelline membranes are collected via pipette. To remove the excess mucus shed during gamete release and soften both the vitelline membrane and inner egg jelly, the collected eggs are passed through 500 µm and 400 µm mesh filters (pluriSelect Life Science). The fertilized and filtered eggs are then passed through three exchanges of filtered sterile seawater (FSW) + 1× penicillin/streptomycin (psFSW). The outer vitelline membrane is refractory to microinjection needles and must be removed. The inner egg jelly

can also clog microinjection needles and should be removed from the egg surface. Acid-sharpened tungsten needles (preferred), or very fine forceps (Dumont #55), are used to remove the vitelline membrane and egg jelly in gelatin-coated dishes. Applying a gelatin coating to dishes prevents the devitellinized single-celled embryos from adhering to plastic and glass surfaces.

First cleavage begins ~60 min after fertilization. With experience, ~60 embryos can be collected, washed and devitellinized for microinjection at the single-cell and two-cell stage. In many cases, the use of both single-cell and two-cell embryos for injection can be insightful. Ctenophores exhibit strict lineage determination during embryonic development[78,80]. Blastomeres separated at the two-cell stage



will develop into viable half animals[75]. Thus, microinjection into one of two blastomeres will affect one half of the developing animal, leaving the other half of the animal as a contralateral uninjected control (Fig. 7b–c′).

Once devitellinized, eggs are transferred via mouth pipette to an injection dish with an egg trough to position embryos for microinjection. Immediately before microinjection, a few drops of FSW are added to fill the egg trough. Devitellinized embryos are then transferred to the egg trough via mouth pipette and gently lined up against the egg trough wall for microinjection.

The use of reliable and uniformly sharp injection needles is key to rapid microinjection of embryos. While many options exist for making suitable microinjection needles, we use a Brown micropipette puller (P-1000, Sutter Instruments) to prepare aluminosilicate needles (AF100–64–10, Sutter Instruments) and then bevel the tips (BV-10-D, Sutter Instruments). Beveling creates a consistent microinjection needle with uniform tip characteristics (Fig. 6a) that are optimized for egg penetration, substantially reducing embryo mortality. Just before injection, beveled needles are loaded via backfilling with the injection cocktail spiked with fluorescently conjugated dextran (Invitrogen) for rapid assessment of injection success and subsequent lineage tracing (Fig. 7b,c). While many options are available for microinjection systems, we mount our loaded needles to a Xenoworks microinjection system (Sutter Instruments) paired to an epifluorescence stereo microscope (Discovery.V8, Zeiss; Fig. 6a). After microinjection, embryos are transferred to gelatin coated plastic dishes for post-injection recovery and incubation to the desired developmental stage for additional analyses. The required preprocessing of embryos, and temporally rapid early cleavage events, make microinjections most efficient for two researchers working in tandem: one collecting and devitellinizing embryos while the other prepares injection reagents and performs the microinjections.

CRISPR–Cas9-mediated *Brachyury* knockdown example

Brachyury, the founding member of the T-box transcription factor family, has well-known roles in embryonic development during gastrulation, mesoderm and endoderm specification, organogenesis and the regulation of morphogenesis[163,164]. Functional characterization of *Brachyury* in cnidarians and *Mnemiopsis* supports a role for *Brachyury* expression in regulating embryonic morphogenetic movements during invagination of the blastopore that will subsequently give rise to the stomodaeum and pharynx[127,163,165,166]. We targeted the *Brachyury* gene (ML174736a) as an example here for our CRISPR–Cas9 genome editing protocol in *Mnemiopsis* (Fig. 7).

Genome editing specificity via the CRISPR–Cas9 system is achieved through the use of sgRNAs that have sequence complementarity to genomic target sites[167]. In this example, we identified *Bra* target sites and assessed potential off-target sites (OTS) in the *Mnemiopsis* reference genome[128,129] using the Cas9 module in Geneious R11 and CasOT[168]. To mitigate against potential OTS activity, target sequences chosen for sgRNAs met one of two criteria: (1) sequence had no possible OTS, or (2) if the sequence had possible OTS, then the OTS needed three or more mismatches. Cas9 activity diminishes with multiple sequence mismatches[169]. sgRNAs were designed according to previous protocols[170–172]. In brief, each crispr RNA (crRNA) oligonucleotide template consisted of a T7 promoter, 20 nucleotides complementary to *Bra* genomic target sequences, and an additional 20

◀ **Fig. 7 | Pharynx formation is disrupted in *Mnemiopsis leidyi* by *Bra* sgRNA/Cas9 microinjection. a**, Schematic gene model for *Mnemiopsis leidyi Brachyury* (*Bra*), ML174736a. The 5′ and 3′ UTRs are black blocks, and exons are white blocks. Colored blocks below the gene model mark the position of genomic sequences used for gRNAs shown in corresponding colors. **b**–**g** in vivo DIC images are matched with **b**′–**g**′ in vivo fluorescent vital dye images where yellow LysoTracker fluorescence marks lysosomal acidic vacuole distribution and blue Hoechst fluorescence marks nucleic acid distribution. For all panels (**b**–**g**′) scale bars are 100 µm, oral is up. TA, tentacular axial plane; PA, pharyngeal axial plane. Asterisks identify the opening of the mouth (stomodeum). Brackets identify the position of the pharynx. In **b** and **c**, the distribution of red fluorescence corresponds with dextran tracer dye microinjected into one of two blastomeres at the two-cell stage. **b**–**b**′, Cas9 protein was microinjected into one of two blastomeres at the two-cell stage to assess untargeted Cas9 activity; no effect on embryonic development was observed. **c**,**c**′, Cocktail of the four *Bra* sgRNAs were microinjected into one of two blastomeres at the two-cell stage; no effect on embryonic development was observed. In **d**–**g**′, *Bra* sgRNA/Cas9 was microinjected at the one-cell stage, resulting in severe morphogenic and developmental defects that phenocopy results from *Bra*-morpholino microinjections[127]. In particular, the pharynx fails to extend aborally to join with the gastrovascular system, resulting in foreshortening of the pharynx and malformation of the stomodeum. In addition to pharyngeal defects, the gastrovascular system develops abnormally, including examples of extrusion of acellular yolk aborally during embryogenesis highlighted by an arrow in **g** and **g**′.

Table 2 | *Mnemiopsis leidyi Bra* guide primer sequences

Guide primer	Template sequence
Bra-gRNA1	GAAATTAATACGACTCACTATA***GGAGGCCGTGCTGGTCGGCAA***gttttagagctagaaatagc
Bra-gRNA2	GAAATTAATACGACTCACTATA***GGACCAGCACGGCCTCTATCA***gttttagagctagaaatagc
Bra-gRNA3	GAAATTAATACGACTCACTATA***GGTTAACGGCGAGTGGGTACC***gttttagagctagaaatagc
Bra-gRNA4	GAAATTAATACGACTCACTATA***GGTGACGTTGGAAGGCGTAGC***gttttagagctagaaatagc
Universal tracrRNA	AAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTgctatttctagctctaaaac

All sequences are oriented 5′–3 ′. Underlined nucleotide sequence corresponds to the T7 promoter. Bold italicized nucleotide sequences correspond to genomic *Bra* targets and include addition of two 5′ G residues to aid T7 polymerase binding. Lowercase nucleotide sequence denotes region of complementary between gRNA primers and Universal tracrRNA primer, which are annealed to form the sgRNA transcription template.

nucleotides complementary to a universal tracrRNA oligonucleotide template (Table 2 and Fig. 7a). Both the crRNA and *trans*-activating crRNA (tracrRNA) oligonucleotides were mixed and annealed to form the sgRNA template. This resulting DNA template was then used to produce sgRNAs for microinjection via in vitro transcription with T7 polymerase (MEGAscript) followed by purification, quantification and storage at −80 °C in single-use aliquots.

By microinjecting a cocktail of four *Bra*-sgRNAs and Cas9 protein, we recapitulated the phenotypes observed in *Mnemiopsis* from a previous study in which Bra activity was reduced via translation inhibiting morpholinos[127] (Fig. 7a). Our microinjection solution consisted of 35% glycerol base mixed with each *Bra*-sgRNA at 100 ng/µl, recombinant Cas9 protein (PNA) at 400 ng/µl, and fluorescent dextran (10,000 MW, Invitrogen) at 0.5 ng/µl as a visual tracer (Fig. 7). Post-microinjection, embryos were transferred via mouth pipette to gelatin-coated plastic or glass dishes and incubated at RT until reaching the cydippid stage (~20 hpf). Cydippids were then incubated in a cocktail of vital dyes for ~1 h at RT before in vivo imaging (Fig. 7b′–g′). To assess mortality associated with embryo preparation and microinjection, we measured the percentage of unmanipulated embryos that developed normally into free-swimming cydippids (85%) compared with the percentage of devitellinized uninjected *Mnemiopsis* embryos that developed normally into free-swimming cydippids (80%) (ref. [96]). Similar ratios for normal development were observed in Cas9 protein only microinjections and sgRNA cocktail only microinjections (Fig. 7b,c). Vital dye labeling of freshly hatched uninjected and control cydippids showed normal pharynx development, tentacle bulb formation and gastrovascular system patterning (Figs. 3i′–k′ and 7b′–c′). These results show that neither devitellinized embryos nor control microinjections into embryo blastomeres had an effect on the normal embryonic development of *Mnemiopsis*.

During gastrulation, *Bra* is initially expressed in ectodermal cells surrounding the blastopore[86]. Expression of *Bra* continues during invagination of ectodermal cells surrounding the blastopore and the subsequent development of the pharynx[86,127]. As development proceeds, the pharynx elongates aborally and ultimately connects with the endodermally derived gastrovascular system (Fig. 3e,g,j). Late stages of pharynx elongation are also accompanied by an increase in embryo size as mesogleal ECM deposition begins between outer ectodermal and inner endodermal tissues (Fig. 3g,j). Previous work using MO to reduce expression of *Bra* transcripts (*Bra*-MO) resulted in abnormal pharynx development, providing evidence for inhibition of normal morphogenesis by *Bra*-expressing ectodermal cells surrounding the blastopore[127]. *Brachyury* has recently been shown to have functionally equivalent roles during pharynx morphogenesis in cnidarians[165,166].

In contrast to normal development, *Bra*-Cas9-injected embryos show consistent pharyngeal elongation defects along with a failure to extrude mesogleal ECM (Fig. 7d–g′). The arrest of pharyngeal elongation, gastrovascular system defects and absence of mesogleal expansion phenocopy Bra-MO knockdown results[127]. Whether mesogleal ECM deposition is causally associated with pharyngeal elongation is unclear, as the mechanisms underlying ECM production are unknown. Additionally, we observed abnormal tentacle bulb development and apical organ development defects also present in Bra-MO knockdown morphants[127]. Our CRISPR–Cas9 *Bra* crispant (G0-injected embryo) results independently phenocopy Bra-MO morphant knockdown results, providing additional independent support for *Brachyury* playing an ancestral role in mediating the development of adhesion boundaries between ectodermal and endodermal germ layers[165,166]. Microinjection of targeted sgRNA/Cas9 reagents produce consistent and robust phenotypes in *Mnemiopsis*.

Materials

Biological materials

- *Brachionus plicatilis* (Reed Mariculture)
- *Mysidopsis bahia* (Aquatic Indicators)
- Zebrafish larvae (e.g., UM Zebrafish Facility)
- Marine fish larvae (e.g., UM RSMAS Experimental Fish Hatchery)
- *Mnemiopsis* (wild caught or shipped from our facility)

Reagents

- Dechlorinated fresh water
- Sea salts (Instant Ocean)
- Prime water conditioner (Seachem Laboratories)
- MitoTracker (Invitrogen, cat. no. M22426)
- LysoTracker (Invitrogen, cat. no. L7528)
- Hoechst 33342 (Invitrogen, cat. no. H1399)
- Dextran 10,000 MW (Invitrogen, cat. no. D22910)
- Agarose (Sigma, cat. no. A9539)
- Cas9 protein with NLS (nuclear localization signal) (PNA Bio)
- EDTA (Sigma, cat. no. E5134)
- Ethanol (Sigma, cat. no. E7023) **! CAUTION** This reagent is highly flammable and an eye irritant.
- Gelatin, unflavored (Knox)
- Glacial acetic acid (Mallinckrodt, cat. no. V193-14) **! CAUTION** This reagent is flammable and a severe eye and skin irritant.
- Glycerol, molecular grade (Sigma, cat. no. G5516)
- MEGAscript T7 transcription kit (Invitrogen, cat. no. AM1333)
- Magnesium chloride (Sigma, cat. no. M2670)
- Nuclease-free water (Ambion, cat. no. 4387936)
- Penicillin (Sigma, cat. no. P7794)
- Streptomycin (Sigma, cat. no. S9137)
- QIAquick PCR purification kit (Qiagen)
- RainX solution (ITW Global Brands)
- Sodium hydroxide (Sigma, cat. no. 567530) **! CAUTION** This reagent is an eye and skin irritant.
- TAE electrophoresis buffer solution
- Taq, HotMasterMix (Quantabio)
- Trizma base (Sigma, cat. no. T6066)
- RG Complete algal concentrate (Reed Mariculture)
- DNA oligos for crRNA and tracrRNA templates (Integrated DNA Technologies)

Equipment

- Pseudokreisel tanks, 12 L, 72 L, 150 L
- Planktonkreisel, 300 L (Midwater Systems)
- Flow meter (Blue-White Industries, model F-44500L-8)
- Glass aquaria for juvenile grow-out, 10 L (Carolina Biological Supply Company, cat. no. 671226)
- Plastic aquaria for juvenile grow-out, 1.5 L (Pioneer Plastics, cat. no. 083CAQUA)
- Rotifer culture system (Reed Mariculture, Compact Culture System)
- Rotifer harvesting sieve, 40 μm (Reed Mariculture)
- TriPour for individual juvenile grow-out, 400 mL (VWR, cat. no. 25384-156)
- Plastic pipettes, 7.5 mL, 4 mL, 1.5 mL (VWR, cat. nos. 414004-004, 414004-007, 16001-192)
- Wet-dry trickle filter (Midwater Systems)
- In-line seawater chiller (TradeWind Chillers)
- Nalgene vacuum filter, 0.2 μm (VWR, cat. no. 150-0020)
- Cell strainers (pluriStrainer, pluriSelect, cat. no. 43-50000-97)
- Cell strainer, 70 μm (Corning, cat. no. 431751)
- Capillary Glass, aluminosilicate with filament (OD 1 mm, ID 0.64 mm; Sutter Instruments, cat. no. AF100-64-10)
- Glass capillary puller, P-1000 (Sutter Instruments)
- Digital microinjector, Xenoworks (Sutter Instruments)

- Micromanipulator, Xenoworks (Sutter Instruments)
- Glass capillary beveler, BV-10-D (Sutter Instruments)
- Glass capillary storage box (Sutter Instruments, cat. no. BX20)
- Glass capillary storage jar (World Precision Instruments, cat. no. E210)
- Dissecting stereo microscope (ZEISS)
- Epifluorescence light source and detection filters (ZEISS)
- Microcentrifuge (Eppendorf)
- Thermocycler (Eppendorf)
- Plastic petri dishes, 35 mm (VWR, cat. no. 351008)
- Glass microscope slides (VWR, cat. no. 48311-950)
- Tungsten wire needles (EMS, cat. no. 73800)
- Handheld LED light source for viewing cydippids
- Airline tubing
- Soda-glass bowls, 8 in, 4 in, 2 in, 1 in (Carolina Biological Supply, cat. nos. 741006, 741004, 741000, 740996)
- Black vinyl material, dark shroud

Equipment setup

Preparation of beveled microinjection needles

Preparing microinjection needles varies widely depending on the type of glass capillaries, puller and heating filament employed. Using Sutter Instruments aluminosilicate glass capillaries (with an internal filament for backloading), browning capillary puller and box style heating filament optimized for pulling short, straight and symmetrical needles, we recommend the following starting parameters for needle pulling: Pull = 60, Velocity = 90 and Time = 200. For detailed guidelines on adjusting parameters, we suggest consulting the 'Micropipette Cookbook' (https://www.sutter.com/PDFs/pipette_cookbook.pdf). For *Mnemiopsis* injections, needles with a relatively shorter taper and finer tip are preferred. We recommend beveling needles to create a consistent, hypodermic-style opening (Fig. 6a, inset). Briefly, using a Sutter BV-10 turntable style beveler, place a pulled needle into the capillary holder and adjust to ~20° angle of attack. Use a Kimwipe dampened with dH_2O to wet the spinning grindstone. Gradually lower the needle tip until it just contacts the stone. Grind until water enters the needle tip. Continue grinding to produce a smooth hypodermic-style tip. After ~2–3 min, use a dry Kimwipe to remove water from spinning grindstone and continue grinding until no water remains within the newly beveled needle tip. Gradually raise the beveled needle from the spinning grindstone. Preparation of each needle requires ~5–10 min. After beveling, the prepared needles can be stored indefinitely at RT in either an electrode storage jar or in a pipette storage box.

Microinjection dish

Dishes used to hold or secure embryos for microinjections vary widely between different animal systems and labs. All are designed to immobilize embryos so they remain stationary during microinjection. Our preferred microinjection dish utilizes an angled trough with ~90° backstop opposite of the needle entry point. The dish is made with common lab ingredients and can be stored between multiple uses. While we have been successful with this type of injection dish, we encourage researchers to make modifications and improvements to meet their explicit experimental demands. Briefly, make enough ~2% agarose solution at a 1:1 volume ratio of FSW:dH_2O to fill several 35 mm plastic dishes. Once the agarose solution is cool enough to handle, add penicillin/streptomycin to a final concentration of 100 units/ml penicillin and 100 µg/ml streptomycin (1×) and pour the molten agarose solution into the dish until the agarose meniscus touches the top edge of the dish. To form a trough impression in the cooling agarose we set a standard glass microscope slide at a 25–35° angle with one end resting just under the top edge of a 35 mm plastic dish creating an 'egg trough' with a steep backstop (Fig. 6b,c). Once the agarose sets, the microscope slide can be removed. If necessary, use a razor blade to trim any excess agarose. The dish can be used immediately or stored at 4 °C saturated with psFSW for later use. Before use, rinse the prepared microinjection dish with FSW a few times. Post-injection, rinse the injection dish with FSW a few times, fill with psFSW and cover with the original lid (or plastic wrap). An injection dish can be used for multiple injection sessions, when stored between sessions at 4 °C saturated with psFSW. To prevent unintended microbial contamination of injected embryos, we recommend replacing agarose-based microinjection dishes every 2–3 weeks.

Procedure

Adult *Mnemiopsis* maintenance ● Timing duration of culture

1 Maintain adult *Mnemiopsis* in a recirculating pseudokreisel at a maximum density of approximately one animal per 10 L for optimal health. The optimal water temperature is 20 ± 2 °C. Ambient room lighting (≥900 lumens) should be on for 8–12 h and off for 12–16 h.
▲ CRITICAL STEP To counteract parasitic infestations and/or outbreaks that require dismantling recirculating systems, prescreen and quarantine ctenophores for several days to prevent introduction of parasites to culture tanks.
? TROUBLESHOOTING

2 Allow animals 2–3 d to acclimate to new light on/off times
▲ CRITICAL STEP Light cycle shifts should not exceed 4 h within a single 24-h period. For example, if the desired on/off time is 8 h from the current on/off schedule, 48 h will be required to shift on/off time to the desired schedule.

3 Maintain rotifer feed cultures in 15 L buckets as described in Box 1.
▲ CRITICAL STEP Maintain optimal automated doser delivery of rotifers to ctenophore tanks by removing old silicone tubing feed lines, replacing with new silicone tubing and flushing any in-line check valves at 6-month intervals.
? TROUBLESHOOTING

4 Feed adult *Mnemiopsis* a continuous supply of rotifers (Box 1) for daily gamete production. Rotifers are transferred directly to the pseudokreisel housing *Mnemiopsis* via peristaltic pumps. For example, ~6 L of rotifer culture is transferred over a 24-h period to a 300 L pseudokreisel. A rotifer-based diet can also, optionally, be supplemented with aperiodic feeding with live copepods and/or live mysid shrimp.
▲ CRITICAL STEP Continuous pharyngeal occupancy of 15–40% is a good visual indicator of sufficient food availability.
▲ CRITICAL STEP If possible, target feed adult *Mnemiopsis* approximately a dozen fish larvae once or twice per day for robust repeatable daily gamete production.
▲ CRITICAL STEP Remove uneaten fish with a fine mesh net, or siphon, after targeted feedings to maintain good water quality.
▲ CRITICAL STEP Remove settled debris and accumulated particulate waste products from the bottom of the tank via vacuum/siphon once or twice per week.
▲ CRITICAL STEP Remove protein skimmer waste and clean cup/column two or three times per week to maintain optimal protein skimmer function.

Spawning *Mnemiopsis* ● Timing 24–48 h

5 We provide spawning details for collecting individual zygotes on a user-defined schedule for immediate experimental use (option A) or for general long-term strain/stock maintenance and propagation (option B).

(A) **Spawn adult *Mnemiopsis* to obtain individual embryos for immediate experimental use**

(i) If possible, provide a fish meal to adult *Mnemiopsis* that will be used for spawning ~24 h before spawning and then again right before the beginning of the dark cycle. This feeding can be performed on animals housed in the main culture tank.

(ii) Place each selected adult *Mnemiopsis* into an 8-inch (1,500 mL) soda-lime glass finger bowl, filled with ASW, in the dark for 3.5 h.
▲ CRITICAL STEP A dark phase of at least 3.5 h is required for reliable user-defined spawning. Refer to Table 1 for temporal variation in *Mnemiopsis* spawning and timing parameters associated with other ctenophore species.
▲ CRITICAL STEP Ensure that animals have been entrained to the desired light regime before spawning, particularly if the chosen spawning time requires a shift of ≥4 h.

(iii) Expose selected *Mnemiopsis* to light, and replace 50–75% of the ASW with clean ASW. This is done by slowly decanting the old ASW to prevent the inadvertent loss of the animal.

(iv) Inspect each animal to monitor gamete maturation status. Imminent sperm release is visually associated with the compaction of mature iridescent sperm around male gonopores between ctene rows. For each spawning cycle, set aside two to six gravid individuals in individual 8-inch soda-lime glass finger bowls. Oocyte shedding from female gonopores should begin ~10–15 min after sperm release has begun. Fertilization occurs during or shortly after oocyte release.

▲ CRITICAL STEP Animals should be washed with ASW during sperm release by replacing 50–75% of the spawning container volume two or three times to prevent polyspermy.

(v) Allow shed oocytes to remain in the spawning bowl for ~10 min to increase egg viability by ensuring both fertilization and the initial expansion of the outer vitelline membrane before zygote collection.

? TROUBLESHOOTING

(B) **Spawn adult *Mnemiopsis* for general long-term strain/stock maintenance and propagation**

(i) To screen adult *Mnemiopsis* for gamete production/maturation, place an adult animal in a 10 L spawning tank with 0.5–1 L of rotifer culture. Target feed *Mnemiopsis* approximately a dozen fish larvae once or twice per day during confinement to the spawning tank. Initial gamete release typically occurs during the first overnight period.

▲ CRITICAL STEP A high concentration of rotifers in the spawning tank is necessary to ensure that newly hatched cydippids have a high probability of prey capture soon after hatching.

(ii) Add 200–500 mL of rotifer culture water to the spawning tank daily.

(iii) Screen spawning tank for the appearance of cydippids daily. This can be done relatively easily with side illumination against a dark background to maximize light scatter contrast through translucent cydippids.

(iv) Remove adult *Mnemiopsis* from the spawning tank, and return to the long-term culture tank after the appearance of desired numbers of cydippids.

▲ CRITICAL STEP Limit water disturbances during the first 1–2 dph to reduce damage to juvenile cydippid tentacles.

? TROUBLESHOOTING

Juvenile *Mnemiopsis* maintenance ● Timing 14–30 d

6 Keep juvenile *Mnemiopsis* cydippids at relatively high densities of >100 cydippids per liter of seawater saturated with rotifers in 10 L spawning tank for the first 7 dph or until they are ~3–5 mm in size (oral–aboral length; ~7 d)

? TROUBLESHOOTING

7 Reduce animal density to approximately five juveniles per liter through metamorphic transition to adult morphology (~10 mm in size) When rotifers are not limiting, sexual maturity can be achieved in as few as 14 dph. Extra juveniles are culled and/or used for downstream processes, for example nucleic acid preparations or protein extractions.

▲ CRITICAL STEP Transfer juveniles to clean 10 L or 1.5 L tanks with ASW once a week.

▲ CRITICAL STEP Maintain high rotifer densities to maximize juvenile growth rates.

? TROUBLESHOOTING

8 Transfer newly metamorphosed adults to larger recirculating pseudokreisel tanks for long-term maintenance.

Preparation of sgRNAs and Cas9 for microinjection ● Timing 3–5 d

9 For each sgRNA, design a custom DNA oligonucleotide template for the crRNA that contains the T7 promoter sequence, the target sequence (excluding the NGG PAM site), and a stretch of sequence that will anneal with the tracrRNA oligo. See Table 2 for oligos used for *MleBra* sgRNAs.

▲ CRITICAL STEP Make sure that the 5′ end of the target sequence in the crRNA oligo contains two G nucleotides to aid T7-mediated transcription. These nucleotides can be manually added to the sequence if they are not already present in the genomic sequence.

10 Order custom crRNA and tracrRNA oligos (IDT) for producing the sgRNA template[170–172] (Table 2). Standard purification is sufficient.

▲ CRITICAL STEP We use a universal DNA oligonucleotide template as the tracrRNA element to anneal to all crRNA templates. The sequence is AAAAGCACCGACTCGGTGCCACTTTTT CAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAAC.

11 For each sgRNA, anneal the tracrRNA and specific crRNA and PCR-amplify the sgRNA by mixing DNA polymerase, appropriate polymerase buffer, dNTPs, tracrRNA and crRNA oligos. An example PCR program is 95 °C for 2 min, followed by 30–40 cycles of 95 °C for 20 s, 58 °C for 10 s, and 70 °C for 10 s (ref. [171]). Reaction volume should be between 50 and 100 µl.

12 Run a small aliquot from each reaction (~2–5 µl) on a 1% agarose gel using standard gel electrophoresis parameters to confirm the correct size, ~100 bp.

13 Purify the remaining PCR product according to the methods listed for the MEGAscript kit.
14 Measure the DNA purity and concentration with a spectrophotometer.
15 Use up to 0.2 µg of PCR-product template and set up the transcription reaction according to the manufacturer's instructions (MEGAscript). The final volume is 20 µl.
16 Incubate the reaction at 37 °C for at least 2 h.
▲ **CRITICAL STEP** Owing to the short sizes of the sgRNAs (~100 bp), it is recommended to incubate reactions overnight (~16–18 h).
17 Add 1 µl of DNase (included in MEGAscript kit), mix gently and incubate at 37 °C for 15 min.
18 Follow the manufacturer's instructions (MEGAscript) for purifying sgRNAs. We typically elute in small volumes (≤20 µl) to obtain highly concentrated (>1,000 ng/µl) sgRNA samples.
19 Quantify sgRNA concentrations with a spectrophotometer or fluorometer, and store small aliquots (double-use, ~2 µl) at −80 °C. PAUSE POINT: sgRNA can be stored under these conditions for ~6 months
20 Resuspend Cas9 recombinant protein in nuclease-free water to 1 ng/µl, and gently mix. Aliquot into single-use tubes, ~2 µl. PAUSE POINT: Reconstituted Cas9 stored at −80 °C should be viable for ~6 months.
21 Dilute 1 µl of each sgRNA to be used for the injection experiment from Step 19 to 600 ng/µl in nuclease-free water. If multiple sgRNAs are to be used in a single injection, they can be diluted in the same tube. Keep the tubes on ice, and immediately return unused sgRNA stocks to −80 °C.
22 In a nuclease-free microcentrifuge tube, gently mix 2 µl of Cas9 and 1 µl of diluted sgRNA (600 ng/µl). Incubate for 5 min at RT.
23 Add 3 µl of 70% glycerol (made in nuclease-free H_2O) and 0.3 µl of fluorescent dextran stock to the sgRNA/Cas9 solution. Gently mix, and centrifuge at ≥16,000*g*, at RT for 2 min to pellet small debris that can clog injection needles.

Embryo and microinjection preparation ● Timing 5–7 h

24 While waiting for ctenophores to spawn in Step 5A, prepare reagents and equipment for embryo processing and microinjections.
25 Back-fill each injection needle with 0.5–1 µl of the sgRNA/Cas9 injection cocktail from Step 23.
26 During the 10 min sperm and oocyte incubation step (Step 4Av), fill: two 3.5-inch soda-lime glass culture dishes with just enough penicillin/streptomycin-spiked FSW (psFSW) to cover the bottom of the dishes; three 1.5-inch soda-lime glass culture dishes halfway with psFSW; and two 35 mm gelatin-coated polystyrene Petri dishes one-third of the way with psFSW.
27 Using a transfer pipette, collect as many embryos as possible from the spawning bowl, and pass through the 500 µm filter into one of the prepared 3.5-inch glass culture dishes.
28 Using a transfer pipette, collect the 500 µm filtered embryos and pass them through the 400 µm filter into the other of the prepared 3.5-inch culture dishes.
29 Continue washing the filtered embryos by transferring them via mouth pipette to one of the prepared 1.5-inch dishes from Step 26.
30 Repeat Steps 27–29.
31 With a mouth pipette, transfer 10–20 embryos to a gelatin-coated dish to remove the vitelline membrane from each embryo.
32 Using fine-needle forceps, or acid-sharpened tungsten wire needles, carefully tear a large opening on one side of the vitelline membrane. Gently push the embryo through the opening.
▲ **CRITICAL STEP** To reduce the likelihood of microinjection needle clogging, gently swirl the dish with the newly devitellinized single cell embryos to break up remaining egg jelly.
? TROUBLESHOOTING
33 Using a mouth pipette, transfer the devitellinized embryos directly into an injection dish or to a second gelatin-coated dish. We recommend the latter, since any devitellinized uninjected embryos should be kept as controls.
34 Repeat Steps 31–33 as needed during the spawning event.

Embryo microinjection ● Timing 1–2 h

35 Insert an injection cocktail-loaded needle from Step 25 into the micropipette holder of the digital injector. Push the needle past the internal O-rings, and gently tighten the holder by hand.
36 Adjust the angle of the needle to between 35° and 45°. Make sure the transfer pressure reads +000 on the digital injector.

37 Add approximately five drops of psFSW to the injection dish trough.

38 Using a mouth pipette, transfer between 20 and 30 devitellinized embryos to the injection dish trough. Ideally, the embryos will fall in a line along the trough backstop.

39 Using a mouth pipette, slowly remove some of the water from the trough and simultaneously array the embryos along the trough backstop (Fig. 6c).

40 Focusing on a single embryo, adjust microscope magnification and center the embryo in the field of view.

41 Switch to low magnification to facilitate alignment of the needle with the embryo in the field of view.

42 Using the micromanipulator, slowly lower the needle until it breaks the surface of the water.
▲ **CRITICAL STEP** Once aligned and immersed, use high magnification to observe the needle tip.

43 Using a combination of fluorescence and low intensity brightfield light, adjust the transfer pressure on the digital injector to achieve a constant slight outflow from the tip of the needle.
? TROUBLESHOOTING

44 Lower the needle to just above the embryo, slightly offset and to the right of center (for a right-side mounted microinjector).

45 In one smooth motion, bring the needle down and left, to pierce and enter the embryonic cell membrane.
▲ **CRITICAL STEP** Use the 'pressure' and 'pulse width' parameters to adjust the pressure and time of each injection pulse, respectively.

46 Inject the microinjection cocktail into the embryo.
▲ **CRITICAL STEP** Observe the initial bolus of injection material. The bolus should begin to diffuse through the embryo shortly after delivery.
▲ **CRITICAL STEP** Typically, a bolus no more than ~5–10% of embryonic volume is desired. It is easiest to estimate the amount of material by eye to account for variation in egg size, cell cleavage state and individual needle-specific adjustments that are needed during an injection session.
? TROUBLESHOOTING

47 Continue injecting remaining embryos in the injection dish. The use of a fluorescent dextran dye tracer makes it easy to determine which embryos have been injected.

48 At the conclusion of the injection session, raise and move the needle out of the injection dish.

49 Add a few drops of psFSW to the injection dish, and use a mouth pipette to gently transfer injected embryos to a new gelatin-coated dish containing psFSW.

50 Repeat Steps 44–49 as needed with remaining devitellinized embryos.
▲ **CRITICAL STEP** For additional experimental injections or changing the needle, reset the transfer pressure to +000.
▲ **CRITICAL STEP** Optimize each new needle by repeating Steps 35–43.

51 Keep injected embryos in a gelatin-coated dish filled with psFSW at 18–20 °C to the desired stage of development.
▲ **CRITICAL STEP** Cooler temperatures will aid post-injection recovery.

Live imaging ● Timing 5–8 h

52 One hour before reaching the desired stage of development, place selected embryos into a single well of a four-well plate. A well sufficiently large enough to hold ~1 mL is suitable for multiple embryos. Additional wells can be used for additional experimental conditions and control embryos.

53 Mix vital dyes in FSW up to 1 mL per number of wells. Hoechst is used at a final concentration of 10 ng/μl and will preferentially stain nuclei. Hoechst 33342 excitation and emission wavelengths are 350 nm and 461 nm, respectively. Lysotracker is used at a final concentration of 100 nM and localizes to acidic vacuole compartments, preferentially staining large vacuolated cells associated with yolk processing and the gastrovascular cavity epithelia. Lysotracker Red DND-99 excitation and emission wavelengths are 577 nm and 590 nm, respectively. MitoTracker is used at a final concentration of 100 nM and preferentially stains mitochondria. In ctenophores, MitoTracker signal is particularly high in ctene row polster cells owing to unique morphology and high density of mitochondria in this cell type. MitoTracker Deep Red FM excitation and emission wavelengths are 644 nm and 665 nm, respectively.
▲ **CRITICAL STEP** Typically an injected fluorescent dextran remains visible for 24 hpf. For detection of Alexa Fluor 488-conjugated dextran, emission and excitation wavelengths are 495 nm and 519 nm, respectively.

54 Incubate embryos in vital dye solutions at RT for 1 h in the dark.
▲ CRITICAL STEP Incubation should be light protected as vital dye fluorescence will diminish under prolonged exposure to ambient light.
55 After incubation, place individual embryos for imaging into individual wells of a 24-well dish with 750 µL of FSW per well. Set aside during microscope slide preparation.
56 Coat microscope glass slides and coverslips with RainX. Allow to briefly dry, and then wipe off until glass surfaces are clear and dry.
57 Place small clay feet on the corners of each coverslip by scraping the coverslip corners over modeling clay. Set these aside.
58 Wash the embryos once with FSW.
59 To mount an embryo, remove a single embryo with a fine-tipped transfer pipette and place onto a RainX-coated slide.
60 Place a similar-size drop of 7.5% $MgCl_2$ onto a coverslip, and cover the embryo.
▲ CRITICAL STEP $MgCl_2$ will temporarily relax embryo muscular contractions, facilitating imaging. Ciliary movement, and thus beating of the ctenes, is not inhibited by $MgCl_2$.
61 While gently pushing on the coverslip, use small movements to adjust the coverslip and orient/position the embryo for imaging.
▲ CRITICAL STEP While orienting the embryo, also apply enough pressure to the coverslip with fingers or forceps to force the embryo to remain stationary. The clay feet will provide enough space for the embryo to remain intact, but just enough force so the embryo does not move during imaging.
62 Image the embryo as needed. Note any phenotypic or morphological abnormalities.
? TROUBLESHOOTING
63 After imaging, gently add enough FSW to flood the space underneath the coverslip. Slowly remove the coverslip with forceps.
64 Transfer the imaged embryo via mouth pipette to the appropriate labeled well of the 24-well dish from Step 4.
65 Repeat Steps 59–64 for each embryo that needs to be imaged.
66 Recovered embryos can be retained for further observations at later developmental stages or individually processed for DNA or RNA extraction.

Troubleshooting

Troubleshooting advice can be found in Table 3.

Table 3 | Troubleshooting table

Step	Problem	Possible reason	Solution
1	Lobes are regularly damaged and/or adults die prematurely	Poor water quality	Check salinity and make sure municipal water sources are pretreated to remove chlorine, heavy metals and other toxins
			Make sure there is adequate filtration and protein skimming; increase frequency of water changes and/or removal of debris/food waste from tank
		Animals are constantly bumping into sides of tank causing physical damage	Adjust gate valve(s) to reduce velocity of recirculating water flow in the pseudokreisel tank
		Adults are not receiving enough food	Increase the volume of rotifers delivered daily
3	Rotifer cultures regularly crash	Rotifer overcrowding	Increase the number of rotifers harvested daily
		Massive infestation of protists/ciliates	Thoroughly rinse and clean the rotifer culture buckets and equipment in direct contact with the cultures weekly
			Passage each rotifer culture through a 40 µm sieve, wash under clean seawater and re-inoculate clean, debris-free rotifer buckets weekly
5A,B	Adult spawning produces few or no gametes	Adults not receiving enough food	Increase volume of rotifers fed daily and provide fish larvae ~24 h before planned spawning
		Adults are not entrained to correct light cycle	Allow adults 2–3 d to acclimate to new light regime before planned spawn

Table continued

Table 3 (continued)

Step	Problem	Possible reason	Solution
	Early cleavage fails, embryos develop abnormally	Polyspermy	After sperm release, replace 50–75% of spawning container volume two to three times
	Eggs are not fertilized and do not cleave	Eggs collected too early	Allow eggs to remain in the spawning container for at least ~10 min before collection
6, 7	Massive cydippid/juvenile die-off	Poor water quality	Check salinity, and make sure municipal water sources are pretreated to remove chlorine, heavy metals and other toxins
		High flow rates and/or current shear forces	Remove potential sources that generate shear forces, such as aerators, from juvenile grow out tanks
32	Embryos killed during devitellinization	Dissecting tool points are too large and/or dull	Use fine, sharpened forceps (e.g., Dumont #55) or acid-sharpened tungsten wire needles
		Dissecting tools contact the devitellinized embryo	Avoid directly touching devitellinized embryos with dissecting tools
	Devitellinized embryos stick to dissecting dish or dissecting tools	Dish surface is sticky	Coat dish and dissecting tools with gelatin
43	Injection cocktail is flowing too quickly out of the needle	Balance pressure set too high	Reduce the balance pressure on the injection controller unit
		Needle opening is too large	Discard needle and use a new one
	No flow of injection cocktail out of the needle	Balance pressure set too low	Increase the balance pressure on the injection controller unit
46	Embryo immediately dies when pierced by the needle	Needle opening is too large	Discard needle, and use a new one with a smaller opening
	Embryo dies when injection cocktail is delivered	Injection pressure set too high	Reduce the injection pressure and/or use a new needle with a smaller opening
62	No phenotypes observed post CRISPR-Cas9 + sgRNAs injection	Poor sgRNA targeting efficiency	Design additional sgRNAs and inject new sgRNA or co-inject several sgRNAs against target locus
		Potential compensation by target gene paralog(s)	Design sgRNA to paralogs and co-inject sgRNA cocktail targeting paralogs

Timing

Steps 1–4, adult maintenance: duration of culture
Step 5A, spawning for immediate experimental use: ~24–48 h
Step 5B, spawning for strain/stock maintenance/propagation: ~24–48 h
Steps 6–8, juvenile maintenance: ~14–30 d
Steps 9–14, preparation of sgRNA template: ~1 h
Steps 15–17, in vitro transcription reaction to generate sgRNA: ~2–18 h
Steps 18 and 19, sgRNA purification: ~3 h
Step 20, Cas9 resuspension and storage: ~5 min
Steps 21–23, preparation of microinjection cocktail: ~20 min
Steps 24–34, embryo preparation: ~2 h
Steps 35–44, microinjection apparatus setup: ~10 min
Steps 45–47, microinjection: ~1–2 h
Steps 48–51, recovery of injected embryos: ~5 min
Step 52, embryo development to desired stage for phenotype analysis: ≤24 h
Steps 53–66, live microscopy of embryos: >2 h
Box 1, rotifer culture maintenance: duration of culture

Anticipated results

Using the protocol described here, we have maintained healthy continuous populations of spawning *Mnemiopsis* in the laboratory (currently >F52) useful for a wide range of studies. Under optimal feeding conditions *Mnemiopsis* sexual maturity is reached within ~16 dph and total life span is ~2 years. Adults are competent to spawn hundreds of embryos daily that can be used for a variety of

experiments and manipulations, including embryonic microinjections[41,42,44,96,138]. We have not observed evidence of inbreeding depression or abnormal embryonic development associated with laboratory strain propagation via self-fertilization.

Using the microinjection protocol, genetic perturbation studies via CRISPR–Cas9 mediated genome editing are relatively straightforward[96]. *Mnemiopsis* embryos are resilient to manipulations required for microinjection with an ~80% survival rate. The use of vital dye stains and live imaging improves phenotypic analyses during embryonic development by facilitating identification and tracking of developmental features and symmetry landmarks over time in single individuals. Importantly, the use of these nondestructive markers for phenotypic characterization allows for the subsequent recovery of single embryos for genotyping assays, including DNA and/or RNA extraction for validating nuclease activity and/or characterizing changes in gene expression[96].

Reporting summary

Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability

The datasets generated during and/or analyzed during the current study are available from the corresponding author on request.

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Conceptualization: husbandry, W.P. and W.E.B.; genome editing, W.E.B. Methodology: husbandry, W.P. and W.E.B.; genome editing, J.S.P. and W.E.B. Investigation: husbandry, all authors; genome editing, J.S.P. and W.E.B. Validation: husbandry, all authors; genome editing, J.S.P. and W.E.B. Visualization: J.S.P. and W.E.B. Resources: husbandry, W.P. and W.E.B.; genome editing, W.E.B. Writing original draft: J.S.P., W.P. and W.E.B. Writing review and editing: all authors. Supervision: W.P. and W.E.B. Project administration: husbandry, W.P. and W.E.B.; genome editing, W.E.B. Funding acquisition: husbandry, W.P. and W.E.B.; genome editing, W.E.B.

Competing interests

The authors declare no competing interests.

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Wild animals	A single wild caught Mnemiopsis leidyi on 23 January 2016 was used as the founder for the captive Mnemiopsis culture
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