Can larval lobster culture be improved through the use of natural feed compounds?

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Final Report to the Atlantic Lobster Sustainability Foundation

Executive summary:

Problem: The growing of larval animals in aquaculture predisposes the animals to high bacterial loading. There are a number of methods that can be used to mitigate the impact of the bacteria on the cultured animals, but many of these will increase the cost of production or are untested in lobsters. It is known that some species of seagrasses have compounds that have antibacterial properties. Feeding these seagrasses has been effective in lowering the surficial bacteria on juvenile lobsters. Thus, the purpose of this study was to assess if seagrasses can be effective in lowering the bacterial loading of hatchery rearing of American lobster (*Homarus americanus*).

Methods: American lobster larvae were collected from females that extruded eggs in the wild. The larvae were equally distributed across 40-l kriesels and fed similarly except that either one of two seagrasses were added to the diets. The seagrasses included *Ruppia maritima,* a plant demonstrated to prevent the growth of some *Vibrio* spp. in culture systems, and *Potamogeton perfoliatus,* a plant demonstrated to be less effective against *Vibrio* spp. in culture. Bacteria levels in the culture vessels were tested by collecting water and tank surface samples. The larvae were raised in kreisels until 4th stage, at which point they were transferred to individual rearing cups, and ongrowing continued. At the 3rd and 4th stages, lobster health was assessed by testing for bacteria levels and lipid storage capability. Larval and juvenile lobster survival and growth were also assessed.

Outcomes: Neither *P. perfoliatus* nor *R. maritima* was shown to be consistently effective against bacteria in water or tank surface samples. This is likely because the experimental system utilized a common holding tank from which water was distributed, diluting the antimicrobial properties of the seagrasses. While *R. maritima* fed larvae had slightly greater survival to metamorphosis than did those fed *P. perfoliatus,* neither seagrass treatment exceeded the survival results of larvae fed an alternate diet (positive control). Bacterial fouling on stage 3 larvae was noticeable, and may be a potential factor to track in determining the health of lobsters reared for enhancement efforts.

Introduction:

Hatcheries are a necessary component of any aquatic animal enhancement program. While hatcheries can increase production of the early stages of marine animals compared to nature, there are several challenges to maintaining high levels of production. The physical environment, including temperature, water quality, and diet need to be appropriate. Above and beyond these needs, and regardless of the hatchery type (flow through, partial or full recirculation), the artificial environment presents several challenges. One of the most significant is that hatcheries have an altered bacterial composition and likely an increased density compared to that of the wild due to a reliance on bacteria in the filtration system. In recirculation facilities this is exacerbated by the presence of filters and sumps that hold extra water volume and have media to which bacteria adhere. In addition, to maximize the efficiency of the hatchery, animals are often held at high densities and the water temperature is warmed to increase the rate animal of growth. These factors provide for conditions conducive to high bacterial growth. Bacteria are a natural component of marine waters. However, in high density they can be deleterious because some of the bacteria are pathogenic. To increase captive animal growth, there are two options. The "Swedish model" (Andreasen et al. 2005) is to optimize the holding of the animals to increase their baseline health making additional treatments unnecessary. The other option is to use various substances to increase performance (survival and growth). In aquaculture, while antibiotics have had success in promoting performance, there is backlash against the overuse of these chemicals. Now aquaculture producers are turning to probiotics, or known bacteria, as a means to increase performance. In 2004, only 12 peer reviewed aquaculture publications examined probiotics, whereas by 2009, the number increased to 35. Yet, while there are examples of beneficial additions of probiotics even for lobsters (Daniels et al. 2007) there are many unknowns as to the specific mechanisms leading to the success. One overall difficulty with probiotic experiments is that they do not consider a negative control, namely decreasing the level of bacteria in a system and thereby increasing the overall health of the culture animals. This is an important facet of this work, as an important lesson learned in the antibiotic studies is that antibiotics do not exhibit prophylactic benefits in healthy animals. Prior work by the Tlusty laboratory demonstrated that early benthic juvenile lobsters carry a large bacterial load, and there was a negative association between bacterial load and the ability to handle a stress challenge (Tlusty et al. 2000). Thus, even common bacteria in the environment will, at some density, become a hindrance, and may decrease health (Tlusty et al. 2008; Tlusty et al. 2007). This leads to the question if there are ways to increase animal health through the natural decrease of bacterial loading. There are a number of technological, high cost methods, including UV sterilization units. However, organisms native to the lobsters habitat such as seagrasses and algae, have adopted chemical defenses against bacterial loading. For example, eelgrass produces zosteric acid, a phenolic acid sulfate ester that prevents settlement by marine organisms (Achamlale and Grignon-Dubois 2008; Bushmann and Ailstock 2006). One- and two-year- old lobsters that have eelgrass included in their diet demonstrated less bacterial colonization on their shells compared to lobsters not fed these plants (Bushmann and Tlusty, unpublished data). Since our laboratory has had initial success in decreasing the bacterial load on older lobsters by adding specific components to their diet, the goal of this proposal is to assess the utility of natural feed additives to improve growth, survival and health of larval lobsters grown in hatcheries. Furthermore, it was determined at meetings of USA-Canadian lobster hatchery biologists (Boston 2005, Maine 2008) that measures of larval health to ensure that newly developed rearing systems will have a positive impact on animal health represent one of the most critical and crucial research priorities. Thus, part of this work also involves an assessment of potential new indices of health in larval lobsters. The specific aims of this work include:

SA 1 – determine if addition of ground seagrass to larval tanks results in lower bacteria ain vessel b) on surface of larvae, and c) evidenced as encysted in hepatopancreas of recently settled early benthic juveniles

SA 2 - determine if addition of ground seagrassresults in greater survival and growth a) through the larval stages and b) after settling to the benthic environment

Methods:

Female and Larvae Collection:

The experiment was conducted twice; the first experiment was preliminary and used to determine appropriate methodologies. For the preliminary experiment, an ovigerous female with newly extruded eggs was collected by diving in New Hampshire waters in the late summer of 2011 and brought to the New England Aquarium's Lobster Research and Rearing Facility (NEAq LRRF). The female was maintained in the LRRF throughout the fall and winter (2011-2012) in a 150L tank and began hatching in the spring of 2012. Newly hatched larvae were collected from the female, counted and then placed into a 40L round upweller tank, or kreisel where they were observed for their growth and survival on the test diets (described below). Due to tank constraints and larval hatching patterns, two kreisels of 100 newly hatched larvae each were established on one date while two more kreisels (each with 100 larvae) were established four days later. Female and larval tanks were all maintained within a 1700L semi-closed recirculating system with incoming seawater from Boston Harbor and passed through two sand filters and a 5 μ mechanical filter prior to use. Water temperatures during the preliminary experiment averaged 14.8 ^{o}C during larval rearing.

For the second experiment, an ovigerous female was again collected by hand and held at the University of New Hamsphire's Coastal Marine Laboratory. The female was transported to the LRRF in the summer of 2012 and started hatching immediately. 600 larvae were once again collected, counted and randomly placed into 6 kreisels. All tanks were within the same water system as previously described, and water temperatures during the larval rearing averaged 16.5^{o}C.

Diet treatments and Feeding Protocol:

Two seagrasses, *Potamogeton perfoliatus* and *Ruppia maritima*, were axenically cultured and grown by P. Buschmann at Anne Arundel Community College (Arnold, MD). The seagrasses were dried and frozen and sent to the LRRF for use in this study. Both seagrasses have antimicrobial properties, but *R. maritima* has shown to be effective against *Vibrio* spp., which are common marine bacteria (P. Buschmann, personal communication).

For the preliminary study, larvae in the kreisels were cared for in a modified version of the standard operating procedure for the LRRF. Larvae were fed three times a day (morning, noon, and evening). In the morning, all kreisels were fed spirulina-enriched adult frozen brine shrimp (Hikari Brand Fish Food, Hayward, CA). At noon, the uneaten brine shrimp were carefully removed from all tanks, and the larvae were then fed one of the seagrasses; two kreisels received *P. perfoliatus* (1Pa and 1Pb) and two kreisels received *R. martima* (1Ra and 1Rb). In the evening, uneaten seagrass was removed from the tanks, and the larvae were again fed spirulina-enriched adult frozen brine shrimp. The amount of food in the evening was about 3 times the amount fed in the morning to maintain the larvae overnight and in an effort to decrease cannibalism. Seagrasses were initially fed to the larvae by cutting it into small pieces, however direct observations showed that the larvae were not consuming the seagrasses in this form. Various methods were tried and eventually it was determined that the best option was to grind the seagrasses into a powder, mixing it into a suspension with seawater (to prevent floating) and then adding it to the tanks in order to allow the larvae to filter feed. This method was employed one week after the start of the preliminary experiment.

For the second study, the larvae were cared for as above, except in the evening, the seagrasses were not removed prior to being fed brine shrimp. This was to maximize the amount of time the seagrasses were in the tanks and maximize the opportunities for the larvae to feed on the seagrasses. The feeding regimen was the same as in the preliminary study with the addition of a positive and a negative control that were added to the experiment. Thus, six kreisels were used for this second study with different noontime feedings. Two kreisels were fed *P. perfoliatus* (2Pa and 2Pb), two were fed *R. maritima* (2Ra and 2Rb), one positive control (fed Cyclop-eeze (Argent Chemical Laboratories, Redmond, WA), a frozen pigment-enriched freshwater copepod, which is the standard noontime diet used for raising larvae in the LRRF) (2+), and one negative control (not fed at noontime) (2-).

Survival, Stage, and Color:
For both experiments, larvae were removed from the kreisels and were staged and counted once a week. Survival curves were calculated and analyzed by a log-rank Kaplan-Meier test (SigmaPlot 12.0, Systat Software, Inc, San Jose CA). Significant results were further analyzed for pair-wise comparisons using a Holm-Sidak method.

Larvae were also analyzed for color by taking a subsample of 10 larvae from each tank and identifying color for these individuals. A primary or dominant color was identified as the carapace color, and when appropriate a secondary color was identified as the abdomen color or a color that was apparent but less dominant. Color was analyzed over a white background and done by only two individuals to minimize subjectivity. Color was analyzed daily throughout the larval stages although some dates were missed. Towards the end of the larval period, only the larvae available in the tank were analyzed, which were less than 10 individuals, due to mortalities and metamorphosis out of the larval stages. The percentage of larvae exhibiting a dominant and secondary color was determined out of the total analyzed in each tank on each day, and the average percentage seen of each color for all tanks was determined.

Juveniles and Growth:
Upon metamorphosis to stage 4 (postlarval stage), lobsters were removed from the kreisels, placed in individual containers (2.7 cm dia), given an identification tag, and placed in a shallow tank within the same semi-closed recirculation system. The identification tags allowed for the lobsters to be monitored daily for molt occurrences, survival, and to determine whether diet treatment at the larval stage influenced these factors. Juveniles were fed twice a day on a diet regimen of frozen spirulina-enriched adult brine shrimp, five-times a week and frozen Cyclop-eeze, two-times a week (LRRF standard protocol).

When possible, postlarvae and juveniles were photographed with a ruler several days after molting in order to monitor growth. However, due to mortalities after molting, it was not always possible to obtain some measurements. Digital images were scaled and carapace length measured using ImageJ software (Abramoff et al. 2004).

Survival curves and growth of the juvenile lobsters, post-metamorphosis, were analyzed by grouping the lobsters by the larval tank (and thus diet treatment) in which they were raised. Survival curves were calculated and analyzed by a log-rank Kaplan-Meier test (SigmaPlot 12.0). Significant results were further analyzed for pair-wise comparisons using a Holm-Sidak method. Growth in the juvenile lobsters was calculated as the percent change in carapace length (CL) after each molt and was analyzed by one-way ANOVA.

Bacteria Levels:
To determine whether the seagrasses had an antimicrobial effect in the kreisels, water was sampled from the kreisels, and growth was tested on two different agar plates. Thiosulfate citrate bile salts sucrose agar (TCBS) is a medium specific for *Vibrio* spp. while marine agar is a medium that is non-specific for marine bacteria. Once a week, a 10 ml water sample was collected from each kreisel. 100 ul of this sample was aliquoted each onto a marine agar and TCBS plate, spread using spread plate technique, and allowed to incubate at room temperature. Initially, a 24 hours incubation time was used, but due to minimal growth after 24 hours, a 48 hour incubation time was instigated. During the second experiment, due to high bacteria growth on the marine agar plates, a 1:10 dilution of each water sample was performed and 100 ul of the diluted sample was aliquoted and spread on marine agar in addition to an undiluted sample. Plates spread with diluted samples were conducted starting on the second weekly sample and thereafter. Bacteria growth was calculated as total number of colony forming units (cfus).

Additionally, kreisels were also tested for bacteria on the tank surfaces. Once a week, the plastic mesh surrounding the drains in each kreisel was removed and a sterile swab was passed 5 times over a 5 cm length of the mesh. The swab was then rubbed onto an agar plate. Marine agar and TCBS plates were both used, and separate swabs were

used for each plate. The plates were marked with 4, 6, 8, 6, and 4 cm lines and the lines were traced with the swab in a specific order creating a single trace, 28 cm long. Growth was calculated after 48 hours of incubation at room temperature as the amount of growth out of the total 28 cm possible growth.

In the preliminary experiment, water and swab samples were started 1-2 weeks after the start of the experiment. In the second experiment, the first samples were taken immediately prior to larval introduction into the tanks.

Histopathology:

During the second experiment, 6 stage-3 larvae and 5 stage-4 postlarvae were collected from each kreisel for histopathology analysis. Larvae and postlarvae were injected with Davidson's fixative in the cephalothorax and then immersed in Davidson's for 12-24 hours before being transferred to 50% ethanol. Samples were sent to the University of Arizona Aquaculture Pathology Laboratory and paraffin sections were analyzed by hematoxylin and eosin-phloxine (H&E) staining. Lipid content of the hepatopancreas (lipid vacuoles in cytoplasm of R-cells), enteric bacterial infection, and bacterial fouling of the exoskeleton were determined and rated on these scales: Lipid content: 0 – 4, 0 = no to few lipid vacuoles, 1 = small and sparsely distributed vacuoles, 2 = mix of cells with no, some, and moderate vacuoles, 3 = cells have numerous vacuoles, 4 = cells are filled to capacity with vacuoles; Bacteria fouling and enteric bacterial infection: 0 – 4, 0 = no signs of infection, 0.5 = trace levels of infection, 1 = signs of infection are present but at low levels indicating either early disease state, 2 = low to moderate number and severity of pathogen caused lesions, 3 = moderate to high number and severity of pathogen caused lesions with potentially lethal prognosis if not treated, 4 = high number of pathogen caused infections/lesions with a lethal prognosis if not treated or managed.

Results:

Preliminary Experiment:

One kreisel (1Rb) fed the *R. maritima* seagrass treatment had 100% mortality by day 25 with no larvae surviving to metamorphosis. The remaining tanks exhibited a long larval period of up to 62 days due to a combination of relatively low temperatures and the time of year the female hatched her eggs.

Kaplan-Meier survival analysis showed a significant difference in the survival curves (log-rank statistic 76.688, df = 3, $p < 0.001$). Pair-wise comparisons showed that the survival curve from tank 1Rb to be significantly different from the survival curves of the other 3 tanks. Tanks 1Pa (*P. perfoliatus* diet) and 1Ra (*R. maritima* diet) were also shown to be significantly different (Table 1).

The larval stages lasted from 25 days (1Rb) to 62 days (1Ra). Tank 1Ra (*R. maritima* treatment) also showed extended times at each larval stage compared to the other three tanks. By day 17, all larvae in tanks 1Pa, 1Pb, and 1Rb had molted to stage 2. The larvae in these tanks showed more variation in molting to stage 3, taking until day 35 (1Pa), 46 (1Pb), or 24 (1Rb) days from hatching to reach stage 3. In contrast, the larvae in tank 1Ra did not all molt to stage 2 until 28 days after the start of the experiment and did not all molt to stage 3 until day 50.

The colors, both primary (dominant) and secondary, were identified in the larvae as blue, tan, clear, and red. Blue was the most prevalent dominant color and was, on average, seen in 60.36% (± 2.1 S.E.) of the total larvae analyzed, while red was the most prevalent secondary color, seen on average in 61.5% (± 2.6 S.E.) of the larvae viewed. Tan was the 2nd most dominant and 2nd most secondary color seen (22% ±1.75 S.E. and 19.1% ± 1.93 S.E., respectively). Clear and red were less prevalent as a dominant color (11.32% ± 1.3 S.E. and 6.37% ± 0.95 S.E., respectively). And, tan, blue and clear were less often seen as a secondary color (19.1% ±1.93 S.E., 14% ±1.9 S.E. and 5.4% ±0.94 S.E., respectively).

A total of 29 postlarvae were collected out of the 400 possible larvae; a 7.25% survival rate from hatching to the stage 4 postlarval stage. From the tanks being fed *P. perfoliatus*, 16 (1Pa) and 6 (1Pb) postlarvae were collected

respectively. From the one remaining tank fed *R. maritima* (1Ra), 7 postlarvae were collected. None of the postlarvae survived past 53 days post metamorphosis. Due to the high mortality rates, growth was not able to be calculated, as many postlarvae died shortly after metamorphosis before initial size could be determined.

Bacteria growth on marine agar from water samples taken from the kreisels showed high variability from one sample date to the next. The two tanks started at the later date (1Pb and 1Rb started 4 days after tanks1Pa and 1Ra) had large amounts of bacteria growth on the first sample date (day 9), regardless of the fact that each tank was on a different diet treatment. The other two tanks, 1Pa and 1Ra, showed low levels of bacteria on their first sample date. These two tanks also showed a slight increase in cfus on day 35. All tanks operating and sampled on day 41-45 of the experiment (tanks 1Pa, 1Ra, and 1Pb) showed an increase in total number of cfus. Tank 1Ra had a higher increase in total number of cfus than the other tanks being fed *P. perfoliatus* (Table 2).

Bacteria growth on marine agar from swabs taken from the inside of the mesh surrounding the tank drains consistently had 28 cm of growth for all samples.

Bacteria growth on TCBS from water samples only occurred three times, on days 8 and 45 from tank 1Ra, and on day 41 from tank 1Pb. Growth was 2 colonies on all three plates. Bacteria growth on TCBS from swab samples only occurred once on day 8 from tank 1Ra with 8 colonies.

Second Experiment:

Larval Stage and Color: The larval stages during the second experiment persisted for 36 to 49 days. Larval stages were identified on days 6, 13, 20, 27, 34, 41, and 48 after the start of the experiment. Stage 1 larvae persisted from the start until day 13, stage 2 larvae were seen from day 6 through day 27, and stage 3 larvae were seen from day 20 through day 48. Larvae in all tanks had reached stage 3 by day 34, although in the positive control tank (2+) this landmark was reached by day 27.

The colors identified during the preliminary experiment were identical to those identified during the second experiment. Blue and tan were the most prevalent dominant colors (42.6% ±1.63 S.E. and 35% ±1.85 S.E., respectively). Clear was seen less frequently (22.08% ±1.39 S.E.), and red was rarely seen as a dominant color (0.35% ±0.22 S.E.). However, red was again the most prevalent secondary color seen (49.1% ±2.18 S.E.), with tan, clear, and blue seen less frequently (20.3%±1.25 S.E., 17.2%±1.14 S.E., 13.4% ± 0.97 S.E., respectively).

Larval Survival:

Larval survival curves were shown to be statistically significant using a Kaplan-Meier analysis (log-rank statistic = 45.856; df = 5, $p < 0.001$). Pair-wise comparisons showed that the negative control tank (2-) and one of the tanks fed *P. perfoliatus* (2Pb) to be similar to each other but each had a significantly different survival curve from every other tank, based on lower survival at day 30 than all of the treatments (Figure 1, Table 3).

The number of larvae surviving to the stage 4 postlarval stage varied with each tank and diet treatment. The positive control tank (2+) showed the highest survival to stage 4 with 54 larvae surviving to metamorphosis. The two *R. maritima* diet treatment tanks (2Ra and 2Rb) had good survival with 45 and 35 larvae surviving to stage 4 respectively. The two *P. perfoliatus* diet treatment tanks (2Pa and 2Pb) had a large variability in survival with 36 larvae surviving to metamorphosis in tank 2Pa but only 11 larvae surviving in tank 2Pb. Tank 2Pb fared worse than the negative control tank (2-) which had 14 larvae survive to the postlarval stage.

Juveniles Survival and Growth:

A total of 195 stage 4 postlarvae were collected from the larval tanks which was a 32.5% survival rate from hatching to stage 4. Of this total, 30 were submitted for histopathology analysis after metamorphosis, and 165 were monitored for growth and survival until the experiment was terminated on November 30, 2012 (up to 131 days post

metamorphosis). By the end of the experiment, only 16 lobsters remained; 142 had died (86% mortality rate) and 7 had escaped from their containers and were not recovered. Mortalities occurred on average at 18 days post metamorphosis (± 1.8 S.E.).

The survival curves post-metamorphosis between juveniles raised in the different larval tanks were statistically significant based on a Kaplan-Meier analysis (log-rank statistic = 19.446; df = 5; $p < 0.002$). Pair-wise comparisons showed only one statistically significant comparison between a tank fed *P. perfoliatus* (2Pa) and the positive control (2+) (Holm Sidak statistic = 10.068; P value = 0.0224; $p < 0.05$); all other comparisons were not statistically significant (Figure 2).

Juvenile lobsters molted up to 4 times in the course of the experiment. Due to low sample sizes as the experiment progressed, analyses were focused on the amount of growth after the first two molts (molts 1 and 2) post-metamorphosis. Two one-way ANOVAs were conducted on the percent change in CL after molts 1 and 2 post-metamorphosis. No significant differences were seen among juveniles raised on different diet treatments as larvae either after molt 1 ($F_{(4,46)} = 0.802$; $p = .53$) or after molt 2 ($F_{(3,15)} = .117$; $p = .948$).

Bacteria Growth:

Undiluted water samples plated on marine agar showed high amounts of variability with no patterns based on diet treatments (Figure 3). One of the tanks on the *R. maritima* diet treatment (2Ra) had two dates with a high total of cfus, but the second *R. martima* diet treatment tank (2Rb) did not follow suit. One of the *P. perfoliatus* diet treatment tanks (2Pa) showed a spike in total cfus approximately one week before the tank was emptied of larvae (from mortality or metamorphosis to stage IV). Four tanks, one of the *P. perfoliatus* tanks (2Pa), both *R. martima* tanks (2Ra and 2Rb), and the positive control tank (2+), showed an increase in total cfus on the 22nd day in the experiment. The other *P. perfoliatus* diet treatment tank (2Pb) and the negative control tank (2-) did not follow suit and had relatively low totals of cfus throughout the experiment (Figure 3).

Total cfus from water samples diluted 1:10 and plated on marine agar did not completely correspond with the bacteria growth seen on their undiluted counterparts (Figure 3). The increase seen on day 22 for tanks 2Pa, 2Ra, 2Rb, and 2+ was still present, although in different degrees as seen with the undiluted samples. The spike in total cfus in 2Pa one week before the end of the larval portion of the experiment is not seen, and instead 2Pb showed this trend (Figure 3).

Swabs taken from the drain mesh from the larval tanks and plated on marine agar showed a consistent 28 cm of growth after a 2 day incubation period. The only exception to this was on day 0 when bacteria growth from all tanks was < 6 cm.

As in the preliminary study, little growth was seen on any of the TCBS plates from water samples or swab samples. Only three plates showed growth from water samples; one plate from one of the *P. perfoliatus* diet treatment tanks (2Pa) had two colonies form on experiment day 0, and two plates from an *R. maritima* treatment tank (2Ra) had 10 and 4 colonies form on day 13 and day 20, respectively. This same tank showed 1 cm of bacteria growth from a swab samples on day 13. The negative control tank (2-) also showed 2 cm of bacteria growth from a swab sample on day 6.

Histopathology:

Lipid vacuole levels were on average higher in stage 3 larvae versus stage 4 postlarvae. Stage 3 larvae from tanks 2Pa (*P. perfoliatus* treatment) and 2+ (positive control) had on average the highest levels of lipid vacuoles (Figure 4), yet these treatments had lower post larval survival than the other treatments (Fig 2). Bacteria fouling on the carapace or appendages were found on all 36 samples of stage 3 larvae compared to only 6 of the 30 stage 4 postlarvae. Fouling levels ranged anywhere from 0.5 to 3 on a 0 to 4 scale. The stage 3 larvae from the positive

control tank had the highest levels of bacteria fouling (Figure 5), but a one-way ANOVA test of the bacteria fouling levels on stage 3 larvae revealed no significant difference ($F_{(5,30)} = 0.716$).

Enteric bacterial infection had a low prevalence; only 7 of the 36 stage 3 larval samples (enteric infection levels 1-3 seen) and only 1 of the 30 stage 4 samples (enteric infection level 2) exhibited infection.

Discussion:

The preliminary experiment showed low survival rates in both the larvae through stage 4 and in the early stage juveniles, post-metamorphosis. Since the low survival was seen among all the tanks, this is most likely due to a combination of a poorly developed brood by the female as well as complications due to the hatch time occurring earlier than is typical in the year (April instead of June/July). Females that hatch in aquaculture facilities have extended hatch times, and the larvae often have extended larval stages (E Annis, pers. comm.). One tank, 1Rb, had 100% larval mortality within 25 days. This high mortality rate in the larvae was not seen in any other tank and is most likely due to an effect of the tank such as improper water flow. Although such inadequacies were not noted by observation during the experiment, this tank was removed from the tank system and not used in the subsequent experiment. During the second experiment, tank 2Pb (*P. perfoliatus* diet treatment) also exhibited a high mortality rate during the larval stage (6% survival to stage 4 postlarval stage) and was equivalent to the mortality rate seen in the negative control tank (2-). Additionally, none of the postlarvae from 2Pb survived past 31 days. While the mortality rate in the negative control tank can be explained by the diet treatment regimen, the mortality in 2Pb was significantly different from its corresponding *P. perfoliatus* diet treatment tank, 2Pa. It is likely that the mortality rate in 2Pb may be due to a tank effect, but to a lesser degree as that seen in 1Rb during the preliminary experiment.

Although the negative control tank, 2-, had a high larval mortality rate (9% survival to stage 4 postlarval stage), it also exhibited the best juvenile survival rate (33% or 3 out of the 9 postlarvae survived to the end of the experiment). We hypothesize that because the larvae were not fed at noontime, they were more likely to cannibalize each other, leading to a high mortality rate but also to more robust postlarvae and juveniles. Cannibalization at the larval stage is seen as problematic due to the decrease in numbers, but often results in healthier and more robust larvae, postlarvae, and juveniles. In contrast, the positive control tank, 2+, had the highest survival rate through the larval stages but exhibited low juvenile survival (only 1 out of 49 postlarvae survived to the end of the experiment). This indicates that the LRRF's standard rearing protocol should be examined and changes in the dietary regimen may be needed to ensure healthy larvae, postlarvae, and juveniles. It also points out the utility of ongrowing larvae lobsters to assess how larval rearing environment translates to performance during the benthic states.

Neither *P. perfoliatus* nor *R. maritima* was shown to be consistently effective against bacteria in water or tank surface samples when tested on non-specific (marine agar) or *Vibrio* specific (TCBS) media. Because each 40L larval tank is within a larger 1700L system, any antimicrobial effects may have been diluted in the larger system. The amount of seagrass fed in each tank was relatively small compared to the size of the 1700L system. Little to no growth was seen on TCBS agar plates in either the preliminary or second experiment indicating that little *Vibrio* spp. reside in this tank system. This would be unusual for a recirculating system, since many *Vibrio* spp. are common in marine environments and tend to proliferate in a recirculating system. *V. algynoliticus* was previously isolated from this system (Tlusty et al. 2008). However, colony formation occurred consistently on plates made with marine agar, indicating that other colony forming bacteria were present during the course of the experiment. Swabs of the tank drains consistently showed 100% (28 cm) of growth after 48 hours, while water samples from the tanks varied considerably. During the preliminary experiment, two tanks (1Pb and 1Rb) showed a peak in total cfus on sample day 9. These two tanks were the only ones tested on this sample day, and 1Pb (*P. perfoliatus* diet treatment) showed a much higher peak than its *R. maritima* counterpart. However, later in the experiment, sample day 40-45, showed a peak in cfus in the 3 tanks remaining at that time, 1Pa and 1Pb (*P. perfoliatus* diet treatment) and 1Ra (*R. maritima* diet treatment). In this instance, the *R. maritima* diet treatment showed a higher total of cfus compared to the *P.*

perfoliatus tanks. The second experiment also exhibited variation in higher total of cfus between tanks fed *P. perfoliatus*, *R. maritima*, and the control tanks. The second experiment also failed to show consistency in bacteria growth from undiluted and 1:10 diluted water samples. These varying results indicate that bacteria levels in water within the LRRF varies greatly and may be determined by other factors not taken into account during this experiment including bacteria levels of incoming water from Boston Harbor, surviving larvae in the tank, amount of debris within the tank including exuvia, dead larvae, and amount of uneaten food.

Histopathology analysis showed that lipid vacuole levels were higher in stage 3 larvae than in the stage 4 postlarvae. Since the stage 4 postlarvae were sampled shortly after metamorphosis (within 24 hours), it is possible that this difference in lipid vacuole levels is due to the physiological changes and stress incurred on the postlarvae during metamorphosis. The sample time after metamorphosis also explains the low number of stage 4 postlarvae seen with bacteria fouling on their carapace or appendages. In contrast, stage 3 larvae were sampled at unknown points after molting to this stage, and may have been at stage 3 for several days before sampling allowing time for bacteria to grow on their shells. The presence of fouling bacteria on all samples of stage 3 larvae also indicates that fouling bacteria are prevalent within the LRRF system. In contrast, bacteria causative of enteric infection are most likely not prevalent in the LRRF system as indicated by the low number of larval and postlarval samples from all tanks that were positive for enteric infection.

Although stage 3 larvae from tanks 2Pa (*P. perfoliatus* diet treatment) and 2+ (positive control) had on average the highest levels of lipid vacuoles, indicating higher nutrient absorption, this did not correspond with higher long term survival, as juvenile survival among all tanks was relatively low.

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Table 1: Preliminary Experiment. Average days (± 1 SE) to metamorphosis for larvae raised on two seagrasses with different antimicrobial properties, *Potamogeton perfoliatus* (1Pa and 1Pb) and *Ruppia maritima* (1Ra and 1Rb). Statistical similarity (denoted by common superscript) was determined from Holm-Sidak pair-wise comparison tests on Kaplan-Meier survival curves.

Tank	Average (± 1 S.E)
1Pa	30.59 ± 1.73^{c}
1Pb	28.47 ± 1.46^{bc}
1Ra	23.26 ± 1.45^{b}
1Rb	14.35 ± 0.41^{a}

Table 2. Preliminary Experiment: Total Colony Forming Units (cfus). Total cfus from water samples taken from each tank and spread on marine agar. Numbers in bold indicate noted increases in total cfus.

Sample Day	1Pa	1Ra	1Pb	1Rb
9			**288**	**71**
15	12	7		
17			17	6
21	8	7		
24			23	25
28	10	5		
31			12	
35	**29**	**45**		
41			**29**	
45	**63**	**164**		
47			7	
51	3	8		
57		8		

Table 3: Second Experiment. Average values (± 1 SE) and P value results from Holm-Sidak pair-wise comparison tests on Kaplan Meier survival curves from larvae raised on different diet treatments in 6 tanks: *Potamogeton perfoliatus* seagrass with some antimicrobial properties (2Pa and 2Pb), *Ruppia maritima* seagrass with antimicrobial properties against *Vibrio* spp. (2Ra and 2Rb), positive control fed Cyclop-eeze (2+), and negative control starved (2-).

Tank	2Pa	2Pb	2Ra	2Rb	2+	2-
2Pa		**0.00413***	**0.762**	**0.896**	**0.367**	**0.0115 ***
2Pb			**0.000061 ***	**0.00536 ***	**7.56E-06 ***	**0.963**
2Ra				**0.729**	**0.751**	**0.000292 ***
2Rb					**0.29**	**0.0142 ***
2+						**4.23E-05 ***
2-						

Figure 1: Second Experiment: Survival curves of larvae in tanks fed at noontime either the seagrasses *P. perfoliatus* (2Pa and 2Pb) or *R. maritima* (2Ra and 2Rb) and a positive (2+) and negative (2-) control tank. Survival is from the start of the experiment (hatching) until metamorphosis to the stage 4 postlarval stage. Survival curves grouped in brackets indicate statistically similar curves by Kaplan-Meier analysis and Holm-Sidak pair-wise comparisons. The two bracketed groups were significantly different from each other (Table 3).

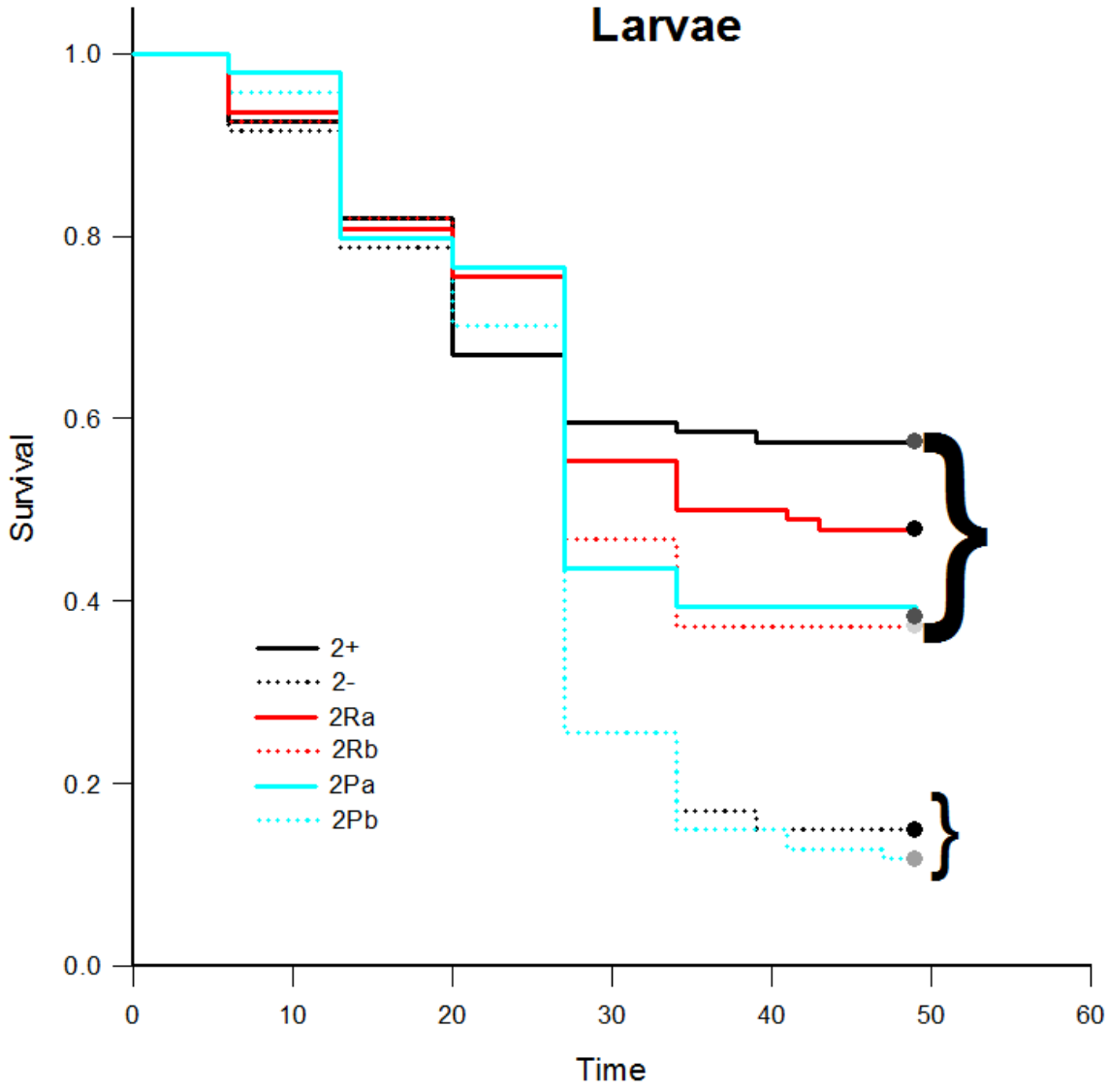


Figure 2: Second Experiment: Survival curves of postlarvae, which were raised as larvae in tanks fed at noontime either the seagrasses *P. perfoliatus* (2Pa and 2Pb) or *R. maritima* (2Ra and 2Rb) and a positive (2+) and negative (2-) control tank. Survival is from post-metamorphosis to stage 4 until the end of the experiment, up to 131 days. Survival curves were statistically significant by Kaplan-Meier analysis (log-rank statistic = 19.446; df = 5; $p < 0.002$), and Holm Sidak pair-wise comparisons revealed only one significant comparison between tank fed *P. perfoliatus* (2Pa) and the positive control (2+) (Holm Sidak statistic = 10.068; P value = 0.0224; $p < 0.05$).

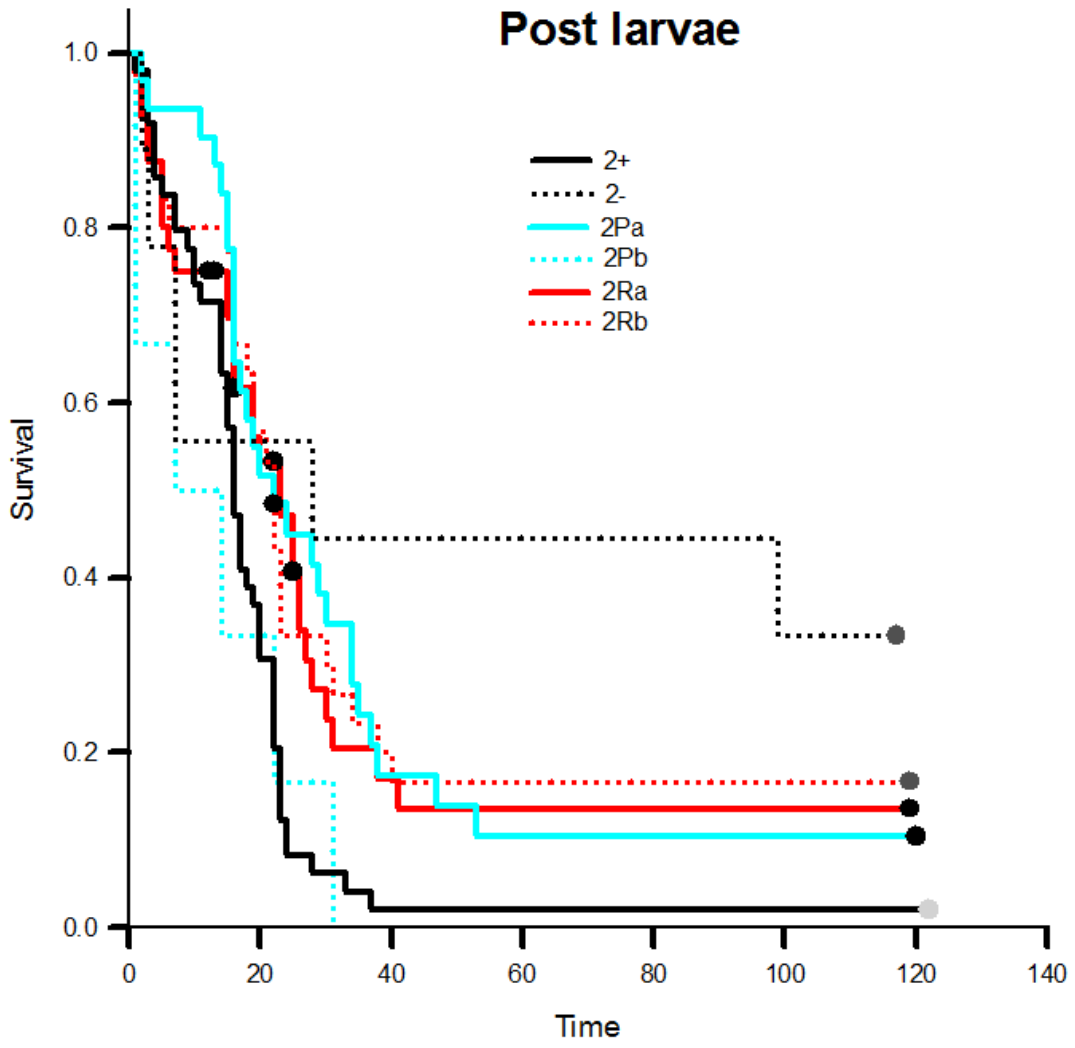
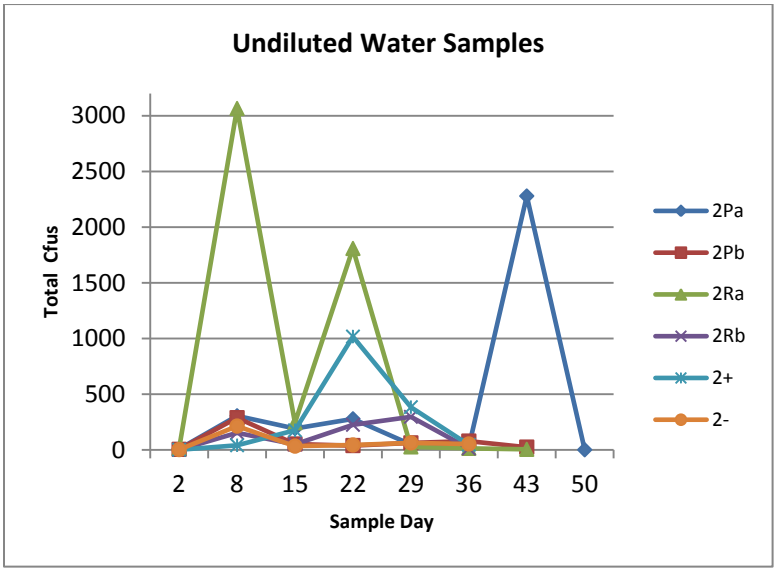


Figure 3. Second Experiment: Total Colony Forming Units (cfus) of water samples taken from tanks and spread on marine agar. On sample day 15, samples were diluted (1:10) and also spread on marine agar plates.



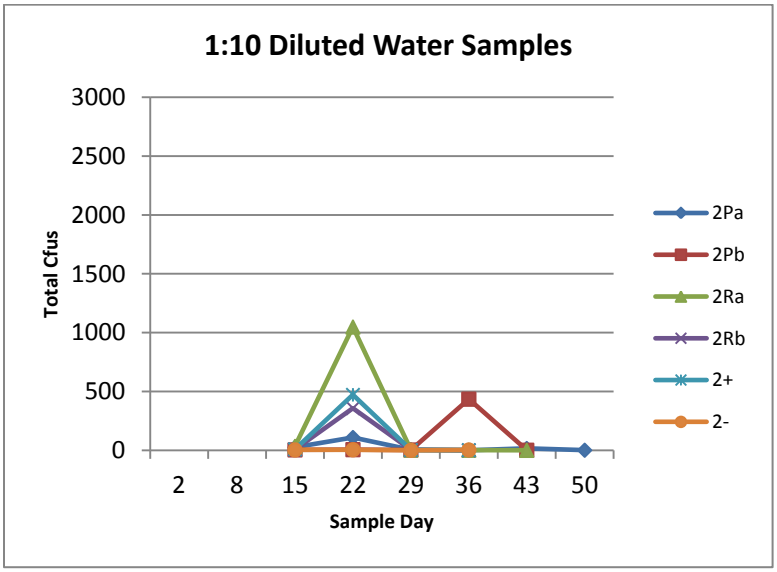


Figure 4. Second Experiment: Lipid vacuole levels of R-cells from the hepatopancreas in samples from stage 3 larvae and stage 4 postlarvae from tanks fed either the seagrasses *P. perfoliatus* (2Pa and 2Pb) or *R. maritima* (2Ra and 2Rb) and a positive (2+) and negative (2-) control tank. Lipid vacuole levels were rated on a scale of 0 – 4, with 0 = no visible vacuoles in R-cells and 4 = R-cell cytoplasm 100% filled with vacuoles. Analysis was conducted by the University of Arizona Aquaculture Pathology Laboratory.

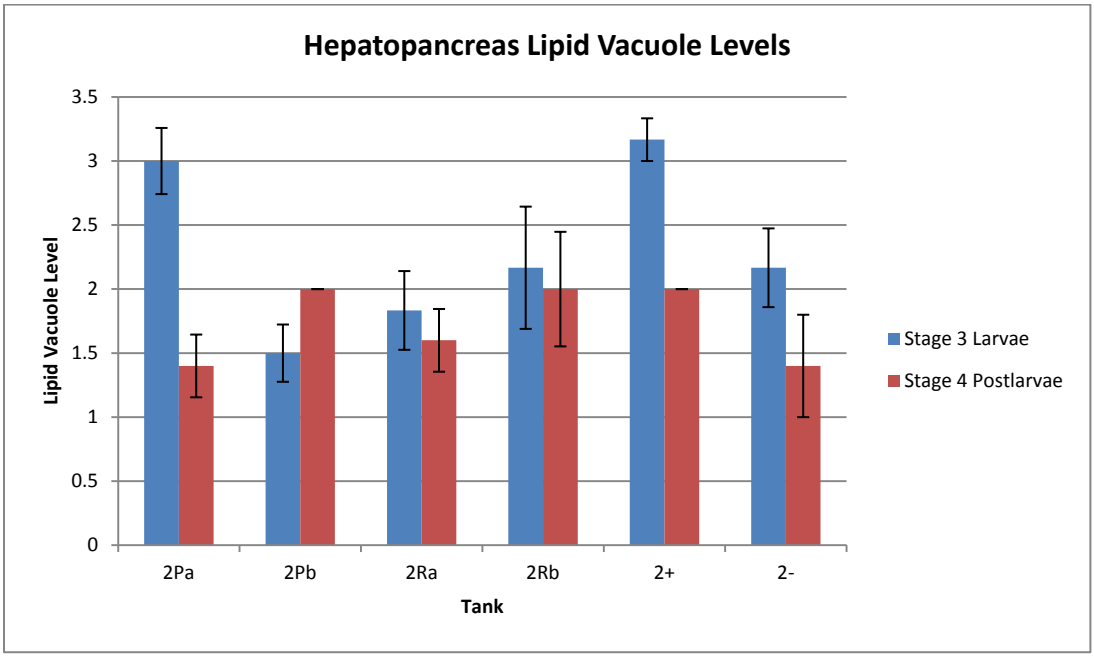


Figure 5. Second Experiment: Level of fouling bacteria assessed on stage 3 larvae (n = 6) and stage 4 postlarvae (n = 5) from tanks fed either the seagrasses *P. perfoliatus* (2Pa and 2Pb) or *R. maritima* (2Ra and 2Rb) and a positive (2+) and negative (2-) control tank. Fouling bacteria levels were assessed on a 0 – 4 scale, with 0 = no signs of infestation and 4 = high number of pathogens. Analysis was conducted by the University of Arizona Aquaculture Pathology Laboratory. All stage 3 larvae sampled had fouling bacteria on their carapace or appendages, while only 6 of the 30 stage 4 postlarvae exhibited fouling bacteria.