Do *Balanus glandula* consume brine shrimp cysts?

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Abstract

The barnacle, *Balanus glandula,* is a good experimental organism for studying the impacts of both temperature and flow rate on filter feeding efficiency. Such knowledge has important implications for understanding the physiological constraints under which organisms operate, information necessary for predicting species' responses to environmental changes. As a first step toward this larger goal, I tested the feasibility of using brine shrimp cysts as prey organisms for *B. glandula* feeding studies. Specifically, the experiment examined the ability of *B. glandula* to ingest brine shrimp cysts and pass them through its digestive tract. This experiment demonstrated that *B. glandula* do ingest cysts, but the number of cysts ingested per individual is highly variable. Approximately half of the individuals tested did not consume any cysts within the 24hr feeding period. Of the ones who did, the number of ingested cysts ranged from 2 to 207. With one exception, all cysts had passed through the guts of the animals within 24hr. These results suggest a reasonable strategy for analyzing feeding efficiency would be to examine the number of brine shrimp cysts in the feces of individual animals following feeding periods at different water flow rates and temperatures.

Introduction

Understanding the impact of climate change on individual species remains an important scientific and public policy goal (Parmesan and Yohe 2003.) The rocky intertidal ecosystem is an increasingly important model for predicting the effects of global climate change on species, communities, and ecosystems (Barry, *et al.* 1995; Harley, *et al.* 2006; Sagarin, *et al.* 1999) Currently available data suggests that climate change has already affected species abundances, resulting in gradual northward shifts in species distribution (Barry, *et al.*; 1995). Such trends fail to take into account complexities of local environments. For example, due to spring tides in the summer months, northern regions experience more extreme climactic conditions than southerly regions. As a result,

climate change may have a different impact on identical species, depending on their local habitat conditions. (Helmuth, *et al.* 2002)

The complexity of an organism's response to global climate change reflects the complexity of its physiological responses to environmental changes or stresses (Helmuth, Kingsolver, and Carrington 2011). As studies by Carrington, Helmuth, and co-workers suggest, temperature is not the only driver of an organism's response to climate change (Barry, *et al.* 1995; Harley, *et al.* 2006; Helmuth , *et al.* 2006) Water flow velocity is another critical factor that determines organism abundance and distribution by affecting recruitment, predation, competition, and growth (Leonard, *et al.* 2011). For example, recruitment and growth of benthic suspension feeders, such as barnacles, is much greater in regions of high versus low water flow rates (Leonard, *et al.* 1998). Clearly, flow rate should be included in any model that aims to predict shifts in species abundance and distribution in response to climate change.

In spite of its importance, the coordinated physiological responses of marine organisms to changes in ocean temperature and water flow are still not clearly understood. Long-term studies that directly examine growth rates and fecundity in natural environments with different temperatures and flow rates would be useful predictors of the local impact of climate change on a species. However, such studies require months or years to complete and normal variations in weather events or patterns may make the results difficult to interpret. An alternative, synergistic strategy would be easily controlled, laboratory-based measurements of feeding rates. Such measurements may have substantive value for predicting local responses of sessile filter feeders to climate change. The experiments presented here provide some of the basic information needed to design effective experimental protocols to measure the effect of water temperature and flow rate on feeding efficiency in *Balanus glandula*, the acorn barnacle (Anderson 1994).

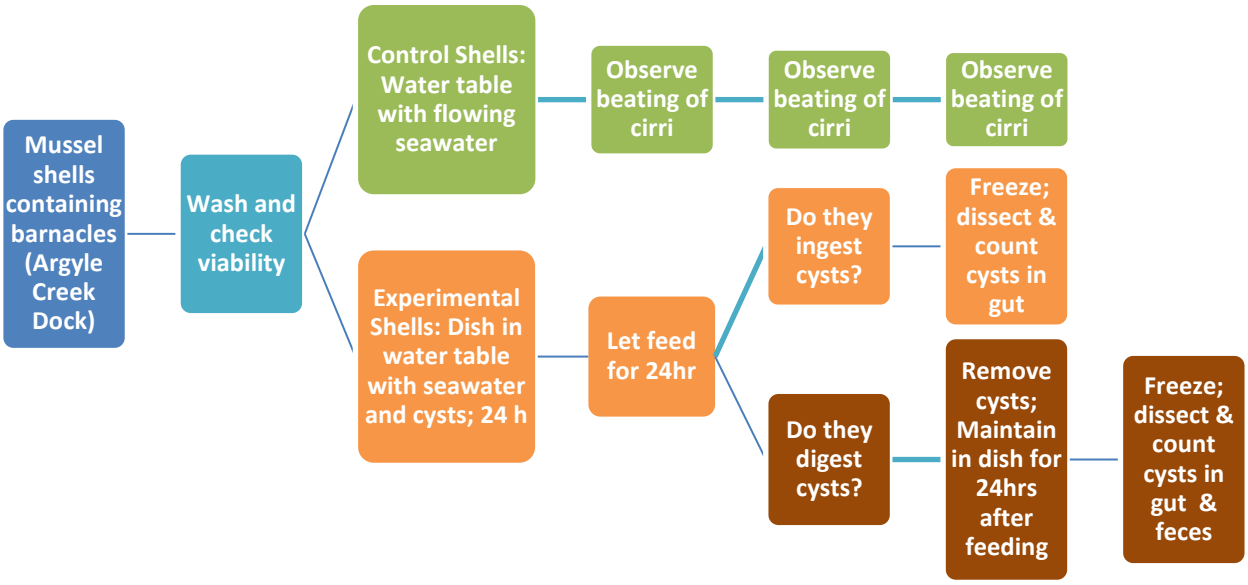
(The following paragraph is based on the Catalog of Life entry written by Soulanille.) *B. glandula* is common on intertidal substrates along the North American Pacific Coast, from Alaska to Mexico. Because *B. glandula* can achieve high densities, their larvae can be an important food source for fish. Adults and juveniles are preyed upon by *Nucella,* limpets, starfish, nemerteans, nudibranchs, ducks, geese, and seagulls. A rich scientific literature is available concerning the biomechanics of barnacle feeding, as well as on larval development and settling behaviors.

Barnacles filter feed using a fan formed from three biramous, setae-covered appendages called cirri. Three shorter cirri clean particles from the feeding fan and transfer them to the organism's mouth (Anderson, 1994). Maximal particle capture is achieved by orienting the fan relative to the direction of the current and altering the pace and shape of the sweeping motions. For example, at low current speeds, barnacles actively feed by periodically sweeping the water with their cirri and at higher current speeds, they passively, and more efficiently, feed by holding the cirral fan in the current for prolonged periods (Trager, Hwang, and Strickler 1990). In addition to delivering food to the sessile organisms, water flow is also important for waste removal and oxygen delivery. In fact, the cirral fan is the chief gas exchange organ for the sessile, gill-less adults (Personal communication, Megan Dethier, 2011).

The experimental goal of the "Team Balanus" 2011 project that included Emily Magley and myself, under direction of Michael Nishizaki, was to examine the efficiency of *B. glandula* ingestion of brine shrimp cysts at different water temperatures and flow rates (Miller 2007; Sanford, *et al.*; Marchinko 2007). Results from initial feeding experiments using a flow chamber suggested that that *B. glandula* were not consuming cysts. (These results are described in Emily Magley's research internship paper, Marine Zoology/Botany Quarter, Spring 2011.) To test this hypothesis, I examined whether or not barnacles ingested brine shrimp cysts and whether the cysts passed through their guts.

Materials & methods

The overall experimental design is outlined below:



Brine shrimp hydration: Fourteen ml of dry brine shrimp cysts (Sanders Brine Shrimp Company, Ogden, UT) were measured in a 15ml plastic screw cap tube and poured into 200-300 ml of seawater in a beaker; the mixture was stirred gently for 15 min using magnetic stirrer and a small stir bar. The cysts were then allowed to settle for 45-60 min. Floating cysts were removed by pouring off the water down to the settled cysts. Cysts were suspended in 50ml for use. One ml volume produced approximately 50,000 hydrated cysts.

***Balanus glandula* collection:** Mussels with *B. glandula* epizoa were collected from the dock at the salt stream/marsh, Argyle Lagoon[1], Friday Harbor Island, WA (N 48' 31.728' W 123' 00.802') during low tide. They were immediately brought back to the labs and maintained in Lab 3 at FHL in

[1] http://faculty.washington.edu/cemills/UWSanJuanPreserves.html

a water table with flowing, unfiltered seawater. After 3 days, the water was filtered to starve the barnacles.

Feeding experiment: On day 6 after harvesting, empty mussel shells containing barnacles were selected from the large pile of mussels in a water table. The shells were washed and placed into a separate, newly rinsed water table with flowing, unfiltered seawater. From this group of ~20 shells, 10 were randomly selected and placed into a large dish containing unfiltered seawater and hydrated brine shrimp cysts. (The concentration of cysts was not measured, but was greater than the 20,000 per ml used in our earlier experiments in the flow chamber. At the end of 24hr, no detectable difference in cyst density was noticeable by eye, suggesting that the cysts were available in large excess as intended.) An airline with an attached sparger was placed into the bowl for aeration and to help maintain cysts in the water surrounding the barnacles (Figure 1). However, even with the bubbling, the cysts settled in approximately 15 minutes. After an overnight incubation with the cysts, the dish was stirred approximately every 2 hours to resuspend the cysts.

Control barnacles were placed directly into the water table next to the dish containing the cyst-feeding barnacles (Figure 2). At the beginning of the feeding period, both control and experimental barnacles exhibited vigorous feeding, rapidly kicking out and retracting cirri. After 24 hours, the cyst-fed barnacles had a much less frequent cirral cycle than that of the control barnacles.

Measurement of cyst ingestion: After 24hr in the presence of brine shrimp cysts, half of the mussel shells with attached barnacles were removed from the dish, washed in flowing seawater, placed into a plastic bag, and frozen at -20°C to anesthetize them.[2] The shells were stored in the freezer for at least 24hr, then thawed by placing in seawater in preparation for dissection. While observing under a dissecting microscope, the tergum and scutum on one side of the aperture were

[2] We had initially assumed that freezing would euthanize the animals. However, movement was noted following thawing. Subsequently, we placed the barnacles on dry ice for a couple of hours or in the -70C freezer for 15 minutes before dissection. Either treatment accomplished successful euthanasia.

removed using watchmaker's forceps. The body sac containing the gut, cirri, and testes was then removed, usually by pulling on the remaining tergum and scutum, along with the cirri. Ovaries and tissue attached to the base were left in the carapace, which was inspected under the dissecting scope to ensure that no other tissues remained behind. Each animal was placed into a separate well in a 24-well microtiter plate and washed clean of uningested cysts changes of seawater squirted onto the animals with a Pasteur pipette. In most experiments, two changes of water were sufficient to remove uningested cysts, which were commonly present within the cirral branches and occasionally inside the mantle cavity.

After washing, the bodies were covered with bleach to digest tissue and release ingested cysts. We tried undiluted bleach as well as 20% bleach, but the undiluted was judged to provide superior clearing of tissue. Cysts exposed to bleach up to 48hrs were not dissolved. The number of cysts was determined after 6-24hr of digestion with undiluted bleach or overnight with 20% bleach.

To count cysts, the fluid in the well was mixed by trituration and then transferred to the lid of a small petri dish with an embossed quadrat. The transfer was monitored under a dissecting scope to ensure that no cysts were left behind. Cysts, which had a "frog egg" appearance and were readily identifiable by eye, were counted at low power on a dissecting microscope. In 20% bleach, some cysts remained opaque or golden-brown following bleach treatment.

Measurement of Cyst Clearance 24hrs after feeding: Shells containing barnacles exposed to cysts for 24hr were removed from the dish and washed extensively in flowing seawater. The dish was rinsed thoroughly and then filled with fresh, unfiltered seawater. The washed shells were returned to the clean dish in the water table, the airline was replaced, and the animals were undisturbed for 24hrs. At this time, they were removed, washed, and frozen as described previously. Feces that remained in the dish were collected and analyzed by microscopy.

Results

The experimental approach appears to be adequate for measuring ingestion of brine s*hr*imp cysts by* B. glandula*.
Analysis of barnacles on Shell 1, Dissected Sunday, May 22, 2011

A shell with four large barnacles was selected as a pilot to determine whether or not bleach would digest barnacle tissue without destroying brine shrimp cysts. The bleach did digest the barnacle tissue, and cysts remained intact and easily identifiable for up to 48hr, indicating that the general experimental protocol was feasible (data not shown).

The number of brine s*hr*imp cysts ingested varies greatly among individual barnacles.
Analysis of barnacles on Shell 2, Dissected Monday, May 23, 2011and Shell 3, Dissected Tuesday, May 24, 2011

Two shells containing barnacles that were frozen after 24hr of feeding on brine shrimp cysts were thawed and the number of cysts in the gut of each barnacle was determined. Nearly half of the barnacles (13 of 30) had not ingested any cysts. Of the 17 barnacles that had ingested cysts, the numbers of cysts varied from 2 to 207, with an average of 73 ±67. (Figure 5).

Barnacle size is not closely correlated with the number of cysts ingested by the individual.
Analysis of barnacles on Shell 3, dissected Tuesday, May 24, 2011

Based on the data from shell 2, barnacles of different sizes may consume different numbers of cysts. Specifically, larger barnacles may ingest more cysts and smaller barnacles may ingest fewer or no cysts. To test this hypothesis, I repeated the experiment, with two modifications:

1. Prior to dissection, the shell was placed on a dry ice block in the dry ice container for 2hrs. This treatment was expected to euthanize the barnacles, an expectation that was supported.
2. Instead of undiluted bleach, the washed body sacs were incubated overnight in 20% bleach. I hoped that lowering the bleach concentration would achieve the same level of digestion, but would be a gentler treatment. Unfortunately, after an overnight incubation, some of the bodies were incompletely digested. The remaining body tissue was teased apart with

dissecting needles to release cysts, but the remaining small bits of tissue in the well made counting cysts difficult. Two drops of undiluted bleach were added and the bodies were incubated for 1 hour prior to counting. This treatment was sufficient to enable counting.[3]

The number of cysts in the barnacles on Shell 3 was similar to those on Shell 2, varying from 0 to 198. No strong correlation appeared between barnacle size and the number of cysts it consumed. In addition, barnacles that had not ingested any cysts were present that were above and below average size.

Most cysts had passed through the digestive tract of barnacles within 24 hours after feeding. Fecal deposits were present in the dish that contained animals exposed to cysts for 24hr, washed free of external cysts, and incubated in unfiltered seawater for an additional 24hrs. When examined microscopically, the feces contained concentrated masses of cysts, which appeared surprisingly intact (Figure 7). To further analyze whether passage through the barnacle gut had had some effect on the cysts, we compared the effect of bleach on cysts in the feces to control hydrated cysts (Figure 7). Following exposure to bleach for 1hr, Cysts that had passed through the barnacles had a "frog egg" appearance in which most of the interior of the cyst appeared empty except for a central translucent mass. In contrast, control cysts became lighter in color, but remained opaque.

Barnacles that had been incubated for 24hr after feeding were dissected and the number of cysts remaining in their guts was counted. Of the 33 animals that were analyzed, 32 contained no cysts and one contained 23 cysts.

[3] Incubation for up to 48 hours in undiluted bleach did not digest the ingested cysts; they were still easily identifiable. However, it is possible that some are more fragile than others; thus, optimizing digestion time should be attempted to ensure that all ingested cysts can be counted after bleach treatment.

Discussion

Results of this simple experiment demonstrate that (1) *B. glandula* consume brine shrimp cysts; (2) the cysts pass through most animals' guts within 24 hours; and (3) the cysts undergo some change as a result of that passage, suggesting the cysts were at least partly digested. As a result, brine shrimp cysts are confirmed as a possible food choice for experiments that analyze the effect of temperature and water flow rates on barnacle feeding efficiency.

The advantages of brine shrimp cysts for feeding experiments include the availability of high quality preparations, ease of use, and simple, rapid quantitation. However, the density of the cysts is likely to confound experiments: the cysts settle under condition tested, even at flow rates of 60cm/sec in an empty flow chamber (see E. Magley's paper describing these results). The settling problem is likely to be even more pronounced at lower flow rates and in the presence of the complex surfaces presented by masses of barnacles. Thus, it appears that a solution must be devised to maintain a particular concentration of cysts within the water column during feeding, so that a constantly decreasing concentration of cysts does not encumber interpretation of feeding rates.

Probably the most challenging issue uncovered by this experiment is the variability of the feeding behaviors and/or efficiency of individual barnacles. Nearly half of the animals examined did not ingest any brine shrimp cysts. Based on direct observation of feeding behavior, it appears unlikely that these animals were simply not feeding at all. Instead, particularly at the beginning of the experiment, most animals appeared to be feeding (i.e. kicking out and retracting their cirri), but some were not ingesting cysts. If confirmed, this observation suggests that individual barnacles may be preferentially feeding on or excluding brine shrimp cysts. The one animal with many nauplii in its gut, but no cysts, is consistent with the possibility that some animals may have specific food preferences or aversions. Alternatively, the orientation of the animal's aperture or location of

neighboring barnacle shells may enhance or prevent feeding on cysts. Thus, at a minimum, any experiments aiming to evaluate feeding efficiency will need to identify and eliminate from consideration those barnacles that are not feeding. An important initial experiment would be to correlate specific feeding behaviors of individuals with numbers of ingested cysts to try to understand the huge individual variation.

The direct analysis of feeding described in this paper also explains why we were unable to detect changes in the numbers of cysts in our first series of experiments using the flow chambers. We placed plates containing ~60 barnacles into a 600ml flow chamber with 20,000 brine shrimp cysts per milliliter. If each barnacle consumed the maximum observed number of cysts (~200/24hr), we'd expect that the plate of barnacles would collectively consume 12,000 cysts over a 24hr period or 500 cysts per hour. Thus, the maximal expected decrease over an hour-long feeding experiment amounts to less than 1 cyst per milliliter, sensitivity well beyond our sampling margin of error.

Suggested experimental protocol: Based on my participation in this interesting series of experiments, I suggest that the following protocol (Figure 9) might solve some of the mechanistic challenges.

1. Difficulty removing barnacles from mussel shells while maintaining the base plate: We originally pried barnacles off of mussel shells using a scalpel. Approximately 90% of the barnacles removed in this manner were unusable, since the base plate remained attached to the mussel shell. Instead, use a Dremel-syle tool to remove barnacles of similar sizes from mussel shells, leaving the base plate attached to a small piece of shell. In this way, all barnacles would be potential subjects for the experiment, saving preparation time as well as eliminating potential for unknown bias associated with discarding 90% of potential research subjects.

2. Settling of brine shrimp cysts: Design the flow chamber to continuously feed in fresh seawater with the desired concentration of cysts. This change would enable the concentration of cysts moving past the barnacle aperture to remain constant. Alternately, switching to a food source that remains suspended would be a feasible alternative. Although the group used Instant Algae in several pilot experiments, it is likely that the data collection should switch from a strategy of measuring decreases in seawater to a strategy that measures increases inside the barnacle gut.
3. Lethality associated with using Z-spar: The base plates of many barnacles were damaged during attachment to the plastic plate used for insertion into the flow chamber. A reasonable alternative would be to use a sand or glass bead-filled shallow tray instead as a "sand trap." Barnacles could be placed into the tray, and then the bases covered with sand or beads up to ½ the height of the barnacle carapace. This change would also allow individual barnacles to be easily recovered, repositioned, or replaced.
4. Measure cysts in feces: Instead of dissecting barnacles to count cysts, measure the cysts in the feces. For example, consider proposed results from barnacles fed for 6 hours in the flow chamber. Individual barnacles feeding at an average rate in ambient seawater should consume 18 cysts over this time. If the barnacles are removed from the sand trap and place them into individual wells of a multiwall plate, the data in this paper suggests that all cysts should pass through the gut within 24hr. Thus, simply covering the barnacles with seawater and waiting 24hr, would allow the number of cysts per barnacle to be rapidly and accurately counted, without any concerns about the impact of bleach. Feces could also be harvested for later quantitation of cyst quantity.

Acknowledgements

Participating in a new research project was both exciting and invigorating. It was a relief to know that I could contribute, even though ecophysiology and barnacles were well outside my sphere of knowledge, not to mention my comfort zone. It was interesting to experience the overwhelming flood of information that comes with a new research project. I was very inspired by the curiosity, hard work, and professionalism that students brought to their projects. Clearly, this model is fantastically effective and I hope to replicate it at the University of Minnesota.

I am very grateful to Megan Dethier and Charlie O'Kelly, who allowed me (a very non-traditional student) be an undergraduate in their classes. They are remarkable teachers, who know so much and share that knowledge so generously and patiently. Adam Summers gave some of the most powerful and fun introductions to the trials and tribulations I've ever seen. I took good notes, so I can do a better job at this when I return to Minnesota. Our TAs, Michael Nishizaki and Hilary Hayford, were remarkable teachers and even more remarkable human beings. They have the patience of Job! They treated us all as colleagues and welcomed us into the world of graduate school, which is an education in itself.

It was wonderful to work on this project with Emily Magley, who is dedicated, curious, and full of great ideas. Mike Nishizaki is a terrific mentor and I appreciate being able to be his assistant for a couple of weeks. Although, I have to say that his standards of productivity are impossible for normal human beings to achieve. He was in lab before we were in the mornings and showed us results of experiments he ran between 10 pm and 3 am, and he still had time to play Frisbee golf at least occasionally.

Finally, I am both deeply grateful and indebted to my fellow undergrads. You were kind so kind and welcoming. I appreciate it all, from your steady hands helping me navigate the Cattle Point

Trail to treating me like a fellow student Because of you, I was able to rediscover the stresses of being an undergraduate, as well as the joys. I will be a better teacher as a result.

Figure 1: *B. glandula* on Mussel Shells Presented with Brine Shrimp Cysts for Food

Mussel shells with attached barnacles were placed into a dish containing unfiltered seawater and hydrated brine shrimp cysts. An airline with an attached sparger was inserted for aeration and to assist in suspension of the cysts, which are visible in this photo as small brown particles suspended in the water.

Figure 2: Control Barnacles Exhibited Vigorous Feeding Behavior (35mm photo)

As a control, mussel shells with attached barnacles were placed into the water table next to the dish with cysts. Note extended cirri (blue arrow).

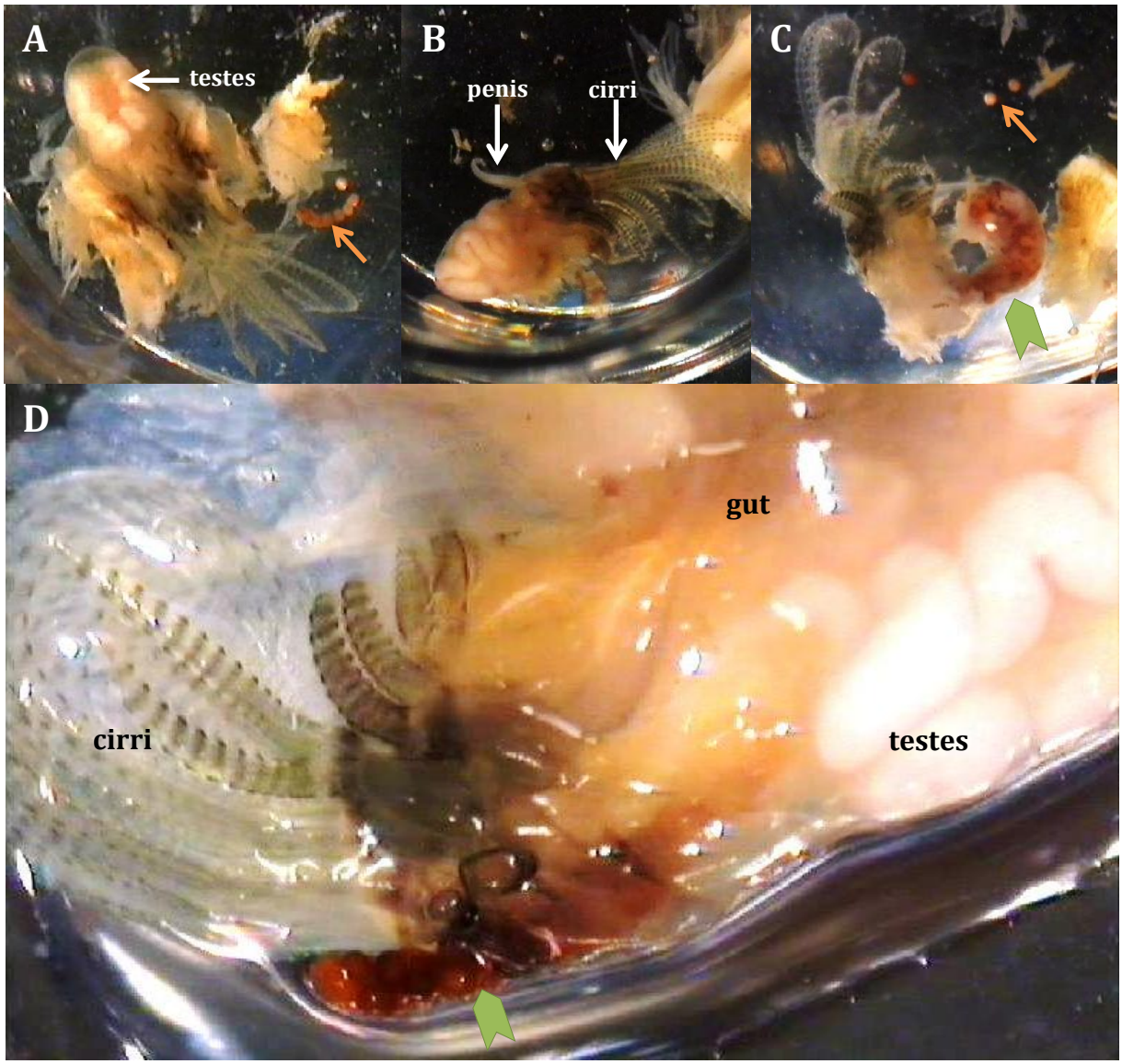


Figure 3: Ingestion of cysts is visible in individual *B. glandula* immediately after removal from their carapace.

After 24 hours of exposure to hydrated brine shrimp cysts, barnacles were frozen overnight, thawed, and removed from their carapaces with forceps, leaving behind the ovaries and tissue associated with the lateral and base portions of the carapace. The thin orange arrows indicate brine shrimp cysts that are external to the organism, probably released from cirri or edges of carapace. The thick green arrows indicate brine shrimp cysts that have been ingested. A & B – No evidence of ingested cysts; C – Gut is visible and engorged with cysts. D – Cysts are being ejected from anus.

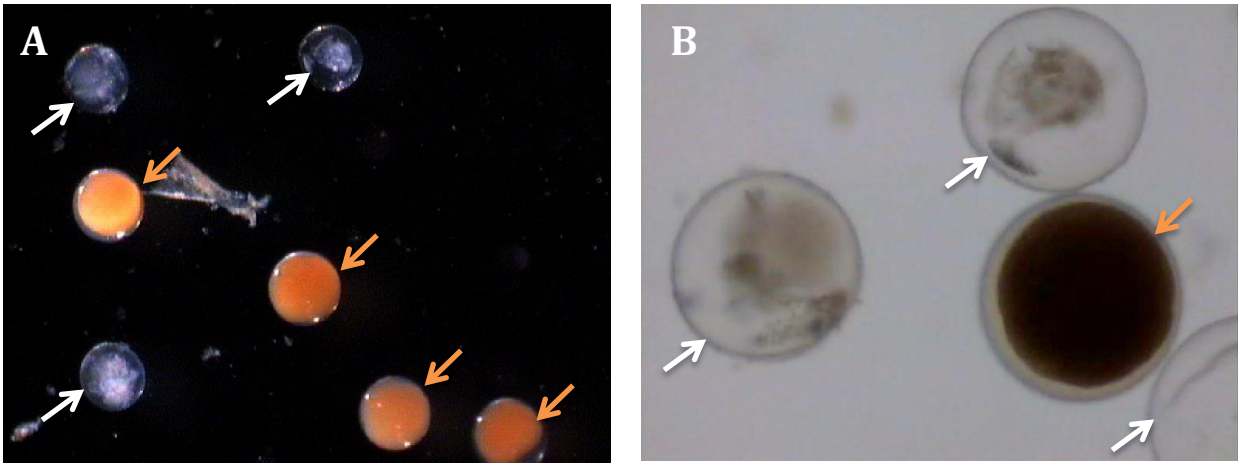


Figure 4: Cysts that have been released from the gut of barnacles can be distinguished from cysts that have not been ingested.

Barnacles that had fed on brine shrimp cysts were placed into bleach to facilitate release of cysts from the digestive system. At the same time, hydrated cysts were also placed into bleach as a control. A drop of both preparations was combined onto a slide so that both types of cysts could be viewed simultaneously. The orange-colored cysts are the control cysts after 6hr of exposure to bleach (orange arrows). The clear spheres with a translucent internal structure are cysts that were released from a barnacle (white arrows) by exposure to bleach for 6hr. A: Dissecting scope, low power; B: Compound scope, 5X objective

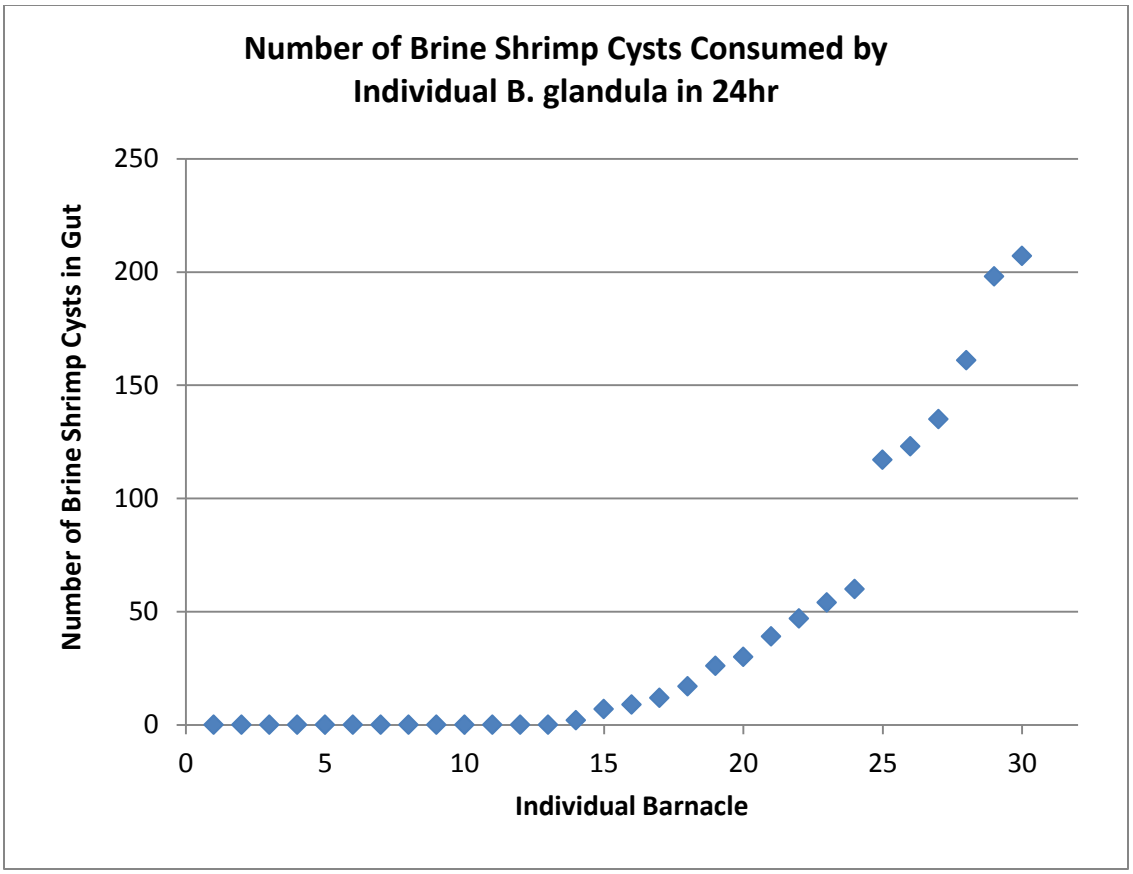


Figure 5: Ingestion of brine shrimp cysts varied greatly between individual barnacles.

Of the 30 barnacles analyzed, 13 had no ingested cysts. Of the 17 barnacles that had ingested cysts, the numbers of cysts varied from 2 to 207, with an average of 73 ±67.

(Data from Shells 2 & 3)

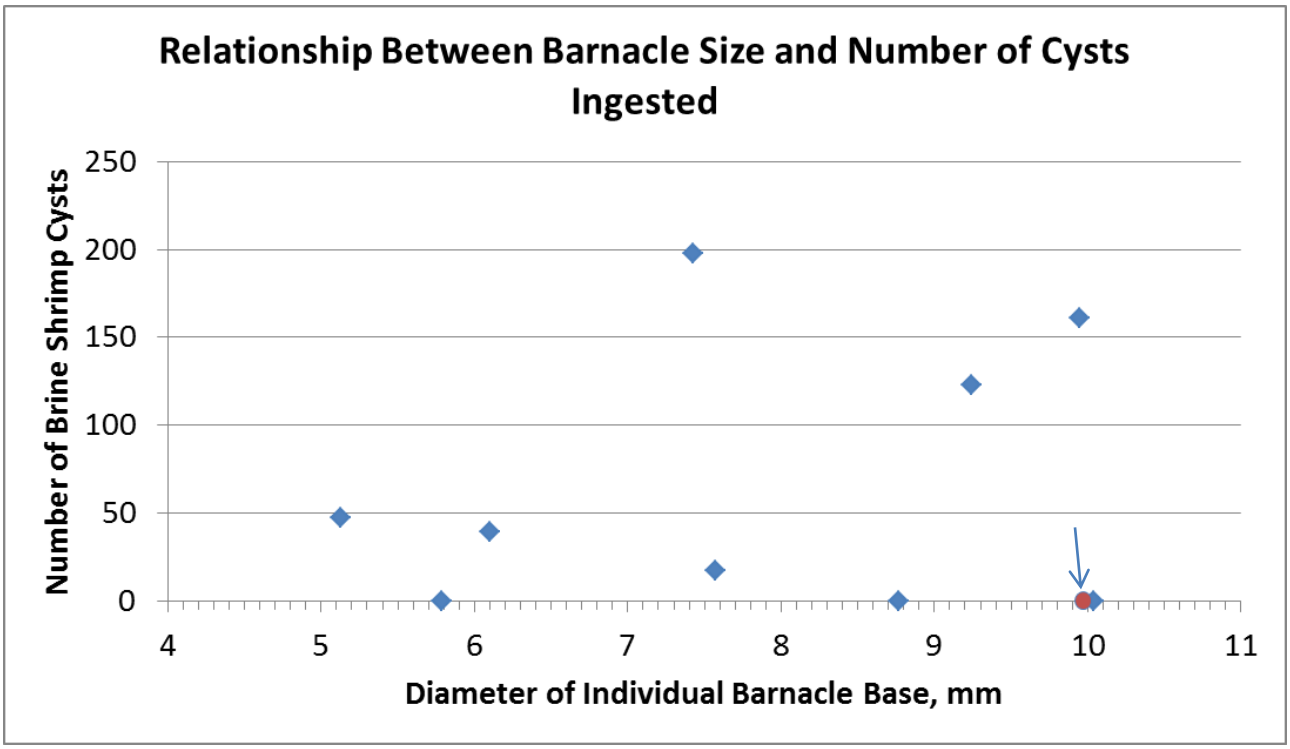


Figure 6: The number of cysts in an individual barnacle was not closely correlated with barnacle size.

The barnacles that had ingested no cysts were both above and below the average size (8mm). The highest number of cysts was found in an animal that was slightly below average size. The data spot in red (see arrow) represents a barnacle that had no cysts in its gut, but instead had ~12 nauplii larvae exoskeletons.

(Data from Shell 3)

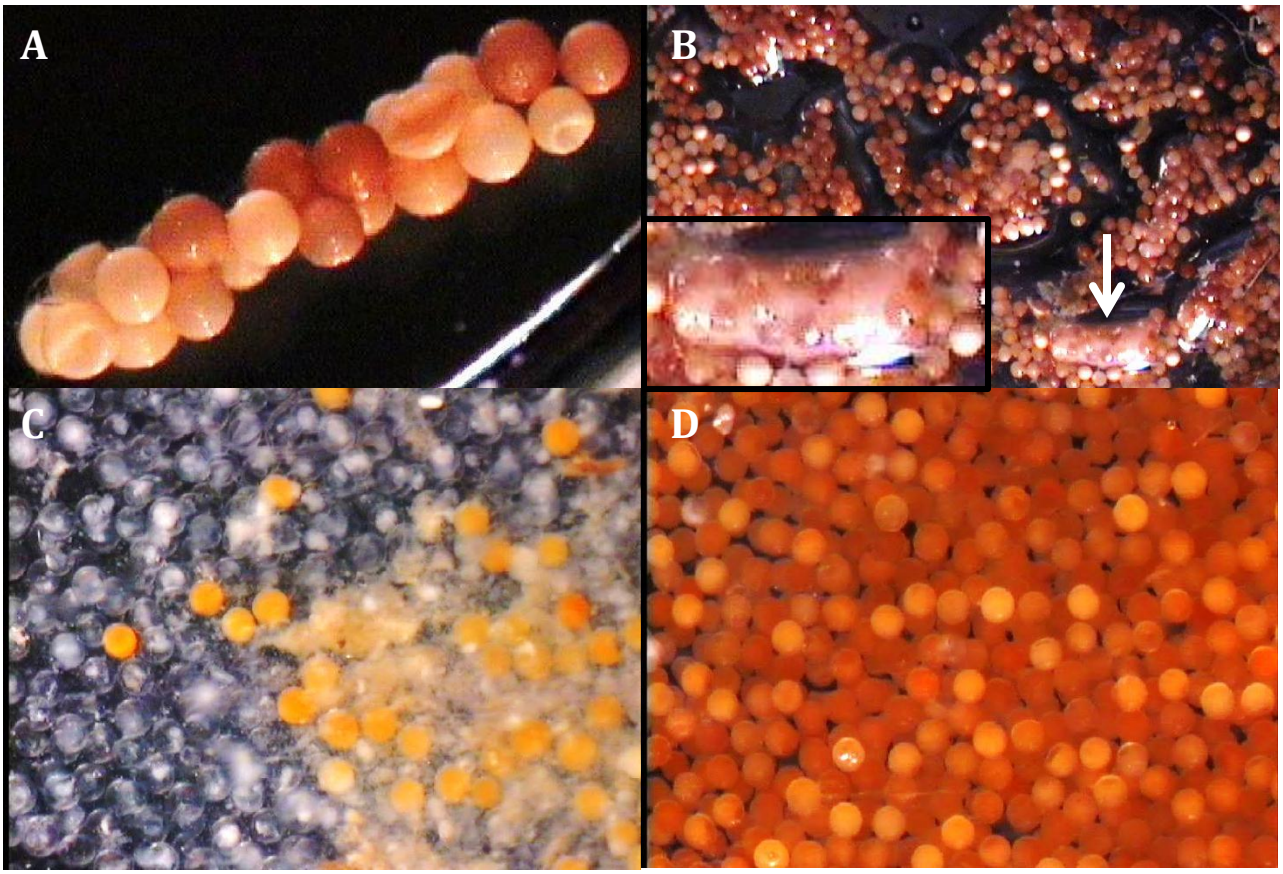


Figure 7: Brine shrimp cysts were passing through the guts of barnacles within 24 hours of feeding.

After 24 hours following removal of cysts, feces were removed and examined under a dissecting microscope. A – A single fecal string showing the typical appearance of closely packed cysts in a mucus cylinder. No feces were observed that did not contain cysts. B – Feces appear to consist almost entirely of brine shrimp cysts. Arrow points to a fecal string that also contains closely packed pink material of unknown nature (see inset). C – Cysts in feces after exposure to undiluted bleach for 1hr. Note the "frog egg" appearance of most cysts. D – Control cysts after exposure to undiluted bleach for 1hr. Note the various colors and lack of any "frog egg" cyst. After longer periods of time, control cysts eventually whiten, but remain opaque.

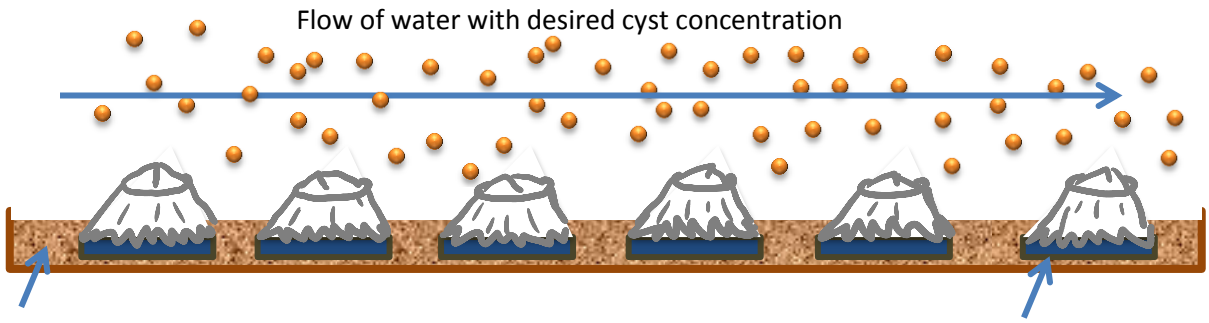


Figure 8: Suggested Experimental Protocol

1. Set up tray of barnacles removed from mussels by circumscribing the shell, maintaining structural integrity of the barnacle base plate.
2. Place into shallow tray (about ½ of the height of the barnacles). Pack barnacles so they do not touch one another. Pour glass beads or sand around barnacles to sufficient depth to hold them in place.
3. Put tray into flow chamber, with an inlet for continuous supply of well-mixed, cyst-supplemented seawater at desired temperature. Include an outlet to allow excess water to flow out of the chamber.
4. Feed for 6hrs at specific flow rate and temperature. (This time frame should allow average feeders to consume 18 cysts and maximal feeders to consume 50 cysts.)
5. Remove barnacles from flow chamber and place into fresh seawater without cysts in individual wells in a multiwall plate. After 24 hours, remove barnacles and count the number of cysts in the feces.

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