The spawning and development of *Mopalia muscosa* from fertilization through metamorphosis at Friday Harbor Laboratories

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REU-Blinks Program for Undergraduates

Summer 2025

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Keywords: chitons, spawning, larval development, metamorphosis

Abstract

Exploring unique physiological processes across animal taxa inspires ideas and innovations. Chitons are marine mollusks that biomineralize teeth with magnetite, an iron oxide mineral. Iron is toxic to animals in high concentrations, so past research focused on how adult chitons use iron. In situ spawning and fertilization of chitons have historically been unreliable, so larval and juvenile specimens have been difficult to obtain to study radula development. Here we describe methods that induced spawning in *Mopalia muscosa* and *Katarina tunicata*, as well as several methods that failed. We maintained cultures of *M. muscosa* through metamorphosis. Our experimental spawning trials and photographic documentation of *M. muscosa* build on past literature to further understand the embryology, development, and metamorphosis of chitons. In the future, the samples we collected across developmental stages will be used to reveal the timeline of morphological and transcriptomic changes of early stages of *M. muscosa*, especially as it relates to radula growth and the start of iron biomineralization.

Introduction

Chitons (Mollusca:Polyplacophora) are marine invertebrates that are a powerful system for studying biomineralization, informing the development of bio-inspired materials and engineering products. Chitons are basal mollusks characterized by a shell consisting of 8 valves and a large muscular foot that they use to cling to rocks and other hard substrates. Most chitons are gonochoristic and spawn by releasing gametes into the surrounding water; they do not exhibit external sexual dimorphism. They are most often found ubiquitously in intertidal regions, but some species are found in the deep sea.

Chiton diversity is high in the Pacific Northwest of the United States (Navarrete et al., 2020).

Chitons feed on algae using their radula, which is a tongue-like organ with rows of iron-coated teeth. Chitons continuously create new teeth and move them towards their mouth like a conveyor belt, so adult chiton radulas contain all stages of tooth development (Kim et al., 1989; Shaw et al., 2002). Mature teeth are coated with magnetite, a mineral of iron oxide (Lowenstam, 1962). Chitons have an iron concentration in their bloodstreams that would be toxic to most animals (Papanikolaou & Pantopoulos, 2005).

It is not known at what stage of development chitons first incorporate iron into their teeth, largely because spawning and rearing chitons have been difficult to achieve. Many techniques known to induce spawning in invertebrates are ineffective on chitons. Past studies tried sperm suspensions, electric shock, UV irradiated sea water, isotonic KCl, nerve tissue extracts, temperature shock, light and dark periods, and hydrogen peroxide – none succeeded (Watanabe & Cox, 1974). Lamprey gonadotropin has been shown to induce spawning in one species of chiton, *Mopalia* sp. (Gorbman et al., 2003). Other studies in yellowfoot limpets and abalone have used salmon gonadotropin-releasing hormone (GnRH) to induce spawning (Hua & Ako, 2013; Mau et al., 2018; Nuurai et al., 2010).

When gametes were collected in past efforts, they produced little information on the development of chitons because chiton eggs are difficult to fertilize. They are protected by ornate hulls to prevent polyspermy and predation (Carlton, 2008), which

makes it difficult to fertilize them in vitro. To date, there has been little photographic documentation of chiton development in most species.

Here, we induced spawning in two species of chiton, *Mopalia muscosa* and *Katarina tunicata*. We describe the development and rearing of *M. muscosa* from fertilization through metamorphosis. We also took samples across development for both transcriptome sequencing and microscopy, which will be used in future studies to provide insight into how chiton feeding structures are related to the environment they are in and how chitons first contend with iron in their radula. Further knowledge of spawning and raising chitons can be applied to additional species in the future, building up our knowledge of chiton development as it relates to biomineralization.

Methods

Specimen collection

This study was conducted at the University of Washington, Friday Harbor Laboratories (FHL) on San Juan Island from June to August of 2025. Two *Mopalia muscosa* chitons collected from Cattle Point (48.4507°N, -122.9634°W) of San Juan Island were used for initial heat stress trials in sunlight. The remaining samples were collected from Deadman Bay (Figure 1) at 48.5131ºN, -123.1468ºW on June 23, 2025, at 9:47 am PDT at low tide (-2.7 feet). All chitons were collected in rocky tide pools on the west side of the beach. The largest chitons were targeted for collection. Chitons were removed from rocks by placing the tip of a flat butter knife under the posterior end and gently lifting. They were immediately placed into 5-gallon buckets filled with water and equipped with air pumps. Once transported back to the lab, they were put into flow tables

with ambient seawater flowing from just outside FHL. The species collected were *K. tunicata* and *M. muscosa.* The largest specimens were placed into individual glass custard bowls and assigned numbers for spawning trials. There were 20 *M. muscosa* and 8 *K. tunicata* separated to observe if post-capture stress induced spawning.

Figure 1: A map of Deadman Bay, where the most chiton specimens were collected from the rocky intertidal at low tide.

Spawning Trials

Eggs were collected on three occasions. One of those occasions was due to an experimental trial inducing spawning. The other experimental trial methods did not yield

eggs (Supplementary Table 1). The largest chitons in healthy condition were used in the trials. The specimens received water changes every day during the week and were free in the flow table if not being used in a spawning trial. Any eggs collected were maintained at a natural temperature between 12-13ºC due to the continuous flow of seawater from around the FHL campus.

A. Post-Capture Stress

Within 24 hours of collection from Deadman Bay, eggs were found in the flow table under a *K. tunicata* specimen as it was lifting the posterior end of its girdle in a spawning stance. Using a transfer pipette, they were moved to embryo-grade glassware filled with seawater filtered through a 2x 100 μm filter bag. Eggs were spawned within a mucus coating, which was placed in the bowl with the eggs. The bowls were on a raised platform to prevent water from entering them while allowing water to flow around the sides to maintain an appropriate temperature.

B. Heat Trial & Flipping on Back

Five *M. muscosa* were placed in separate bowls. Water was taken from a heated tank set to 26ºC and used to fill a large plastic bowl. The chitons were taken out of their glass bowls and plunged into the heated water until submerged. To maintain steady temperatures, after 10 minutes, the plastic bowl was placed into the heated tank. After 25 minutes, the chitons were returned to their bowls in the flow table to abruptly shift back to ambient seawater temperature. The water temperature and chitons' condition were recorded every 5 minutes after the initial 10 minutes during heat stress (Figure 2). Then, 1.5-2 hours after heat stress, the chitons were placed on their back for 10 minutes, then flipped back over. No chitons flipped themselves during this time.

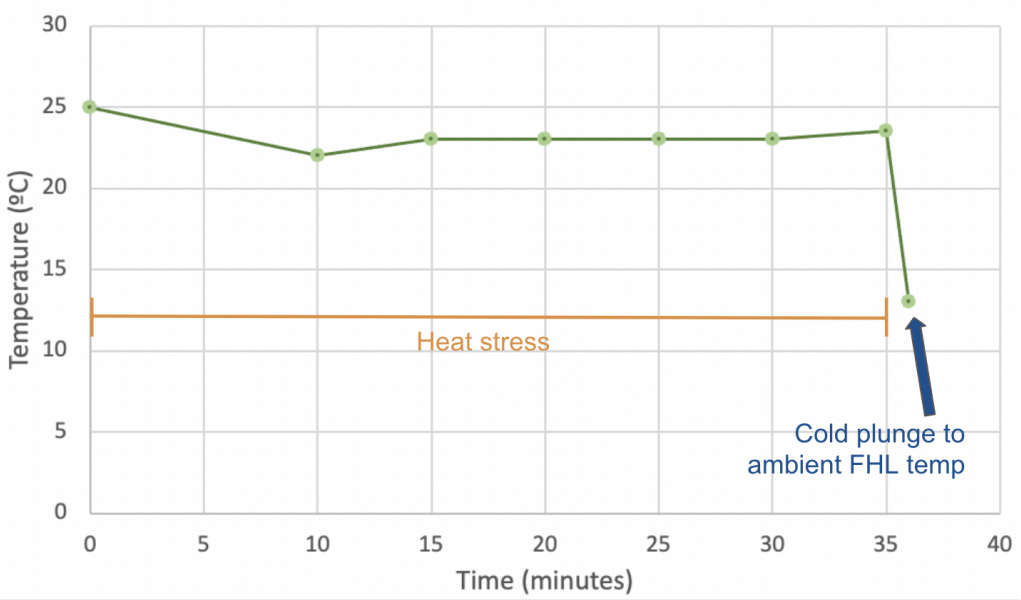


Figure 2: Recorded temperature over time during the heat stress trial for *Mopalia muscosa*. Chitons were plunged into 25ºC water and then returned to ambient seawater (13ºC).

C. Independent Spawning

Upon moving a female *K. tunicata* to another flow table, it was observed in spawning position with a fold in its posterior girdle. Eggs were found two hours later in the flow table and collected into embryo-grade glassware filled with filtered seawater. Another *K. tunicata* that was in the tank with the spawning female had a white substance ventrally near its gonopore, possibly sperm.

Fertilization

Chiton eggs will only fertilize if spawned by a female, but sperm can be collected via dissection. After eggs were collected, chitons of the same species were dissected to find sperm. The *K. tunicata* sperm was observed to be mobile under a compound microscope. Sperm wash was made using one drop of sperm in 10 mL of filtered

seawater in a sterile 15 mL Falcon tube. Approximately 2mL of sperm wash was placed in the bowls with the eggs. After 30 minutes, the water was decanted from the bowls to remove sperm, and then the bowls were filled with filtered seawater. The eggs were observed for fertilization and development under dissecting and compound microscopes.

Upon collection of the second round of *K. tunicata* eggs, they were split into 4 embryo-grade glassware dishes. A male conspecific was dissected to collect sperm. Two of the bowls were fertilized with 5 mL of sperm wash, obtained from 1 drop of sperm in 10 mL of filtered seawater. The other two bowls were fertilized with 5 mL of sperm wash from ½ drop of sperm in 10 mL of filtered seawater. After 30 minutes, the water was decanted, and filtered water was added to the bowls. After another 10 minutes, the eggs were washed again to remove sperm and prevent polyspermy.

A female *M. muscosa* laid eggs after the heat stress from the heated tank and being on its back for 10 minutes. The eggs were found in and around its bowl. The eggs and the mucus were placed into a glass bowl with filtered seawater using a transfer pipette. They were observed under a microscope to confirm which species the eggs belonged to. The eggs were then transferred to 4 embryo-grade glassware to be fertilized. A male chiton conspecific was dissected, and its sperm were used to fertilize the eggs collected. The eggs were fertilized by the same procedure used to fertilize the second fertilization of *K. tunicata* eggs collected.

Rearing

There were frequent observations of the eggs and daily water changes to minimize predation. After the *M. muscosa* eggs hatched into trochophores, they were transferred to

7 new embryo-grade glassware with filtered water to maintain a clean culture, using Pasteur pipettes so as not to rupture the trochophores (Strathmann, 2017). The stages of development were documented and photographed as they appeared. Every other day, debris was manually removed from the trochophore culture using a Pasteur pipette. If there were abundant ciliates or predators, the trochophores were moved to a new embryo-grade glassware with filtered water, also using a Pasteur pipette.

Chitons have been shown to settle in response to crustose coralline algae (CCA) in their environment, as well as to oyster shells with a bacterial film or limpet shells with an algal film (Strathmann, 2017). Chiton shells covered in CCA and green algae were placed in the bowls as a settlement cue. Pieces of an oyster shell with algae were placed in a few days later. Bacterial film buildup in the bowls was cleaned to avoid ciliates, but some was left for the chitons to develop on. Some chiton species can settle with bacterial films in their dishes if there is not an overwhelming amount, including *M. muscosa* (Strathmann, 2017). We chose to mix settlement cues rather than test isolated cues to maximize the settlement of our cultures.

Data Collection

At each developmental stage, samples of the chitons were taken, from unhatched fertilized eggs through juvenile development. Samples were placed in RNAlater for future transcriptomics and in paraformaldehyde for morphological studies using SEM microscopy and CT scanning.

Photographs were taken with a combination of a Nikon Eclipse E600 microscope, a Canon Rebel camera with a 150mm reduction lens, and an iPhone 14 held to the

microscope ocular lens. Photographs were taken on both compound and dissecting microscopes to document development.

Results

The only experimental spawning trial that yielded eggs was placing the *M. muscosa* in a heated tank, then flipping them on their back. None of the other experimental spawning trials resulted in spawning, including the salmon GnRH injections (Supplementary Figure 1). The eggs collected from the *K. tunicata* were from specimens in the flow tables, not in any active experiments.

All of the chiton eggs collected were yellow under the dissecting microscope and black with dense yolk under the compound microscope (Figure 3). Unfertilized eggs were more opaque and cloudy.

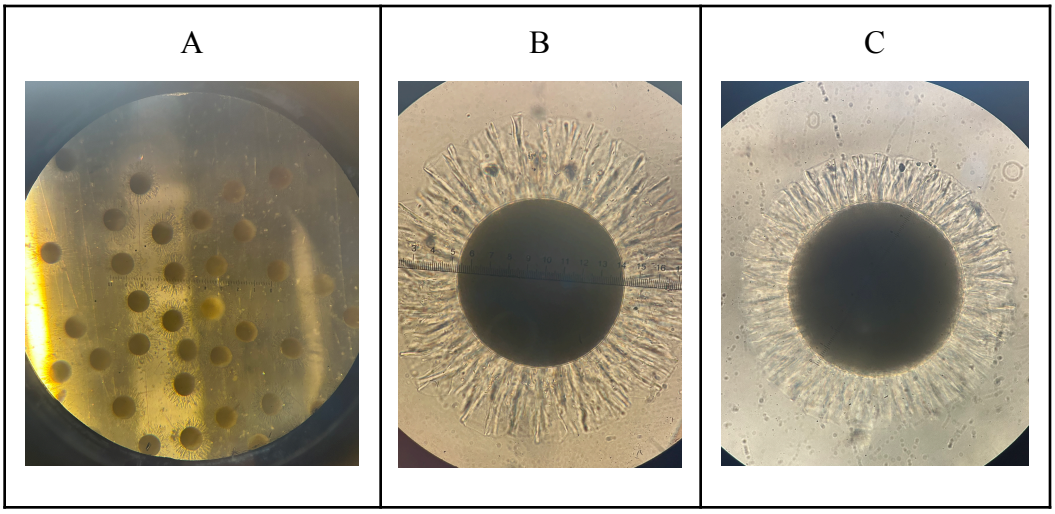


Figure 3: A) Picture of *K. tunicata* eggs under a dissecting microscope. B) A picture of a *K. tunicata* egg under a compound microscope. C) Picture of an *M. muscosa* egg under a compound microscope. All eggs were about 0.5mm in width, including hulls.

Katarina tunicata

No stages of development were confidently identified from the first fertilization of *K. tunicata* eggs. Several stages of pre-hatching development of the *K. tunicata* embryo were photographed, including the expulsion of the second polar body, the 2-cell stage, the 8-cell stage, and a blastula (Figure 4). These stages were all observed at similar times after experimental fertilization. An odor came from the eggs during water changes. Cellular contractions in the periphery of the yolk due to calcium signalling were observed 17 hours after fertilization. The eggs were observed for 5 days, but no hatching occurred despite indications of embryonic development in the yolk.

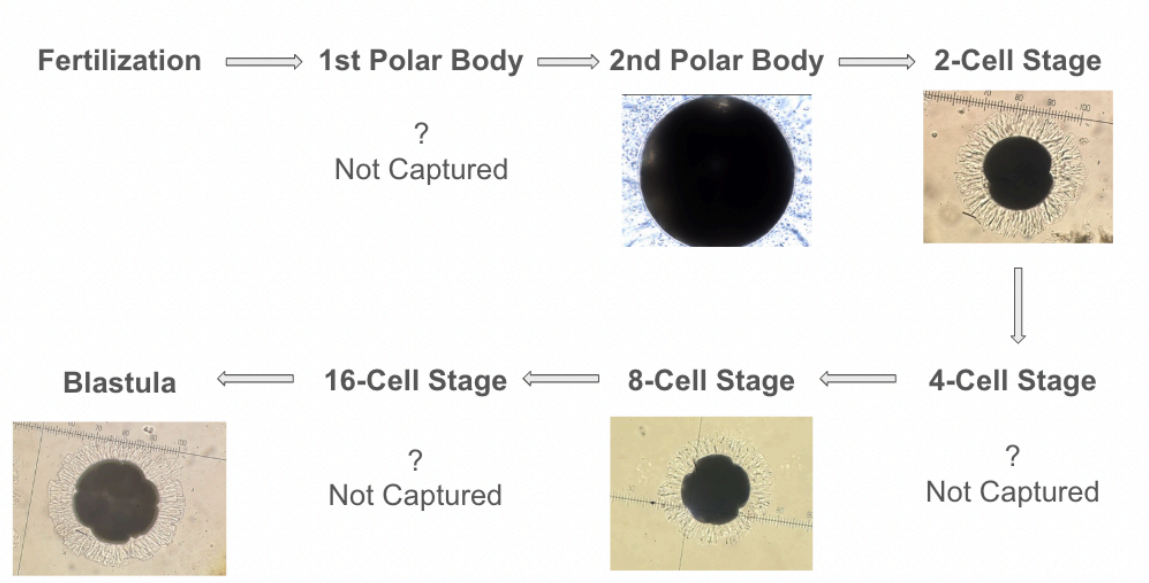


Figure 4: Photographs of *K. tunicata* pre-hatching, with approximate developmental stages. All eggs were about 0.5mm in width, including hulls.

Mopalia muscosa

The *M. muscosa* eggs did not display any visible signs of cellular division, but contractions in the periphery of the yolk were observed 4 hours after fertilization (Figure

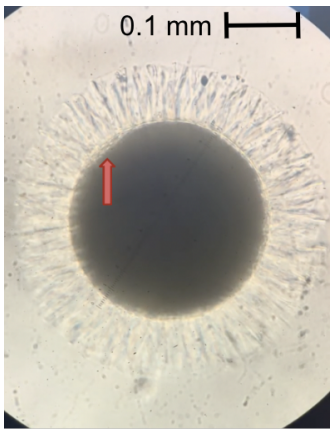
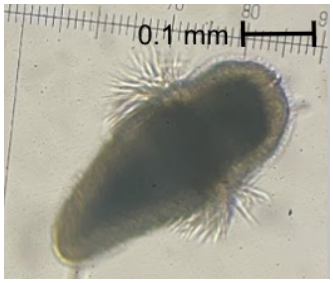
5A). Some eggs shed their hull, leaving only the yolk. The color of the egg was similar to that of the *K. tunicata* eggs, but its hull was shorter (Figure 3).

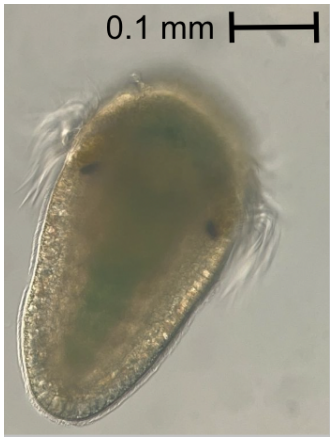
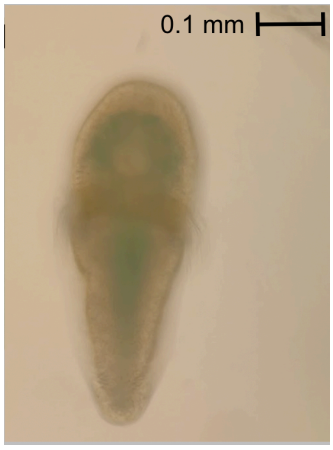
Hatching of *M. muscosa eggs* occurred 17-18 hours post-fertilization. There was a high hatch rate, and trochophores were continuously moved to cleaner bowls as they hatched. Trochophores were breaking through their shells and swimming around using prototroch cilia. They were green and torpedo-shaped with a band of cilia, the prototroch, and an apical tuft at their anterior end (Figure 5B). When swimming, they spun in a circle and propelled forward. Larval trochophores were found swimming towards the surface of the water. On day 3, the ocelli, larval eyes, were visible (Figure 5C). On day 5, there were 7 plates, and the muscular foot was beginning to develop. At this point, the trochophores were both crawling and swimming (Figure 5D). Their bodies became more elongated as they began crawling. On day 6, the larvae were competent to settle and spent the majority of their time on the bottom of the dish (Figure 5E). At this stage, the plates were more developed, and conglobation was observed when disturbed.

After 9 days, the larvae were settling on the edges of the dish and no longer crawling. The apical tuft and prototroch were lost (Figure 5F). They were feeding on bacterial film growing on the bowl and no longer relying on their yolk. They became less green and more transparent. Setae began to be visible on the periphery of the dorsal side of their girdle. This stage of development started to quickly attach their foot to the interior of pipettes when being moved. On day 16, pieces of a chiton valve covered in crustose coralline algae (CCA) and green algal turf were placed in each bowl as a settlement cue. On day 17, the chitons were found congregating under and crawling and feeding on top of the settlement cue, mainly on the green algal film (Figure 5G). Many

transitioned from torpedo-shaped larvae to settled with a more oval form and expanded plates. Many were feeding on algal turf. On day 21, chitons taken from CCA were observed and had undergone metamorphosis (Figure 5H). They began development of their 8th valve, indicating a transition to being juveniles. Ocelli were still present at this stage, but more visible from the ventral side of the body.

Photos and the corresponding expected and observed times to reach each developmental stage were recorded (Figure 5).

Stage of Development	**Expected Time at Ambient FHL Temperatures**	**Expected Time at 13.5-15.8ºC California**	**Observed Time**	**Photos**
A. Calcium signalling contractions (red arrow) in the yolk of the fertilized egg	N/A	N/A	4 hrs	
B. Hatching - trochophore	30 hrs	20 hrs	17-18 hrs	

C. Trochophore with ocelli	2.5 days	3.5 days	2.5-3 days	
D. Trochophore with plate and foot (swimming and crawling)	4 days	6 days	5 days	
E. Larvae competent to settle (mainly crawling) - ventral view of foot	-	-	6 days	

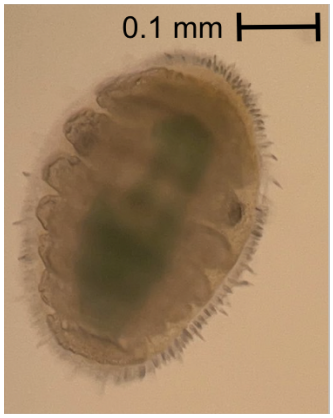
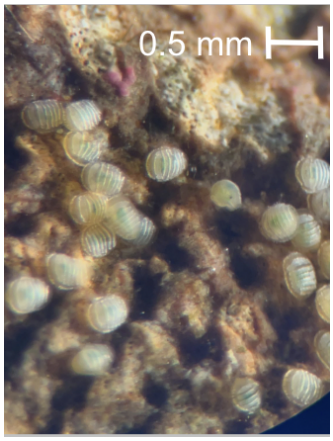
F. Larvae settling (plates 2-7) - feeding begun, apical tuft and prototroch lost	9 days	11.5 days	9 days	
G. Larvae settling on settlement cue (chiton valve with CCA and green algal turf)	N/A	N/A	17 days (settlement cue introduced on day 16)	
H. Developing 8th plate (metamorphosis to juvenilehood)	N/A	N/A	21 days	

Figure 5: A time series of stages captured through development and settlement labelled A-H, including expected and observed times of reaching each stage (Strathmann, 2017). N/A indicates that the time to reach that stage of development was not recorded in Strathmann et al.

Rearing Observations

Bacterial films began growing quickly despite daily water changes. The film on the bottom of the dish trapped chitons in it, so a pipette was used to untangle them and then remove the film. The film attracted ciliates if not removed. The chitons seemed to settle quickly when there was some bacterial film present, but if too much film grew, then it trapped larvae, and they died. Once the settlement cue was introduced, the chitons congregated on the green algal turf in earlier stages, then moved on to CCA in more mature stages. Cultures had varying stages of development, with later developmental stages being reached more quickly in cultures with a higher density of trochophores (e.g., 100 trochophores/bowl).

Hatching seemed to be quicker than expected at ambient FHL temperatures, but reaching developmental stages seemed to be on similar timelines (Strathmann, 2017).

Discussion

Chiton development has long been difficult to track due to a lack of knowledge about their spawning patterns, difficulty fertilizing their eggs through their ornate hulls, and difficulty tracking developmental changes in their dense yolk. This has led to a deficit in knowledge in their development through embryology and metamorphosis. Several techniques to induce spawning in the chitons were tested, building upon the work of existing literature to understand spawning cues in chitons. The combination of thermal stress induced by heat and flipping them on their back is the only experimental spawning trial that resulted in eggs being laid. Further experimental trials should be done on how to induce spawning in male chitons to collect viable sperm more easily and reliably.

Despite the *K. tunicata* eggs not hatching, this study provides an understanding of developmental cues that may be seen in the embryonic development of *K. tunicata*. The first trial of *K. tunicata* fertilization likely did not hatch due to polyspermy. The second trial of *K. tunicata* eggs likely did not hatch because of unclean cultures, as fecal and algal matter were entangled in the spawning mucus wrapped around the eggs. If rearing chitons, all fecal matter should be discarded from eggs to ensure the stability of the rest of the culture.

This study is the first photographic documentation of the development of larval and juvenile *M. muscosa* recorded in the literature. This documentation can be used to confirm and expand on past observations on *M. muscosa* development. Further studies will build upon the samples collected to track changes in the transcriptome and morphology of the chitons through the timeline of their development. The growth of the radula may shed light on long-standing questions about biomineralization in chitons. By producing larvae, we can examine the larval radula and its composition in more detail than previously possible. This is relevant for advancing bioinspired products and understanding how chitons withstand the toxicity associated with high concentrations of iron.

Acknowledgments

I would like to thank Dr. Rebecca Varney for her guidance and mentorship. Thank you to Dr. Billie Swalla for her support and for allowing us to use her microscopes to photograph the chitons' development. I would also like to thank the 2025 REU-Blinks coordinators, Dr. Cassandra Donatelli, Dr. Amy Cook, and Dr. Adam Summers, for

providing me with the opportunity to conduct this research at FHL. Thank you to Dr. Eric Edsinger for providing us with the salmon GnRH used in the hormone spawning trial. Lastly, a huge thank you to my family and friends for their constant encouragement.

This project was funded by the National Science Foundation (NSF) Grant DBI-2149705.

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Supplementary

Spawning Trials

I. Heat - Ambient Sunlight

Two *M. muscosa* specimens were placed into separate glass bowls. They were placed in direct sunlight for 2.5 hours in the afternoon and checked every 20 minutes to record the temperature and observe their condition. At every checkpoint, their physical appearance was recorded, and their attachment to the bowl was tested by pushing on them with one finger. Once their suction was weak, they were plunged into the flow table. The specimens were observed for the release of any gametes over 24 hours.

II. Heat - Heated Tank

A tank was set up in the lab with a heater set to 26ºC and an air pump. The two *M. muscosa* used in the heat trial using sunlight were placed in the warm water bath by submerging their bowls into the heated tank. They were in the heated tank for 8 minutes and then plunged into the flow table. They were observed for any gametes released over 24 hours.

The heated tank was also used on five *K. tunicata* samples. These chitons were placed into a warm water bath at 23ºC for 25 minutes. They were checked every 5 minutes to check the temperature and observe their condition. They were observed for 48 hours after the trial for gametes.

III. Placing in Sperm Wash

Sperm collected from dissected male *K. tunicata* was placed into 15mL Falcon tubes. Leftover sperm not used to fertilize eggs were placed into a large glass bowl full of seawater in the flow table. The 10 largest *K. tunicata* individuals were placed in the

sperm water for 10 minutes each. If no gametes were released after 10 minutes, then the chiton was placed in the flow table, and a new specimen was placed in the water. The flow table was observed for any gametes over the following 24 hours.

IV. Hormone Injections

A trial 0.2mL injection of seawater from the flow table was injected into an *M. muscosa* chiton to test if the volume had any adverse effects. The needle was placed through the foot of the chiton and then held for 10 seconds after releasing the fluid to minimize spillage upon removing the needle. The chiton did not have any negative reaction over the following 24 hours, so hormone calculations were made to use a volume of 0.2mL of liquid.

Salmon GnRH solutions were created to inject chiton and induce spawning. There was 1mg of freeze-dried OvaRH (sGnRHa) manufactured by Syndel. Sterile water was used to dissolve the OvaRH powder. This solution was placed into plastic vials to freeze and use later. There were vials with 20µL of solution and a couple of vials of 50 µL of solution placed in a 4ºC freezer.

Vials were removed from the freezer and thawed. For the *M. muscosa* hormone trial, 980µL of sterile water was added for each 20µL vial thawed. The vials were inverted 5 times to mix thoroughly. Each chiton was injected with 0.2mL and then placed in its respective bowl and observed for any negative conditions. They were injected with a second dose 24 hours after the initial dose.

For the *K. tunicata* hormone trial, the hormone injections were doubly concentrated. Vials were thawed, then 480µL of sterile water was added to each vial.

They were inverted 5 times to mix. Chitons were injected with 0.2mL of the hormone solution and placed into their respective bowls for observation of condition and gametes.

Table 1: Summary of methods tested to induce spawning and their results.

Treatment	**Time or Dose**	**Species**	**Number of Samples**	**Results**
Heat - sunlight	2.5 hours	*Mopalia muscosa*	2	No spawning
Heat - tank heater	8 minutes 26ºC then plunged in cold water	*Mopalia muscosa*	2	No spawning
	25 minutes (23ºC)	*Katarina tunicata*	5	No spawning
Stress from field collection	*24 hours*	*Mopalia muscosa*	10+	No spawning
	24 hours	*Katarina tunicata*	10+	1 female specimen spawned
Placing in sperm water	10 minutes	*Katarina tunicata*	10	No spawning
Salmon GnRH injections	1 dose of 0.2mL (20µL in 500µL H_2O)	*Katarina tunicata*	5	No spawning, 2 specimens died
	2 doses of 0.2mL (20µL in 1000µL H_2O) 24 hours apart	*Mopalia muscosa*	5	No spawning, 5 specimens died
Heated tank & flipping on back	35 minutes (22-25ºC), 2 hours later placed on its back for 10 minutes	*Mopalia muscosa*	5	1 female specimen spawned