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The sex lives of ctenophores: the influence of light, body size, and self-fertilization on the reproductive output of the sea walnut, *Mnemiopsis leidyi*

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Ctenophores (comb jellies) are emerging as important animals for investigating fundamental questions across numerous branches of biology (e.g., evodevo, neuroscience. and biogeography). Several ctenophore species including, most notably, *Mnemiopsis leidyi*, are known as invasive species, adding to the importance of studying the ecology of these animals. Despite the growing interest, relatively little is known about ctenophore reproduction. Like most ctenophores, *M. leidyi* is a simultaneous hermaphrodite capable of self-fertilization. In this study, we assess the influence of light on spawning, the effect of body size on spawning likelihood and reproductive output, and the cost of self-fertilization on egg viability in *M. leidyi*. Our results suggest that *M. leidyi* spawning is more strongly influenced by circadian rhythms than specific light clues, and that body size significantly impacts spawning and reproductive output. We also find a lower percentage of viable embryos from *M. leidyi* that were spawned alone versus those that were spawned in pairs, suggesting that self-fertilization may be costly in these animals. These results provide critical insight into the reproductive ecology of these ctenophores and provide a fundamental resource for researchers working with *M. leidyi* in the laboratory.

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The sex lives of ctenophores: the influence of light, body size, and self-fertilization on the reproductive output of the sea walnut, *Mnemiopsis leidyi*

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Abstract

Ctenophores (comb jellies) are emerging as important animals for investigating fundamental questions across numerous branches of biology (e.g., evodevo, neuroscience. and biogeography). Several ctenophore species including, most notably, *Mnemiopsis leidyi*, are known as invasive species, adding to the importance of studying the ecology of these animals. Despite the growing interest, relatively little is known about ctenophore reproduction. Like most ctenophores, *M. leidyi* is a simultaneous hermaphrodite capable of self-fertilization. In this study, we assess the influence of light on spawning, the effect of body size on spawning likelihood and reproductive output, and the cost of self-fertilization on egg viability in *M. leidyi*. Our results suggest that *M. leidyi* spawning is more strongly influenced by circadian rhythms than specific light clues, and that body size significantly impacts spawning and reproductive output. We also find a lower percentage of viable embryos from *M. leidyi* that were spawned alone versus those that were spawned in pairs, suggesting that self-fertilization may be costly in these animals. These results provide critical insight into the reproductive ecology of these ctenophores and provide a fundamental resource for researchers working with *M. leidyi* in the laboratory.

Introduction

Ctenophores (comb jellies) are fascinating planktonic animals most easily recognized by eight rows of cilia that they use as their primary means of locomotion. Recent work suggests ctenophores are the sister group to the rest of all animals and therefore are especially informative as to the state of the most recent common ancestor of animals (Dunn et al. 2008; Hejnol et al. 2009; Ryan et al. 2013; Borowiec et al. 2015; Chang et al. 2015; Whelan et al. 2015) but see (Pisani et al. 2015). This phylogenetic position, the availability of nuclear and mitochondrial genome sequences (Pett et al. 2011; Ryan et al. 2013), and the ease with which embryos can be collected and observed (Pang & Martindale 2008b) has made the ctenophore *Mnemiopsis leidyi* (Fig. 1) an emergent model system in which to study animal evolution and development (Pang & Martindale 2008a). In addition, since the invasion of *M. leidyi* into European waters from its native range on the Atlantic seaboard (Vinogradov et al. 1989; Reusch et al. 2010) has had profound impacts on European fisheries (Kideys 2002; Oguz, Fach & Salihoglu 2008; Finenko et al. 2013), interest is high in the biogeography and invasion dynamics of *M. leidyi*. Despite the growing importance and utility of *M. leidyi*, the reproductive ecology of these animals is not very well understood.

The reproductive biology and life-history of *M. leidyi* has likely played a major role in its ability to invade and establish populations in foreign waters. *M. leidyi*, like most ctenophores, are simultaneous hermaphrodites that have the ability to self-fertilize and have been observed to produce thousands of eggs a day (Baker & Reeve 1974; Costello et al. 2006; Lehtiniemi et al. 2012). Offspring may develop from egg to reproductive adult in as few as 13 days (Baker & Reeve 1974; Costello et al. 2012). *M. leidyi* may even produce viable gametes as juveniles (Martindale 1987).

A number of studies have described the spawning behavior of *M. leidyi* (Baker & Reeve 1974; Pang & Martindale 2008b). Early research suggested that *M. leidyi* spawns as a response to darkness (e.g., sunset) (Freeman & Reynolds 1973), while more recent protocols have stated that *M. leidyi* use light cues to trigger spawning, readily releasing gametes upon exposure to light after spending at least three to four hours in darkness (Pang & Martindale 2008b).

Adult *M. leidyi* vary dramatically in body size and this variation can affect both the likelihood to spawn and the number of eggs produced (Baker & Reeve 1974; Finenko et al. 2006). Animals are more likely to spawn as they grow larger (Baker & Reeve 1974) and larger animals generally produce more eggs per day (Baker & Reeve 1974; Finenko et al. 2006). However, the threshold size before spawning begins has varied from 15mm (Finenko et al. 2006) to 32mm (Baker & Reeve 1974) across studies. It is unclear whether this wide variation in initial spawning size is due to population-specific differences, seasonal timing, or other factors.

While self-fertilization may provide the benefit of allowing *M. leidyi* to reproduce when conspecifics are not present, it may also come with the cost of inbreeding depression. Inbreeding depression has been shown to affect the viability of offspring in many systems (Charlesworth & Charlesworth 1987; Crnokrak & Roff 1999; Herlihy & Eckert 2002). Rates of self-fertilization and inbreeding depression may be especially high in recently established populations where the population size and genetic diversity are low. Thus, establishing the degree to which self-fertilization is costly in *M. leidyi* has particular significance for the management of areas where these ctenophores are invasive. However, to our knowledge, the costs associated with self-fertilization in *M. leidyi* have never been thoroughly investigated.

In this study, we aim to describe the reproductive cues, effect of body size on spawning, and potential costs of self-fertilization in *M. leidyi*. We first investigate spawning cues by placing individuals under different light regiments. We then describe how body size influences spawning likelihood, egg production, and egg viability. Finally, we test whether self-fertilization in *M. leidyi* is costly by comparing the viability of eggs from ctenophores spawned individually to those spawned with a partner. If self-fertilization is costly, we predict that the offspring of *M. leidyi* spawning alone will have lower viability than those spawned in groups. Taken together, this study will provide a detailed description of the reproductive ecology of *M. leidyi* , supply critical information for studying the invasive impact of these ctenophores, and become a pivotal resource for establishing *M. leidyi* as a model system in the laboratory.

Materials & Methods

Collection

We carefully collected a total of 218 *M. leidyi* from the surface waters of Port Orange and St. Augustine, FL using a cteno-dipper (beaker on a stick) and transported them in buckets to the Whitney Laboratory for the Marine Biosciences in St. Augustine, FL between June and October 2015. Upon arrival, the ctenophores were transferred first to a large beaker with filtered sea water and then placed in 4” diameter circular glass dishes filled with 250 mL of filtered sea water. We labeled each bowl with a unique identification number and measured the polar length of every ctenophore along the oral/aboral axis to the nearest mm using calipers. Most ctenophores were released after spawning although a few were used for DNA and RNA extraction.

Light effects on spawning and egg production

We tested the protocol described in Pang and Martindale (2008b) using a subset of 64 *M. leidyi* that we had collected that day (N = 25) or collected and kept overnight in a large kreisel aquarium (N = 39). We did not monitor animals for spawning while they were in the kreisel. Between the hours of 10:00 and 18:00, we placed these animals in dishes in the dark for three to four hours. Upon exposure to light, bowls were monitored over the next two hours for the presence of eggs.

We conducted a separate set of experiments to test the importance of light cues for spawning on a subset of the *M. leidyi* that we had collected from Port Orange (N=66). On the day of collection, we separated each ctenophore into individual 4” diameter bowls filled with 250 mL of filtered seawater and haphazardly assigned individuals to one of four treatments: A) constant light (N = 21), B) 11 hours of light and then four hours of darkness (N = 15), C) seven hours of light and then eight hours of darkness (N = 12), or D) constant darkness (N = 18). All treatments began at 18:00 and ended at 9:00 the next day, at which point we exposed all of the animals to light and immediately recorded whether eggs were present in each bowl.

Size effects on spawning, egg production, and egg viability

In many systems, body size strongly influences reproductive output. We designed an experiment to test the effect of body size on spawning likelihood, egg production, and offspring viability. We tested the effect of size on spawning likelihood using the ctenophores already spawned in the previous light cues experiment (N=66) and an additional 52 *M. leidyi* (total N = 118) that we collected. We measured the length of every ctenophore along the oral/aboral axis to the nearest mm using calipers and then placed each in their own bowl with 250 mL of filtered seawater. We left the additional 52 animals that had not already been spawned overnight in either constant darkness (N = 26) or in a room with no artificial lights and an uncovered window to experience natural changes in light (N = 26). We immediately recorded whether eggs were present in each bowl on the following morning at 9:00. Since *M. leidyi* typically spawn hundreds of eggs, we only considered bowls with at least 15 eggs as having a true spawn. We calculated the effect of size on spawning likelihood using logistic regression and visualized the data with a cubic spline.

To collect the eggs of the ctenophores that spawned, we poured the water and eggs from each bowl through a 70-µm filter. The eggs of each ctenophore were then pipetted into separate 2” diameter bowls filled with filtered seawater. Eggs were allowed to settle in the bowl before we counted eggs.

A number of the ctenophores produced thousands of eggs, making a direct count of all eggs difficult. To address this challenge, we developed a protocol to allow us to estimate the number of eggs in each 2” bowl. We drew a 2” diameter circle and placed a square within the circle so that each point on the square touched the edge of the circle (Fig. 2). Finally, we divided the square into eight equal sized triangles that we labeled 1 – 8. For each ctenophore, we counted the number of eggs in two randomly selected triangles. Two triangles comprise 15.91% of the total area of the circle, and so to estimate the total number of eggs in the dish we multiplied the combined egg count by 6.285. Estimated egg production was log-transformed to increase normality. We then evaluated the correlation between body size and estimated egg production using linear regression for the individuals that spawned (N = 30). The reason the eggs from more *M. leidyi* spawns were not counted is because we developed the counting method halfway through the study.

To determine egg viability, we recounted the number of eggs in each dish after 24 hours. *M. leidyi* typically develop into juvenile cydippids within 18-24 hours after fertilization (Martindale & Henry 2015). Juveniles can easily be distinguished from undeveloped eggs due to ciliary movement, and since viable embryos can swim away from their original triangle into the water column, we counted the number of undeveloped eggs in the same triangles as in the egg production assay. We then estimated the number of undeveloped eggs in the entire dish using the method described above. Using this estimate we calculated the percent of undeveloped eggs (estimated undeveloped eggs / estimated total eggs) and subtracted that number from one to determine the percentage of viable eggs. We used linear regression to assess the effect of body size on egg viability (N = 30).

Costs of self-fertilization

If self-fertilization is costly, we would expect *M. leidyi* that were spawned alone to have reduced offspring viability compared to those that were spawned in pairs. To test for such a cost, 80 *M. leidyi* were randomly placed by themselves or with another individual in a 4” diameter bowl with 250 mL of filtered seawater. Individuals were spawned overnight and the next day we estimated the number of eggs present in each bowl and the percent of viable offspring 24 hours later (see above). We compared estimated egg production and egg viability from ctenophores spawned alone (N = 30 for egg production, N = 29 for egg viability) to ctenophores spawned in pairs (N = 25) using Student’s t-test.

All statistical analyses were run in JMP 11.0 (SAS Institute, Cary, NC).

Results

Spawning cues

Following the recent spawning protocol (Pang & Martindale 2008b), we placed *M. leidyi* in the dark for three to four hours between the hours of 10:00 and 18:00 before exposing them to light. After two hours in light, only five of 39 (12.8%) animals had produced any eggs. Furthermore, the few ctenophores that did spawn often released only a few eggs (median = 18 eggs, range 12 – 177 eggs).

We next tested the role of light cues in *M. leidyi* spawning. We kept ctenophores in individual bowls overnight in four treatments with varied light cycles and checked each bowl for eggs the following morning. Almost every ctenophore spawned overnight; we found no difference between ctenophores kept in constant light (20/21 [95%] spawned), four hours of darkness (15/15 [100%] spawned), eight hours of darkness (12/12 [100%] spawned), or constant darkness (17/18 [94%] spawned).

Size effects on spawning and egg viability

As *M. leidyi* grow larger, the likelihood of spawning significantly increases (Fig. 3, Logistic regression, $N = 118$, $\chi^2 = 62.0$, $p < 0.0001$). All but three ctenophores larger than 30mm spawned overnight, while only one ctenophore smaller than 26mm produced eggs.

We saw large variation in the number of estimated eggs spawned (range = 25-3934 eggs, median = 484 eggs). Larger individuals generally produced more eggs (Fig. 4, $N = 30$, $r^2 = .38$, $p < 0.001$). We also found a weak but insignificant positive correlation between body size and egg viability (Fig. 5, $N = 29$, $r^2 = 0.12$, $p = 0.07$).

Costs of self-fertilization

We compared the egg production between *M. leidyi* that spawned alone ($N = 30$) with *M. leidyi* that spawned in pairs ($N = 25$). We found no difference between treatments in the estimated number of eggs produced (Fig. 6, Student's t-test, t-ratio = 0.005, $p = 1.0$). However, we did find that a higher percentage of offspring from individuals that spawned in pairs ($N = 25$) had developed after 24 hours when compared with individuals that spawned by themselves ($N = 29$, Fig. 7, Student's t-test, t-ratio = 2.3, df = 52, $p = 0.025$).

Discussion

The ctenophore *Mnemiopsis leidyi* has become an emerging model from which to study evolution and development, especially for understanding early animal evolution (Pang & Martindale 2008a). Additionally, the invasion of *M. leidyi* in European waters has had devastating impacts on fisheries (Shiganova 1998; Kideys 2002; Finenko et al. 2013) and has led to strong interest in these animals. Understanding *M. leidyi* reproductive ecology is a necessary step in establishing it as an important model in the laboratory and may allow for improved management of these animals in afflicted areas.

Previous work has suggested that *M. leidyi* uses light cues to induce spawning (Freeman & Reynolds 1973; Pang & Martindale 2008b; Martindale & Henry 2015); however, our attempts at replicating this spawning cue failed. Instead, we found that almost every *M. leidyi* over a certain size spawned overnight regardless of the light/dark cycle; even those individuals that were placed under constant light consistently spawned. This result suggests that *M. leidyi* spawns using a circadian rhythm rather than specific light cues, at least when initially brought into the lab. Sequencing data indicate that the *M. leidyi* genome contains a number of orthologs involved in animal circadian rhythm including *Clock* and ARNTL. These and other circadian rhythm genes have been associated with reproduction and reproductive timing in a number of systems (Boden & Kennaway 2006; Leder, Danzmann & Ferguson 2006; Liedvogel et al. 2009). Functional genomic analyses into how these circadian-rhythm genes affect spawning could potentially provide solid evidence linking circadian rhythms and *M. leidyi* spawning. Given the phylogenetic position of ctenophores as the sister lineage to the rest of animals (Dunn et al. 2008; Ryan et al. 2013; Borowiec et al. 2015; Chang et al. 2015; Whelan et al. 2015), such a study would also address to what extent the genetic circuitry underlying animal circadian rhythm was present in the last common animal ancestor.

Previous spawning protocols were described for *M. leidyi* populations near Woods Hole, Massachusetts (Pang & Martindale 2008b). To our knowledge, spawning protocols have not previously been described for *M. leidyi* in the Atlantic waters of northern Florida. While these two *Mnemiopsis* populations had previously been classified as a separate species (Massachusetts = *Mnemiopsis leidyi*, Agassiz 1865, northern Florida = *Mnemiopsis mccradyi* Mayer, 1900), they are now generally considered to be separate populations of the same species (Pang & Martindale 2008a; Bayha et al. 2015), although this has yet to be extensively tested genetically. Populations within species may differ in their reproductive timing or cues (e.g. Partecke, Van't Hof & Gwinner 2004; Moore, Bonier & Wingfield 2005) and so it could be that the spawning behavior we observed is unique to the northern Florida population of *M. leidyi*. Alternatively, spawning behavior could change across seasons with changes to day length or water temperature.

Body size plays an essential role in ctenophore reproduction. Spawning occurs almost exclusively in larger *M. leidyi* (>30mm), although a few individuals smaller than 30mm spawned and a few animals larger than 40mm did not spawn (Fig. 3). Interestingly, this result differs from *M. leidyi* reproduction in the Caspian Sea where individuals begin spawning at 15 mm and the most common size of spawning individuals is between 20 and 30 mm (Finenko et al. 2006). Why these populations differ in size of reproduction is unclear, but they may be influenced by water temperature, resource abundance, or the low salinity of the Caspian Sea (Finenko et al. 2006). The differences in the non-native *M. leidyi* might also be a result of selection for body size or age of reproductive maturity due to selective pressures imposed by ship-ballast transport.

Not surprisingly, larger individuals in our study produced more eggs than smaller individuals (Fig. 4). Body size may correspond to nutritional status rather than age (Reeve, Syms & Kremer 1989) and so larger ctenophores may simply be those well fed enough to produce gametes. The production of gametes is costly (Hayward & Gillooly 2011) and smaller ctenophores preferentially allocate resources to somatic growth rather than gamete production (Reeve, Syms & Kremer 1989). Since larger individuals consume more prey (Bishop 1967; Finenko et al. 2006) they likely have more resources available to produce eggs than smaller individuals.

Body size may also affect offspring viability. We found that the percentage of developed eggs after 24 hours increased as individuals grew larger (Fig. 5), although this result was marginally not significant. If body size truly does affect offspring viability it may be due to sperm volume. If sperm are limited, especially in small individuals, larger animals may simply have more sperm available to fertilize eggs. Alternatively, larger animals may provision more resources to their eggs than smaller animals, which may increase egg viability or development speed. This possibility could be tested by comparing the size of eggs across body sizes.

Most ctenophores are simultaneous hermaphrodites with the ability to self-fertilize (Martindale & Henry 2015), but it is unknown whether self-fertilization is costly in these animals. Self-fertilization may lead to inbreeding depression which has been shown to have a suite of negative effects, such as reduced fecundity or viability, in many systems (Charlesworth & Charlesworth 1987; Crnokrak & Roff 1999; Herlihy & Eckert 2002). We have shown that *M. leidyi* individuals spawning alone had a lower percentage of developed offspring after 24 hours than ctenophores that spawned in pairs (Fig. 7). What contributes to this apparent cost to self-fertilization is unclear. It could be that spawning pairs simply fertilize more eggs than individuals spawning alone, which could occur if sperm are limited. Another possibility could be that the percentage of eggs fertilized did not differ between treatments but that fewer fertilized eggs developed for individuals spawning alone. Although we did not differentiate between unfertilized eggs and non-developing embryos in this study, we did commonly observe embryos that appeared to have arrested development after only a few stages of cell division. This possibility is consistent with a reduction in offspring viability due to inbreeding depression.

Interestingly, ctenophores in pairs did not produce more eggs than those spawning alone (Fig. 6). The average size of the ctenophores did not differ between treatments, suggesting that, when paired, ctenophores either reduce the number of eggs spawned or only one of the two ctenophores spawned eggs. This latter option, referred to as egg-trading, may indicate the intriguing possibility that ctenophores alternate between releasing sperm and eggs when in pairs or groups. Egg-trading has been reported in other simultaneously hermaphroditic systems including sea slugs, tobacco fish, and polychaetes (Leonard & Lukowiak 1984; Sella 1985; Petersen 1995). This behavior could be used to reduce the chance of self-fertilization in *M. leidyi*. However, the underlying assumption of egg-trading is that individuals spawn with the same partners multiple times; we would not expect this to be the case in *M. leidyi* under natural circumstances since movement is largely governed by water flow.

Our results also suggest that individuals may be more efficient when spawning alone than with others. Despite the reduced percentage of developing eggs, more viable offspring were produced per individual when spawned alone than when paired. However, we only spawned each ctenophore once. Individuals spawning alone may require a longer refractory period for gametogenesis before spawning again than paired individuals that alternate between releasing eggs and sperm. Comparing the reproductive output and viability between paired and single individuals over multiple days could provide more resolution on the costs associated with self-fertilization.

The ability to self-fertilize almost certainly enhances the capability of ctenophores to spread when invading new areas. However, the costs to self-fertilization that we've demonstrated may at least slow down their invasive capabilities. These costs may be especially high at the initial stages of an invasion when population numbers and genetic diversity are low. Our self-fertilization experiment only examined one stage of development (i.e., 24 hours after spawning) in one generation and yet we still found evidence that self-fertilization is costly. Additional costs likely do not appear until later in life or after multiple generations of self-fertilized offspring. An experiment investigating the multi-generational effects of self-fertilization may provide a clearer picture of the hurdles, or lack of hurdles, *Mnemiopsis* faces when initially invading a new area.

Conclusions

Due to their evolutionary position as sister taxa to all other animals (Ryan et al. 2013), ctenophores in general, and *M. leidyi* in particular, are quickly emerging as new model systems from which to understand evolution, development, regeneration, and even human disease (Pang & Martindale 2008a; Maxwell et al. 2014). Understanding the reproductive ecology of ctenophores is a necessary step in establishing these animals as tractable models for these areas of research. This study has reinforced the importance of body size in *M. leidyi* reproduction and has provided the first suggestions that self-fertilization may be costly in ctenophores. However, ctenophore reproduction in natural systems is still very much a mystery. For example, little is known about how common it is for *M. leidyi* to self-fertilize in the wild. We have shown that spawning likely follows a circadian rhythm, which may be a mechanism to increase the odds of out-crossing if all animals spawn simultaneously. If self-fertilization is indeed costly, additional adaptions to increase the chance of out-crossing are likely. This work provides a fundamental resource for researchers working with *M. leidyi* in their laboratory, as well as, a jumping-off point from which future studies of *M. leidyi* reproductive biology can be launched.

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Fig. 1 *Mnemiopsis leidyi*

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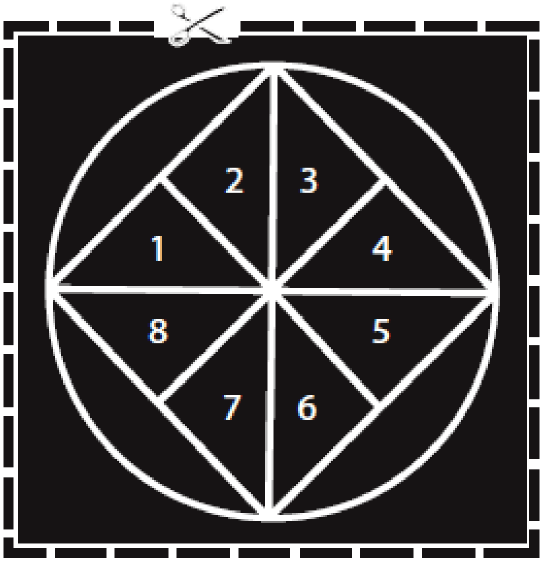

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Fig. 2 The diagram used to estimate egg numbers. Each triangle (labeled 1 – 8) represents 7.96% of the total area of the circle. We counted the eggs in two triangles and then multiplied the total by 6.285 to estimate the total number of eggs in the dish. Scaled to actual size used for 2” bowls.

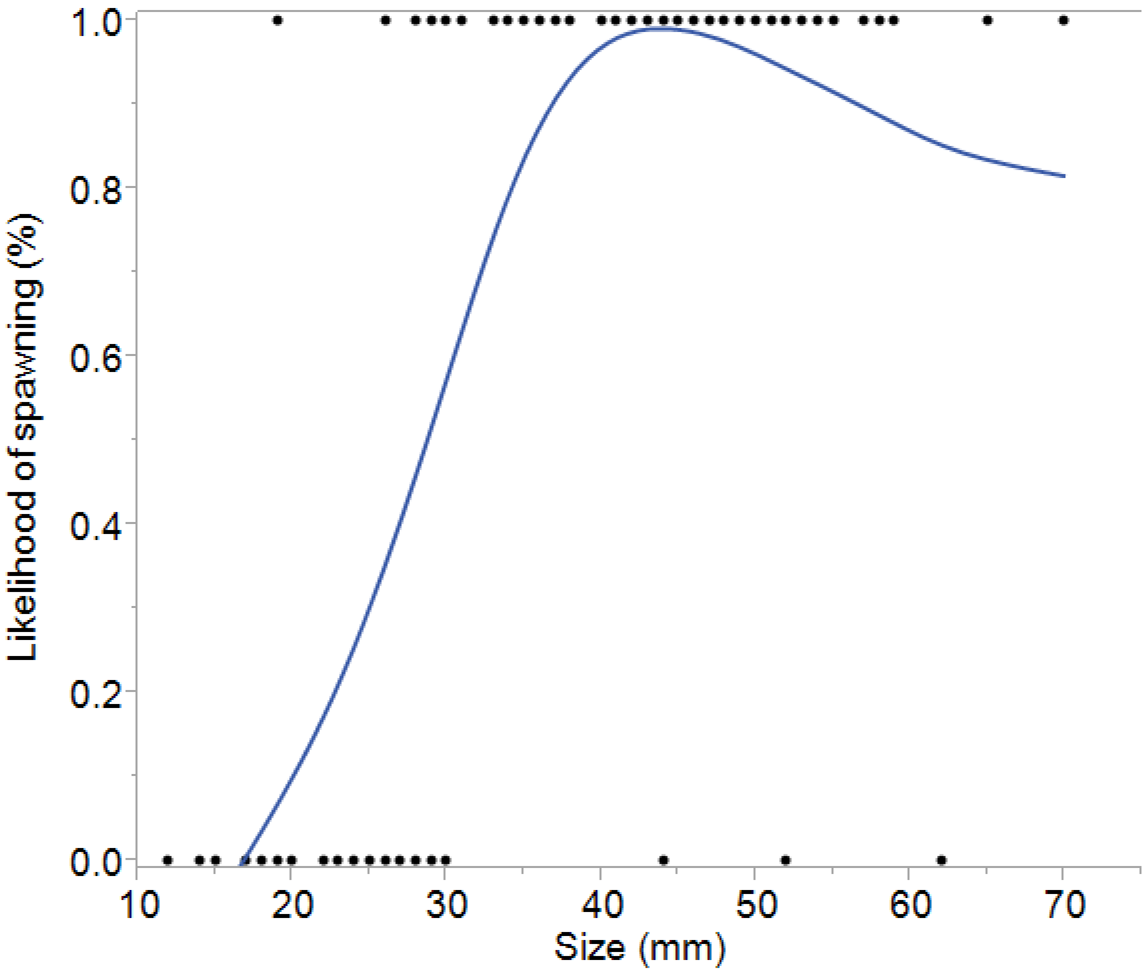

Fig. 3 Cubic spline showing the effect of body size on the likelihood to spawn. Individuals smaller than 26mm rarely spawned while those larger than 30mm almost always spawned. Lambda value of cubic spline set to 1.

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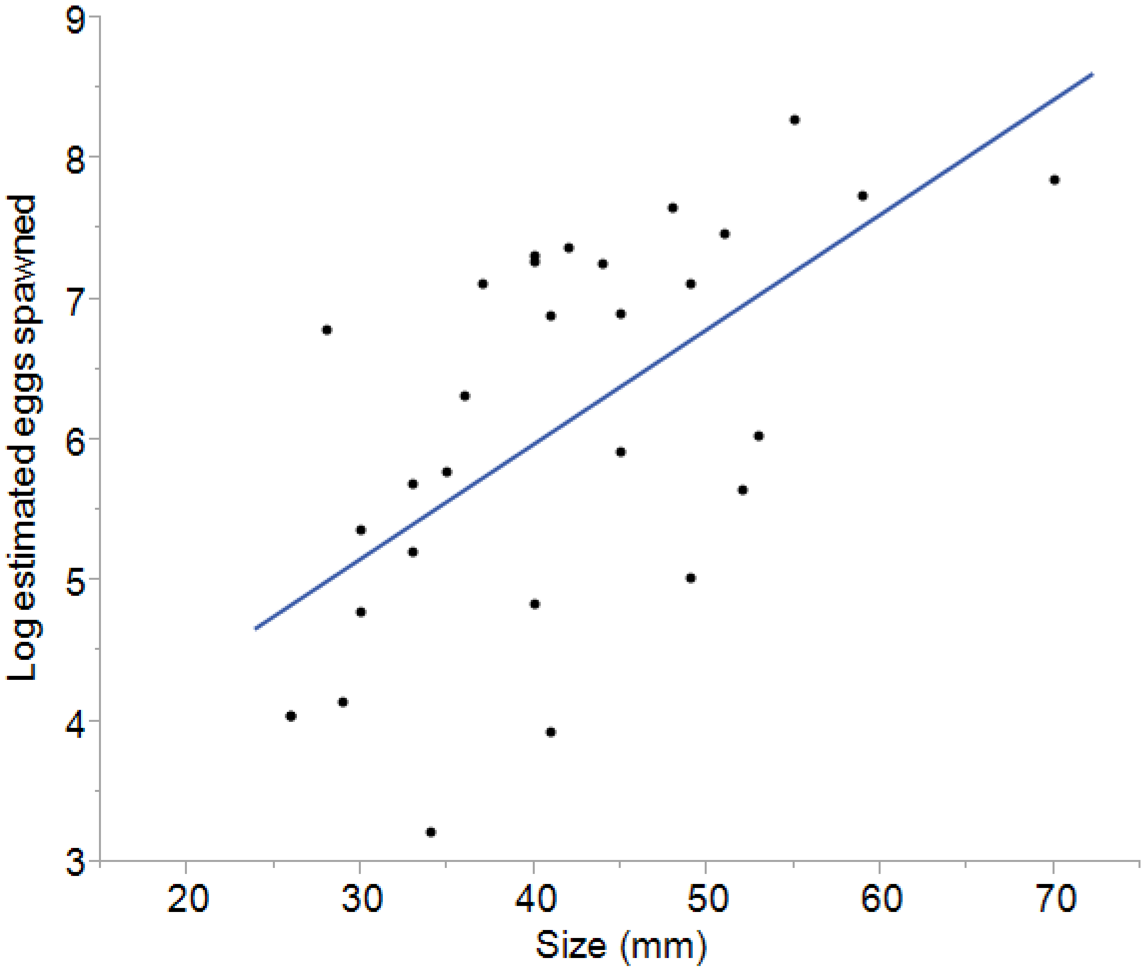
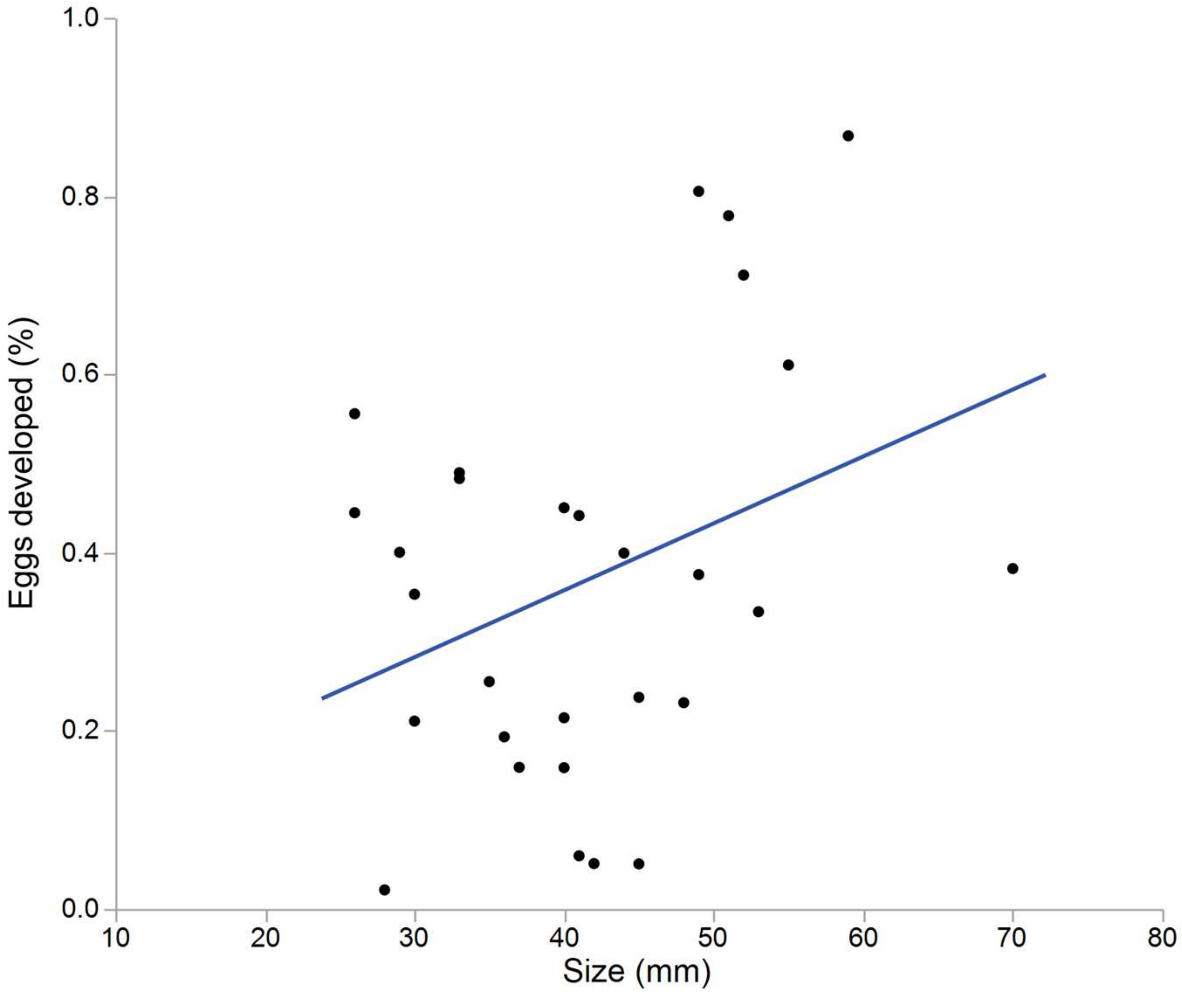


Fig. 4 The effect of body size on egg production. Larger individuals generally produced more eggs than smaller individuals (N = 30, $r^2 = .38$, $p < 0.001$). Only those animals that spawned 15 or more eggs are included in the analysis and figure.



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418 Fig. 5 Correlation between body size and egg viability. Body size positively correlated the percentage of eggs that developed after 24 hours, although the result was marginally not significant (N = 29, $r^2 = 0.12$, $p = 0.07$).

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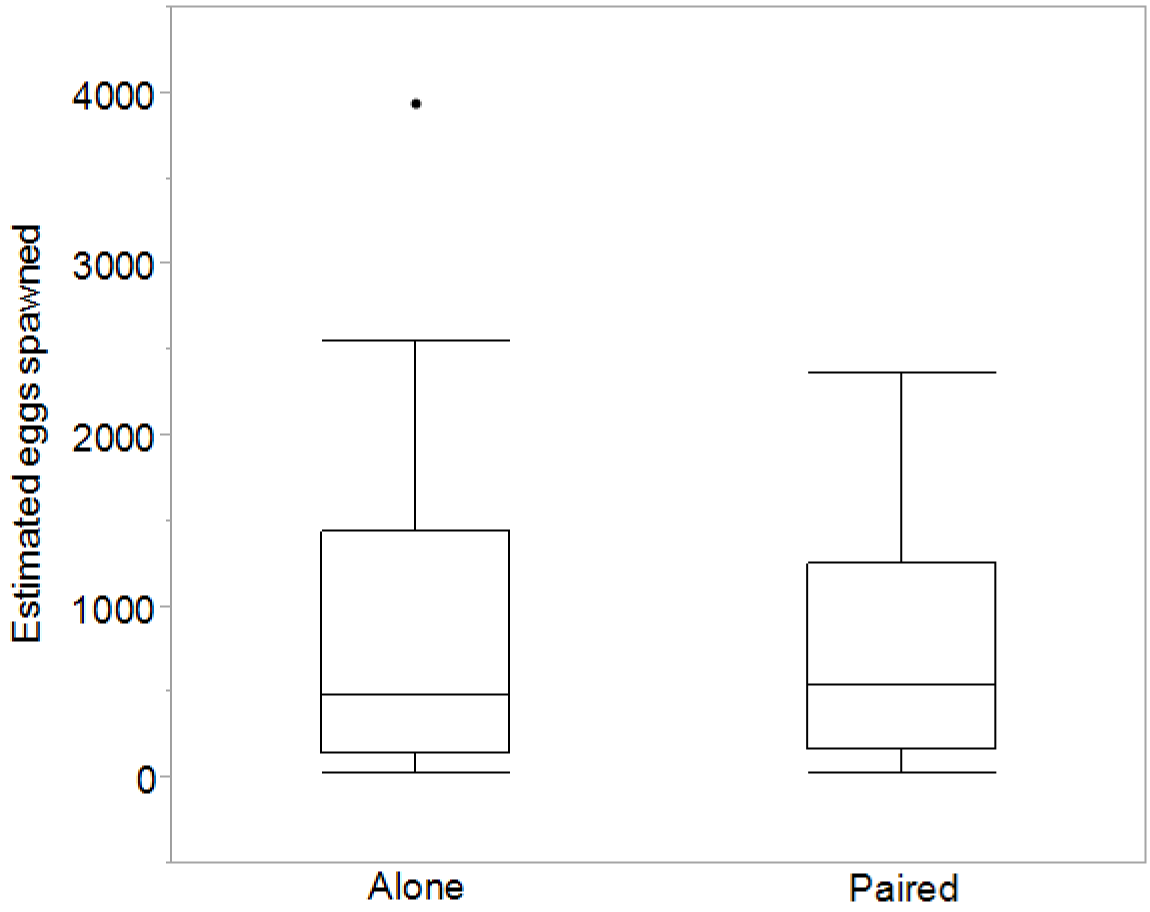

Fig. 6 The estimated number of eggs spawned for individuals spawning alone (N = 29) and in pairs (N = 25). Surprisingly, two *M. leidyi* spawning together did not produce more eggs than individuals spawning alone (Student's t-test, t-ratio = 0.005, p = 1.0). The data point above the Alone box plot indicates an individual that spawned an estimated 3,934 eggs. Removing that data point does not change the overall findings of the analysis.

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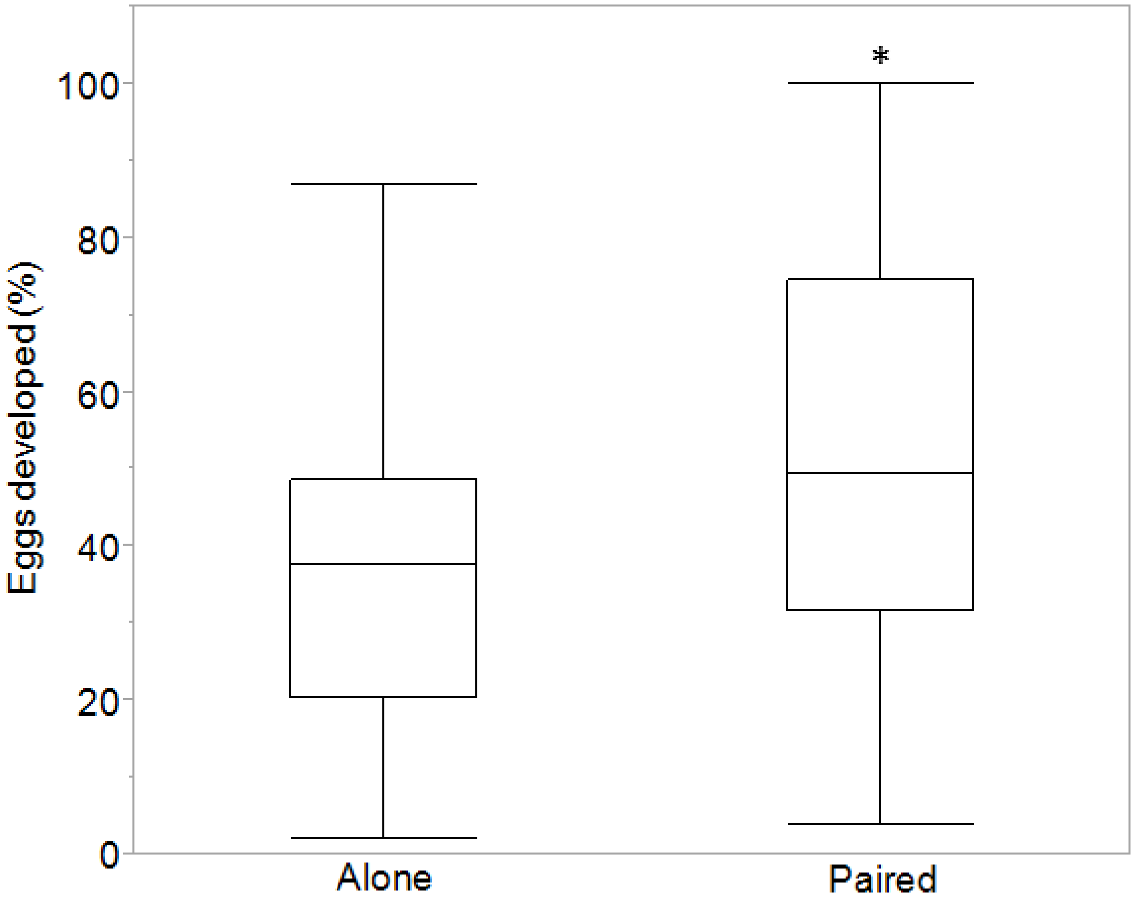

Fig. 7 The percentage of eggs developed after 24 hours for individuals spawning alone (N = 29) and in pairs (N = 25). A higher percentage of eggs developed for *M. leidyi* in pairs, possibility suggesting a cost to self-fertilization (Student's t-test, t-ratio = 2.3, df = 52, p = 0.025). Asterisk indicates significant difference across treatments.