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# Optimizing abalone outplant strategies using larval *Haliotis kamtschatkana* in the San Juan Archipelago, Washington

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**Optimizing abalone outplant strategies using larval *Haliotis kamtschatkana* in the San Juan Archipelago, Washington**

By

Katie Mills-Orcutt

Accepted in Partial Completion  
of the Requirements for the Degree  
Master of Science

ADVISORY COMMITTEE

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Katie Mills-Orcutt

July 2019

**Optimizing abalone outplant strategies using larval *Haliotis kamtschatkana* in the San Juan Archipelago, Washington**

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Presented to  
The Faculty of  
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In Partial Fulfillment  
Of the Requirements for the Degree  
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## Abstract

Prior studies testing the use of *Haliotis kamtschatkana* larvae as a means of stock enhancement in the San Juan archipelago have shown no success in inducing larvae to settle in densities required for successful reproduction. I conducted an experiment to test the effectiveness of new methods for outplanting larval abalone in hopes of creating a protocol Puget Sound Restoration Fund could use to restore wild populations. Using two sites within the San Juan archipelago, I outplanted abalone into pre-constructed larval abalone modules (LAMs) and sampled over four months, testing retention strategies. Three types of LAMs were used at each site to determine whether 125 $\mu$ m Nitex tenting was necessary for retention of larvae after outplanting. In total, nine LAMs were placed at each site: three with Nitex tenting (tented LAMs), three without tenting (open LAMs), and three which received no larval abalone (control LAMs). All larval abalone were treated with 5 $\mu$ M of settlement cue gamma-aminobutyric acid (GABA) at time of outplanting to stimulate metamorphosis into their benthic life phase. Larvae of the same families that were outplanted were also reared in LAMs at Shannon Point Marine Center so that survival could be compared to survival in field LAMs and so that emigration could be estimated. Shell lengths of field abalone were measured four months after outplanting and were compared to hatchery reared individuals of the same families to better understand if hatchery life compromises early growth. Four months after outplanting abundance counts of settled pinto abalone were significantly greater ( $p < 0.001$ ) in LAMs with 125 $\mu$ m Nitex tents encasing the LAMs for 24 hours after seeding, compared to open and control LAMs. Open LAMs had some settlement (0-12 abalone), while tented LAMs all contained settled juveniles (16-35 abalone). Control LAMs (which had not been seeded during outplanting) remained unsettled by any abalone, suggesting no adult reproductive abalone were present at my study sites. Emigration measurements from laboratory LAMs showed 8% (tented) and 14% (open) of total seeding densities settled on aquarium walls during final sampling, often clustered together. Shell length measurements during final sampling showed no size difference in outplanted abalone compared to hatchery controls suggesting hatchery conditions do not stunt growth rates of newly settled pinto abalone, however, more research needs to be done in order to better understand if behavioral differences exist between hatchery-reared juveniles and juveniles that were outplanted as larvae. In conclusion, I believe outplanting pinto abalone as larvae has significant potential to supplement current wild stocks in the San Juan archipelago as a cost-effective alternative to traditional juvenile outplanting.

## **Acknowledgements**

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## Introduction

Abalone species worldwide have steadily declined over the past century, forcing the closure of fisheries (both commercial and recreational) for over 75% of historically harvested populations (McShane 1996; Prince 2005). Concerns regarding unsustainable harvest, global climate change, environmental disturbances, and the spread of infectious disease such as withering foot syndrome all might be contributing factors responsible for the closure of many once robust abalone fisheries and prompting restoration groups worldwide to intervene in species management (Neuman et al. 2018; Sweijd et al 1998; Tegner et al. 2001). In North America seven species of abalone were historically abundant along the west coast (Geiger 1999) and supported viable subsistence fisheries for centuries (Cox 1962). However, anthropogenic and ecological pressures over the past century have resulted in a dramatic population decline of all seven species forcing the closure of abalone all fisheries in the United States. Within this range, only one species of abalone (*Haliotis kamtschatkana*) lives in Washington state.

Pinto abalone (*Haliotis kamtschatkana*), once abundant throughout their range from Alaska to Point Conception, California, have undergone a precipitous decline in the past half century, particularly within Washington waters (Woodby et al. 2000; Rothaus et al. 2008). Due to both poaching pressure and significant recreational harvest until 1994 in the Pacific Northwest, populations now fall below densities required for successful reproduction at all sites monitored by the Washington Department of Fish and Wildlife (WDFW; Figure 1). Originally thought to be resilient to over-exploitation due to high fecundity and widespread distribution, we now know that *H. kamtschatkana*'s slow-growing, sessile lifestyle makes large-scale harvest incompatible with sustainable populations (Bouma et al. 2012). Often described as a boom-bust species, recognition

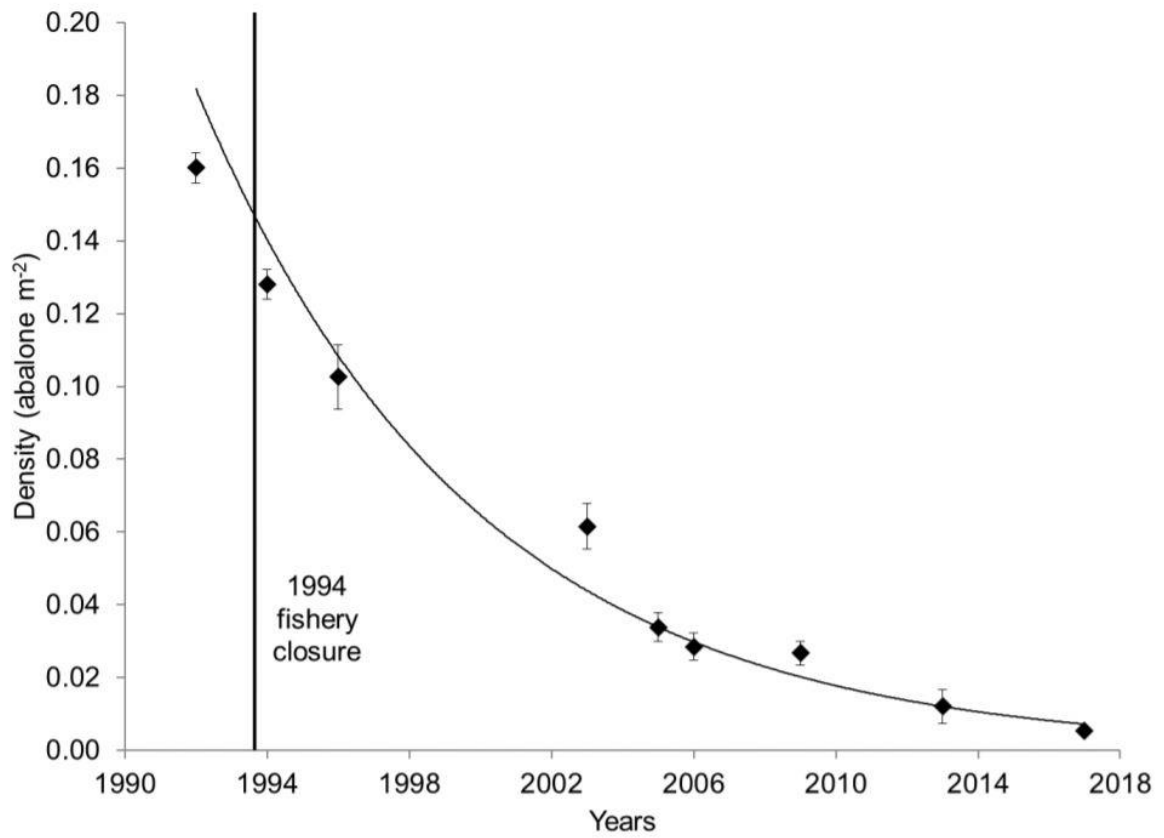


Figure 1. Change in mean density of pinto abalone of 10 index sites established in the San Juan archipelago. Points are mean density of abalone m<sup>-2</sup> with bars representing the standard error of the mean. <https://wdfw.wa.gov/sites/default/files/2019-05/B.%20Pinto%20Abalone%20Summary%20Sheet.pdf>

that populations are being over-exploited is typically delayed due to the highly aggregate nature of abalone and harvesters' tendency to remove an entire aggregation before moving on to another aggregation (Jamieson 1993; Babcock and Keesing 1999). As broadcast spawners, abalone depend on a variety of factors to ensure fertilization success including: number, density and distribution of spawning individuals, synchrony of gamete release, currents and turbulence, and gamete properties (Levitan and Sewell 1998; Courchamp et al. 2008). When any of these factors shift, it can move a population below its threshold density and successful fertilization will no longer sustain population growth (Berec et al. 2007). The last commercial fishery of pinto abalone closed in 1995, yet populations still continue to decline throughout their range (Hankewich et al. 2008). Due to the failure of pinto abalone populations to rebound despite fishery closures, experts believe increased management of local abundances might be the only possibility for populations to rebuild in the San Juan archipelago (Campbell 2000). As of 2019, pinto abalone are listed as Endangered Species under the Endangered Species Act (WDFW 2019).

Restoration groups worldwide are testing various methods for increasing wild stocks of abalone, the most common being the outplanting of hatchery-reared individuals. This process involves gathering broodstock individuals from the wild and spawning them in captivity to create as many juvenile individuals possible, which will later be released into the wild. Primary focus is currently centered around outplanting hatchery reared juveniles that range from between six to eighteen months old (Schiel 1992). Rearing the juveniles in the hatchery for this long is commonly thought to boost their chance of survival once outplanted into the wild, due to the extremely fragile morphology of young abalone. However, while outplanting juveniles of this age range is a viable option, new studies on numerous abalone species have shown limited outplanting success due to high mortality and low recapture rates (Schiel 1993; McCormick et al. 1994). In California, USA,

less than 1% of outplanted juvenile *Haliotis rufescens* survived to harvestable size despite continued stock supplementation, and most individuals were never seen after outplanting (Rogers-Bennett and Pearse 1998). In Australia, 0-57% of juvenile greenlip abalone (*Haliotis laevis*) survived after being outplanted at different depths over nine sites with different substrata, suggesting that multiple variables are playing a part in the extreme variation found between trials (Dixon et al. 2006)

The low survival rates of outplanted juveniles might be the result of morphological and behavioral differences between hatchery reared and wild populations (Schiel and Welden 1987). Juvenile wild abalone have been observed grazing on multiple species of diatoms, consuming up to 30% of their body weight per day (Hahn 1989). Hatchery juveniles, however, are typically settled in high densities and compete for food resources during early life stages (Bouma, personal communication 2018). Competition for resources during rearing might account for slower growth rates observed in hatchery settings and potentially account for the low survival of hatchery outplants. Altering key outplanting elements such as abalone developmental stage and condition upon outplanting might therefore have considerable potential to increase outplanting success (Dixon et al. 2006).

One such element yet to be tested on a full scale in the San Juan archipelago is the release of larval abalone instead of juveniles. Outplanting larvae would reduce rearing time in the hatchery to as little as seven days due to the short larval phase of pinto abalone, freeing up hatchery personnel to raise another batch of larvae or focus on other aspects of restoration. It might also increase the likelihood of the abalone reaching adulthood and reproducing if extended hatchery life does induce behavioral or morphological differences that lead to mortality after outplanting. Outplanting abalone larvae as a restoration tool has not been tested as extensively as juvenile

outplanting, so little is known about the conditions needed for success in the San Juan archipelago. Studies of larval outplanting on other species have yielded large differences in settlement (Tong et al 1987; Preece et al. 1997; Shepherd et al. 1995), suggesting that strategies for outplanting are species and site-specific, and requiring that even successful methods performed elsewhere must be tested within the San Juan archipelago before full-scale implementation by restoration groups can occur.

The most comprehensive outplanting of larvae to date using pinto abalone was tested in Barkley Sound, British Columbia, Canada (Read et al. 2012). Researchers released different densities of larvae into square meter sections of rocky bottom habitats using Nitex tents to retain individuals for the first two days after outplanting (Hansen 2006). Larvae were also treated with gamma-aminobutyric acid (GABA) 30 minutes prior to release to induce settlement. After a year of surveys to determine survival, researchers concluded that success of outplanting larvae was comparable to outplanting juveniles, provided larvae were released in quantities of approximately 50,000 individuals  $m^{-2}$  (Hansen 2006). This study did not however test whether Nitex tents were necessary for larval settlement.

Given the necessity for managers to supplement pinto abalone stocks in the San Juan archipelago, optimizing methods for local outplanting is crucial for successful restoration. If outplanting larvae is possible, researchers at pinto abalone hatcheries could switch from using juvenile to larval stocks and thus increase hatchery potential. To create a protocol for outplanting larvae, I tested whether Nitex tenting is necessary for retaining outplanted larvae that have been exposed to GABA. Nitex tenting can only remain in place a short time before fouling and preventing water flow to settled abalone, which presents a time commitment to hatchery managers because they must return to outplant sites and remove tents following outplanting. I predicted that

larval abundances would be greatest within seeded structures tented with 125 $\mu$ m Nitex screen compared to those with no tenting. I also compared the shell lengths of outplanted larval abalone to hatchery abalone of the same families to determine if growth rates were different between populations of *H. kamtschatkana* in the field and in a hatchery setting.

## Materials and Methods

To examine whether larval *H. kamtschatkana* could be successfully outplanted in the San Juan archipelago, and to determine whether tenting larval abalone modules (LAMs) increased outplanting success, I seeded larval abalone in LAMs with and without Nitex tenting at two sites in the field and in the laboratory at the Shannon Point Marine Center (SPMC). Laboratory LAMs were seeded using the same methods as with field LAMs and were used to compare survival in field and laboratory conditions and to estimate emigration of settled individuals. I tracked the number of juvenile abalone present in the LAMs and the size of the juveniles for four months. Abalone of the same families that were outplanted were also reared at the hatchery in Manchester, WA so that growth could be compared between hatchery and outplanted individuals.

### *Spawning and rearing larvae*

I spawned abalone for this study at a hatchery managed by the Puget Sound Restoration Fund (PSRF) in Manchester, WA using a protocol developed by Morse et al. (1977) that was adapted for *H. kamtschatkana* (Appendix 1). Adaptations to the protocol increase the spawning success of pinto abalone and include applying a heat shock that is 3°C warmer than the holding tank temperature and leaving the abalone in a hydrogen peroxide solution for slightly longer than the recommended three hours (Bouma, 2011 unpublished). Volitional spawning of pinto abalone is typically from April to July (Campbell et al. 2003), however ripe individuals are found year-round at the PSRF hatchery (Bouma personal communication). The spawning event used for this study was in early July and was attempted using six females and six males, however only one female was induced to spawn on that particular day. Once all eggs and sperm were collected, I evenly divided the eggs between each of the spawned males giving me six distinct families for

outplanting. After the eggs were fertilized, blastulae were divided into two groups, one to remain at the hatchery and another for outplanting. The blastulae designated for outplanting were transported in coolers to SPMC where they were reared for 8 days in 12°C filtered seawater. Two 75-liter rearing tanks were set up side by side with flow through seawater so that every 12 hours all larvae would be moved via water flow from one tank to the other. This was done so that rearing tanks could be drained and cleaned without the necessity to filter the delicate larvae onto mesh screens. Once a tank was cleaned water inflow and outflow were switched and all larvae would be moved into the newly cleaned tank (Figure 2).

To determine when the larvae were ready for outplanting, I assessed the development of larvae each day (Figure 3). Starting on day seven, a subsample of larvae was exposed to 5µM GABA and observed for 30 minutes. Of that subsample, only about 75% of the larvae settled within the time frame required for outplanting so rearing was extended to eight days. On day eight, all larvae that were subsampled settled within 30 minutes, indicating readiness for outplanting.

### ***Field sites and larval abalone modules***

I chose two sites in the San Juan archipelago, one off the beach of the Shannon Point Marine Center, Anacortes, WA and one off the east end of Young Island in Burrows Bay, WA (Figure 4). At each site, divers secured 50 m downlines between eight and ten meters deep, with larval abalone modules (LAMs) attached every five meters. Each downline had three LAMs encased in 125µm Nitex tents that were seeded with larvae (tented LAMs), three LAMs without tenting that were seeded with larvae (open LAMs), and three controls (no tenting and no seeded larvae), for a total of six LAMs for each treatment and eighteen LAMs in total between the two sites.





Figure 2. Larval rearing tanks at Shannon Point Marine Center. Top tank is the hatching tray, which blastulae were placed in after being transported from hatchery site in Manchester, WA. After 24 hours all fertilized eggs hatched and larvae migrated to the top of the tray where they were carried via water flow to the rearing tanks below.

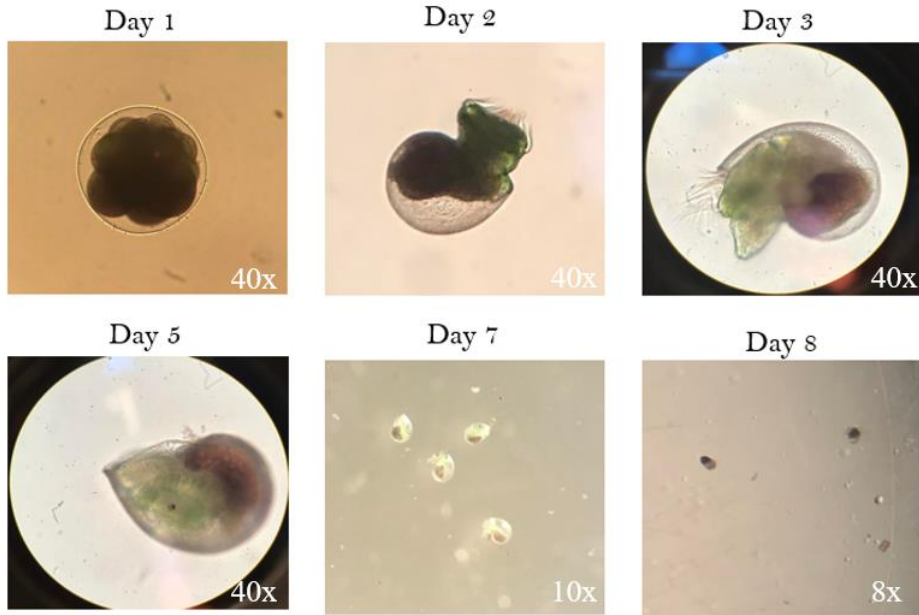


Figure 3. Larval abalone development over eight days of rearing at Shannon Point Marine Center. Day seven shows abalone which have not yet settled (still swimming around) after 30 minutes of GABA exposure. Day eight shows settled abalone (shells rotated so that their foot is grasping the substrate).

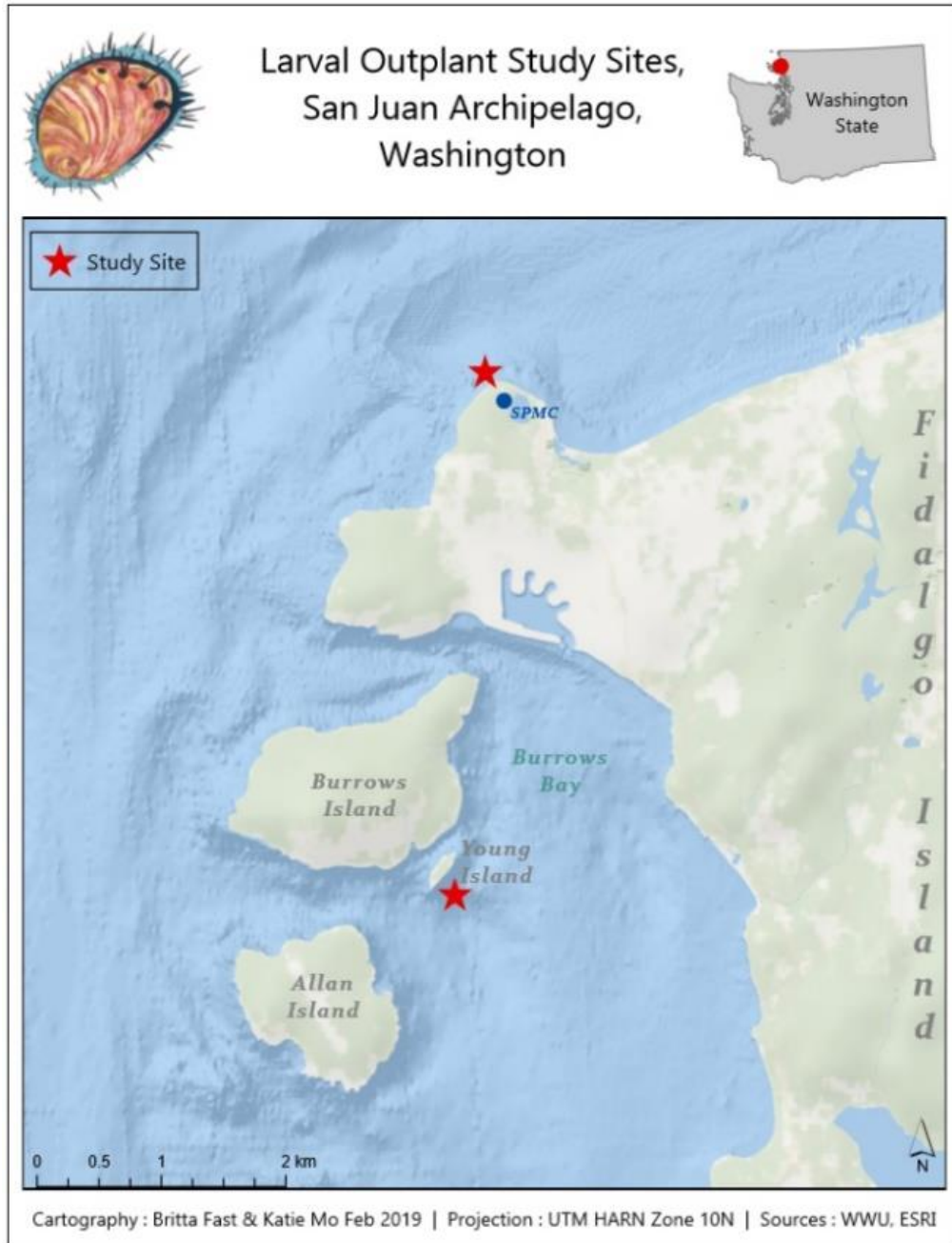


Figure 4. Map of the two study sites used for outplanting larvae in the San Juan archipelago and the location of laboratory controls at the Shannon Point Marine Center (SPMC).

I also seeded six laboratory LAMs (three tented and three open) to compare survival rates between lab and field conditions. Each of these LAMs was contained in a Perspex aquarium (50 x 45 x 35cm) supplied with a constant flow of unfiltered seawater. Since each LAM was in its own aquarium, I could count the number of juvenile abalone on the aquarium walls, allowing me to estimate emigration from the LAM. Control LAMs were not used in the laboratory because no migration between LAMs or natural settlement was possible.

Each LAM consisted of a plastic milk crate (48 x 33 x 28 cm) fastened with a lid and lined with a corrugated polycarbonate bottom. Inside the crates, four stacks of four polycarbonate tiles (10 x10 cm) were used to increase settlement area and allow for repeated sampling (Figure 5). This basic design was used for open and non-seeded controls, while tented LAMs were covered with 125µm Nitex screens. To ensure LAMs would have suitable diatom growth prior to seeding of larvae, I conditioned them in a large tank of flow-through seawater at SPMC for two months preceding outplanting.

### ***Outplanting larvae***

Following eight days of rearing, I removed larvae from the rearing tanks and placed them into transport containers at concentrations of 6,500 individuals per 100 mL seawater. Once at each study site, larvae were exposed to 5 µM GABA 30 minutes prior to outplanting and randomly divided into plastic syringes, one for each LAM at each site. I seeded the sites using SCUBA protocols outlined by Puget Sound Restoration Fund (PSRF). Upon descent, divers first secured the Nitex tenting to the LAMs in that treatment. Then, moving from one end of the downline to the other, divers seeded each module by slowly pushing the larvae out of a syringe. Syringes were refilled and expelled three times with ambient seawater to ensure that as many of the larvae present

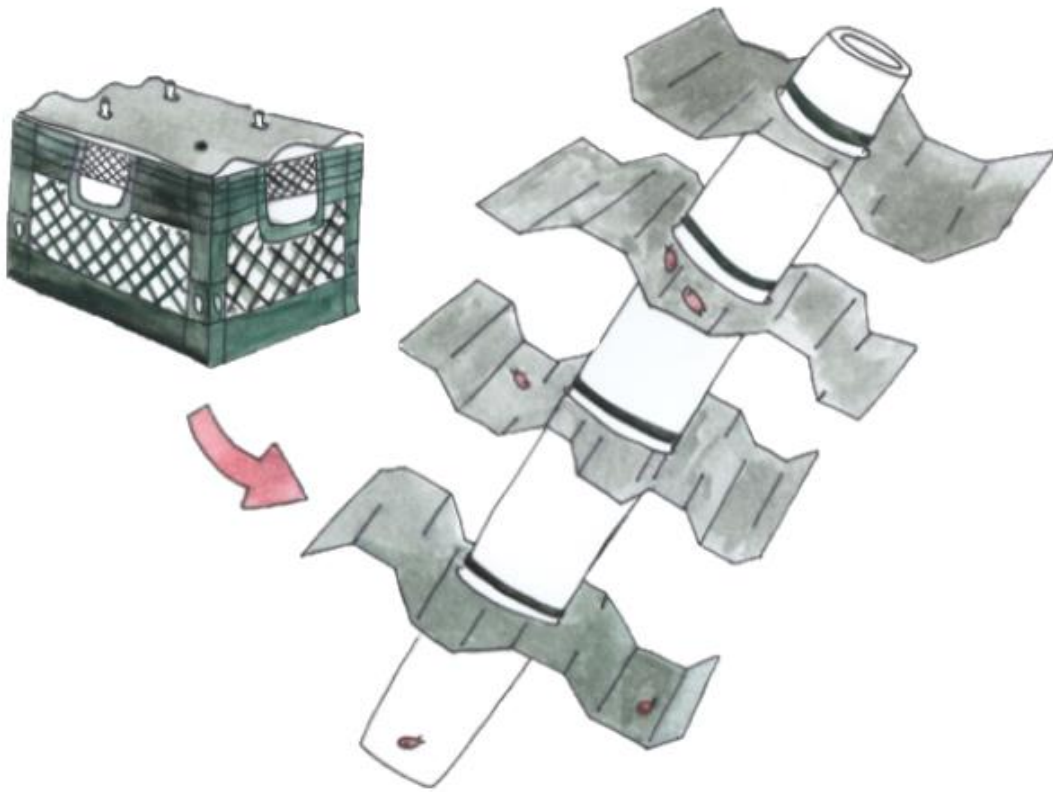


Figure 5. Illustration of LAM showing one of the four settlement plate stacks being removed during a sampling. Each month one stack was removed until the final month when the final stack and entire LAM was brought to the lab for analysis.

had been outplanted before moving to the next LAM. Precautions were taken by divers to outplant during slack tidal currents, however a slight current was detected by the divers at depth so seeding took place from the up current side of each LAM. While seeding was underway, a second set of divers secured the downline with large rocks to prevent movement of LAMs in the strong San Juan archipelago currents. Divers returned to the sites 24 hours after outplanting and removed the Nitex screens from the tented LAMs before screens could become fouled and limit water flow inside the module. Each screen was placed into a bag when it was removed from the LAM and was taken to the lab to determine if any abalone had settled on the Nitex. To count any abalone settled on the screens, the Nitex was rinsed with fresh water and particulate matter was condensed onto 20 cm round 125  $\mu\text{m}$  filters. Filters were then examined under a microscope and settled abalone were counted.

### *Sampling larval abalone modules*

The LAMs were sampled every month for four months. During the first three samplings, divers removed one stack of polycarbonate tiles from each LAM. Stacks were cut free from the polycarbonate lid that secured each module and transferred to a 7.5-liter Ziplock bag which was sealed underwater. Stacks were transported in a cooler to the lab where the number of settled juveniles on each stack was counted. Laboratory LAMs were sampled using the same methods; however, no transportation was necessary prior to analysis.

The fourth and final survey was conducted after four months and was spread out over two days to fully remove all LAMs and downlines from the two sites. At each site, the last polycarbonate stack from each LAM was removed as described above, then all of the LAMs at the site were brought to the boat and transported to the lab. That same day, the final stack of

polycarbonate tiles as well as the lid, bottom, and crate were thoroughly checked for juvenile abalone. Laboratory LAMs were surveyed in the same manner except that aquarium walls were also checked for abalone.

To calculate outplanting success in the field and laboratory LAMs, the total number of abalone from all sampling days was used and divided by the number of larvae that was outplanted (~6500). By combining counts from all sampling days, I assumed that animals removed during previous samplings would have survived to the four-month-mark. Sub-sampling was conducted for the first three months of the trial in order to have some preliminary data on LAMs in case they were lost or destroyed by ocean conditions. Although some of the animals counted during the first three months of subsampling likely would have emigrated or died before the four-month final count, including them in the final count had no effect on overall survival percentages due to the magnitude of survival.

### ***Shell length***

In addition to the number of abalone in each LAM, I also measured the shell length of each abalone retrieved during the final survey from field sites [Shannon Point Beach (n=29) and Young Island (n=44)], as well as the shell lengths of abalone from the same families being reared in the hatchery (n=60). Digital images were taken of the shell at 40x magnification, and then shell lengths were measured using ImageJ version 1.8.0 (Schneider et al. 2012; Figure 6). Hatchery abalone were measured using electronic calipers to 0.1 mm accuracy. Abalone outplanted in laboratory LAMs at Shannon Point Marine Center were excluded from the shell length analysis because the conditions they were kept in differed from hatchery settings (water temperature and pH were unaltered from ocean intake pipes).

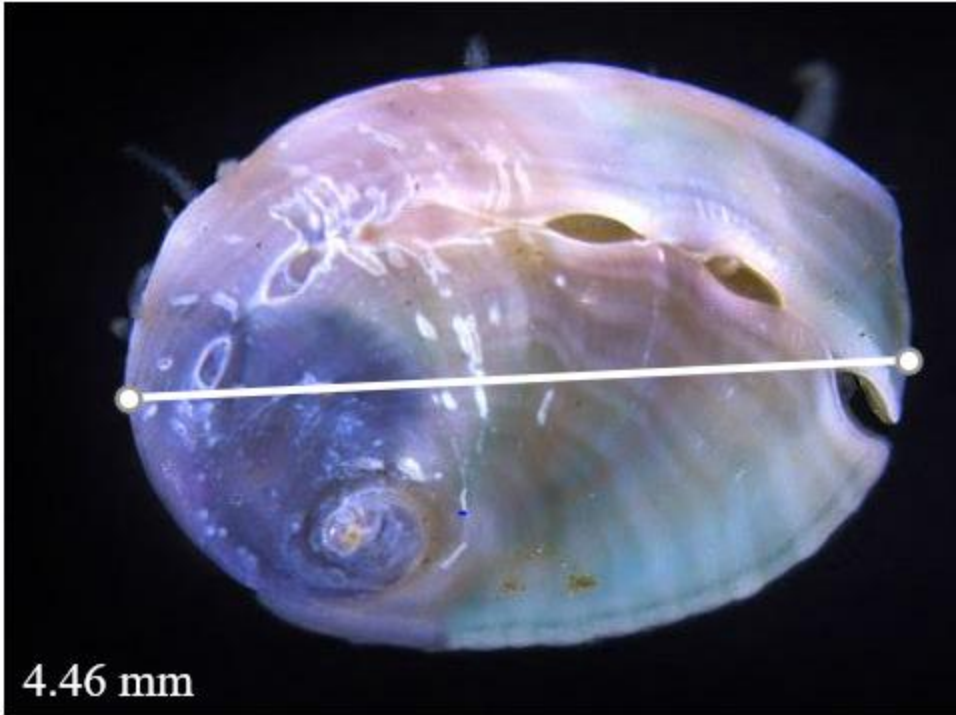


Figure 6. Shell length of outplanted abalone measured using ImageJ, taken at the four-month-mark.



### *Statistical analyses*

I analyzed abundance counts generated from the four months of sampling using a generalized linear mixed-effects model with a Poisson error distribution and log link function with the statistical package R, which is appropriate for count data. All abundance subsample data (from the first three months) and final count data (from month four) were compiled for statistical analysis. I chose to include the subsample data from months one-three since statistically it had no effect whatsoever to include or ignore these abundance counts due to the overall percent survival, and I would rather use all the data I collected. LAM type (i.e., treatment) was examined as a fixed factor, while site was a random factor. Due to the magnitude of individuals found on laboratory LAMs compared to field LAMS, I analyzed these separately in order to not mask any trends in field LAMs. One unseeded control LAM was lost during the final sampling and could not be analyzed. However, because no control LAMs had any settled abalone on them during any sampling in my study, I still included data from the first three samplings of the lost LAM in my analyses.

I analyzed shell length of four-month-old abalone to compare individuals from the field to hatchery individuals of the same cohort using an ANOVA with the statistical package R. A Levene's test to ensure equal variance for sites with different sample sizes was used to meet the assumption of equal variance. Site (SPMC beach, Young Island, or hatchery) was the predictor variable and shell length as the response variable. A Tukey test was then conducted to determine if any differences were observed between sites.

## Results

### *Abalone abundance on Nitex tents*

On average, tenting from field LAMs had approximately twice the number of settled individuals compared to tenting from laboratory LAMs (Table 1). The number of settled abalone found on the Nitex screens was less than 3% of the abalone initially seeded in the LAMs.

### *Abalone abundance in LAMs*

The number of settled abalone at both field sites (Shannon Point Beach and Young Island) was significantly higher within tented LAMs compared to open or control LAMs (Poisson GLMM,  $z = 7.48$ ,  $p < 0.001$ , contrasting tented and open LAMs; Figure 7A). Four months after outplanting, I did not find any abalone in the control LAMs (which had not been seeded during outplanting). Open LAMs had some settlement (0-12 abalone), while tented LAMs all contained settled juveniles (16-35 abalone).

Laboratory LAMs (which were tested separately from field LAMs in order to not mask any trends seen in the field) also had significantly higher abalone abundances within tented LAMs compared to open LAMs ( $z = 9.71$ ,  $p < 0.001$ ; Figure 7B). In addition, laboratory LAMS had approximately 10x the number of settled abalone compared to field LAMs.

### *Emigration from laboratory LAMs*

Emigration or settlement outside the LAMs was measured for laboratory LAMs by counting individuals found on aquarium walls. For tented LAMs, the number of abalone found on the aquarium walls was 8% of the number of abalone found on the LAM, indicating that total outplant success was approximately 3.2% of the initial 6,500 larvae (Table 2). The number of

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Table 1. Number of *H. kamstchatkana* settled on Nitex tents one day after outplanting (n=3)

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Site	Number of abalone (mean $\pm$ std)	% of abalone outplanted
Lab	81 $\pm$ 2.76	1.2%
Shannon Point Beach	177 $\pm$ 3.36	2.7%
Young Island	182 $\pm$ 3.83	2.8%

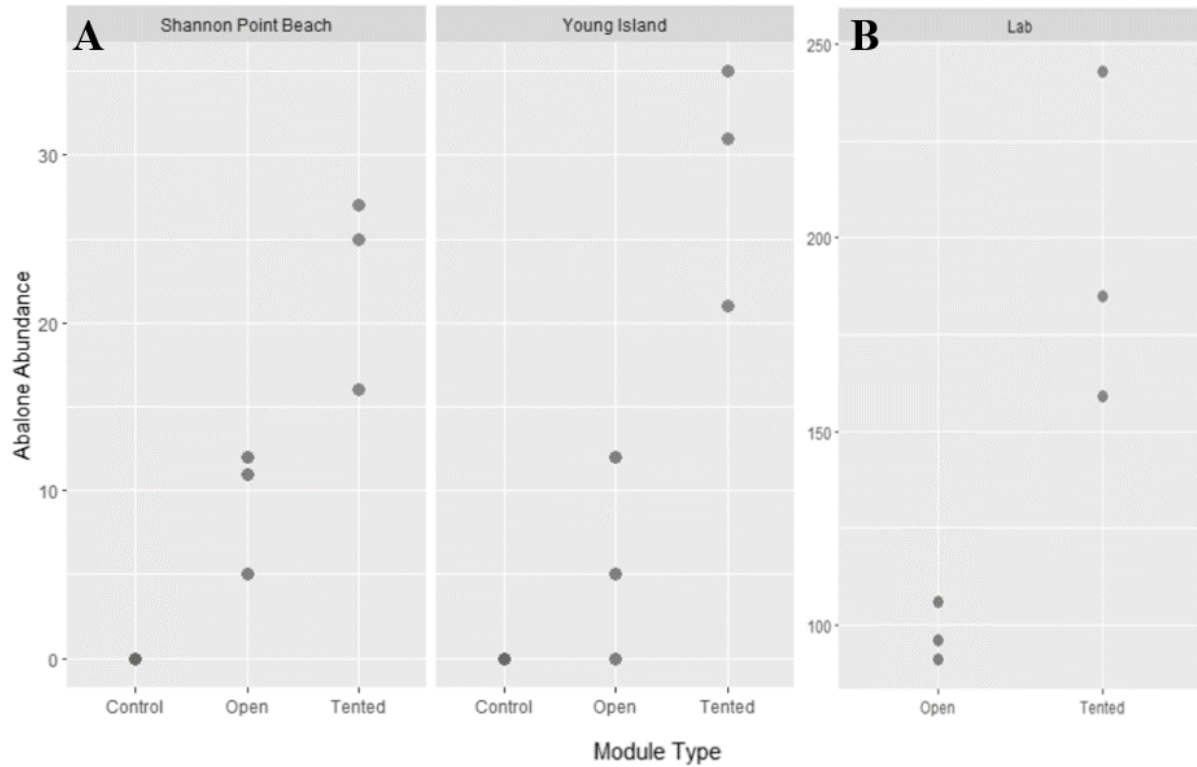


Figure 7. Effect of module type on *H. kamtschaticana* settlement at (A) two sites in the San Juan archipelago and (B) in the laboratory. Tented LAMs had significantly higher settlement rates compared to open LAMs. ( $p < 0.001$ ). Control LAMs had no settled abalone. No control LAMs were used in the laboratory setting since natural settlement and juvenile migration between LAMs was impossible. Note the different scales used for abalone abundance at field sites and in the laboratory.

abalone found on the aquarium walls surrounding open LAMs was 14% of the abalone found on the LAM, for a total outplant success of 1.7% (Table 2).

### ***Shell length***

I found no significant difference in shell length between outplanted abalone at the two field sites and abalone raised in the hatchery ( $F = 1.55$ ,  $p = 0.86$ ; Figure 8).

Table 2. Estimated outplant success of *H. kamtschatkana* in lab and field LAMs after four months. Emigrated individuals were abalone found on aquarium walls surrounding lab LAMs that were no longer living on or within LAMs. Emigrated individuals were not included in final abundance counts. Numbers are means  $\pm$  standard deviation.

Site	LAM type	Number of seeded abalone	% of seeded abalone settled on LAM	% of seeded abalone that emigrated
Lab	Open	~6,500	1.50 $\pm$ 0.08	0.20 $\pm$ 0.11
Lab	Tented	~6,500	2.96 $\pm$ 0.54	0.21 $\pm$ 0.17
Shannon Point Beach	Open	~6,500	0.08 $\pm$ 0.07	unknown
Shannon Point Beach	Tented	~6,500	0.46 $\pm$ 0.07	unknown
Young Island	Open	~6,500	0.14 $\pm$ 0.04	unknown
Young Island	Tented	~6,500	0.35 $\pm$ 0.09	unknown

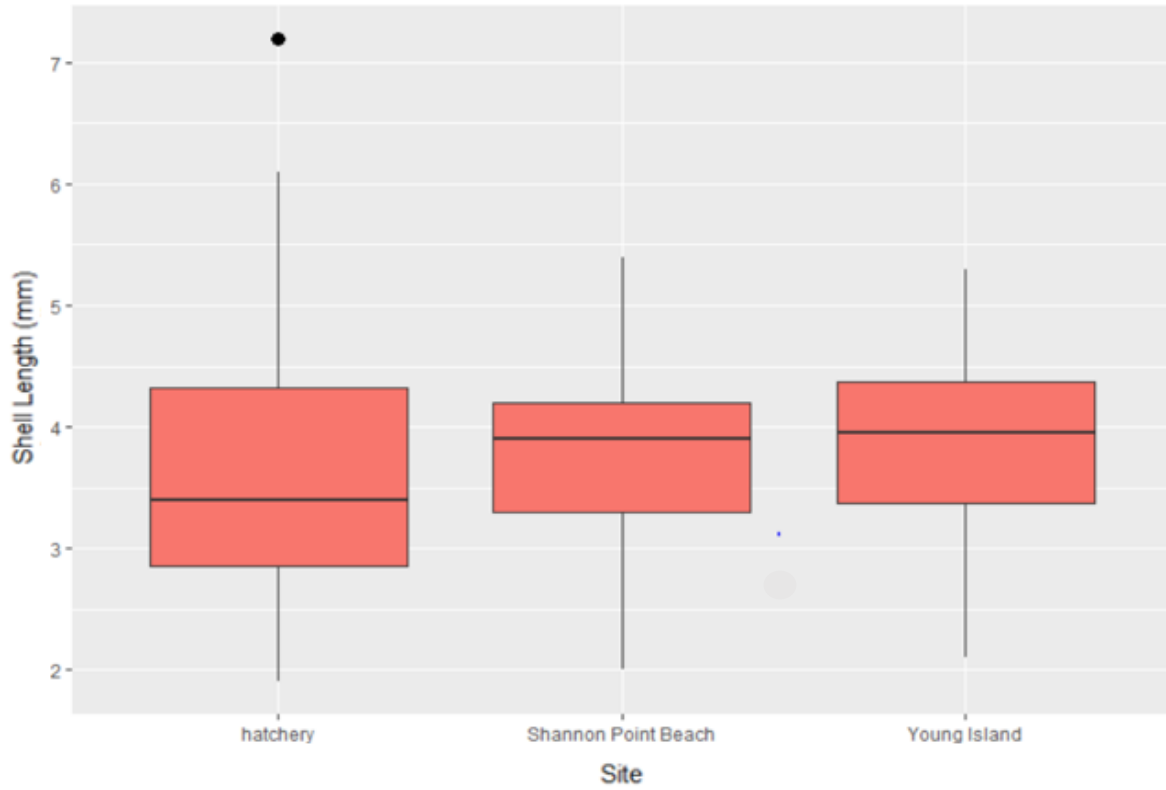


Figure 8. Shell lengths of all *H. kamtschatica* counted during the final sampling four months after outplanting [Shannon Point Beach (n = 29), Young Island (n = 44)], and from the hatchery (n = 60).

## Discussion

Outplanting pinto abalone larvae in the San Juan archipelago has the potential to supplement current wild stocks of abalone and increase the cost effectiveness of hatcheries. With a cost of ~\$12,000/month to rear juvenile abalone in preparation for outplanting (Josh Bouma, personal communication), switching to larval release could free up hatchery personnel and funds for other aspects of restoration. I found that outplanting larvae is most successful when 125  $\mu\text{m}$  Nitex tenting is used for 24 hours to retain larvae at the outplant site. Although this represents added time for hatchery personnel, it appears necessary for increasing the success of outplanting larval *H. kamtschatkana*. Tented LAMS contained approximately 3.5x the number of juvenile abalone compared to open LAMs, and no abalone were found in the unseeded control LAMs. Additionally, in a pilot study conducted by the Puget Sound Restoration Fund in 2012, eight-day old hatchery-reared individuals were outplanted into modules filled with natural benthos (rocks encrusted with crustose coralline algae), using GABA as a settlement cue, but without tenting the modules (Bouma, DNR final report, 2013). These outplant sites were surveyed approximately a year and a half later and no abalone were found within or surrounding any of the modules (WDFW, unpublished data).

Tenting has proved successful in other studies as well. A study in Barkley Sound, British Columbia, used Nitex tenting as a retention tool for larval *H. kamtschatkana* and found that the lowest density tested (50,000 individuals  $\text{m}^{-2}$ ) was optimal, with approximately double the individuals  $\text{m}^{-2}$  compared to background settlement (Hansen 2006). However, investigators were not able to test whether tenting affected settlement at the site because there were no untented treatments. Preece et al. (1997) found mixed results with tenting for larvae of two species of



Australian abalone. For *Haliotis rubra*, tented plots yielded higher densities of juvenile abalone when larvae were seeded at low (1,600 individuals m<sup>-2</sup>) and high (80,000 individuals m<sup>-2</sup>) densities, but not at an intermediate (16,000 individuals m<sup>-2</sup>) density. However, tenting improved juvenile abundance at all experimental densities of larval *Haliotis laevis*. In general, tenting appears to improve outplanting success for larval abalone and although it presents an additional time commitment for restoration work because the tents must be retrieved after seeding, the successful settlement found in my study and others makes it an economical choice for hatchery managers. If tent removal could be optimized further by attaching tents to buoys for topside removal the following day, the process could become even more cost effective.

Another factor to consider is Nitex mesh size used during outplanting. Hansen (2006) used 200 µm mesh and found considerably fewer abalone (~2) settled on the tents at the time of removal compared to my study (~180). Pinto abalone larvae are typically around 200 µm in length at time of metamorphosis, which led Hansen to suggest that the larvae might have been forced through the mesh due to the current at her study site. The smaller mesh size of 125µm used in my study could account for the additional larvae settled on my Nitex tents, especially because there was a slight current detected by divers during outplanting. Currents likely pushed larvae to the sides of each module instead of allowing them to settle down as they did in the lab after being exposed to GABA. Nitex tents could have also trapped dead larvae against the sides, which were then brought to the lab and counted as settled individuals. The laboratory modules seeded using the same procedures as field modules had significantly fewer settled abalone found on tenting material (~80 individuals) further suggesting that minimizing currents during outplanting is important for successful outplanting of larvae.

Another important factor to consider when outplanting is how to promote settlement of larvae. In one early experiment in New Zealand, researchers extended the duration of the larval phase by four days, then released *H. iris* larvae in a sheltered bay with abundant crustose coralline algae (a preferred food of juveniles) in an attempt to increase recruitment. They found juvenile densities were 1.5 - 8 times higher than background settlement densities after three months, with 12 - 68 individuals m<sup>-2</sup> (Tong et al. 1987). PSRF typically rears abalone larvae for seven days before settling them in hatchery tanks with the assistance of GABA. My study extended the larval phase to eight days however due to an almost 2°C decrease in water temperature during rearing at the Shannon Point Marine Center and low settlement success following GABA treatment on day seven. GABA is commonly used in hatchery settings as a potent inducer of larval settlement, a process that begins with the search for a suitable substratum and ends with metamorphosis (Stewart et al. 2008). Synthesized GABA mimics the naturally produced response when the foot of the abalone interacts with crustose coralline algae, indicating a suitable habitat for settlement. Exposure time and concentration of GABA used to induce settlement is debated among abalone hatcheries, with some choosing low concentrations (1µM) over extended time frames of up to 96 hours (Yu et al. 2010) and others using high concentrations (10 µM) for as little as 30 minutes (PSRF, unpublished data). Based on our understanding of how pinto abalone react to GABA and the short time frame in which we needed them to settle, I choose to use a midlevel concentration of 5 µM for thirty minutes prior to outplanting. Concern over latent effects in prolonging metamorphosis from the larval stage and exposing larvae to high levels of GABA (Roberts and Lapworth 2000) requires long-term monitoring of larval outplants yet to be tested in the San Juan archipelago.

Hatcheries and restoration groups are also trying to maximize survival of outplanted larvae by optimizing the seeding density. In Australia, a study testing different release densities found a 4.5% increase in recruitment compared to control plots when *H. rubra* larvae were released at densities of 16,000 individuals m<sup>-2</sup>, yielding about 40 individuals m<sup>-2</sup> 19 days after outplanting (Preece et al. 1997). These researchers also released larval *H. laevis* into tented plots at densities of 2,000 and 120,000 individuals m<sup>-2</sup> and found that the higher density yielded the most juvenile abalone (6.5 ± 1.2 individuals m<sup>-2</sup> after 49 days). Preece et al. (1997) suggested that, given the likely density-dependent mortality of newly settled abalone, release of larvae at lower densities over larger plots is likely the optimal seeding method for *H. rubra* and *H. laevis*. Hansen (2006) tested outplant densities of 50,000 and 100,000 individuals m<sup>-2</sup> and found the lower density was most effective, yielding ~3.5 individuals m<sup>-2</sup> 13 months after outplanting. I used a density of about 13,000 individuals m<sup>-2</sup> (the surface area of each LAM was approximately 0.5 m<sup>2</sup> with all sides of the LAM and the settlement plates). On average, open and tented LAMs had 7.5 and 25 abalone respectively, or 15 and 50 individuals m<sup>-2</sup> after four months. Thus, outplanting success in my study was comparable to that of other studies on larvae. In addition, given the results of my study and of Hansen's (2006) work on pinto abalone, it is possible that seeding densities between 13,000 and 50,000 could further optimize recruitment and this should be tested before full-scale implementation occurs.

Previous studies that have successfully implemented larval outplanting have also compared recruitment rates of outplants to natural settlement found within sites. I found no natural recruitment within any unseeded open LAMs used as controls, suggesting that no adult reproductive abalone were present at my two sites. This is further evidence of the need for outplanting pinto abalone if wild stocks have any chance of recovering.

One metric yet to be monitored and accounted for during previous larval outplanting studies is the possibility of emigrated individuals surviving and reproducing in the wild. Laboratory LAMs in my study were seeded using the same methods as field LAMs and monitored to better understand survival and emigration of outplanting larvae with my methods. In restoration projects, emigration is often ignored when modeling survival because the goal of outplanting is to create dense aggregations of adults (Rossetto et al. 2013). Emigration can be expected with all abalone outplanting. However, due to the challenging nature of measuring it accurately, it is often incorporated into mortality estimates (Carson et al. 2018). Although emigrated individuals could potentially contribute to future population growth, researchers have considered it unlikely that emigrated individuals would find reproductive partners unless they return to the experimental area due to the density dependent nature of abalone reproduction (Carson et al. 2018). In my study, emigration in laboratory LAMs was measured as the number of individuals found on the walls of the flow through tanks holding the LAMs but not still settled on or within the LAM in that tank. Interestingly, these emigrated individuals were often found clustered together, suggesting that they may have a greater chance at reproductive success than originally thought. Thus, emigration may need to be considered when modeling outplant success since these individuals may not only survive but may form clusters capable of reproducing.

Pinto abalone restoration in the San Juan archipelago is currently transitioning from a pilot phase focused on data collection towards an active restoration phase focused on production. Between 2011 and 2016, approximately 11,000 juvenile abalone were released over ten sites in the San Juan archipelago and surveyed in 2017 for survival. The best site had 65 individuals present and the worst had none (Carson et al. 2018). Adding up all ten sites, 243 outplanted abalone were located during the 2017 surveys. This equates to approximately 2% survival success,

although this estimate is likely low due to emigration and cryptic individuals not spotted during the surveys. In comparison, I found an average of 0.4% survival in my tented LAMs four months after outplanting. However, mortality rates within the hatchery leading up to juvenile outplanting must be considered to better compare the effectiveness of the two methods. If less than 20% of abalone survive from settlement to time of outplanting in the hatchery, then survival percentages would be roughly even for larval and juvenile outplants. These survival estimates, along with the added cost of rearing juveniles until outplanting, are all factors that need to be considered by hatchery managers. This comparison however implies that individuals in both my study and previous PSRF juvenile outplants will survive to reproduce, and given the shorter time frame of my larval outplant study this assumption is likely not accurate. It does though give hatchery managers a framework to assess if larval outplanting should be implemented on a larger scale in the San Juan archipelago.

Another factor for restoration groups to consider when deciding between larval and juvenile outplanting is the potential latent effects from rearing abalone for 12 – 18 months in the hatchery (Pechenik 2006). One metric for understanding if the high densities of abalone settled in hatchery tanks are causing competition is shell length and differences in growth rate. PSRF hatchery personnel have observed that some cohorts of abalone grow at slower rates than anticipated, delaying juvenile outplanting (Bouma, personal communication 2018). In my study, I measured shell length of hatchery-produced abalone four months after outplanting and compared their growth to individuals of the same families reared in the hatchery. This allowed me to better understand if rearing abalone in the PSRF hatchery slowed their growth rates during this critical timeframe. I found no significant difference in shell length between hatchery and outplanted individuals.

Apart from the high costs of producing and rearing juveniles for outplanting, there is concern that hatchery reared abalone behave differently than wild animals (Schiel and Welden 1987; Olla et al. 1998), which could explain the high mortality levels recorded on outplanted juveniles (Tegner and Butler 1985). Lack of predator exposure and habituation to humans during early life stages has been found to change behaviors such as response to nearby movement, direct contact, and predator cues, which might in turn cause increased mortality of hatchery-reared populations (Hansen & Gosselin, 2016). If this trend is true for pinto abalone, then outplanting as larvae may ameliorate unwanted hatchery learned behaviors and better prepare abalone for life in the wild. More research needs to be done in order to better understand if behavioral differences exist between hatchery-reared juveniles and juveniles that were outplanted as larvae.

Outplanting larval *H. kamtschatkana* could provide significant advancement for abalone restoration in the San Juan archipelago. The benefits of outplanting larvae instead of juveniles include cost savings for the hatchery and potentially better survival due to behavioral differences between juveniles outplanted as larvae and juveniles reared in the hatchery. Tenting appears to be necessary to increase successful settlement of larval pinto abalone and the use of GABA to induce settlement, while not directly tested in my study, also likely increases settlement success. Considering the relatively small number of larvae outplanted in each LAM in my study, I would recommend experimenting with different outplanting densities to determine if higher densities result in better outplant success. Long-term monitoring at sites where outplanting larvae has occurred will also be necessary to determine if animals of reproductive age have been added to wild abalone populations.

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## Appendix 1

### PINTO ABALONE (*HALIOTIS KAMTSCHATKANA*) AQUACULTURE METHODS SPAWNING PROTOCOL

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As commonly used in abalone aquaculture, the hydrogen peroxide protocol developed by Morse et al. (1977) is employed to induce spawning activity in Pinto abalone. Several adaptations to the protocol have increased spawning success including allowing the abalone to feed on fresh macroalgae (primarily *Nereocystis leutkeana* and *Palmaria mollis*) throughout the year, applying mild heat shock by spawning the animals at a temperature that is 3 °C warmer than the holding tanks and also leaving the abalone in the hydrogen peroxide solution for slightly longer than the recommended 3 hrs. Induced spawns are coordinated with either full moon or new moon lunar cycles. Before selection for spawning, ripeness of broodstock abalone was determined using a gonad index of 0-3:

0 = no development of gonad, sex indeterminate.

1 = gonad beginning to show signs of swelling and gamete production, male gonad beige to orange in color, female gonad green to purple in color.

2 = gonad swelling up to level of shell margin, partially mature, may spawn.

3 = gonad swelling above level of shell margin, fully mature and spawnable.

The following section gives a detailed description of our spawning protocol:

1. All seawater used during spawning should be UV-irradiated and filtered to 5 µm.
2. Prepare buffer for raising pH of seawater to 9.1. Wearing gloves, add 24.2 g of Tris buffer to 75 mL of distilled water to make a 2 M solution. After the Tris has dissolved, add water to a final volume of 100 mL. A typical spawning event requires 8 spawning buckets each filled with 7 liters of filtered seawater. This will require 370 mL of 2 M Tris solution. To make 375 mL of 2 M Tris, add 90.75 g of Tris to 281 mL of distilled water. After Tris has completely dissolved, add distilled water to achieve a final volume of 375 mL.
3. Prepare hydrogen peroxide solution. Wearing gloves, add 20 mL of 30% H<sub>2</sub>O<sub>2</sub> to 80 mL of distilled water to make a 6% H<sub>2</sub>O<sub>2</sub> working solution. Use H<sub>2</sub>O<sub>2</sub> stock opened < 1 month from a batch < 1 year old. A typical spawning event requires 8 spawning buckets each filled with 7 liters of filtered seawater. This will require 224 mL of 6% H<sub>2</sub>O<sub>2</sub>. To make 250 mL of 6% H<sub>2</sub>O<sub>2</sub>, add 50 mL of 30% H<sub>2</sub>O<sub>2</sub> to 200 mL of distilled water.
4. Use a spatula or thin sheet of rigid plastic to remove abalone from holding tanks without causing injury or excessive stress. Select animals with a gonad index of 3 for spawning, animals with a gonad index of 2 may be spawned if necessary. Ideally, more females should be selected than males (e.g. 3 males and 5 females).
5. Measure shell length, weight and record tag number of each animal.

6. Place ripe abalone in spawning buckets filled with a known volume of filtered 14 °C (or the temperature of broodstock holding tanks) seawater and place buckets in a 17 °C (or 3 °C warmer than broodstock holding tanks) water bath so that the temperature in the buckets acclimates slowly to spawning temperature. Add mild aeration to each bucket. Let the animals acclimate to the buckets for 10 minutes.
7. Initiate spawning attempt with the females 15 minutes prior to the males. Add 6.6 mL of the 2 M Tris solution per L of seawater (e.g. 46.2 mL of 2 M Tris per 7 L spawning bucket). Mix well and wait 15 minutes.
8. Add 4 mL of the 6% H<sub>2</sub>O<sub>2</sub> solution per L of seawater (e.g. 28 mL 6% H<sub>2</sub>O<sub>2</sub> per 7 L spawning bucket) and mix well.
9. Turn all lights off or cover tanks and keep noise levels low.
10. Leave abalone in solution for up to 3 hrs (or a few minutes longer if necessary). If any abalone begin spawning while still in the solution, remove immediately, rinse well and place in bucket of fresh seawater.
11. After 3 hrs, drain water from spawning buckets, thoroughly rinse both the abalone and the bucket to remove all peroxide and refill with fresh aerated seawater.
12. Cover buckets or keep lights turned off.
13. Begin observations for spawning behaviors every 20 minutes. Abalone should begin spawning within 2.5 hrs of removal from the peroxide solution. Usually, gamete release will begin shortly after removal from the peroxide solution. Behaviors that could indicate impending spawning include climbing to the top of the bucket (possibly above water level), raising and forceful lowering of the shell, splayed extension of all epipodial tentacles and general activity.
14. Gametes liberated from one sex can potentially trigger spawning in the opposite sex. Therefore, several mL of sperm can be added to the female spawning buckets to stimulate egg release and vice versa. For single parent crosses, this method should be avoided or minimized. If used, rinse abalone that spawn thoroughly with fresh seawater to remove foreign gametes from that individual.
15. Additional temperature stress can also induce spawning if abalone will not release gametes. Partially empty spawning bucket, refill with fresh seawater that is 3 °C colder, wait 15 minutes and observe. Repeat with fresh seawater that is 3 °C warmer if necessary.
16. When abalone begin to release gametes, remove aeration from bucket.
17. Always be very cautious to avoid cross-contamination of gametes between spawning buckets!
18. Allow male to release sperm for at least one hour. When sperm concentration in spawning bucket appears high, use a plunger to mix and suspend sperm in bucket, then use a tri-pour beaker to remove a 1 L sample from bucket.
19. Immobilize and count a sub-sample of sperm from the 1 L beaker: Using a cryovial or centrifuge tube, combine 950 µL of sperm with 50 µL of Lugol's stain and mix. Add 10 µL of sperm/Lugol's to each side of a hemacytometer and observe under microscope. Count the total number of sperm in five of the grid squares (4 corners and center), repeat this count for the other side of the hemacytometer and take the average of the two. Use the following formula to calculate sperm density in your sample: # sperm counted  $\times 5 \times 10^4 / 0.95 = \# \text{ sperm/mL}$ .

20. Cover the tri-pour beaker sperm sample, label beaker with sperm density and from which male the sample was obtained. If the sample will not be used immediately, place in 4° C refrigerator until eggs are available. Sperm will remain viable for up to 12 hours or longer if refrigerated.
21. Allow female to release eggs for 1.5 hrs. Siphon eggs (which will be negatively buoyant) out of the spawning bucket into a clean bucket through a submerged 300 µm filter screen to remove mucus and debris and onto a 75 µm filter screen to retain eggs. Gently rinse eggs from 75 µm screen into a clean bucket using a squirt bottle. Label bucket with tag ID from female. A sample of eggs can be observed under a microscope to ensure they are perfectly spherical and healthy.
22. Estimate number of eggs from female: Eggs should be held in blue bucket which has volume demarcations labeled on the inside. With the eggs held in a known volume, use a clean plunger to gently distribute eggs throughout bucket. Use a P1000 pipette to take 1 mL samples (5 total) from the evenly distributed bucket. Place samples in tissue culture plate wells and observe/count under microscope. Take the average of the five egg counts and multiply by total volume to acquire estimate of total number of eggs.
23. Eggs should be fertilized within two hours of release from female. After two hours, egg membranes harden and viability decreases significantly.
24. Based on available gametes, carefully determine which crosses are to be made, adhering to a strategy of either single-parent crossing or maximizing a partial factorial cross matrix.
25. Eggs should be fertilized at a ratio of 500 sperm/egg. Using egg count estimate and sperm density estimate, calculate the volume of sperm to be used from the 1 L beaker set aside earlier. Add sperm to egg bucket, plunge gently and allow 5 minutes for fertilization.
26. Thoroughly rinse fertilized eggs on a submerged 75 µm filter screen to wash away excess sperm, then rinse fertilized eggs retained on screen into hatching trays filled with UV-irradiated, 5 µm filtered 14 °C seawater. Eggs should settle on the bottom of the hatching tray in a single layer, densities higher than this will cause anaerobic conditions and high mortality.
27. One hr after fertilization, observe a sub-sample of fertilized eggs under a microscope to ensure that polar bodies are present. If polar bodies are not observed it may be possible to re-attempt fertilization by adding more sperm.
28. Two hrs after fertilization, first cell divisions should be evident. At this point, subsamples can be taken from the hatching tray and fertilization success can be estimated by counting unfertilized eggs and embryos in each sample.
29. Eggs will hatch between 12-36 hrs post-fertilization.