



## Main Manuscript for

Ctenophores are direct developers that reproduce continuously beginning very early after hatching

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## Abstract

A substantial body of literature reports that ctenophores exhibit an apparently unique life history characterized by biphasic sexual reproduction, the first phase of which is called “larval reproduction” or “dissogeny”. Whether this strategy is plastically deployed or a typical part of these species’ life history was unknown. In contrast to previous reports, we show that the ctenophore *Mnemiopsis leidyi* does not have separate phases of early and adult reproduction, regardless of the morphological transition to what has been considered the adult form. Rather, they begin to reproduce at small body size and spawn continuously from this point onwards under adequate environmental conditions. They do not display a gap in productivity for metamorphosis or other physiological transition at a certain body size. Furthermore, nutritional and environmental constraints on fecundity are similar in both small and large animals. Our results provide critical parameters for understanding resource partitioning between growth and reproduction in this taxon, with implications for management of this species in its invaded range. Finally, we report an observation of similarly small-sized spawning in a Beroid ctenophore, which is morphologically, ecologically, and phylogenetically distinct from other ctenophores reported to spawn at small sizes. We conclude that spawning at small body size should be considered as the default, on-time developmental trajectory rather than as precocious, stress-induced, or otherwise unusual for ctenophores. The ancestral ctenophore was likely a direct developer, consistent with the hypothesis that multiphasic life cycles were introduced after the divergence of the ctenophore lineage.

## Significance Statement

Ctenophore cydippid “larvae” are not larvae at all and begin adult reproduction at an early age (~14 days vs. ~60 days) and small size (~1 mm vs. ~100 mm) relative to attainment of what was considered the adult stage. This overturns the previous understanding of the ctenophore life cycle, which was believed to be a unique form of biphasic life cycle with two separate sexually reproductive periods. Practically, these results clarify ecological controls regulating ctenophore reproduction and will aid management of this invasive species. Additionally, the two-week egg-to-egg generation time will open new avenues of research in this understudied but informative taxon.

## Main Text

### Introduction

Whether indirect development has a single or multiple origins, when it arose, and whether the adult or larval body plan is ancestral are classic and ongoing debates in zoology (1–4). Competing hypotheses include: “adult-first” hypotheses with either a single intercalation of larvae (5) or multiple gains of a larval form (4, 6), or a “larva-first” hypothesis suggesting a common origin of an ancestral larva with later diversification of adult forms (7). Broad definitions of indirect development include some combination of morphological, physiological, or ecological differences between the larval and adult forms, the magnitude of which is at least partly a matter of philosophy (8). However, most working definitions include a major post-embryonic morphological or ecological change before which the animal is not sexually reproductive. Asexual reproduction at pre-adult stages is widespread in marine invertebrates and includes diverse strategies such as budding, fission, or parthenogenesis (9–11), but almost never the production and fertilization of mature gametes. Thus, spawning does not necessarily preclude identification of a life stage as larval but a true sexually reproductive phase in larval life followed by a normal adult phase is an extraordinary claim. A lineage of marine animals called ctenophores has been cited as a rare – and potentially sole – example of true sexual reproduction in “larvae”, involving maturation of both male and female gametes and fertilization. This phenomenon has been noted in literature as far

back as 1892 (12), explicitly called “larval reproduction” or “dissogeny” and observed in several phylogenetically distant species of both tentaculate cydippid and lobate ctenophores (13–20). In favor of this view, it appears from published literature that small “larval” and large “adult” spawning are discontinuous (Supplemental Table S1) in distantly related species and under diverse experimental conditions. The reported pause between reproductive phases correlates with temporary degeneration of the gonads as well as a period of overall morphological change in at least some species. Various secondary morphologies appear to have arisen multiple times in ctenophores’ evolution (21, 22). However, species that change little as they grow (life-long cydippids) are also understood to exhibit “larval reproduction” (19, 20).

Phylogenomic studies often place ctenophores as the sister group to all other animals (23–29); although this placement remains controversial as other studies place sponges in this position (30–33). Either reconstruction requires losses or multiple independent origins of complex traits; a multiphasic life history is one complex trait that would require multiple origins or a loss in ctenophores under the latter hypothesis if ctenophores lack larvae. Thus, whether ctenophores are direct developers is a key consideration in the debate over when, how, and how many times multiphasic life history evolved, regardless of which tree topology is used. Furthermore, while it is conventional for the ctenophore literature to refer to “larval” ctenophores or a “cydippid larva” stage, if ctenophores lack a larval stage then this terminology should be amended to be more precise. Finally, if both cydippid and lobate stages represent adults, then the introduction of this morphological transition represents a type of post-embryonic development distinct from a larva-to-adult transition.

Ctenophore life history thus has implications for broader research on animal origins, life history evolution, and ctenophore ecology. If spawning at a small size is physiologically distinct from later spawning, it might imply that ctenophores have cryptic larvae, which would provide evidence for the hypothesis that the last common ancestor of animals had separate larval and adult stages. Moreover, ctenophores would be a unique representative of true sexual reproduction during a larval life stage. Available alternative hypotheses, that size at sexual maturation in ctenophores is a plastic trait that responds to environmental factors or epigenetic programming, or that small size at maturation is a genetic trait favored by selection in certain environments (such as a bottleneck during invasion) are of interest in their own rights. Finally, if the observed small size of reproducing individuals in pioneer populations is a product of resource limitation, predation pressure, or some other pressure specific to low-density populations then successful colonization should be signaled by the reliable appearance of large-size individuals, making the size distribution of animals a key indicator of invasion success.

Our new results in the ctenophore *M. leidyi* are consistent with the hypothesis that reproduction at small and large sizes in ctenophores reflects a single, continuous process of maturation and resource acquisition, contradicting prior studies of small-size reproduction in ctenophores. Despite their morphological transition at larger sizes, there is no pause in reproduction. The observation of exclusively small-sized populations of ctenophores in certain contexts, particularly as new invaders, likely reflects their invasion ecology rather than an adaptation to the novel environment. The mechanism and meaning of secondary morphologies that appear in some ctenophores bear further investigation, but morphological change alone is not metamorphosis.

## Results

### ***Progeny of cydippids develop normally and become fertile around the same time as parents***

The parental cydippids’ gonads are visibly enlarged (**Error! Reference source not found.**A-F). As previously reported (13, 18), only 4 of the 8 pairs of gonads become visibly enlarged, suggesting

that only half the full complement of gonads becomes fully mature at this stage. However, it is unknown whether all gonads are equally active in larger individuals as well.

These cydippid stage *M. leidy* produce normal embryos that begin as 1-cell zygotes enclosed in a fertilization envelope (**Error! Reference source not found.**G, H). The embryos develop into morphologically normal cydippids on a similar timeline to embryos produced by large lobate stage *M. leidy*, although the percentage of embryos that develop normally is lower: mean 19.8% of cydippids' self-fertilized progeny (N = 746 embryos from 4 biological replicates) vs 90.9% of lobates' self-fertilized progeny develop into morphologically normal, free-swimming cydippids by 24 hours post-fertilization (hpf) (N = 129 embryos from 4 biological replicates). These F1 offspring cydippids themselves become fertile around the same developmental stage as their parents. We have observed embryo production through the F3 generation.

***Cydippids' reproductive output is best modeled with a time-explicit negative binomial model***

While previous reports suggested a minority of individuals are capable of small-size spawning, most isolated individuals >1 mm spawned at least once during our observation period (34/56 from 4 biological replicates over 4-11 days observation) and almost every individual observed for 6 or more days produced at least one embryo. However, daily spawning is rare; among all isolated individuals observed on at least 4 days that spawned at least once (32 individuals from 4 biological replicates) only a single individual produced an embryo every day of the observation period. When actively spawning cydippids (~1 mm and up) spawn, they usually produce only 1-2 embryos per day (**Error! Reference source not found.**A) under this culture regime. This pattern of irregular spawning persists; however, as they grow past ~5 mm, they may occasionally produce several dozen embryos in a 24-hour period.

Following the approach of a recent analysis of small-size spawning in another ctenophore (19), we plotted parental body size versus clutch size when zero reproductive output days are dropped and confirmed that our species also exhibits a similar positive correlation between size and fecundity (**Error! Reference source not found.**B). However, moving forward, we wanted to use a model that accommodates these highly skewed, zero-inflated data. We thus formulated a de-novo, hierarchical stochastic model of births randomly occurring over time according to a set of biological hypotheses regarding the drivers of offspring production. This hierarchical model is a modification of a pure birth process (34) that incorporates individual heterogeneity in birth rates. Using this model has the dual advantage that 1) by modeling the birth process as a temporal biological process, it explicitly incorporates the embedded time-dependency in the counts done over time and 2) admits the inclusion of birth rate heterogeneity as a random effect that ultimately recapitulates naturally an excess of 0's in the observed counts. Traditional Generalized Linear Models to test the effect of different experimental and biological factors on the accumulation of births should not be used here because they erroneously ignore the biological time dependency in the counts, and therefore may result in excessive Type I errors in hypothesis testing as well as severe model choice errors (34). The full hierarchical model derivation, our Maximum Likelihood parameter estimation, and our non-parametric bootstrap model selection approaches using Evidential Statistics and the Bayesian Information Criterion (BIC) (35–39) are detailed in the Supplemental Materials. Supplemental Figure S1 shows a visual representation of the modeling process, and its panel C shows embryo production and parental body size over time for three real individuals. Our R code permits similar visualizations for any individual in our data set.

We found that the model using parental body size was slightly outperformed by models using other parameters, notably days in the experiment for the raw data. However, the median bootstrap difference between the lowest and the second lowest BICs suggested much stronger evidence for biological replicate + body size (median  $\Delta\text{BIC1}$  7.742626,  $\Delta\text{BIC2}$  5.493028) than experimental day

(median  $\Delta\text{BIC1}$  = 3.846758,  $\Delta\text{BIC2}$  = 12.474642) as a driver of 24 hpf offspring production (Supplemental File Data S1).

Crucially, there is no pause in spawning as previously hypothesized, as illustrated by plotting embryos or cydippids produced by parental body size category (**Error! Reference source not found.**C, D). Previous studies (detailed in Supplemental Table S1) reported a pause in reproductive effort beginning at a certain size threshold (beginning by 3.0 mm in all cases, range ~0.6-2.8 mm body width or 0.75-2.8 mm body length). However, by following single individuals we observed that spawning continues across sizes 1.0-8.0 mm; we document concurrent spawning and growth during the observation period, suggesting that there is not a binary switch between growth and reproduction.

#### ***Incubation temperature is positively correlated with reproductive output***

We used two temperatures near the extremes of typical temperatures used to culture *M. leidyi* and known to permit normal growth (40). We found that rearing temperature is positively associated with both the number of eggs produced per parental cydippid and the number of eggs that develop into normal offspring by 24 hours post-fertilization (hpf) (**Error! Reference source not found.**). The best-supported model for embryo production includes both temperature and number of parentals (embryo median  $\Delta\text{BIC1}$  = 3.608418, median  $\Delta\text{BIC2}$  = 6.382366 vs all other selected models median  $\Delta\text{BIC1}$  = 2.615262 and  $\Delta\text{BIC2}$  = 6.779690). The same model was also best supported for cydippid production (cydippid median  $\Delta\text{BIC1}$  = 2.637563, median  $\Delta\text{BIC2}$  = 4.1911 vs all other selected models  $\Delta\text{BIC1}$  = 1.948039 and  $\Delta\text{BIC2}$  = 6.665559).

#### ***Fecundity is relatively insensitive to culture density***

We hypothesized that culture density might play a role in inducing small-size spawning, whether as a response to stress or a mechanism such as community spawning. We tested cultures of 1, 2, 4, and 8 individuals in a constant volume of 50 ml (**Error! Reference source not found.**). The per capita fecundity slightly decreased when the parental density in the culture increased; this density-dependent effect was most apparent in a single biological replicate that had higher overall fecundity. Most of the effect is driven by the transition from one to two individuals per culture. Importantly, animals cultured singly spawn, so there is not a requirement for presence of a conspecific to spawn at small size. The best-performing model for both embryo and normal cydippid production incorporates biological replicate and number of parentals into its parameters, indicating extremely strong support for this model (embryo median  $\Delta\text{BIC1}$  = 8.553733, median  $\Delta\text{BIC2}$  = 11.56055 vs all other selected models median  $\Delta\text{BIC1}$  = 2.527544 and  $\Delta\text{BIC2}$  = 5.399919; cydippid median  $\Delta\text{BIC1}$  = 9.143399,  $\Delta\text{BIC2}$  = 11.699243 vs all other selected models median  $\Delta\text{BIC1}$  = 3.381177 and  $\Delta\text{BIC2}$  = 4.849772).

#### ***Fecundity is conditional on nutrition***

Several preliminary observations indicated that spawning at small sizes is affected by diet. First, we found that cydippids transitioned onto artemia rather than rotifer-based diets entirely ceased spawning after approximately 3 days. We also observed that when our standard cultures (35-50 individuals per ~200 ml culture in standard finger bowls) were fed with rotifers raised on complex commercial algal feed vs non-enriched feedstock, the bowls of cydippids fed the non-enriched rotifers ceased spawning entirely after approximately 5 days. These preliminary experiments suggested the hypothesis that diet is an important factor in cydippid spawning, especially as starvation for a similar duration (2-4 days) has been shown previously to dramatically reduce spawning in lobate stage *M. leidyi* at similar temperatures (41, 42).

To identify the nutritional requirements of cydippids to spawn, we purchased commercial single- and multi-species algal feeds with different nutritional composition to enrich their prey rotifers' diets. Diets based on these feeds or similar commercial options have been used in similar experiments with different focal taxa (e.g., (43, 44)). To identify macronutrients required for cydippid-stage spawning, we tested four prey rotifer diets with varying macronutrient (protein, carbohydrate, and

lipid) and essential fatty acid (docosahexaenoic acid, DHA; eicosapentaenoic acid, EPA; and arachidonic acid, ARA) contents. Using manufacturer-reported nutritional data (Supplemental Table 2), we analyzed the relationship of prey animals' dietary macronutrients and essential fatty acids to predator ctenophore fecundity. We found evidence that lipids and particularly the fatty acid DHA positively correlate with fecundity (**Error! Reference source not found.**). The protein content of these diets is relatively similar and thus carbohydrate and lipid content are inversely related. Broad ranges of EPA, DHA, and ARA were provided by these diets. The general results were similar whether scoring embryos produced or number of normal 24 hpf offspring produced and for DHA or total lipids (**Error! Reference source not found.**). The best supported model incorporated biological replicate and feed DHA concentration (embryo median  $\Delta\text{BIC1} = 2.069955$ , median  $\Delta\text{BIC2} = 3.772602$ , median  $\Delta\text{BIC3} = 6.110557$  vs all other selected models  $\Delta\text{BIC1} = 3.302318$ ,  $\Delta\text{BIC2} = 4.145272$ ,  $\Delta\text{BIC3} = 12.803460$ ); cydippid median  $\Delta\text{BIC1} = 3.36046$ , median  $\Delta\text{BIC2} = 5.75557$  vs all other selected models  $\Delta\text{BIC1} = 5.170143$  and  $\Delta\text{BIC2} = 6.36331$ ).

#### ***The ctenophore Beroe ovata also spawns at a small size***

We also observed small size spawning in another ctenophore from a lineage that does not undergo a morphological transition as it grows, *Beroe ovata*. This species is thought to preferentially outcross so we did not isolate individuals but 3 dishes containing 2, 3, or 4 animals as small as 0.3 x 0.7 mm and as large as 2 x 3 mm spawned over several days and produced normal 24 hpf offspring. We counted as many as 33 normal 24 hpf offspring produced by a single pair in one day.

## **Discussion**

With adequate nutrition, *M. leidyi* will spawn reliably and continuously, beginning at small sizes (~1 mm and up, compared to maximum sizes reached by adults of >60-100 mm), regardless of their overall morphology *i.e.*, cydippid versus lobate body shape. Warm temperatures and high-quality nutrition, especially higher content of the essential fatty acid DHA, further increase their fecundity (**Error! Reference source not found.**). Where these conditions occur in the wild, small-size spawning may contribute to *M. leidyi* recruitment – perhaps considerably, given the potential for geometric population growth. Since temperature and food availability are two major factors that control reproductive output in a range of marine invertebrates, and have been amply demonstrated in the literature as key factors in *M. leidyi* spawning at larger size (17, 41, 42, 45, 46), their apparent sufficiency to induce spawning at small body size in *M. leidyi* suggests that small-size spawning is a typical part of this animal's life cycle rather than a rare, inducible phenomenon or otherwise physiologically distinct from later reproduction. Experiments reported here were carried out with animals collected throughout more than a calendar year and from several locations; thus, we are confident that we did not sample only individuals sharing a unique genetic predisposition to small-size spawning and that seasonality is not an important factor.

Moreover, small-size spawning appears to be continuous with, and physiologically indistinguishable from, spawning at larger sizes. Our bootstrap simulations agree with prior work that clutch size generally scales with parental body size, consistent with the observation that body size determines ~20% of the egg production rate in larger *M. leidyi* (47). However, in contrast to prior reports of small-size spawning, we show clearly that there is no stereotyped pause in spawning in the previously reported size range. We highly sampled animals in the hypothesized size range for this pause of ~2.2-8+ mm (Table S1), which overlaps with the morphological transition from cydippid to lobate in this species of ~2-5 mm (Figure 2C), and found that spawning continues at these sizes, conclusively ruling out a pause. The morphological differences between cydippid and lobate stages primarily affect feeding structures and are known to affect feeding behavior (48–50), but the continuity of reproduction suggests that this morphological and ecological transition is not homologous with, or even analogous to, metamorphosis despite its apparent irreversibility (41) and is unrelated to adulthood *per se*. Rather, these successive forms

may be better understood as serial eco-morphs, perhaps even arising plastically at some point in the past.

It remains possible that other ctenophore species pause spawning in this size range. However, we believe that technical differences in experimental design adequately explain why we did not observe a pause in spawning where others have. First, we fed all experiments such that at least some food remained after 24 hours. Anecdotally, we observed that even heavier feeding might increase fecundity, but at the expense of higher mortality due to fouling in closed cultures. At least one previous report of such a gap was shown under controlled experimental feeding conditions (16) where a constant amount of prey was provided for all individuals regardless of size; intermediate-size animals simply may have been inadequately nourished for reproduction. Second, we made more and longer-term daily observations of both individuals and groups. The robustness of this data set overcomes limitations imposed by the inherent stochasticity in reproduction rates. All individuals >1 mm we observed over 6 or more days spawned at least once in that time. Most published reports using wild-caught small-size animals observed the number of embryos produced in a short (24–48 hour) period after collection (19, 20). In our observation, it is extremely rare for *M. leidy* cydippids to spawn daily, with gaps of several days between spawns being common, so shorter sampling periods would create the impression that not all small individuals spawn.

This reproductive strategy was likely present early in the ctenophore lineage. The phylogenetic distribution of species in which spawning at an extremely small size relative to its potential maximum has been reported, including our new observation in *B. ovata*, is consistent with small size spawning in the stem lineage leading to the last common ancestor of *Mnemiopsis*, *Pleurobrachia*, *Mertensia*, and *Beroe* (**Error! Reference source not found.**C). We thus propose elimination of the terms “dissogeny”, “larva”, and “metamorphosis” from the ctenophore literature, as these reproductively mature animals are clearly adults despite their size. We recommend that ctenophores be described by their body size, type, and reproductive status as needed for clarity (e.g. “pre-reproductive cydippids”).

However, this is not to say that differing environments do not differentially favor reproduction at smaller versus larger sizes, or that small-size spawning is not an important driver of their invasion biology. On the contrary, evidence from the field suggests that local conditions may dramatically favor reproduction at small body size in other species of ctenophores. In a *Mertensia ovum* population newly colonizing a warmer region, 95% of animals found in zooplankton sampling nets were under 1.1 mm (20), suggesting that this population is maintained entirely by small-size spawning. Similarly, temporospatial variation was observed in whether small-size or large-size individuals contribute to recruitment in a species colonizing cooler waters, *Pleurobrachia globosa* (19). The relative abundance of cydippid-stage *M. leidy* increases with water temperature and dramatically so above 25°C in its invaded region (51). Thus, spawning at small sizes may be an important driver of ctenophore ecology, particularly during invasion, as one introduced individual's offspring can grow exponentially after only ~2 weeks post-fertilization.

High juvenile attrition (52) or heterogenous environmental conditions (49) selectively favoring early reproduction are both plausible mechanisms for the observed importance of early reproduction to recruitment of newly established ctenophore populations. However, many ctenophores, especially the focal species of this study, are well adapted for survival under varying abiotic conditions (51, 53–56). Furthermore, their reproductive output at large body sizes is enormous at thousands of eggs per day, accounting for as much as 10% of their total carbon (57). Thus, the life-history theory that low-density populations are generally advantaged by faster time to reproduction (10) seems to better explain why pioneer populations disproportionately recruit through early spawns. As a population approaches the carrying capacity, intraspecific competition becomes relatively more important compared to sparse populations. However, the prey concentration required for egg production in large animals is typically exceeded in *M. leidy*'s

introduced range (41) so the drivers of this observed effect in the wild will be a fruitful area for future study.

While precise consensus definitions for direct versus indirect animal development remain elusive, we propose that absence of sexual reproduction is likely one universal feature, as “dissogeny” or “larval reproduction” in ctenophores was the only specific example of such proposed in the scientific literature. Definitive absence of a larval life stage should reassure researchers who use ctenophores to study aspects of their biology such as regeneration and development, since small-size individuals represent an adult life stage and embryos produced by parents of any size represent normal development. On the other hand, the adult morphological transition from cydippid to lobate exhibited in some ctenophores represents a new study system for post-embryonic development. We also found an effect of dietary DHA on ctenophore fecundity, which has been observed in many bilaterian animals but our new results suggest may be a pan-metazoan trait. Furthermore, since juvenile cydippids can be spawned reliably with improved nutrition, ctenophores will become a more accessible developmental model system even to researchers located inland or otherwise far from ctenophore species of interest. We have seen that *M. leidy* can be raised reliably to reproductive age in 2 weeks rather than months and that it is possible to keep reproductively active, overlapping generations of animals in a compact footprint and without complex culturing systems. *M. leidy* may become a viable candidate for a ctenophore genetic model system, permitting establishment of transgenic lines and direct investigation of previously inaccessible developmental phenomena such as maternal effects and development of the primordial germ cells.

## Materials and Methods

### Animals

Lobate-stage *Mnemiopsis leidy* and similarly sized *Beroe ovata* were collected in the waters surrounding The Whitney Laboratory for Marine Bioscience in Marineland, FL, USA from floating docks on site, near Flagler Beach, Anastasia Island, or Indian River Lagoon. Each biological replicate reported is the product of a captive spawn of two or more unique wild-caught individuals; no parental lobate was used in more than one replicate. Animals spawned overnight based on their endogenous circadian cycle. Hatchlings were reared in 2L glass beakers or ~18L HDPE containers in UV-sterilized filtered sea water (FSW). All experiments reported here were from the offspring of animals collected between December 2019 and March 2021.

### Cultures

Depending upon the experiment, cydippids were reared in 2-5 gallon HDPE buckets, 1-4 L glass beakers, 50 ml glass beakers, glass finger bowls, 6-well plates, or 24-well plates. Constant-volume density experiments were performed in 50 ml glass beakers with 1-8 animals. Other isolation experiments were performed in 24-well plates. Bulk culture experiments such as food type were performed in glass finger bowls with ~10-40 animals.

Experiments were kept on a 12-hour light cycle at 28°C unless otherwise noted. In all cases cydippids were fed daily such that a slight excess of prey animals remained in the culture container between feedings and water was changed completely each day before feeding. For the experiments shown in Supplemental File S6 (replicates AA and AC) shown in Figure S1C, some animals were fed more heavily to speed body growth but at a cost of greatly increased mortality in culture. Animals in these growth-specific experiments were measured daily and thus exhibit slight fluctuations in size. The reproductive output vs. growth experiments shown in Figure 2C and D were measured twice weekly so their growth patterns appear more stable but this reflects lack of measurement.

A biological replicate is defined as the progeny of 1 or more unique wild-caught lobates (in practice typically ~5-10) spawned in the same container on a single day and never reused for any other replicate. These bulk cultures were kept at ambient room temperature and light conditions and fed daily with rotifers fed RGcomplete. To set up an experiment, animals were collected from the bulk culture into a single container, measured, and transferred to one of the experimental containers at random (alternating/rotating between each treatment container until either the goal number was reached or there were no more animals left).

Whether body length or width is used varies across studies, but we found body width easier to measure. We measured approximate body diameter in the equatorial tentacular plane, similarly to (19). The conversion factor to body length, which has been used in other studies, is ~0.8 (16, 19). However, since our method to repeatedly estimate body size allowed us to track many more measurements, we recommend it to others.

To measure *M. leidy* cydippid size, we used known-diameter polypropylene disposable pipettes. We cut gradually tapering plastic transfer pipets at different points along their length with razor blades to obtain the desired internal diameter as measured with a mini-ruler (Ted Pella, Inc., Redding, CA) in half-millimeter steps below 4 mm and 1 mm steps above 4 mm. Body sizes reported are rounded up to the nearest size category. Animals were transferred under a dissecting microscope to estimate body size. *Beroe ovata* body size was measured as width x length using a mini-ruler.

#### Rotifer care and feeding

Cydippids were fed primarily rotifers (*Brachionus plicatilis*, L-type, Reed Mariculture, Campbell, CA) fed with RGcomplete (Reed Mariculture) unless otherwise noted; alternative rotifer feeds were purchased from the same supplier. Rotifers were maintained as described in (58) and fed daily with the appropriate microalgal feed.

To identify macronutrients required for cydippid-stage spawning, we tested four prey rotifer diets: 1) *Brachionus plicatilis* (L-type) rotifers fed a commercial, complex microalgal diet (RGcomplete); 2) *B. plicatilis* fed a high-EPA single algae commercial feed, *Nannochloropsis* concentrate; 3) *B. plicatilis* fed a high-DHA single algae commercial feed, *Isochrysis* concentrate; 4) *B. plicatilis* fed a mixture of equal parts of the two single-algae diets.

Manufacturer-reported nutritional content of the algal feeds is available in Supplemental Table S2.

#### Imaging

Live images were captured with either Zeiss Stemi 508 microscope equipped with a Ximea camera system Inc. 12.4 MP camera system (MC124CG-SY-UB) for the whole-animal view of parental cydippids or a Zeiss Axio Imager M2 coupled with an AxioCam (HRc) digital camera using ZEN software for all other images.

#### Data analysis

Data analysis was performed in R (59). All raw data, as .csv files, are available in Supplemental Files S2-S6. All R code used to generate the results is available, in standard R GLM notation for ease of use, on GitHub (<https://github.com/jmponciano/ctenophores>). Details of the statistical models are available in the appendix.

For each experiment, we fitted a different set of models for the mean number of births of embryos occurring over time. We then carried out model selection using the Bayesian information criterion (BIC) to compare the relative fit of these models to the data to choose amongst possible explanatory models, including a null model for each. To test the reliability and support in the resulting chosen “best models” for every experiment, we adopted the recent non-parametric

bootstrap model selection methodology of (35). To do that, we performed sampling with replacement of each of the data sets while preserving the structure of the data and generated a set of 100 non-parametric bootstrap samples each time to generate nonparametric bootstrap (n=100) results and compared the difference in the first two BICs under the best-supported model vs. all other models (see Supplemental File Data S1 for results of all models tested, including bootstrap results and first two delta-ICs for best model versus all other models).

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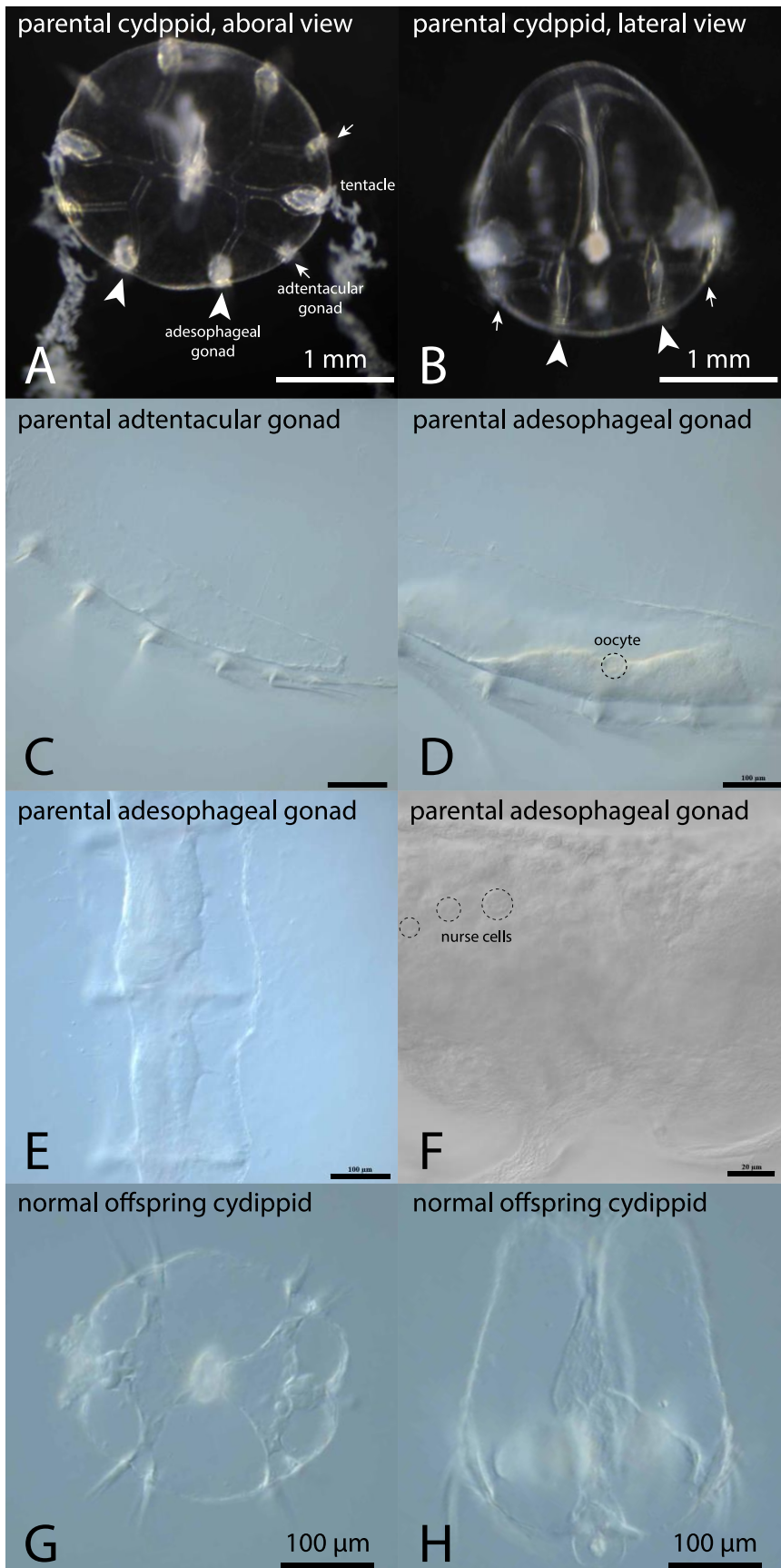
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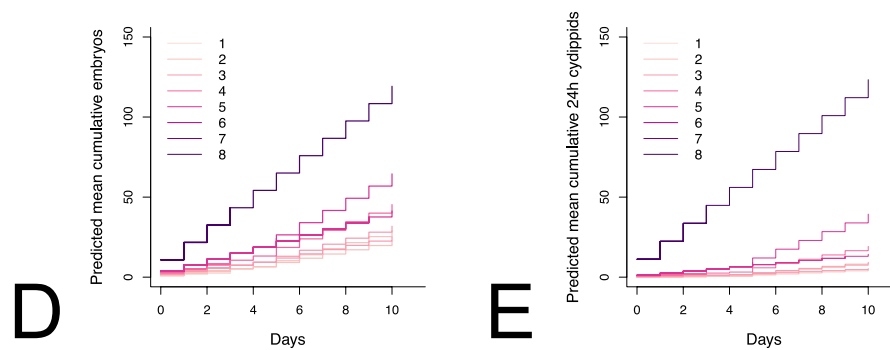
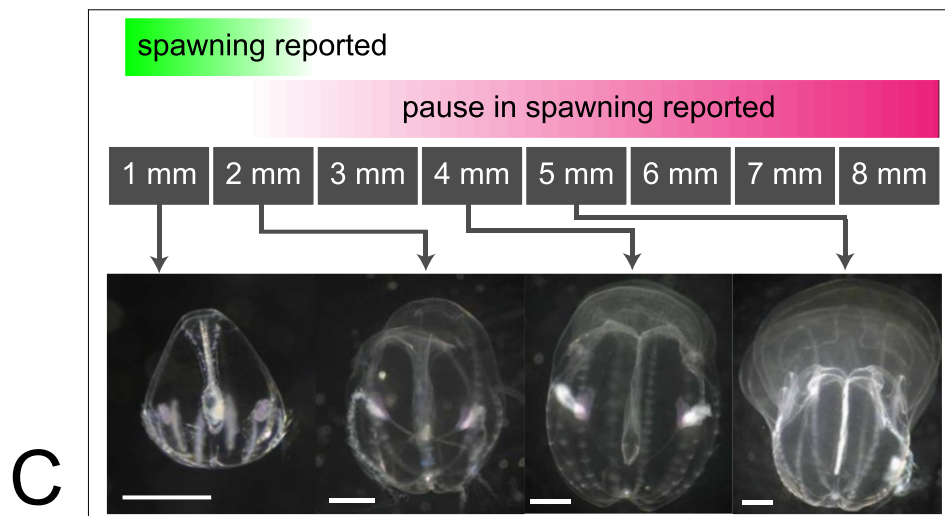
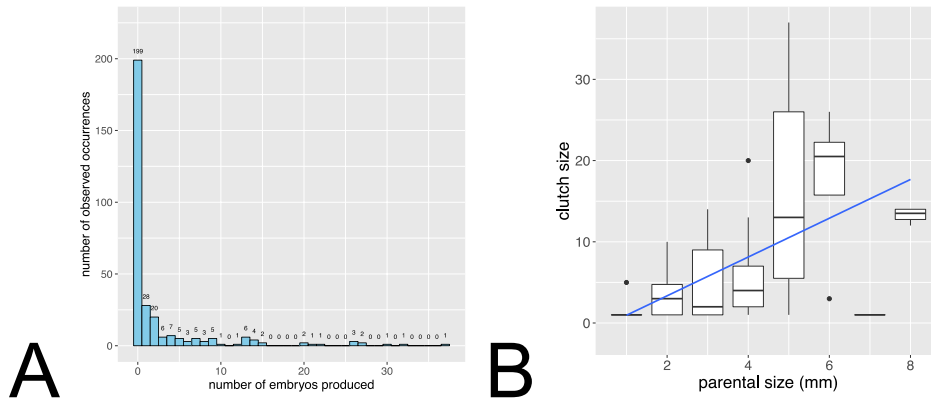
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## Figures and Tables



**Figure 1.** Appearance of reproductive cydippids' gonads and progeny. A. Apical (aboral) view of a reproductively mature cydippid. Four of the eight gonads are visibly enlarged (large arrowheads); the adtentacular (closer to the plane of the tentacles) four gonads are visibly smaller (small arrows). B. Lateral view of a reproductively mature cydippid. Oral pole faces up. Enlarged adesophageal (orthogonal to the plane of the tentacles) gonads face the viewer. C. Unenlarged adtentacular gonad under higher magnification (side view). D. Enlarged adesophageal gonad in the same animal (side view). Arrowhead highlights a visible immature oocyte. E. Face-on view of a gonad in a live, reproductively active cydippid. F. High magnification view of ovary in reproductively active cydippid. Arrowheads highlight individual large cells, likely nurse cells. G. Apical (aboral) view of a live offspring of a cydippid at ~24 hpf. H. Live offspring of a cydippid ~24 hpf. Oral pole faces up.

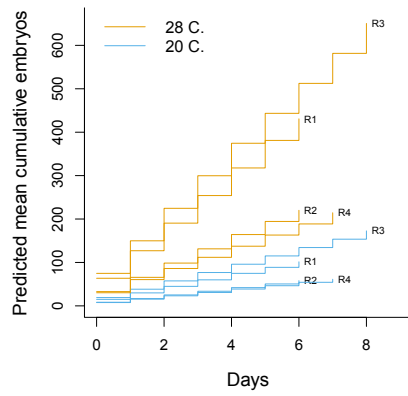


**F**

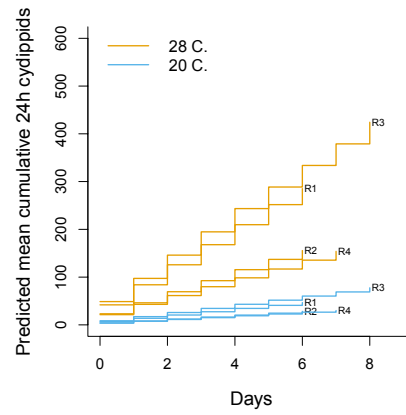
| model        | embryos BIC (bootstrap) | cydippids BIC (bootstrap) |
|--------------|-------------------------|---------------------------|
| day          | 894.5517 (0.40)         | 444.4695 (0.38)           |
| bio.rep      | 899.5539 (0.21)         | 452.0115                  |
| bio.rep+day  | 901.5416 (0.12)         | 451.5707 (0.25)           |
| bio.rep+size | 901.8521 (0.27)         | 447.3678 (0.37)           |
| size         | 912.2451                | 454.0638                  |
| null         | 918.2136                | 467.377                   |
| number       | 923.7704                | 472.8107                  |
| tech.rep     | 1027.007                | 625.2783                  |

**Figure 2.** Daily reproductive output of individual *M. leidyi* at body size 1-8 mm. A. Histogram of embryos counted in daily observations of 56 individuals from 4 biological replicates. Numbers above each bar indicate the number of times a given clutch size was produced. B. Clutch size (embryos per clutch on days when any embryos are produced) increases with parental body size (N = 98 clutches produced by 32 individuals from 4 biological replicates) when 0 spawn days are excluded; blue line shows the mean and shading around the line shows the 95% confidence interval. 7 mm individuals were relatively under-sampled and so we believe this to be an outlier. C. The transition from cydippid to lobate morphology begins around 2 mm and is apparent by around 5 mm, which overlaps with the appearance of lobate morphology. Bars in each panel represent 1 mm. D, E. Staircase graphs showing predicted mean cumulative reproductive output of isolated individuals at body size 1-8 mm. Panel D shows embryos produced and panel E shows daily normal hatched offspring produced at 24 hpf. Each colored line represents reproductive output at a different parental body size in mm; lines do not represent specific individuals. Specific individuals' outputs across different body sizes can be seen in Supplemental Figure S1C. The hypothesized "gap" period of 2-4 mm is highly sampled in the data set but no such pause is apparent. Repeated measures of 68 isolated individuals at various body sizes 1-8 mm diameter, totaling 307 daily observations, from 7 biological replicates. F. The two columns represent the BIC results for all models evaluated in the size experiments shown in D and E; bootstrapping results are in parentheses. The model with the lowest BIC should be understood as the model that most closely represents the generating process given the data; the nonparametric bootstrapping results indicate the uncertainty around selection of that model (see Appendix for model derivation).

A



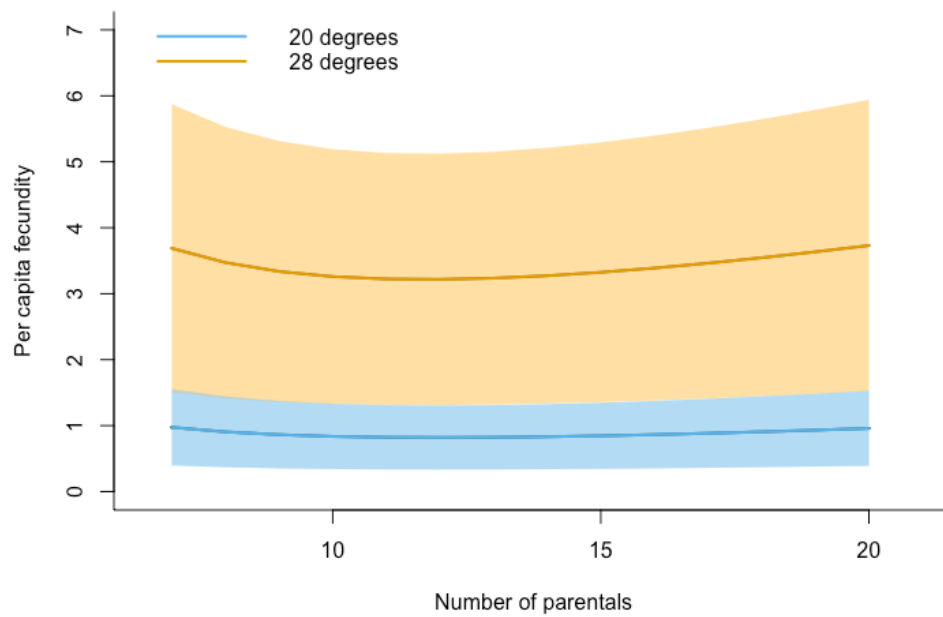
B



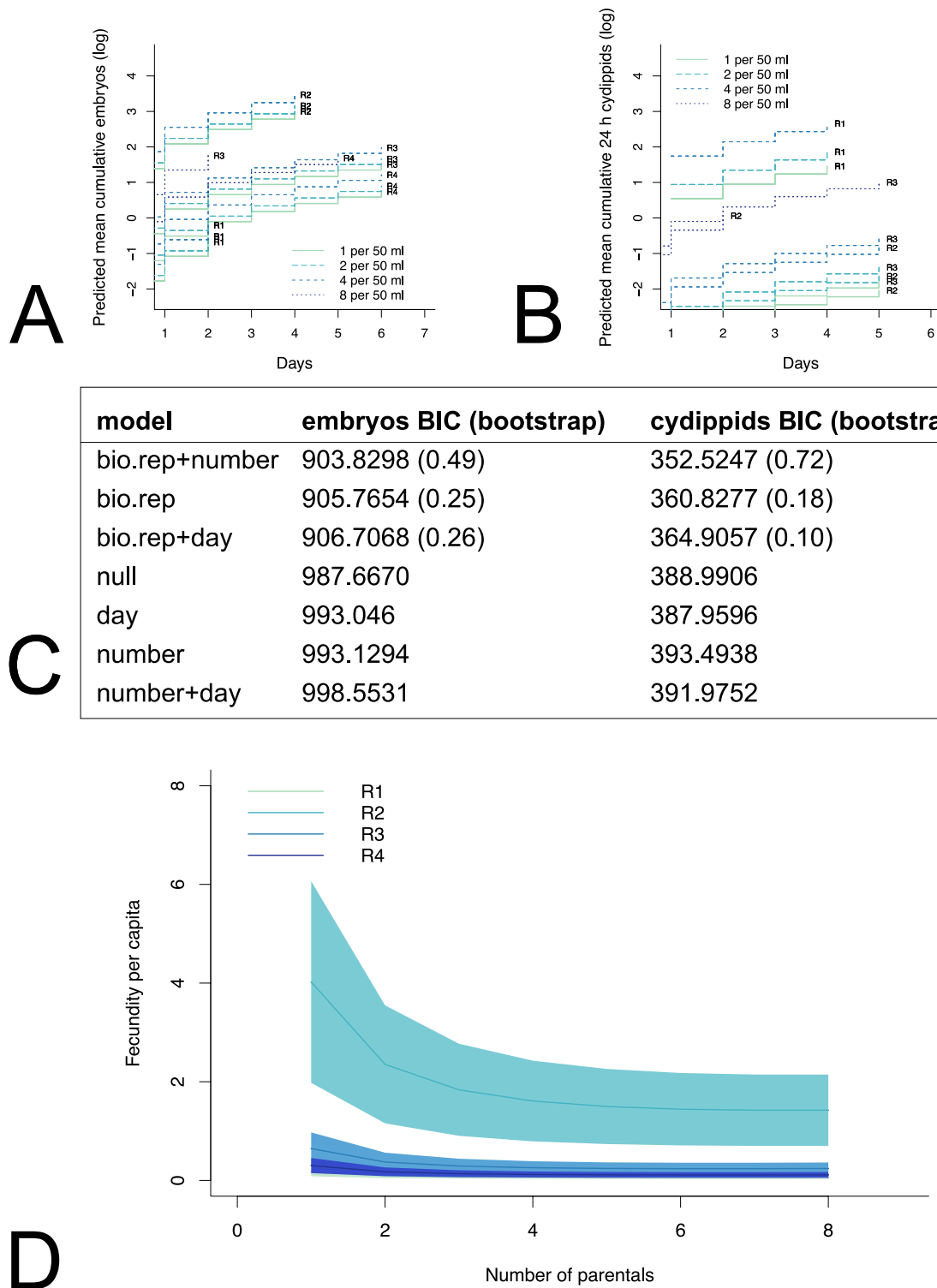
C

| model          | embryos BIC (bootstrap) | cydippids BIC (bootstrap) |
|----------------|-------------------------|---------------------------|
| number+temp    | 532.1247 (0.60)         | 464.9463 (0.53)           |
| bio.rep+temp   | 537.3869                | 468.101 (0.02)            |
| temp           | 538.3136 (0.13)         | 468.4962 (0.30)           |
| number+day     | 544.7987 (0.27)         | 483.7533 (0.06)           |
| day            | 546.3797                | 482.9868 (0.09)           |
| number         | 550.5957                | 489.5525                  |
| null           | 552.014                 | 488.5174                  |
| bio.rep        | 556.1047                | 494.4993                  |
| bio.rep+number | 556.2008                | 495.9482                  |

D

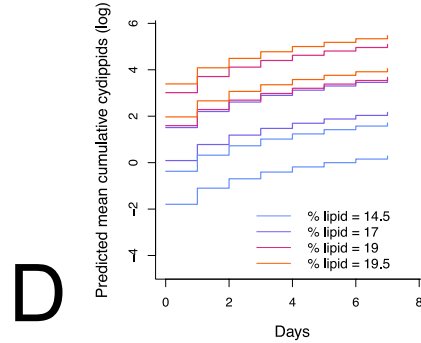
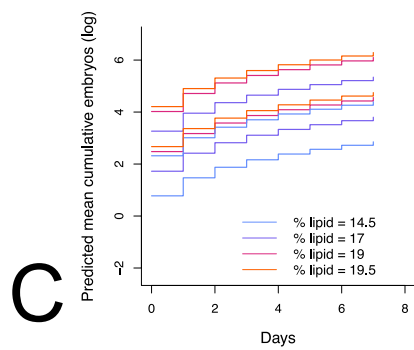
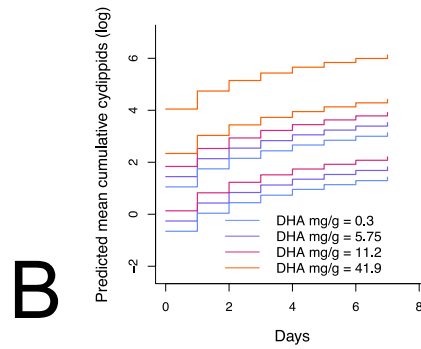
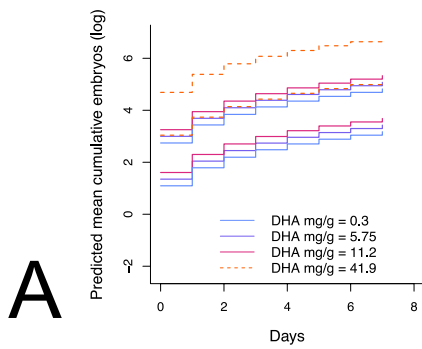


**Figure 3.** Environmental temperature affects fecundity, as shown by predicted mean cumulative reproductive effort reproductive output at two temperatures, 20°C and 28°C indicate a daily increase from ~0.88 embryos/parent to ~2.05 embryos/parent between the two temperatures. Repeated measures of 133 parental cydippids cultured in 4 groups of 7-20 animals from 4 biological replicates, totaling 63 total daily observations. A. Reproductive *M. leidyi* cydippids produce more embryos and B. more normal 24 hpf offspring when incubated at the warmer temperature. C. The two columns represent the BIC results for all models evaluated in the temperature experiments shown in A and B; bootstrapping results are in parentheses. The model with the lowest BIC should be understood as the model that most closely represents the generating process given the data; the nonparametric bootstrapping results indicate the uncertainty around selection of that model (see Appendix for model derivation). D. Per capita fecundity at 20°C and 28°C. Shaded area (transparent) shows 95% confidence interval; by chance, the CIs of the samples are very closely apposed.



**Figure 4.** Fecundity increases with culture density. A, B. Predicted mean cumulative reproductive output at 4 culture densities (1-8 animals per 50 ml culture). 297 individual observations of N=60

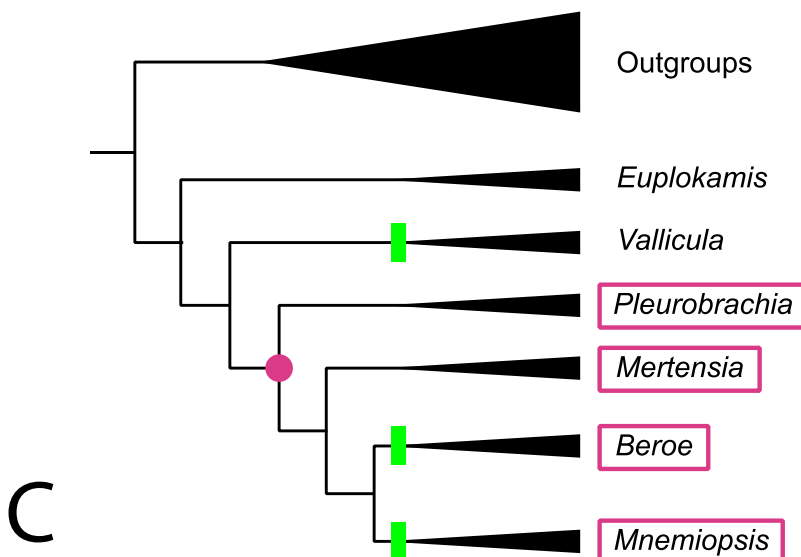
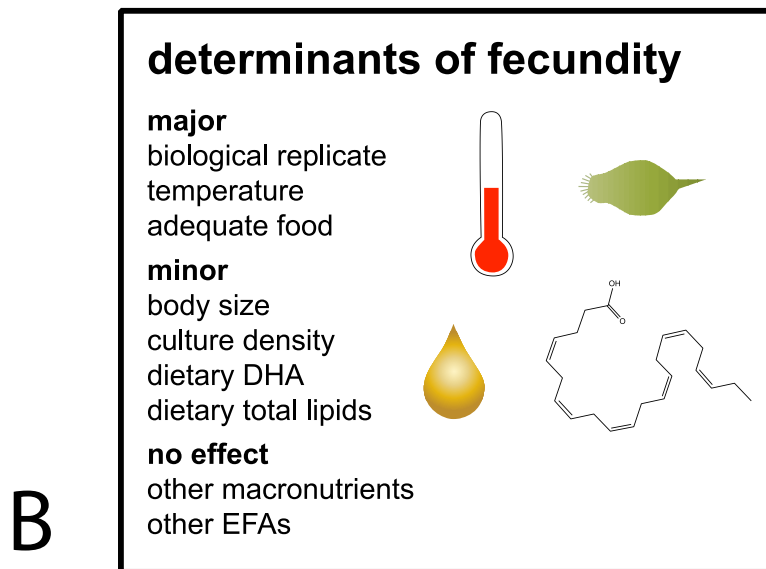
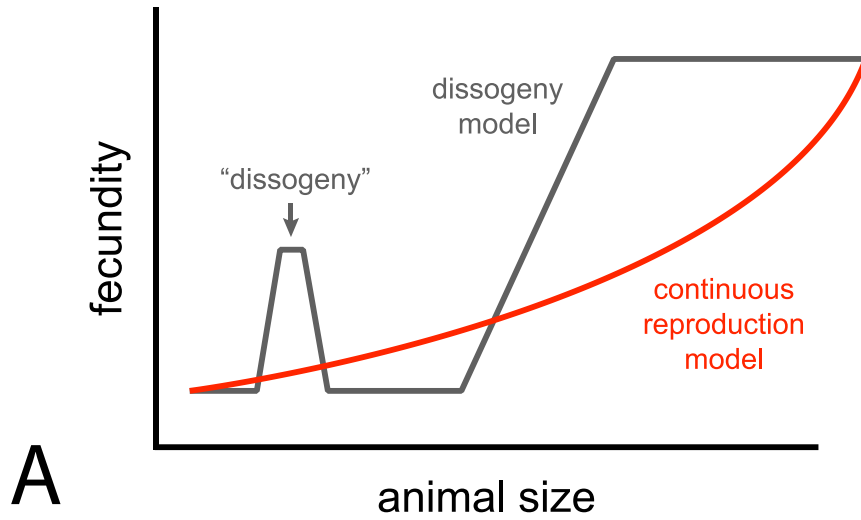
treatment replicates totaling 174 animals from 4 biological replicates. C. The two columns represent the BIC results for all models evaluated in the density experiments shown in A and B; bootstrapping results are in parentheses. The model with the lowest BIC should be understood as the model that most closely represents the generating process given the data; the nonparametric bootstrapping results indicate the uncertainty around selection of that model (see Appendix for model derivation). D. Per capita fecundity for each biological replicate. Shaded area shows standard error.



**E**

| model             | embryos BIC (bootstrap) | cydipids BIC (bootstrap) |
|-------------------|-------------------------|--------------------------|
| bio.rep+DHA       | 480.6468 (0.46)         | 326.6361 (0.71)          |
| bio.rep+num+DHA   | 484.7735 (0.06)         | 330.7605                 |
| lipid+DHA+bio.rep | 484.7840 (0.25)         | 329.5366                 |
| bio.rep+lipid     | 487.3263 (0.20)         | 325.7526                 |
| bio.rep+num+DHA   | 493.2619                | 329.5366 (0.01)          |
| number+DHA        | 918.2136                | 339.5698                 |
| DHA               | 923.7704                | 337.9548                 |
| number+lipid      | 496.4327 (0.03)         | 335.2860 (0.04)          |
| bio.rep           | 496.8494                | 351.4429                 |
| lipid             | 497.2842                | 332.6167 (0.04)          |
| food.type         | 499.2041                | 336.6597 (0.02)          |
| null              | 501.7295                | 352.6024                 |
| carbohydrate      | 502.2316                | 353.3290                 |
| number            | 502.7014                | 354.7691                 |
| parental.dpf      | 502.7569                | 355.5525                 |
| protein           | 505.0470                | 356.3046                 |
| ARA               | 505.3080                | 356.6440                 |
| EPA               | 506.3734                | 356.4496                 |
| day               | 506.3784                | 356.7601                 |

**Figure 5.** Rotifer (prey) nutrition affects *M. leidyi* capacity to spawn at small sizes (1-3 mm). Repeated measures for 8 days of N=148 animals in cultures of 12-20 individuals per treatment from 2 biological replicates, totaling 64 daily observations. A. Predicted mean cumulative reproductive output of embryos at 4 dietary levels of DHA. B. Predicted mean cumulative reproductive output normal 24 hpf offspring cydippids at 4 dietary levels of DHA. Dashed line is used where two treatment lines overlap (19.5% in panels B and D) to facilitate visualization. C. Predicted mean cumulative reproductive output of embryos at 4 dietary levels of total lipids. D. Predicted mean cumulative reproductive output normal 24 hpf offspring cydippids at 4 dietary levels of total lipids. E. The two columns represent the BIC results for all models evaluated in the diet experiments shown in A-D; bootstrapping results are in parentheses. The model with the lowest BIC should be understood as the model that most closely represents the generating process given the data; the nonparametric bootstrapping results indicate the uncertainty around selection of that model (see Appendix for model derivation).



**Figure 6.** Revised model for ctenophore sexual maturation. A. Ctenophores do not have two separate phases of reproduction as described in previous literature (“dissogeny model”). Rather, hatched ctenophores become fertile early and slowly increase their reproductive output (“continuous reproduction model”). B. Several variables tested affect fecundity similarly in small-size *M. leidyi* as in published reports on large-size animals. These reproductive characteristics – responsiveness to nutrition, temperature, and culture density, continuous and gradually increasing reproductive capacity, and the commonness of small-size spawning – all clearly suggest that *M. leidyi* are functionally adult from a body size  $\geq 1$  mm, regardless of morphology. C. Small-size reproduction mapped onto a cladogram of the Ctenophora. Tree topology based on (25). Pink boxes containing a genus name indicate a branch with one or more species that have been shown to reproduce at a small size (~0.5-1 mm) (16, 19, 20, 47); pink circle indicates the most recent common ancestor of these taxa. Green bars indicate lineages with at least some species exhibiting morphologies other than cydippid at some developmental stage, as reconstructed in (22). Beroids do not have a cydippid form at any stage. Reproduction at similarly small sizes has not been ruled out in the other ctenophore lineages.