The Effect of Temperature on Larval Survival and Development of The Giant California Sea Cucumber *Parastichopus californicus* in a Hatchery Setting

by

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ABSTRACT

THE EFFECT OF TEMPERATURE ON LARVAL SURVIVAL AND DEVELOPMENT OF PARASTICHOPUS CALIFORNICUS IN A HATCHERY SETTING

Kendra Baird

The current global demand for sea cucumber products cannot be met through commercial harvesting alone, which has led to a recent increased interest in developing aquaculture techniques for sea cucumbers. Currently, there is no commercial-scale aquaculture of the Giant California sea cucumber *Parastichopus californicus,* and development is in the early research stage. The goal of this experiment was to determine the optimal temperature range *P. californicus* larvae should be reared at in hatcheries. The experiment was conducted at Puget Sound Restoration Fund's Kenneth K. Chew Center for Shellfish Research and Restoration hatchery located at NOAA's Manchester Research Station in Manchester, WA. Larvae were reared in five temperature treatments (12°, 15°, 18°, 21°, and 24°C) for 24 days, and survival and developmental stages were measured and scored, respectively. Larvae developed quicker with an increase in temperature, but survival was lower with the temperature increase. Survival was best at 12°C, with a mean survival of 14%, but took the longest to develop. The 24°C treatment had negligible survival, but almost all larvae counted were at the final pentactula phase on day 24. Of the temperatures tested for rearing *P. californicus* in a hatchery setting, 12°C is recommended for obtaining the highest survival for maximum production. Understanding proper larval conditions for maximizing larval growth and survival will further the advancement of aquaculture of *P. californicus.*

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INTRODUCTION

There has been an increase in demand for sea cucumber products in recent years, primarily from Asia. The current global demand cannot be met through commercial harvest alone, due to limited fishing opportunities over concerns for the sustainability of the fishery. The sustainability of sea cucumber fisheries is a major concern because sea cucumbers are especially vulnerable to overfishing because they can be easily and effectively targeted in shallow waters, display a slow growth rate, late age of maturity, and a low recruitment rate, which results in a slow population replenishment (Bruckner, 2005; Bruckner et al., 2003; Uthicke and Benzie, 2000; Uthicke et al., 2004). Many countries such as Japan and New Zealand have been able to recover populations by limiting fishing opportunities. However, the sustainability of the local sea cucumber *Parastichopus californicus* fishery in Washington State is a major concern because recent surveys have found that in areas of Puget Sound 90% of the historic populations have been harvested out. This has resulted in a decrease in fishing opportunities through emergency closures and decreased quotas set and enforced by Washington Department of Fish and Wildlife (WDFW).

P. californicus is the largest of the 12 species of sea cucumbers found in Puget Sound, with an average length of 30 cm, but can reach up to 50 cm (Clark, 1922). Recent studies indicate that the feeding behavior of sea cucumbers plays an important role in their local ecosystems (Uthicke 2001a). They process large volumes of benthic sediments from which they assimilate bacterial, fungal, and detrital organic matter. Their feeding results in the horizontal redistribution and bioturbation of sediments, and the recycling of nutrients (Crozier, 1918; Haukson, 1979; Lawrence, 1982; Moriarty, 1982; Slater and Carton, 2009; Uthicke, 1999, 2001b). As sea cucumbers feed, they turn over the top layers of sediment and transform sediments into finer particles, inhibiting the buildup of detritus material that contributes to anoxic conditions (CITES, 2002; Michio et. al, 2003). These findings suggest that the loss of *P. californicus* have a large impact on the nutrient cycling in Puget Sound.

P. californicus currently supports a valuable commercial fishery in both Canada and the United States (Bruckner, 2006; Department of Fisheries and Oceans Canada statistics). Currently, the United States sea cucumber fishery is managed by individual states within state waters, and by NOAA Fisheries in coordination with Regional Fishery Management Council in waters 200 miles off the coast (Bruckner, 2006). The Washington State fishery is a co-operative management agreement between WDFW and treaty tribes, with quotas being split about half and half. Washington management measures for sea cucumbers include seasonal closure from April-June during spawning season, spatial closures, license of collectors, and annual quotas set for each management zone (Bruckner, 2005).

Managing the fishery is difficult because sea cucumbers are difficult to size, sex, age, and tag. Enforcing fishery management techniques such as minimum and maximum sizes and the harvest of only males would be difficult due to their plasticity, seasonal weight variance, and lack of external evidence of dimorphism (Carson et al., 2016). Past attempts to tag sea cucumbers have been unsuccessful, which makes it difficult to obtain growth, mortality, and longevity measurements (Carson et al., 2016). Currently, the Washington Department of Fish and Wildlife assesses populations through abundance, harvest weight, and catch-per-unit effort (CPUE) measurements, obtained by video and dive surveys, and harvest logbooks (Carson et al., 2016).

The development of aquaculture for *P. californicus* would relieve pressures on wild stocks, provide additional income and products for shellfish growers, provide more information on the biology of the species, and allow for restoration projects in areas where the wild populations have significantly declined. While the demand and market for sea cucumbers exists, there is still little information regarding broodstock conditioning, larval and juvenile production, and grow out, impairing the development of hatchery methods (Zamora and Jeffs, 2013). Research on *P. californicus* has primarily focused on stock assessment, fisheries management, and the use as a deposit feeder in Integrated Multi-Trophic Aquaculture (IMTA) to reduce organic accumulation.

Commercial aquaculture has only been developed for a few species of tropical sea cucumbers globally (Duy, 2012; Gamboa et al., 2012; Huiling et al., 2004; Renbo and Yuan, 2004; Xiyin et al., 2004). Currently, there is no commercial level production of *P. californicus*, but the Alutiiq Pride Shellfish Hatchery in Alaska has started to develop spawning and larval grow-out techniques. They have made advancements in overcoming transport stress of adults during collection, holding and conditioning of broodstock, spawning, larval culture, settlement, and nursery culturing (Development of red sea cucumber (*Parastichopus californicus*) poly-aquaculture for nutrient uptake and seafood export, unpublished). The non-profit organization Puget Sound Restoration Fund (PSRF) has started to develop aquaculture techniques for sea cucumbers, with a focus on continuing to advance the develop of methods for broodstock maintenance, spawning methods, larval culture, and nursery culturing for grow out.

One of the most difficult early life stages to grow in hatcheries is the larval phase. Early life stages are the most sensitive to environmental conditions, and therefore, it is extremely important to know the optimal ranges of physical stressors such as salinity and temperature, as well as, nutritional requirements and the ideal diets. Larvae experience the most damage from temperature extremes (Strathmann and McEuen, 1987). Most species can tolerate a relatively wide ambient temperature range within their natural habitat, although optimal performance is typically restricted to a specific thermal range (Meng et al., 2009; Pawson, 1966). The range varies between species and life stages, but typically broadens with development (Costlow et al., 1960; Crisp and Ritz,

1967). Studies have shown that echinoderm larvae display a high sensitivity to temperature, which results in increased mortality, delayed development, reduced growth, and reduced metabolic rate (Bressen et al., 1995; Przeslawski 2005; Roller and Stickle, 1989, 1993). This is hypothesized to be due to multiple factors including, but not limited to; oxidative stress, lysosomal destablilisation, increased lipid peroxidation, a change in immune ability, and the decrease in concentrations of soluble proteins, soluble sugars and ions (Chang et al., 2006; Deschaseaux et al., 2010, 2011; Liu et al., 2013; Wang et al., 2012).

Studies have examined the effect of temperature on the survival and growth of other tropical species of sea cucumbers. *Apostichopus japonicas* and *Holothuria spinifera* larvae rapidly develop at higher temperatures and slower with delayed metamorphosis at lower temperatures (Asha and Muthiah, 2005; Li Li et al., 2011). Hamel and Mercier (1996) discovered lower temperatures increased the duration between developmental stages, but did not increase mortality at each developmental stage for the sea cucumber *Cucumania Grandosa*. Liu et al. (2010) observed that *A. japonicas* larvae grew faster with increasing temperatures within a suitable range, but extreme temperatures restrained development. High temperatures resulted in weak-digestion, decreased-absorbing of nutrients, increased oxygen consumption rate and ammonia excretion rate (Villarreal and Ocampo, 1993).

In order to grow *P. californicus* at a large-scale production level in hatcheries, it is imperative to know the effects of temperature on survival, growth, and development in order to determine the ideal temperature range that larvae should be reared in. The objective of this study is to determine the optimal thermal range that *P. californicus* larvae should be reared at in hatcheries. The application of this research will further the advancement of the development of aquaculture techniques for *P. californicus* and provide important information to Puget Sound Restoration Fund (PSRF) in order to grow *P. californicus* in their hatchery for future restoration efforts throughout Puget Sound.

LITERATURE REVIEW

For my thesis I researched the effect of temperature on *P. californicus* larval survival and development in order to determine the thermal range that larvae should be reared in for optimal growth and survival in hatcheries. There has been an increased interest in developing aquaculture techniques for *P. californicus* in recent years due to the increased demand for sea cucumber products. Wild populations in Washington State have dramatically declined due to over harvesting, and as a result, fishing opportunities have been limited, creating a further demand to develop the methods and techniques for farming. Techniques have been developed for many tropical species globally, but there is little information pertaining to farming *P. californicus*. My thesis was completed in partnership with Puget Sound Restoration Fund (PSRF). PSRF will use the findings from

this study to develop aquaculture techniques for rearing larvae and juveniles at their Kenneth K. Chew Center for Shellfish Research and Restoration hatchery in Manchester, WA, in order to out plant juveniles throughout Puget Sound for restoration work at areas where populations have experienced significant declines.

The literature review will provide information on the natural history, reproduction, and larval development of *P. californicus* to understand the biology and ecology of the species. Next, the history of the global and local fisheries will be examined to understand the changes that have occurred over time to the wild populations and the relationship to the market and market potential in Washington State. The aquaculture techniques that have been established for related tropical species and the recent advancements in aquaculture of *P. californicus* will be briefly discussed in order to understand the current state of development and discuss where future research efforts should be focused. Finally, the effect of temperature on echinoderm and sea cucumber larvae will be discussed in order to understand the importance of this physical variable for the production success of hatcheries.

Natural History

Ecology

At least 12 species of sea cucumbers are native to Puget Sound (Strathmann and McEuen, 1987). *P. californicus* is the largest species with an average length of 30 cm, but can reach up to 50 cm (Clark, 1922). They are typically reddish-brown in color but sometimes appear pinto or albino (Figure 1) (Cameron, 1985; Strathmann and McEuen, 1987). They are found off the west coast of Northern America and range from Baja, CA to British Columbia (Cameron, 1985). This particular species is found in Rocky low intertidal and subtidal zones up to 249 meters deep, and prefer areas away from strong wave action such as quiet bays (Cameron, 1985; Ricketts and Calvin, 1968). They can migrate randomly, covering 3.9-100 meters in a day (Cameron and Fankboner, 1989; Muse, 1998). They are epibenthic and prefer sand and shell substrate, kelp beds, and rocks (Fankboner and Cameron,1985; Feder et al., 1974; Woodbey et al., 2000). Population densities may reach 0.5 individuals per m^2 in the wild (Sloan, 1985).

Figure 1. Photograph of *Parastichopus californicus.* Photo Credit: Kendra Baird.

Like other sea cucumbers, *P. californicus* eviscerate their gut, respiratory tree, circulatory system, and gonads when they are subjected to predation or physical stress as a defense mechanism (Garcia-Arraras and Greenberg, 2001). It takes only a few weeks to regenerate the lost organs, but it can take 1-3 months to regenerate sufficiently to be fully functional (Cameron, 1985; Swan, 1961). *P. californicus* atrophy their internal organs on a seasonal basis in the fall, as they enter a state of winter dormancy, and regenerate organs over a period of several weeks in the spring (Fankboner 2002; Fankboner and Cameron, 1985; Swan, 1961).

Recent studies are indicating that the feeding behavior of sea cucumbers plays an important role in their local ecosystems (Uthicke 2001a). *P. californicus* are deposit feeders and process large volumes of benthic sediments from which they assimilate bacterial, fungal, and detrital organic matter. Their feeding results in the horizontal redistribution and bioturbation of sediments, and the recycling of nutrients (Crozier, 1918; Haukson, 1979; Lawrence, 1982; Moriarty, 1982; Slater and Carton, 2009; Uthicke, 1999, 2001b). Methews et al. (1990) suggests that sea cucumbers turn over the top few millimeters of sediment for over 90% of the sea floor. Studies further suggest that sea cucumbers may affect the physical nature of the sea floor. Eradication of sea cucumbers in localized areas resulted in the hardening of the sea floor, which subsequently eliminated habitat for benthic and infaunal organisms (CITES, 2002).

As sea cucumbers feed they turn over the top layers of sediment and transform sediments into finer particles, inhibiting the buildup of detritus material that contributes to anoxic conditions (CITES, 2002; Michio et. al, 2003). Studies in tropical coral reef ecosystems have shown that sea cucumbers play an important role in the nutrient cycling of nitrogen and phosphorus. The sediments are typically high in nitrogen and phosphorus, but studies found that the presence of sea cucumbers resulted in the translocation of

nitrogen and phosphorus into the water column, where they become available to other organisms to utilize (Uthicke, 2001a,b).

Natural predation is not limiting on sea cucumber populations, but known predators include the sea stars *Solaster dawsonii* and *pycnopodia helianthoides*, fish such as kelp bass *Paralabrax clathratus* and California sheephead *Semicossyphus pulcher*, and crabs (Fankboner and Cameron, 1985; Quast 1968; Sewell, 1990; Washington Department of Fisheries, 1976; Woods, 1993). Sea cucumbers also serve as a host for many parasitic and symbiotic organisms. The scale worm *Arctonoe pulchra* is commonly found living on the ventral side within the tube feet, the flatworm *Anoplodium hymanae* lives in the body cavity, the flatworm *Wahlia pulchella* lives in the upper intestine, the gastropod *Enteroxenos parastichopoli* attaches to the intestine, and the snail *vitriolina Columbiana* attaches to the outside skin. (Cameron, 1985; Fankboner and Cameron, 1985; Kozloff and Shinn, 1987; Shinn, 1983, 1984).

Spawning

P. californicus reach sexual maturity at four years of age (Cameron and Fankboner, 1989). Sexes are separate with no external evidence of sexual dimorphism, and occur at a 1:1 ratio in the wild (Cameron, 1985). Individuals spawn repeatedly during the season (Washington Department of Fisheries, 1976). Adults may migrate to shallower water for spawning, with spawning frequently observed 5-12 meters below the surface by divers (Cameron 1985; Courtney, 1927). Spawning events may correlate with bright sunshine days and high phytoplankton blooms (Cameron, 1985; Cameron and Fankboner, 1986; Muse, 1998).

The spawning season in the wild has been observed to extend from late April to August, with the peak of the season occurring May to mid July in the San Juan Islands (Cameron, 1985; Courtney,1927; Bovard and Osterud, 1918**;** Johnson and Johnson, 1950; MacGinitie and MacGinitie 1949; McEuen, 1986; Mortensen, 1921; Smiley 1984, 1986a, 1986). Spawning has been observed in August in Newport Bay, CA and June through August in British Columbia (Cameron and Fankboner, 1986).

During spawning adults assume a distinctive posture as they release the gametes into the water column. One third to one half of the anterior end is raised vertically from the substrate with the “head” curved forward to the substrate (Figure 2). The gonophore opens at the point of maximum elevation above the substratum on the anterior dorsal surface of the animal (Cameron, 1985). Gametes of each sex are easily distinguishable from one another in situ. Sperm is cream colored and occurs in a thick cloud. Oocytes are orange and disperse immediately in water, which can make it difficult to notice when a female has begun to spawn (Cameron, 1985). Females have a significantly larger gonad index (the calculation of the gonad mass as a proportion of the total body mass) at

maturity than males, which indicates that females have a much greater reproductive effort than males (Cameron, 1985).

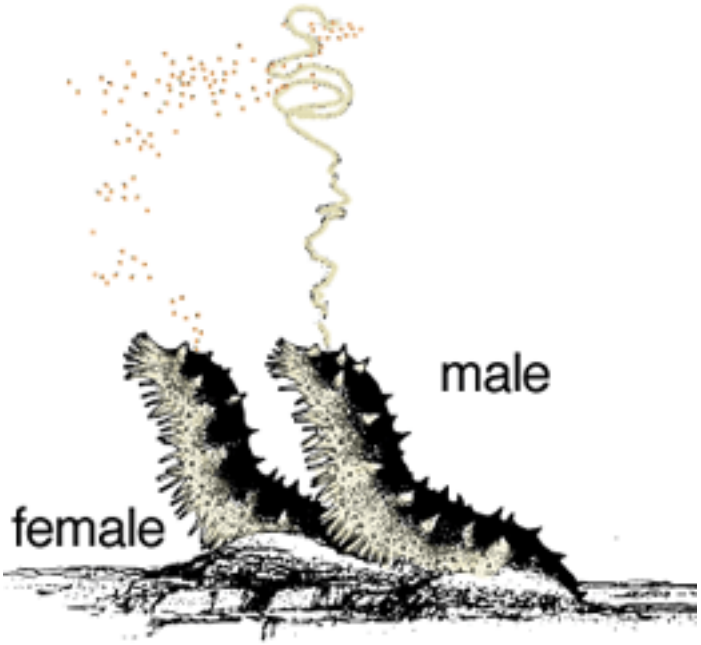

Figure 2. Illustration of a male and female *P.* californicus releasing gametes during spawning. Credit: Cameron, 1985.

Eggs

Large females can have fecundities up to 8.92×10^6 (Strathmann and McEuen, 1987). Oocytes are 185-210 μm in diameter and surrounded by a jelly coat about 30 μm thick (Cameron, 1985; Strathmann and McEuen, 1987) (Figure 3). The oocytes possess a distinct germinal vesicle and nucleolus (Cameron, 1985). Oocytes are negatively buoyant and will sink into the water column or to the bottom of tanks; therefore must be syphoned out of spawning tanks during spawning events in hatcheries (Cameron, 1985). Oocytes collected from excised ovaries are enclosed within an ovarian capsule. Once they are exposed to seawater the oocytes exit the capsule, becoming distorted during the process (Cameron, 1985). Strip spawning is not effective with *P. californicus* because only less than one percent of oocytes undergo germinal vesicle breakdown within the ovarian tubules on their own, indicating they need external triggers to begin meiosis (Cameron, 1985).

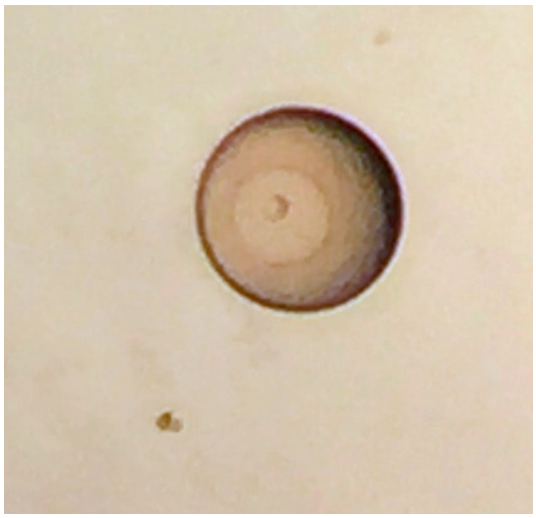

Figure 3. Photograph of an oocyte collected in the lab taken with an Iphone and a compound scope. Credit: Kendra Baird.

Larval Phases

Most benthic marine invertebrates have a pelagic planktonic larval phase characterized by high dispersal and high mortality (Chen, 2003; Miller, 1995; Rumrill, 1990; Smiley, 1986b). This period may extend from three weeks to several months for *P. californicus*, and larvae can develop asynchronously (Cameron, 1985; Miller, 1995; Strathmann and McEuen, 1987). The development schedule for *P. californicus* at 11°C is reported by Strathmann and McEuen (1987) and is included in Table 1. The development rate of larvae is strongly influenced by temperature and food supply, therefore, it is expected to vary in different conditions (Miller, 2001). The advancement of aquaculture and hatchery methods for this species will be dependent upon determining these factors, along with other physical and biological parameters such as salinity and stocking density. Planktrophic larvae feed primarily algal cells through the capture of particles, and through absorption of dissolved organic molecules. Food is processed in the gut and fecal matter is expelled through a ventral anus. Because the development of *P. californicus* requires feeding on external matter, eggs are smaller and contain little lipid reserves compared to other species (Miller, 2001).

2 cell	32 cell	64 cell	Blastula	Gastrula	Early larvae	Early doliolaria	Settlement	Armored
2.75h	10.75h	12 h	19h	27 h (290 um)	3.25-4.25d (1120 um)	52 d (410-550 um)	60-61d (245-365 um)	61-65 d (299 um)

Table 1. Developmental schedule for *P. californicus* larvae at 11±0.5°C reported by Strathmann and McEuen (1987). Measurements in parenthesis indicate the size.

The first larval phase of *P. californicus* is auricularia (Figure 4, Table 1). This phase typically lasts 35-52 days. Individuals range from 425-1,120 µm in length during this phase (Strathmann and McEuen, 1987). Early auricularia develop a looped band of cilia used for swimming and feeding. Further development produces arms and lobes over which the single, continuous band is lopped. The width of the band varies and there are 3-7 cilia across the band, one cilium per cell. Encircling the mouth is an aboral band of cilia that is continuous with a ciliary band at the top of the esophagus (Strathmann, 1971, 1974, 1975). Calcareous ossicles are present in the posterolateral lobes. The larva is typically transparent with occasional tinting of the ciliary band (Strathmann and McEuen, 1987).

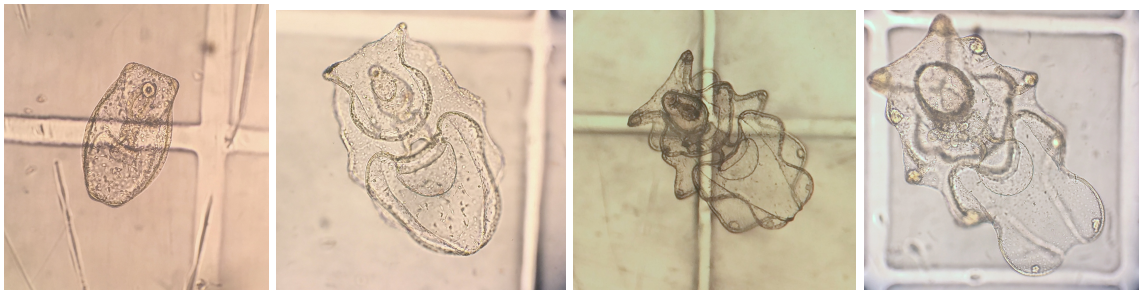

Figure 4. Photographs of early auricularia developing into late auricularia. Photos were taken with an Iphone and a compound scope. Photo Credit: Kendra Baird.

As auricularia begin to metamorphose into simplified doliolaria, the single ciliary band rearranges into five transverse rings of cilia (Strathmann and McEuen, 1987). Further reduction results in the compact barrel-shaped doliolaria (Figure 5). The metamorphosis to doliolaria results in a 90% decrease in body volume, and individuals range from 410-550 µm in length (Strathmann and McEuen, 1987). During metamorphosis the mouth withdrawals to form the atrium and the larvae do not feed during this stage. This is a very brief larval phase that only lasts 24-48 hours (Cameron, 1985; Strathmann and McEuen, 1987).

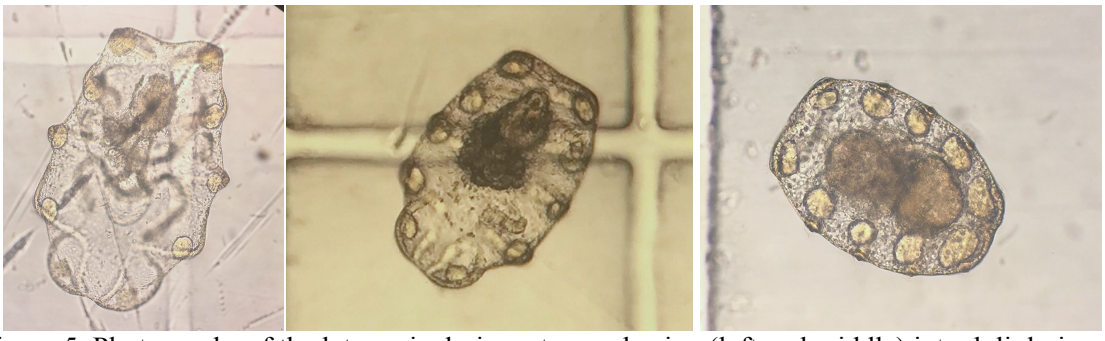

Figure 5. Photographs of the late auricularia metamorphosing (left and middle) into doliolaria (right). Photos were taken with an Iphone and a compound scope. Photo Credit: Kendra Baird.

The final larval stage is the pentactula phase, which is marked by the emergence of a single tube foot at the posterior end (Figure 6). The period to settlement followed by

completion of metamorphosis may be as long as 131 days (McEuen, 1986). Individuals range from 245-1,120 μm in length during this phase (Strathmann and McEuen, 1987). Ciliated bands persist for a short while, and larvae at this stage may be found swimming or crawling (Cameron, 1985). Calcareous ossicles begin to form and cover the larval surface at metamorphosis (Miller, 2001). 3-5 days after metamorphosis, button ossicles form and the organisms take on a spiny appearance. During or just after settlement, the pentactulae grow a single ventroposterior podium (Strathmann and McEuen, 1987). They use the knobbed "sticky" tip of the podium to attach to substratum at settlement (Strathmann and McEuen, 1987). McEugene (1986) suggests that settling larvae probably attach to undersides of rock in calm coves, bays, and fjords, since that is where juveniles 1-11cm long have been observed. In southern British Columbia, juveniles are regularly observed in dense mats of filamentous red algae, on polychaete tubes, and in crevices on rock walls (Cameron, 1985). Settled Juveniles will continue to grow into adults and sexually mature at four years old.

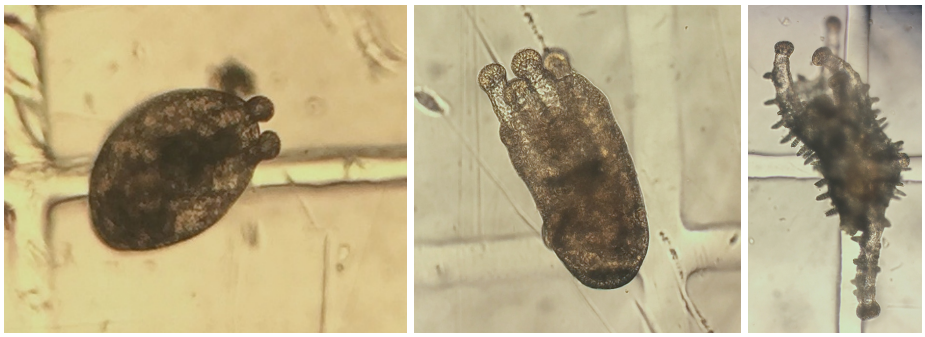

Figure 6. Photographs of pentactula larvae developing over time. Photos were taken with an Iphone and a compound scope. Photo Credit: Kendra Baird.

Sea Cucumber Market

Sea cucumbers are a delicacy in many Asian countries and are also used for nutritional supplements, arthritis treatments for humans and pets, and traditional medicinal treatments (Bruckner, 2005; Chen, 2003; Feindel, 2002; Fredalina et al., 1999). Modern medical research has discovered many biochemical products that can be extracted from sea cucumbers, many of which have medicinal potential for antibacterial, antiviral, anti-oxidant, anticoagulant, antimicrobial, and cancer fighting compounds (Avilov et al., 1998; David and MacDonald, 2002; Findlay et al., 1983; Haug et al., 2002; Kelly, 2005; Li et al., 2008; Mamelona et al., 2007; Roginsky et al., 2004; Silchenko et al., 2007; Tipper et al., 2003; Trotter et al., 1995, 1997; Yayli and Findlay, 1999; Zhong et al., 2007).

Most sea cucumbers harvested in the United States are exported to Asia, to the countries of China, Hong Kong, Taiwan, and Korea (Bruckner, 2005). There are also small commercial markets of *P. californicus* in North America in cities such as New

York, San Francisco, and Vancouver (Bruckner, 2005). The global demand for sea cucumber products has driven the price to increase in recent years. In the early 1980's the value was USD $0.07 per kg and in 2005 had risen to USD $0.82 per kg (Bruckner, 2005). Processed sea cucumbers can be sold for up to USD $9.00 per kg (Bruckner, 2005). The total ex-vessel revenue (the value established by determining the average price for an individual species, harvested by a specific gear, in a specific area) for Washington and California fisheries has varied from USD $1,000,073 in 1999 to a maximum of USD $4,848,999 in 1993, with the majority of the revenue associated with the Washington State fishery (Bruckner, 2005).

Sea cucumbers can be sold raw or preserved. Traditional methods of preserving include drying, smoking, canning, or freezing (Bruckner, 2005). They are prepared by being gutted and then boiled or roasted. The end with the tentacles is removed and the body wall is slit lengthwise to remove the viscera and scrape the muscles off the body wall (Washington Department of Fisheries, 1976). The body wall and muscles are typically boiled, dried, and salted before export (Bruckner 2005). The longitudinal muscles are one of the most common parts consumed in the United States, because they can be prepared like clams. Intestine and gonads can be processed in high-priced delicacies, and were sold for up to USD $23.20 per pound and USD $45.30 per pound in 1974, respectively (Washington Department of Fisheries, 1976).

Sea Cucumber Fisheries

In the United States commercial fisheries of sea cucumbers began in Washington State in 1971, followed by California in 1978, Alaska in 1981, Maine in 1988, and Oregon in 1993 (Bruckner, 2005). *P. californicus* currently supports a valuable commercial fishery in Canada and the United States. In 2003, landings were 807.4 metric tons in Alaska, 132.6 metric tons in California, 0.312 metric tons in Washington, and 520.7 metric tons in British Columbia (Bruckner, 2005). In 2007, the landings in Canada were 623 metric tons worth CAN $2.65 million (Hannah et al., 2012). In 2011, the landings in the United States were 1,101 metric tons worth USD $3.4 million (Department of Fisheries and Oceans Canada statistics).

The sustainability of sea cucumber fisheries is a major concern in the United States. Sea cucumber populations are especially vulnerable to overfishing because they can be easily and effectively targeted in shallow waters by harvesters, and display slow growth, late age maturity, and low rate of recruitment, which results in slow population replenishment (Bruckner, 2005, Bruckner et al., 2003; Uthicke, 2004; Uthicke and Benzie, 2000). As broadcast spawners, they are prone to the Allee effect, meaning that a low population size or density below the critical population threshold will result in a population crash to extinction. If the populations are not dense enough, and individuals are too spread out from one another, gametes cannot reach each other during spawning,

which results in a population collapse and inhibits recovery (Allee, 1938; Bruckner, 2005; Courchamp et al., 1999; Uthicke et al., 2009; Uthicke and Benzie, 2000).

The loss of sea cucumbers not only affects the marine ecosystem, but the decrease in fishing opportunities can have a major impact on the economies of coastal communities and a loss of cultural value. The fishery provides income, food, and is culturally important to many Native American tribes (Kinch et al., 2008). In 1988, the sea cucumber fishery became the most highly valued fishery outside of tuna fishing season and represented 80% of the value of all non-fish marine products in the Maldives (Joseph, 2005). The First Nations of Canada and the native communities in Alaska still harvest sea cucumbers for subsistence and social and ceremonial use (Mathews et al., 1990; Wein et al., 1996). In 1990, yearly harvests ranged from 150 to 700 sea cucumbers per household (Mathews et al., 1990).

In the United States sea cucumber fisheries are managed by individual states in state waters and by NOAA Fisheries in coordination with Regional Fishery Management Council in waters 200 miles off the coast (Bruckner, 2006b). Stock assessments and management of sea cucumbers are hindered by large gaps of knowledge about biological information such as the amount of time spent in larval phases, recruitment, and minimum density needed to successful reproduction, which is necessary for sustainable management (Toral-Granda et al., 2008). Managing the fisheries is difficult because sea cucumbers are difficult to size, sex, age, and tag. Enforcing fishery management techniques such as minimum and maximum sizes and the harvest of only males would be difficult to enforce due to their plasticity and lack of external evidence of dimorphism (Carson et al., 2016). Sea cucumbers can expand and contract in length and diameter, expel or retain water, and their weights vary seasonally due to atrophy (Carson et al., 2016). Past attempts to tag sea cucumbers have been unsuccessful, which makes it difficult to obtain migration, growth, mortality, and longevity measurements (Carson et al., 2016). WDFW's population assessments are determined by abundance, harvest weight, and catch-per-unit effort (CPUE) measurements, recorded in dive surveys and harvest logbooks (Carson et al., 2016).

Some countries have successfully managed sea cucumber fisheries despite the management hurdles. Japan was able to recover populations by limiting fishing through implementing fishery laws, rights systems, permits, and fishery co-operatives and restocking depleted areas (Akamine, 2004). New Zealand established a conservative harvesting limit, with a total allowable commercial catch of 35 metric tons (Ministry of Fisheries, 2011), which is relatively small when compared to 1,000, 6,000, and 400 metric ton harvest limits for Japan, Korea, and British Columbia, respectively (Hamel and Mercier, 2008).

After British Columbia experienced the boom-and-bust pattern they reduced quotas, added license restrictions, and implemented adaptive management and subsequently, catches are beginning to recover (Hand et al., 2008). Alaska sets their harvest limit based on the lower 90% bound of a biomass estimate. Areas are fished on a 3-year rotation schedule and separate areas are left closed as controls (Clark et al., 2009). In California, both *P. californicus* and *Parastichoous parvimensis* are harvested. A special permit was required from 1992 to 1993 for sea cucumber harvest. Separate permits for each gear type and a limit on the total number of permit were implemented in 1997. There are no restrictions on catch, but trawling is prohibited in some conservation areas (Rogers-Bennett and Ono, 2001; Schroeter et al., 2001). Surveys have reported that harvested sites in California showed densities that were 50-80 percent lower than in non-fished areas (Bruckner, 2006). In Oregon, the annual fishery lasts for three weeks in October (DFO, 2002). The commercial fishery is a small limited–entry dive fishery that is managed by individual quotas (DFO, 2002). All landings are monitored by an independent industry-funded firm (Bruckner, 2006).

Washington State Sea Cucumber Fishery

Commercial exploitation of *P. californicus* in Washington State has reduced populations from historic levels, with a decline of up to 90% in some areas. The fishery was established in 1971 and occurred without restrictions until 1987. The early 1980's commercial harvest and value was low (125-181 metric tons per year and USD $0.06-0.13 per kg). The annual harvest began to increase in 1988 from 952 tons and peaked in 1992 at 1880 metric tons (Bruckner, 2005). Following signs of overfishing, the state implemented a rotational harvest from 1987 to 1992 (Bradbury, 1994). And in 1994 seasonal harvest and specific harvest districts were finally adopted (Bruckner, 2005).

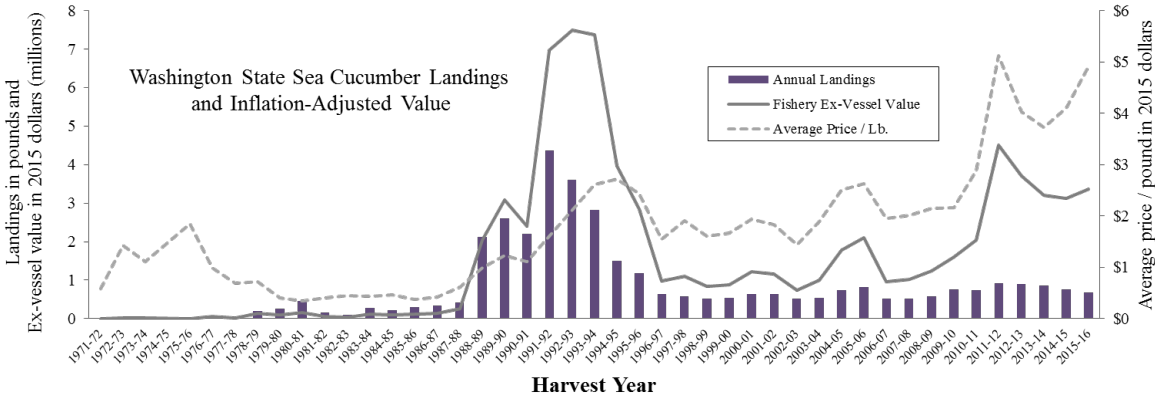

Figure 7. Graph of Sea Cucumber landings with inflation adjusted value over time in Washington State. Credit: WDFW.

The fishery in Washington State is a co-operative management agreement between WDFW and treaty tribes, with quotas being split about half and half. Washington management measures include seasonal closure from April-June during spawning season, spatial closures, license of collectors, and annual quotas set for each management zone. Seven areas have been closed, two for human health reasons. There is a prohibition on trawling and shrimp areas. Regulations for the trawl fishery include 1) a ban on trawling in waters less than about 20 m deep; 2) temporal closures during soft-shell Dungeness crab (reproductive) periods; 3) specific fishing locations; and 4) restrictions on gear type and size, including maximum beam width for beam trawl gear and minimum mesh size for otter trawl gear. Fish receiving tickets are submitted to WDFW after each fishing trip. This data is used to determine when the annual tribal and commercial harvest quota is reached. Fishermen also submit monthly harvest logs that include the date, vessel name or boat registration number, location fished, pounds landed, average depth of harvest, number of divers, and total diver hours spent fishing. There are 46 licensed commercial divers, with a license reduction program initiated in 2002, with a goal of reducing the total number of licenses to 25 (Bruckner, 2005). Recreational licenses also allow individuals to harvest up to 10 animals a day for personal use. There are also a low number of Scientific Collection Permits granted, which allow individuals and organizations to collect a small number of individuals for research purposes.

Each management zone designated by WDFW has individual quotas set using models and estimates from catch-effort data and video surveys and dive surveys. WDFW uses the 5% biomass rule to set the harvest limits, as suggested by Uthicke (2004), for the sustainable fishery of *P. californicus*. Only 5% of the population should be harvested to minimize the effect on the population and ensure the sustainability of the fishery. Surveys have determined that District 1 (Figure 8) has a current biomass estimate of 6,000,000 pounds. The 2013 quota was 11.4% of the biomass. WDFW is lowing the harvest rate by 1% a year, until the ideal 5% is reached. The current 2016 quota is at 8%. Populations in District 2 (Figure 8) have stabilized and may be rebounding in the in the western half of the district. Current biomass estimate is 3,886,516 pounds, which indicates that District 2 can support continued harvest. However, the eastern area of the district was found to have a much lower biomass than the west, and as a result, the district has now been split into two separate management zones with a 128,000-pound quota for the west and a 42,000-pound quota for the east to meet the 5% harvest rate for each section of District 2. WDFW also designated no-take zones in the Strait of Juan de Fuca to act as a reservoir and to allocate a reference site where there is an absence of harvest. Surveys have determined that the Central Puget Sound District (District 3) (Figure 8) has been subjected to a high level of historic harvest and has greatly reduced the biomass in comparison to the virgin state. The biomass estimate for District 3 is 183,000 pounds, 7% of the historic biomass. Harvest has been closed in District 3 until evidence of recovery is obtained. South Puget Sound (District 5) (Figure 8) biomass is estimated to be 437,135

pounds. The quota is now set at 22,000 for the 5% harvest rate. WDFW has focused recovery efforts on continuing to monitor activities, develop the ability to tag to estimate age, growth, mortality, and behavior, as well as research population genetic information. These efforts will help develop and enforce regulations for sustainable management of *P. californicus* in Washington State (WDFW, unpublish data).

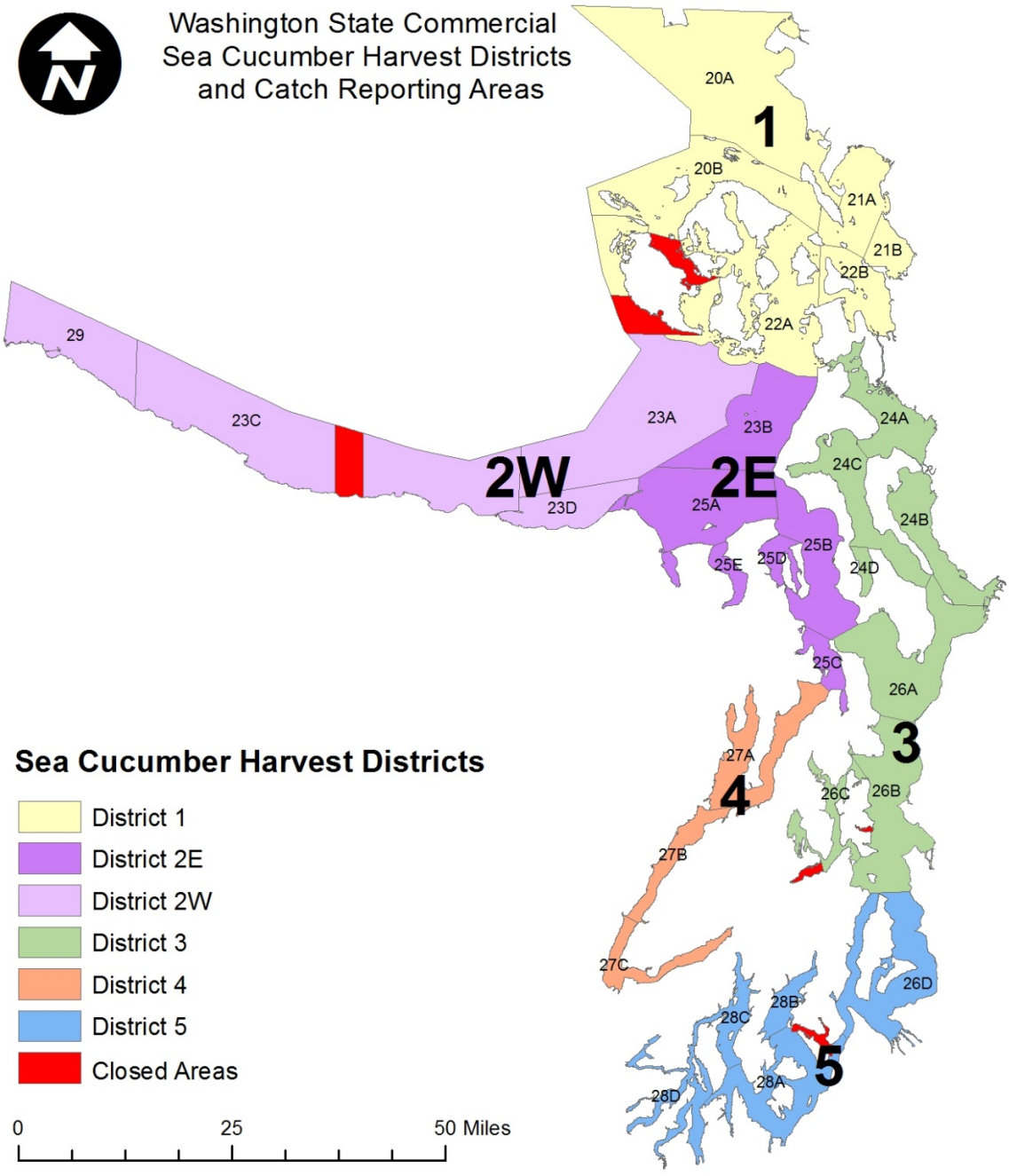

Figure 8. Map of the harvest districts in Washington State managed by WDFW. Credit: WDFW.

Aquaculture

The current demand for sea cucumber products cannot be met through commercial harvesting alone, which has led to an increased interest in developing aquaculture techniques for sea cucumbers globally (Ren et al., 2016). There has been progress made and aquaculture techniques have been established for tropical species in countries such as New Zealand and Australia, for the temperate species Japanese sea cucumber *Apostichopus japonicus*; and the tropical sandfish *Holothuria scabra* (Duy, 2012; Gamboa et al., 2012; Huiling et al., 2004; Renbo and Yuan, 2004; Xiyin et al., 2004). Hatchery and grow-out technology is still being developed for many other species of interest including; *Autralostichopus mollis*, *Isostichopus fuscus*, *Athyonidium chilensis*, *Cucumaria frondosa*, *Stichopus horrens*, *Holothuria fuscogilva*, *Actynopiga spp*., and *Parastichopus californicus* (Guisado et al., 2012; Jimmy et al., 2012; Nelson et al., 2012; Mercier et al., 2012; Paltzat et al., 2008). However, large-scale commercial aquaculture has not yet been established for *P. californicus*, and it remains in the early developmental stage (Zamora and Jeffs, 2013). There is still little information regarding broodstock conditioning, larval and juvenile production, and grow out. Current research is primarily focused on stock assessment and fisheries management, and the use as a deposit feeder in Integrated Multi-trophic Aquaculture (IMTA) to reduce organic accumulation. (Zamora and Jeffs, 2013).

The Alutiiq Pride Shellfish Hatchery in Alaska was one of the first hatcheries to start developing aquaculture techniques for *P. californicus*. They began developing spawning and larval grow-out techniques in 2010. They have since made advancements in overcoming transport stress of adults during collection, holding and conditioning broodstock, spawning, larval culture, settlement, and nursery culturing (Development of red sea cucumber (*Parastichopus californicus*) poly-aquaculture for nutrient uptake and seafood export, unpublished). Puget Sound Restoration Fund (PSRF) received Salton Kennedy Grant from NOAA in partnership with Pacific Shellfish Institute, University of Washington, Alutiiq Pride Shellfish Hatchery, and Washington Department of Fish and Wildlife to research *P. californicus*. PSRF will develop aquaculture techniques for sea cucumbers, with a focus on continuing to develop methods for broodstock maintenance, spawning methods, larval culture, and nursery culturing. The purpose of their research is to develop aquaculture techniques for future restoration work. They hope to grow out juveniles at the Kenneth K. Chew Center for Shellfish Research and Restoration hatchery to out plant to areas of Puget Sound that have experienced significant population declines. The findings from this study will contribute to the advancement of PSRF's work. Determining the optimal temperature range to rear larvae at for maximum survival and development will improve production success.

Spawning

The first step of developing hatchery techniques for *P. californicus* is to determine reliable methods for inducing spawning in order for hatcheries to have access to large number of gametes and subsequent larvae. Many species of sea cucumbers, if collected early in the spawning season, will release gametes within 1-2 weeks in the laboratory (McEuen 1986, Strathmann and McEuen 1987). At the height of the spawning season, *Psolus Chitonoides* frequently spawns in early morning hours in response to increase in light intensity, *Cucumaria miniata* discharges gametes in late morning and afternoon, and *Eupentacta quinquesemite* spawns during evening and early night hours. Stopping seawater flow and warming the water triggered spawning in *Cucumaria lubrica* and *Cucumaria pseudocurat*. Jordan (1972) found that decreasing seawater temperature by 2°C, then warming it again with the addition of dense concentrations of *Dunaliella sp*., induces *Calceolaria frondosa* to spawn.

Alutiiq Pride Shellfish Hatchery has had success with inducing spawning in *P. californicus* by raising the seawater temperature at least 2-4°C. Males began to release sperm first, and females began about an hour after the first male began. Females release oocytes for 20-30 minutes (Ren et al., 2016). During spawning, slight disturbances typically do not completely stop the release of gametes, and individuals resume spawning shortly after being handled. Isolating individuals into separate buckets is possible to collect gametes in order to manipulate crosses if the sea cucumbers are carefully handled and rinsed.

If methods for inducing spawning are not successful, then it may be possible to induce meiosis through chemical and artificial means. Studies have found that meiosis can be induced in oocytes of *P. californicus* by using radial nerve factors (RNF) extracted from holothuroids or *pycnopodia* and prepared in accordance to the methods and Smiley (1984), Strathmann and Sato (1969), and Maruyama (1986). Unlike other echinoderms, 1-methyladenine does not induce maturation in *P. californicus*, but purine does show potential. Smiley (1984) found that low concentrations of dithiothretol (10mM for 10 minutes or less) can be successful in inducing maturation in oocytes, but only a low percentage develop normally into larvae**.** Until methods for inducing meiosis in oocytes has resulted in a high percentage of normally developed larvae, artificial fertilization of ova will not be a practical option for hatcheries.

When obtaining gametes via dissection is recommended to slit the body wall lengthwise, away from the mid-dorsal axis to avoid severing the gonoduct. When very ripe, ovarian tubes spontaneously release oocytes through the gonoduct into the dish (Strathmann and McEuen, 1987)**.** Active sperm can be obtained through dissection and using forceps to open the testis tubule. Sperm can be also activated by adding aqueous NH_4Cl to final concentration of 7-10 mM (Smiley, 1986b). The optimal time for

insemination is immediately following germinal vesicle breakdown. High levels of polyspermy are noted to occur when sperm is added to ripe oocytes after the formation of the first polar body (Smiley, 1986b). It is recommended to collect fertilized eggs with a syphon, and collect onto a wet 45-60μm screen. It also recommended to rinse the fertilized eggs with seawater to remove excess sperm.

Larval Culture

Larval culture of *P. californicus* is currently hindered by poor survival to metamorphosis. The rate of development for larvae is variable and influenced by maturation rate, time of fertilization, water temperature, stocking density, quantity and quality of food, and individual variation in developmental rate (Strathmann and McEuen, 1987). Cultures of embryos and larvae should be stirred occasionally to prevent coalescence in dense aggregations in the tanks or bowls, although, they can be reared successfully in static systems with frequent water changes every 1-5 days (McEugen, 1986; Strathmann and McEugen 1987). The recent study by Ren et al. (2016) determined that *P. californicus* larvae performed well on mono-species microalgal diets containing *Chaetoceros calcitrans*, *Chaetoceros muelleri*, *Dunaliella tertiolecta*, *Pavlova lutheri*, and *Tahitian sp*., but performed best with bi-species microalgal diets containing *C. calcitrans*. It is recommended to have a stocking density between 0.3-1.0 individual per ml (Agudo, 2006; Palzat, 2008). Studies have found that survival to settlement in sea cucumbers is dependent upon stocking densities, however, more studies are needed to understand the relationship between stocking densities and survival during different planktonic phases (Battaglene et al., 1999; Duy, 2012; Purcell, 2012).

Juvenile Care

Methods for growing and feeding newly settled sea cucumbers have been heavily adopted from methodologies for culturing herbivorous gastropods, such as, abalone (Battaglene et al., 1999). Once sea cucumber larvae began to settle and metamorphose into the early juvenile stage they are normally transferred to nursery raceways, ponds, or off shore sea pens (Chen, 2004; Purcell et al., 2012). On shore nursery systems are the preferred method for rearing juvenile *p. californicus* because environmental conditions, feeding, and predation can be controlled. Lavitra et al. (2010) found that the growth rates of juveniles in nursery tanks were highest at lower stocking densities, and were relatively unaffected by sediment quality. Pond cultures are primarily used in tropical regions and China. They can be very cost effective if pre-existing ponds, such as ones used in shrimp production, can be utilized. However, creating new ponds can be expensive and labor intensive (Renbo and Yuan, 2004). Not all species perform well in ponds (Mercier et al. 2012, Purcell, 2012).

Ocean-based aquaculture or "sea ranching" is a method to culture sea cucumbers held in pens, suspended from a raft in cages or placed directly on the sea floor (Chen, 2004; Purcell and Simutoga,, 2008; Renbo and Yuan, 2004). This method has also been used in China and other tropical regions (Chen, 2004; Renbo and Yuan, 2004; Mills et al., 2012). The pens prevent the sea cucumbers from migrating, theft, and helps to designate ownership (Purcell et al., 2012). Research has also evaluated the potential to release juvenile sea cucumbers without any confinement (Bowman, 2012; Fleming, 2012; Juinio-Menez et al., 2012; Purcell, 2012; Purcell and Simutoga, 2008,). Studies have demonstrated that *A. mollis* and *H. scabra* will not move great distances and can be harvested years later, if the habitat conditions and size of the site are adequate (Mercier et al., 2000; Purcell and Kirby, 2006; Slater and Carton, 2010). However, survival of the released juveniles is a major concern because the majority is not surviving market size in the wild (Juinio-Menez et al., 2012; Purcell and Simutoga, 2008).

Developing cost-effective methods and technology will be important for scaling up the aquaculture of *P. californicus* to commercial levels of production (Purcell et al., 2012). The development of broodstock management, larval rearing, nursery culture, and techniques for transporting juveniles are key to successful commercial aquaculture development. If more reliable methods for obtaining high quality gametes throughout the year in large numbers can be developed, then there is more potential for the success of aquaculture for *P. californicus*.

Integrated Multi-Trophic Aquaculture

Recent studies indicate that *P. californicus* may have the potential as an organic-extractive species, to be co-cultured under salmon *Salmo salar* and sablefish *Anoplopoma fimbria*, or under suspended Pacific oysters *Crassostrea gigas* (Ahlgren 1998, Hannah et al., 2012, Paltzat et al. 2008). Results of these studies yielded high growth and survival. Integrated multi-trophic aquaculture (IMTA) is the culture of two or more compatible species, which occupy different trophic levels, in one system (Bardach, 1986; Zamora and Jeffs, 2012). Successfully integrated IMTA systems closely mimic natural ecosystem functions (Folke and Kautsky, 1992). Species at lower trophic level consume the different types of waste produced by the species at the higher trophic levels. Examples include macroalgae can absorb dissolved nutrients, filter-feeding shellfish can consume fine particulates, and deposit feeders can consume heavier particulates (Chopin et al., 2001). Studies suggest that sea cucumbers have the potential to reduce fish farm waste, mitigating the negative environmental impacts of fish pens, while providing an additional remunerative product to the companies.

Commercial-scale IMTA systems have already been established in other areas of the globe. In Canada blue mussels *Mytilus edulis* and kelp *Saccharina litissima* and *Alaria esculenta* are grown adjacent to Atlantic salmon *S. salar* (Neori et al., 2007; Reid

et al., 2009; Ridler et al., 2007). In China, *S. japonicus* grew well when co-cultured with scallops *Chlamys farreri* and *Argopecten irradians* and Pacific oysters *C. gigas* in both closed and open systems (Zhou et al., 2006). Shrimp are also commonly raised in co-culture with sea cucumbers (Martinez-Porchas et al., 2010; Yaqing et al., 2004). In New Zealand, *A. mollis* had high survival and growth rates when cultured below the green-lipped mussels *Perna canaliculus* (Slater and Carton, 2007).

The Effect of Temperature on Sea Cucumber Larvae

One of the most important variables for hatcheries to control during larval culture is seawater temperature. Echinoderms display a high sensitivity to thermal stress (Przeslawski, 2015). Sea cucumbers are ectothermic and they depend on surrounding seawater temperature in regulate their internal temperature, which in turn, controls most of the biochemical and physiological processes (An et al., 2007). Most species can tolerate a relatively wide ambient temperature range within their natural habitat, although optimal performance is typically restricted to a specific thermal range (Meng et al., 2009; Pawson, 1966). The range varies between species life stages, but typically broadens with development (Costlow et al., 1960; Crisp and Ritz, 1967).

Early life stages are the most sensitive to environmental conditions, and experience the most damage from temperature extremes (Strathmann and McEuen, 1987). The developmental rates of marine invertebrates generally increase with temperature until a lethal maximum is approached (Strathmann and McEuen, 1987). Studies have shown that echinoderm larvae show increased mortality, delayed development, reduced growth, and reduced metabolic rate in response to temperature stress (Bressen et al., 1995; Przeslawski 2005; Roller and Stickle, 1989, 1993). This is hypothesized to be due to multiple factors including but not limited to: oxidative stress, lysosomal destablilisation, increased lipid peroxidation, a change in immune ability, and the decrease in concentrations of soluble proteins, soluble sugars and ions (Chang et al., 2006; Deschaseaux et al., 2010; Deschaseaux et al., 2011; Liu et al., 2013; Wang et al., 2012). A study by McEdward (1985) suggests that larvae cannot increase their feeding capacity at high temperatures to meet the energy demands of the increased metabolism for the accelerated development that occurs at higher temperatures thus, leading to deformation and mortality.

Studies have examined the effect of temperature on the survival and growth of other species of sea cucumbers. *A. japonicas* and *H. spinifera* larvae rapidly developed at higher temperatures and slower with delayed metamorphosis at lower temperatures (Asha and Muthiah, 2005; Li Li et al., 2011). The extended larval phase exposes organisms to the hazards present in planktonic life (Davis & Calabrese, 1969; Garcia de Severyn, 2000, Stickney 1964). Hamel and Mercier (1996) discovered for the sea cucumber *Cucumania Grandosa,* lower temperatures increased the duration between developmental

stages, but did not increase mortality at each developmental stage. Liu et al. (2010) observed that larvae grew faster with increasing temperatures in suitable range, but extreme temperatures restrained the development of larvae in *A. japonicas*. High temperatures resulted in weak-digestion, decreased-absorbing, increasing of oxygen consumption rate and ammonia excretion rate (Villarreal, 1993). Li Li et al. (2011) found that thermal stress affected mid-auricularia *A. japonicas* larvae more than early and late auricularia, and the metamorphic period between the auricularia and the doliolaria stage had the highest tolerance to temperature and salinity changes. The low survival of mid-auricularia larvae is due to their poor ability to adjust to physiologically in response to changes in environmental conditions (Kashenko, 2002). In order to grow *P. californicus* at a large-scale in hatcheries it is imperative to know the effects of temperature on survival, growth, and development in order to determine the ideal temperature range that larvae should be reared at.

METHODS

This study was conducted in collaboration with Puget Sound Restoration Fund (PSRF) at the Kenneth K. Chew Center for Shellfish Research and Restoration hatchery located at National Oceanic and Atmospheric Administration's (NOAA) Manchester Research Station in Manchester, WA.

Broodstock

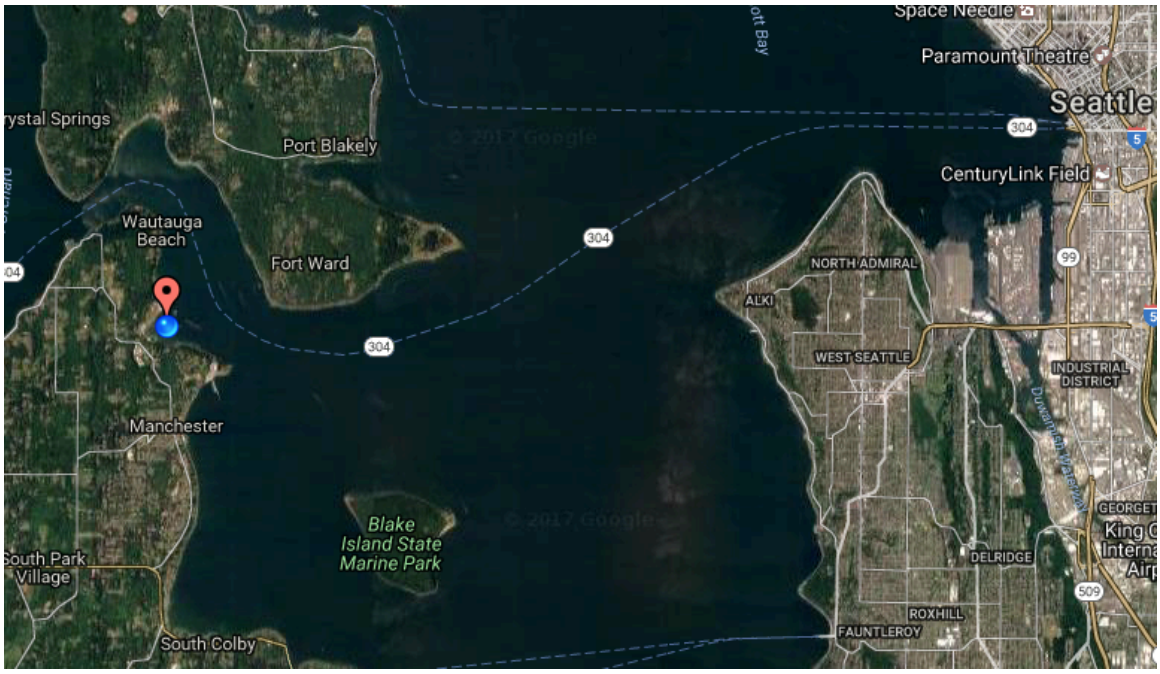

Figure 9. Map of Washington with Clam Bay indicated by the red marker.

Divers collected 74 large adult *P. californicus* from Clam Bay (47.5712079°, -122.5481897°) on March 12, 2016 (Figure 9). Four additional adults were also collected

on May 14, 2016. Divers targeted larger individuals in the wild in order to collect adults that were most likely to be sexually mature and ready to spawn. The sea cucumbers were placed in 5 gallon buckets and transferred to the hatchery within 1 hour of harvest. The sea cucumbers were then housed in outdoor 4200 L tanks on a flow through system with ambient seawater filtered to 5 µm. The tanks were lightly aerated with oxygen distributed through a manifold built from PVC pipe and placed on the bottom on the tank. Cylinder blocks and macroalgae were also placed inside the tanks. The sea cucumbers were observed to accumulate mostly in the cylinder blocks and on the tank walls. Half of the tank lid was left open each day in order to allow the broodstock to be exposed to sunlight in case photoperiod affects gonad development and reproductive conditioning, and to allow natural algae to grow as a secondary food source (Figure 10).

Figure 10. Photograph of outside broodstock holding tank. Photo Credit: Kendra Baird.

The waste from the sea cucumbers was syphoned out once a week, and algae was only scrubbed off the tank once it became noticeably thick. The broodstock were fed Otohime B1 and C2 fish diet but displayed a preference for the algae that grew naturally on the tank walls. Evisceration was rare, but organs were removed from the tank as soon as they were observed in order to limit stress on other individuals. Once an individual eviscerates they are not able to spawn for the remainder of the season and must wait a year to regenerate their gonads.

Spawning

Agudo et al (2016) was referenced for methods to induce spawning. The dates for spawning were chosen within a few days of a new moon because divers had previously observed *P. californicus* spawning in the wild around a new moon. When we attempted to spawn we brought in 29-30 individuals from the outside holding tanks and placed them inside a clean 660 L tote (Figure 11). We used a combination of many different methods to stimulate spawning. For thermal stimulation, the seawater was heated to 16-19°C before adding the broodstock to the spawning tote. For food stimulation, we added combinations of *Pavlova pinguis, Chaetoceros muelleri*, and *Chaetoceros sp.* to the water until it was visibly cloudy (Figure 12).

Figure 11. Photograph of broodstock inside the spawning tote during a spawning attempt. Photo Credit: Kendra Baird.

Figure 12. Photograph of broodstock in the spawning tank with algae in the water to stimulate spawning. Photo Credit: Kendra Baird.

Our attempts to spawn on 05/19/2016 and 05/20/2016 using thermal stimulation and algae only resulted in 3 and 5 males spawning, respectively. We used different individuals on the 20th than previously used on 19th. On 06/01/2016 Fish Biologist Rick Goetz from NOAA assisted with an ultrasound on two individuals (Figure 13). We attempted to sex the individuals and assess gonad development. However, we were unable to identify the organs through ultrasound. A male was dissected and sperm was extracted. The testes were observed to be very full, which led us to believe that we were at the peak of the spawning season. However, we attempted another spawn using thermal and food stimulation in addition to adding the extracted sperm to the water, but still only 4 males spawned. Eviscerated eggs were collected from the out side holding tanks and we attempted to strip spawn using the eviscerated eggs and the extracted sperm from the previous dissection. The sperm was diluted and combined with the eggs and the fertilized eggs were carefully rinsed with seawater to wash off excess sperm. After two hours the

fertilized eggs were counted and examined. Only 0.4% had been fertilized and there was a 0% survival.

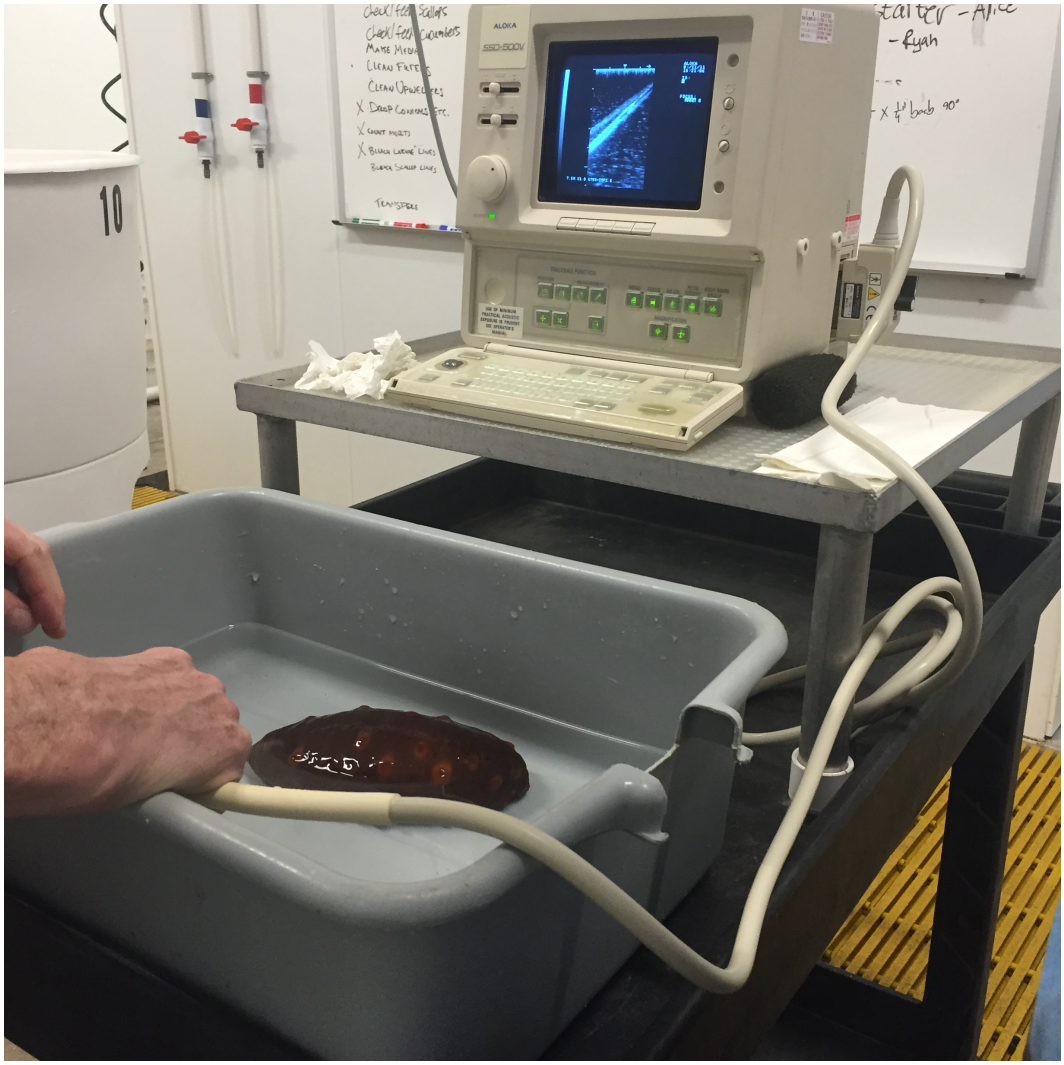

Figure 13. Photograph of NOAA biologist Rick Goetz assisted with an ultrasound to determine gonad development. Photo Credit: Kendra Baird.

Concerned that thermal shock and the addition of algae were not drastic enough to induce spawning, additional spawning methods were implemented in hopes of getting females to spawn. For the spawn on 06/17/2016, individuals were weighed and only those over 782 grams were used for spawning, as the literature suggested these individuals should be at least 4 years old and therefore sexually mature (Figure 14, Table 2). A fluorescent light was also shone on them for 24 hours prior to spawning in addition to thermal and food stimulation (Figure 15). Only one male spawned during this attempt. On 06/20/2016 the spawning totes were drained and the broodstock was left out of the water and dried for 30 minutes before being lightly sprayed with a chemical sprayer, and

the tank refilled with 16°C seawater and algae added (Figure 16). No sea cucumbers spawned during this attempt.

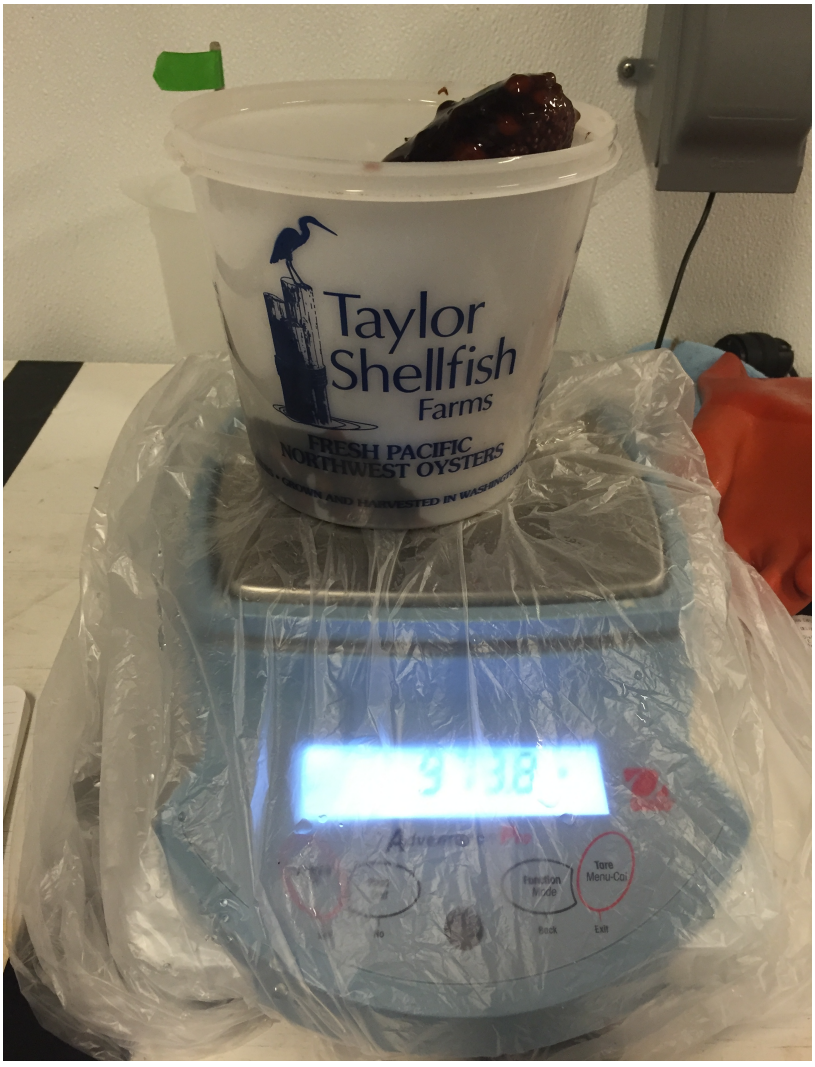

Figure 14. Photograph of a sea cucumber being weighed on a scale. Photo Credit: Kendra Baird.

Number	Weight (grams)	Date Collected
1	169.4	3/12/16
2	199.1	3/12/16
3	285.2	3/12/16
4	330.5	3/12/16
5	338.4	3/12/16
6	346.3	3/12/16
7	347.7	3/12/16
8	363.2	3/12/16
9	375.3	3/12/16

10	383.0	3/12/16
11	415.2	3/12/16
12	418.9	3/12/16
13	419.7	3/12/16
14	421.8	3/12/16
15	422.8	3/12/16
16	436.4	3/12/16
17	439.3	3/12/16
18	453.1	3/12/16
19	476.5	3/12/16
20	484.0	3/12/16
21	526.0	3/12/16
22	528.8	3/12/16
23	533.3	3/12/16
24	543.5	3/12/16
25	546.7	3/12/16
26	570.3	3/12/16
27	578.3	3/12/16
28	587.6	3/12/16
29	593.1	3/12/16
30	611.6	3/12/16
31	617.7	3/12/16
32	621.6	3/12/16
33	622.4	3/12/16
34	628.3	3/12/16
35	658.0	3/12/16
36	658.8	3/12/16
37	659.1	3/12/16
38	661.8	3/12/16
39	666.1	3/12/16
40	673.3	3/12/16
41	673.5	6/14/16
42	683.1	3/12/16
43	688.7	3/12/16
44	700.0	3/12/16
45	723.5	3/12/16
46	745.3	3/12/16
47	758.6	3/12/16
48	769.2	3/12/16

49	771.6	3/12/16
50	**783.1**	3/12/16
51	**787.6**	3/12/16
52	**791.4**	3/12/16
53	**820.6**	3/12/16
54	**829.5**	3/12/16
55	**834.0**	3/12/16
56	**843.7**	3/12/16
57	**867.0**	3/12/16
58	**871.4**	3/12/16
59	**898.8**	3/12/16
60	**954.3**	3/12/16
61	**971.1**	3/12/16
62	**973.8**	3/12/16
63	**985.2**	3/12/16
64	**1,033.4**	3/12/16
65	**1,088.8**	3/12/16
66	**1,105.7**	3/12/16
67	**1,106.8**	3/12/16
68	**1,122.8**	3/12/16
69	**1,152.2**	6/14/16
70	**1,202.3**	3/12/16
71	**1,220.1**	3/12/16
72	**1,304.6**	3/12/16
73	**1,305.8**	3/12/16
74	**1,325.5**	3/12/16
75	**1,390.0**	3/12/16
76	**1,816.6**	6/14/16
77	**2,028.9**	3/12/16
78	**2,110.1**	6/14/16

Table 2. Table of the weights of the broodstock collected. Bold indicates the 29 individuals that were used for spawning on 6/17/16.

Figure 15. Photograph of fluorescent light being shone on the broodstock in the spawning tote for 24 hours prior to spawning attempt. Photo Credit: Kendra Baird.

Figure 16. Photograph of the broodstock being dried for 30 minutes during a spawning attempt to induce spawning. Photo Credit: Kendra Baird.

On 07/15/2016 the heated water line set at 16°C was turned on to one of the outside holding tanks and the broodstock began to spawn. A single female began releasing oocytes. Males in the holding tank next to it also began spawning, even though they were on separate systems and on ambient water. Individuals were collected from the outside tanks and brought into the hatchery and placed inside the spawning tank filled with 16° C seawater. 5 males and 1 female spawned. The fertilized eggs were carefully syphoned out of the tank and collected onto a 48 µm submerged screen (Figure 17). The fertilized eggs were carefully rinsed with seawater to remove excess sperm to prevent polyspermy. The eggs were carefully collected into a tri-pour beaker, homogenously mixed, and sub sampled to determine the density and total count. PSRF conducted two more spawns on August 4, 2016 and September 7, 2016, and were able to get successful spawns with only a temperature stimulation with the seawater temperature increased to

20°C. Males were noted to spawn first, and females begun to release eggs within an hour after the first male began to spawn. When sea cucumbers spawned they maintained a unique posture, with their anterior end raised in the water column and the head curled forward parallel to the surface of the tank (Figure 18). Sperm was thick and milky white and easily distinguished in situ. Eggs were slightly orange, appeared granular, and dissipated quickly making it difficult to notice the release. Females and males occasionally swayed their bodies when releasing gametes. Table 3 contains a summary of all the spawning attempts conducted during this study.

Figure 17. Photograph of eggs being syphoned out of the tank after being released by a female. Photo Credit: Kendra Baird.

Figure 18. Photograph of a male spawning and displaying the typical posture seen while releasing gametes. Photo Credit: Kendra Baird.

Date	Time	Stimulation	Treatment	♂	♀	Successful?
05/19/16	0931-1600	Thermal Algae	19°C *Pavlova pinguis* & *Chaetoceros muelleri*	3	0	No
05/20/16	0916-1245	Thermal Algae	20°C *Chaetoceros muelleri* & *Chaetoceros sp.*	5	0	No
06/01/16	1010-1345	Thermal Algae Gonad Extraction	20°C *Chaetoceros muelleri* Extracted sperm was added to the water	4	0	No
06/17/16	0900-1552	Weight Light Thermal Algae	Individuals > 782 grams Exposed to light for 24 hours prior to spawning 20°C *Chaetoceros muelleri*	1	0	No
06/20/16	0945-1407	Desiccation Water Pressure Thermal Algae	For 30 minutes Sprayed lightly 16°C *Chaetoceros muelleri*	0	0	No
07/15/16	1640-1830	Thermal	16°C	5	1	Yes*
08/04/16	1430-1630	Thermal	20°C	5+	3	Yes
09/07/15	1330-1600	Thermal	20°C	3	1	Yes

Table 3. Table of spawning attempts. The date, time of day, stimulation used, number of males and females that spawned, and if the spawn was successful is noted. * indicates the spawn used to inoculate the experiment.

Experiment: The Effect of Temperature on Larval Survival and Development

System

This experiment was comprised of five different water baths held at five different temperature treatments (12°, 15°, 18°, 21°, and 24°C). The range was chosen to replicate the temperature range that a hatchery would be able to chill and heat to. Teco TK500 chillers were set with a temperature error range of +/- 0.01°C. Each system included two connected 39" x 18.5" x 11.5" totes filled with freshwater to maintain a constant temperature, and an external 500 gph Danner Mag Drive pump was connected with a hose and PVC pipe to circulate water through the system (Figure 19). There were six replicates for each temperature treatment, each consisting of 4L containers. In addition, there were three 2 gallon buckets, which held clean saltwater for the future water changes heated or chilled to the same temperature as the replicates, per system. The replicate containers were filled with 3L of UV sterilized and 1µm filtered seawater and to maintain a constant temperature, the level of the outside freshwater bath was filled to the 2.5 L on the out side of buckets to remain negatively buoyant. Each replicate was lightly aerated

with oxygen from an air stone. The totes, hoses, and pipes were insulted, and both totes contained a Styrofoam lid to insulate temperature and ensure all systems on the top and bottom of the tables had the same amount of light. One table held the 24°C water bath system, and the other table held the 12°, 15°, 18°, and 21°C systems (Figure 19, 20, 21).

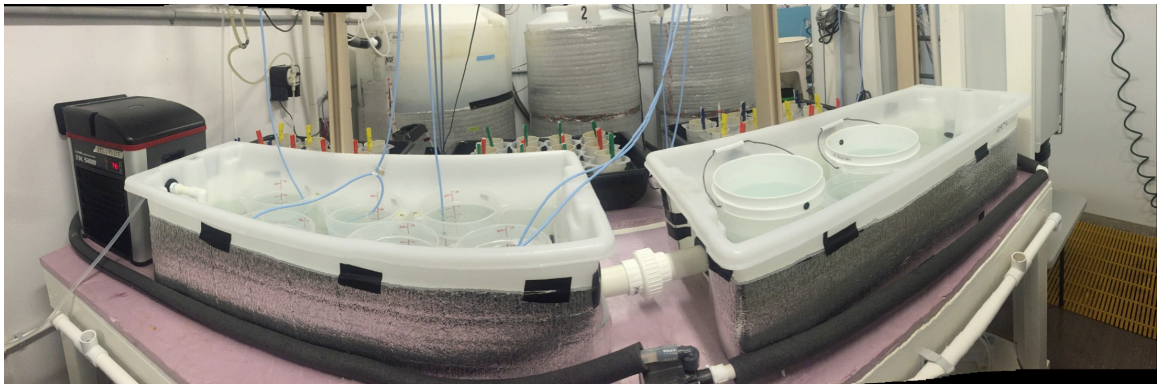

Figure 19. Photograph of the 24°C system without the lids. The first tote (left) connected directly to the inflow from the Teco TK500 chiller and contained the six experimental replicates. The second tote (right) contained three 2 gallon buckets filled with clean water for future water changes. The freshwater flowed out from the right tote the external pump (pictured in the bottom center), which pumped directly back to the chiller. Photo Credit: Kendra Baird.

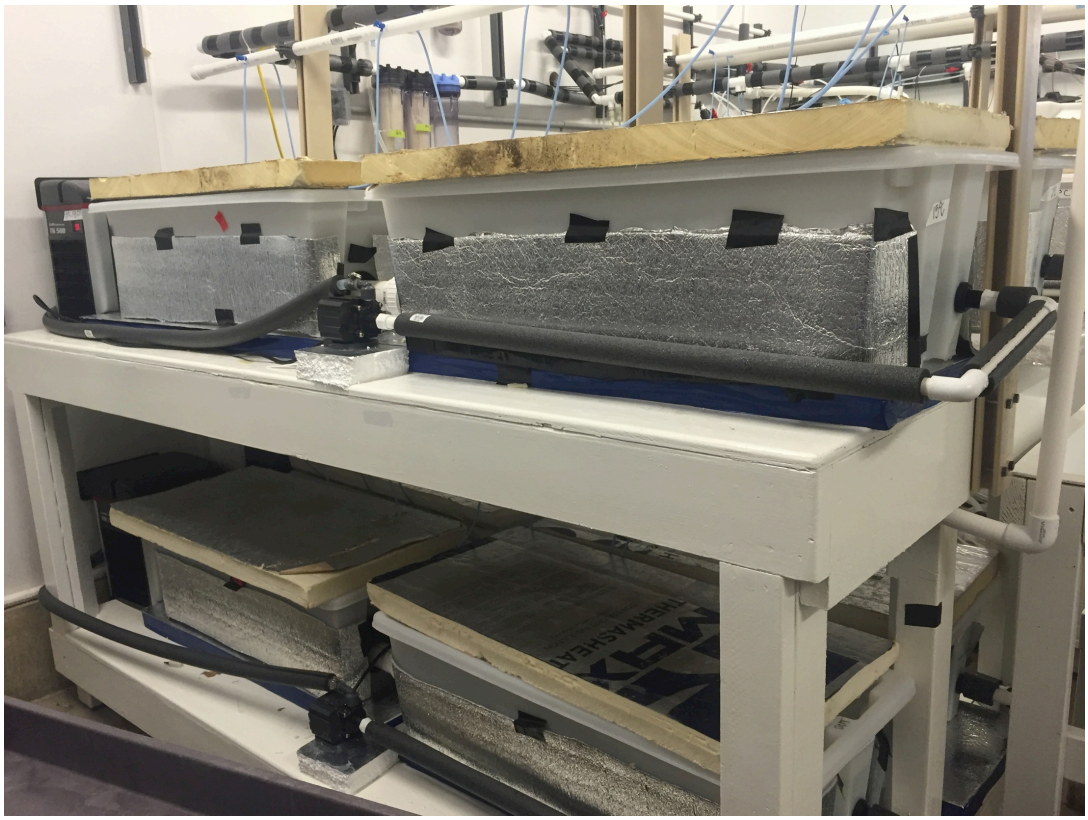

Figure 20. A photograph of the table that contained four of the systems. The 12°C system is on the bottom shelf and the 15°C system is on the top shelf. Photo Credit: Kendra Baird.

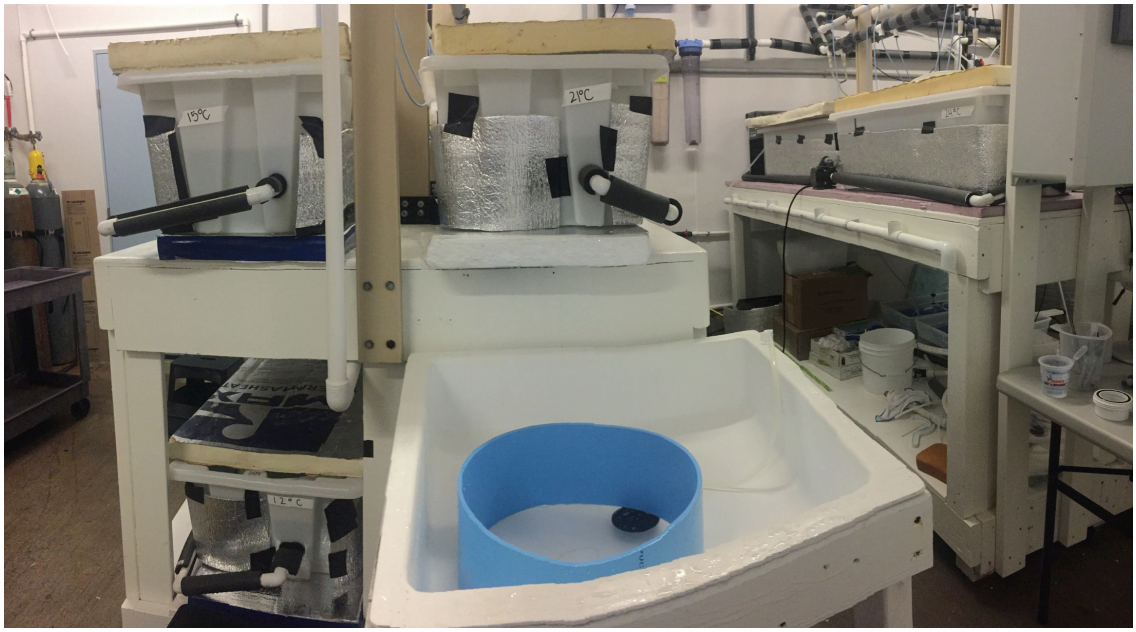

Figure 21. Photograph of the two tables that contained all of the systems. The bottom left corner is the 12°C system, the upper left corner is the 15°C system, the middle upper system is the 21°C system, the bottom middle is the 18°C system (not pictured, behind the larval sink), and the 24°C water bath is on the top of the second table on the right. Photo Credit: Kendra Baird.

Inoculation

Fertilized eggs from the July 15, 2016 spawn were used. The spawn was a cross between one female and five males. Each replicate for each treatment was inoculated with 3,000 fertilized eggs for a stocking density of one individual per milliliter on 7/15/16. All five water baths were held at 16°C until the embryos hatched into the fist larval stage auricularia on July 18, 2016 (day three post fertilization) to ensure that temperature did not affect hatching rate or survival to hatchment. The water was not changed and the larvae were not fed until the experiment started on July 18, 2016.

Larval Maintenance

Once the experiment began on 7/18/16 The larvae were fed either *C. muelleri* or *Chaetoceros calcitrans*, which ever was available, and *Pavlova sp.* at a density of 30,000 cells per ml every other day after water changes. During water changes the larval sink was plugged and filled about an inch with water from the 2 gallon buckets held in the same water bath as the replicates, in order to keep the temperature that the larvae were exposed to consistent throughout the experiment and to limit stress on the organisms. Once the water level was too high in the sink after a couple of water changes, the sink was drained slightly.

During water changes, the larvae were carefully poured from the containers onto the submerged 48-60 µm screen. The smallest available mesh size was used to relieve the pressure on the gelatinous larvae, when held on the screen. The bucket was back rinsed to ensure all larvae were emptied from the bucket and were not stuck on the side of the dry

container. Water changes were a time intensive and careful process. The larvae were never pulled directly out of the water, to make sure they were not squished against the screen. As the screen was slowly lifted, the larvae were carefully rinsed with a squirt bottle to the water line until all the larvae were accumulated to one section of the screen. If samples were being taken, the larvae from the screen were poured into a 250 ml cup for subsampling before being returned to the cleaned container. Each container was scrubbed with the industrial chemical Vortex to kill bacteria and pathogens and then rinsed with freshwater, before being refilled with 3 L of clean seawater. Each 2-gallon bucket was also treated with the cleaning agent Vortex and rinsed with freshwater before being refilled and returned to the water bath. The screens were sprayed with freshwater between each replicate and treated with Vortex between each temperature treatment.

Sampling

Samples were taken on days 0, 5, 11, 17, and 24. Samples were taken once a week and the experiment continued until competent pentactula larvae were observed. The experiment was concluded on day 24 to ensure that larvae were not settling on the containers of the higher temperatures, as they wouldn't be countable. Larvae were concentrated into a 100 ml volume, homogenized with a home made larval plunger, and a 6 ml subsample was collected from each replicate, for a minimum of 100 individuals to be counted. If samples were not counted the same day as collected, they were fixed in Lugol's iodine solution for counting and scoring the following day. Larvae were observed under a Zeiss Srerri DV4 dissecting scope.

Survival was determined by comparing the number of survivors (i.e. present larvae) to the initial number of larvae, and accounting for sampling loss. Development was determined by scoring the developmental stage (e.g. auricularia, dolioloria, pentactula) of each larva. Larvae were considered doliolaria when they shrunk into the barrel shape and rings of cilia were observed. Pentactula was marked by the emergence of a tube foot.

Statistical Analysis

Mean survival and percentage of larvae at each developmental stage were calculated for each replicate container and used in subsequent statistical analyses. One-way analysis of variance (ANOVA) was performed to examine the effects of temperature on survival and development using the software JMP Pro 12 for Mac. Differences among treatment means were determined by Tukey's tests, the differences being considered significant when $P<0.05$.

RESULTS

Survival

Out of all treatments, the coolest temperature generally had the highest number of individuals surviving over the duration of the experiment. Further, for all treatments, survivorship declined with time. Each replicate for each treatment was initially inoculated with 3,000 individuals. On days 11, 17, and 24 the larvae reared at 12°C had the highest survival of the treatments, with the mean number of individuals surviving being 1178, 897, and 435 individuals, respectively, declining with time (Table 4). The survival of the larvae reared in 24°C was the highest of all treatments on day five (2175 individuals) but then drastically decreased to 164 individuals on day 11 (which was the lowest value of all treatments). Survival was negligible on days 17 and 24 (Table 4). With the exception of day 5, higher survival over all time periods correlated with the colder the temperature treatment, with the highest survival at 12°, 15°, 18°, 21° and 24°C respectively (Table 4, Figure 22).

The effect of temperature on survival was statistically significant. The one-way ANOVA on differences of survival of larvae reared in different temperatures indicated a high level of significance on all of the sampling days; with $p=0.0024$ on day five and $p<0.0001$ on days 11, 17, and 24 (Tables 5, 6, 7 and 8). Day 5 ($F(4,25)=5.5661$, $p=0.0024$) pair-wise comparison test indicated a statistically significant difference between the 18° and 24°C, and 21° and 24°C treatments (Table 5). Day 11 ($F(4,25)=8.9448$, $p=0.0001$) pair-wise comparison test indicated a statistically significant difference between the 12° and 21°C, 12° and 24°C, 15° and 21°C, and 15° and 24°C (Table 6), with the cooler temperature always having the highest number of surviving individuals.

Day 17 ($F(4,25)=22.3659$, $p=< .0001$) pair-wise comparison test indicated a statistically significant difference between the 12°C and all the other treatments, and between 15° and 21°C, and 15° and 24°C (Table 7), with the cooler temperature always having the highest number of surviving individuals. Day 24 ($F(4,25)=15.0585$ $p<.0001$) pair-wise comparison test also indicated a statistically significant difference between the 12°C and all other treatments as well, and between the 15° and 24°C treatments (Table 8), also with the cooler temperature always having the highest number of surviving individuals

Days	Temperature (°C)				
	12	15	18	21	24
5	1800 ± 84.8	1675 ± 189.4	1433.3 ± 153.9	1436.1 ± 157.6	2175 ± 110.0
11	1177.8 ± 108.2	966.7± 209.9	644.4 ± 73.7	352.8 ± 158.7	163.9 ± 108.9
17	897.2 ± 93.2	483.3 ± 106.2	261.1 ± 55.6	127.8 ± 68.7	Nil
24	434.5 ± 57.0	188.7 ± 48.4	131.7 ± 18.7	104.2 ± 51.8	Nil

Table 4. Mean (±S.E., n=6) survival of the number of individuals of *P. californicus* larvae observed at different temperatures.

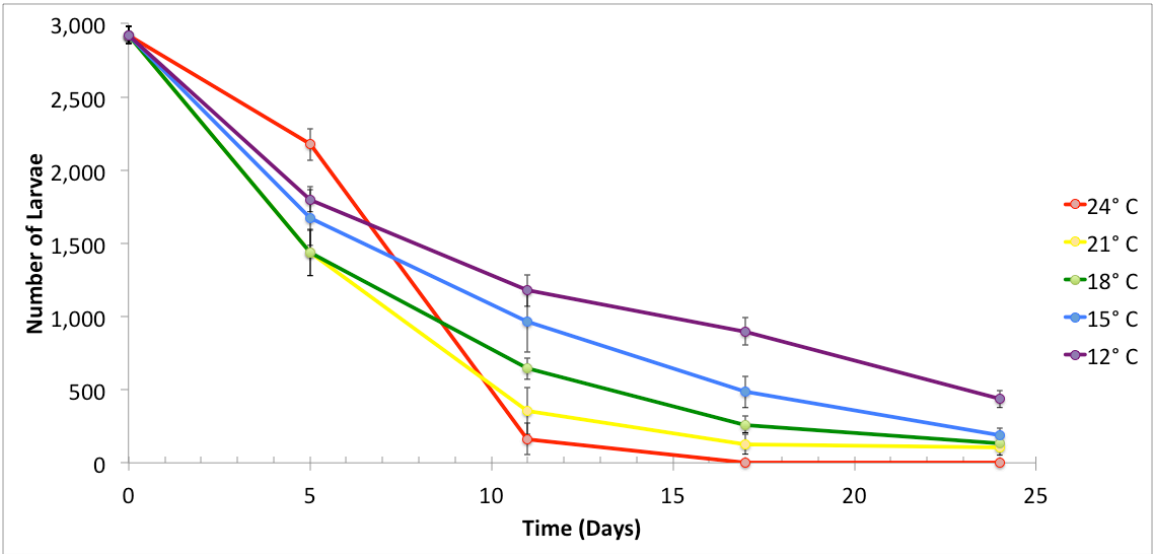

Figure 22. Graph of the mean survival of *P. californicus* larvae at different temperatures.

Treatments	sum of squares	*df*	Mean square	F ratio	P value
Between groups	2261537	4	565384	5.5661	< 0.0024
Within Groups	2539398.1	25	101576		
Total	4800935.2	29			
HSD					
	24				
18	0.0038				
21	0.004				

Table 5. ANOVA table on survival of *P. californicus* larvae on day 5 at different temperatures with results of Tukey post-hoc HSD test.

Treatments	sum of squares	*df*	Mean square	F ratio	P value
Between groups	4217314.8	4	1054329	8.9448	< 0.0001
Within Groups	2946759.3	25	117870		
Total	7164074.1	29			
HSD					
	21	24			
12	0.0028	0.0002			
15	0.0351	0.0036			

Table 6. ANOVA table on survival of *P. californicus* larvae on day 11 at different temperatures with results of Tukey post-hoc HSD test.

Treatments	sum of squares	*df*	Mean square	F ratio	P value
Between groups	2981629.6	4	745407	22.3659	< 0.0001
Within Groups	833194.4	25	33328		
Total	3814824.1	29			
HSD					
	15	18	21	24	
		<.000			
12	0.0049	1	< .0001	< .0001	
15			0.0187	0.0009	

Table 7. ANOVA table on survival of *P. californicus* larvae on day 17 at different temperatures with results of Tukey post-hoc HSD test.

Treatments	sum of squares	*df*	Mean square	F ratio	P value
Between groups	623834.2	4	155959	15.0585	< .0001
Within Groups	258921	25	10357		
Total	882755.2	29			
HSD					
	15	18	21	24	
12	0.0026	0.0002	< .0001	< .0001	
15				0.0305	

Table 8. ANOVA table on survival of *P. californicus* larvae on day 24 at different temperatures with results of Tukey post-hoc HSD test.

Development

The effect of temperature on development was statistically significant. The 12°C treatment had the highest mean percentage of the first larval stage of auricularia and the lowest mean percentage of the third larval pentactula stage throughout the experiment (Table 9). The larvae were first observed to metamorphose to dolioloria on day 11 in the 18°, 21°, and 24°C treatments (Figure 23, Table 9). The highest occurrence of dolioloria was in the 18°C treatment, with a mean percentage of 29.4% (Table 9). The larvae were observed to metamorphose into the final pentactula stage on day 17 in all the treatments, with the highest occurrence in the 18°C treatment at a mean percentage of 59% (Figure 23, Table 9). On day 24 the highest mean percentage of larvae at the pentactula stage correlated with the highest temperatures occurring at 98%, 85%, 80%, 74%, and 46% in 24°, 21°, 18°, 15°, and 12°C, respectively (Figure 25, Table 9).

The one-way ANOVA on differences of percentage of larvae at each developmental stage in different temperatures indicated a high level of significance between auricularia on day 11 with p=0.0176, between auricularia and pentactula on day 17 with p <.0001 for both, and between dolioloria on day 24 with p=0.0021 (Tables 10, 11, 12, and 13). Day 11 auricularia (ANOVA; F(4,25)=3.6621, p=0.0176) pair-wise comparison test indicated a significant different between the 12° and 21°C treatments, and between the15° and 21°C treatments (Table 10). Day 17 auricularia (ANOVA;

F(4,25)=15.3509, p<.0001) pair-wise comparison test indicated a statistically significant difference between 12°C and all other temperature treatments, and between 15° and 24°C (Table 11). Day 17 pentactula (ANOVA; F(4,25)=14.157, p<.0001) pair-wise comparison test indicated a statistically significant difference between 15° and 12°C, between 15° and 24°C, between 18° and 12°C, between 18° and 21°C, and between 18° and 24°C treatments (Table 12). Day 24 auricularia (ANOVA; F(4,25)=2.6162, p=0.0593) pair-wise comparison test indicated a statistically significant difference between the 12° and 24°C treatments (Table 13).

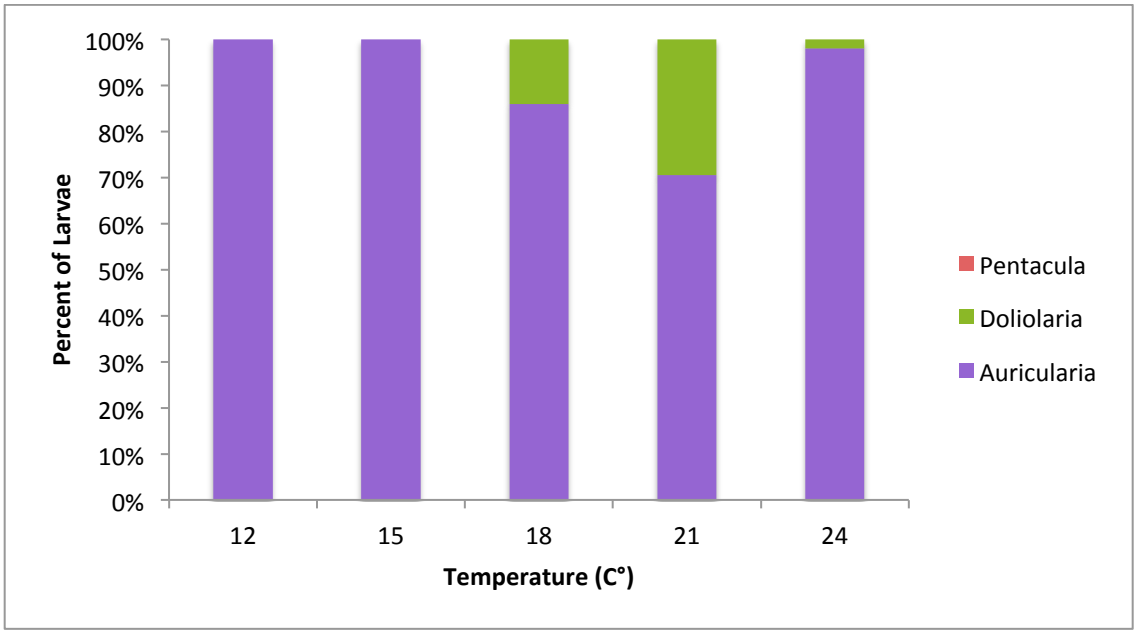

Figure 23. Stacked bar graph of the mean percentage of larvae at each developmental stage reared in different temperatures on day 11.

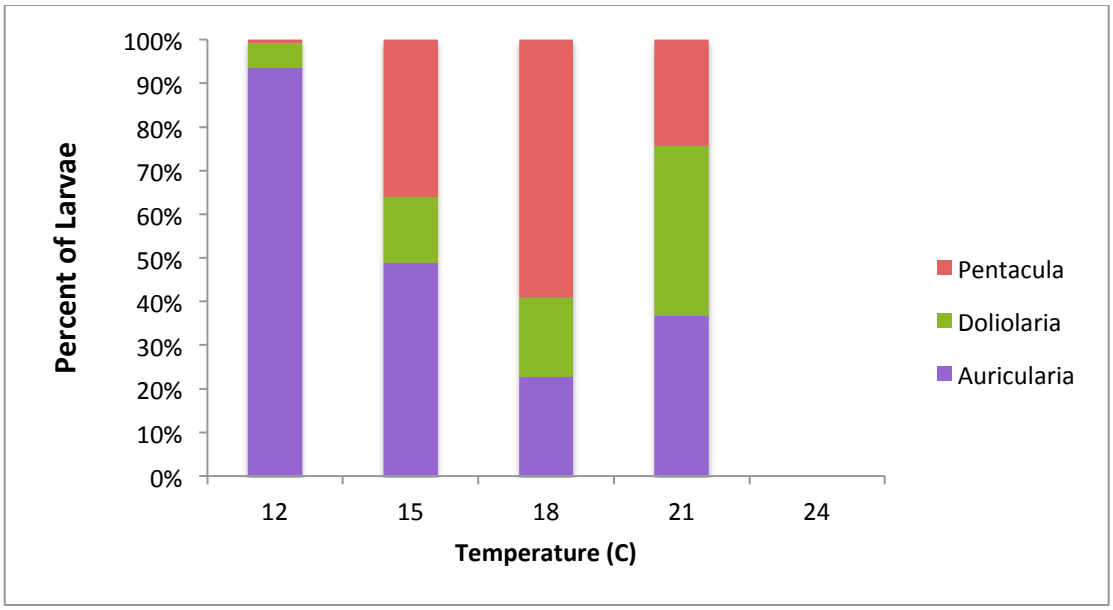

Figure 24. Stacked bar graph of the mean percentage of larvae at each developmental stage reared in different temperatures on day 17. *There were not enough live larvae on day 17 to get a count.

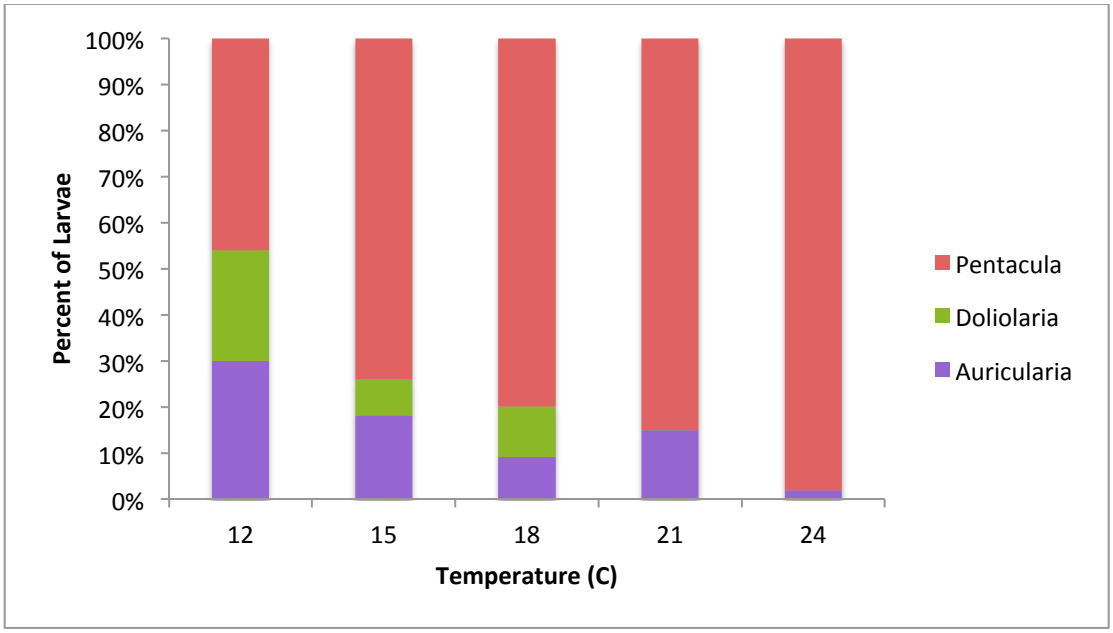

Figure 25. Stacked bar graph of the mean percentage of larvae at each developmental stage reared in different temperatures on day 24.

Days	Developmental Stage	Temperature (°C)				
		12	15	18	21	24
5	Auricularia	100±0	100±0	100±0	100±0	100±0
	Doliolaria	0±0	0±0	0±0	0±0	0±0
	Pentacula	0±0	0±0	0±0	0±0	0±0
11	Auricularia	100±0	100±0	86±4.4	70.6±16.7	98.1±20.7
	Doliolaria	0±0	0±0	14±04.4	29.4±9.8	1.9±1.6
	Pentacula	0±0	0±0	0±0	0±0	0±0
17	Auricularia	93.6±1.3	48.8±11.9	22.8±2.3	36.7±16.2	0±0
	Doliolaria	5.6±1.6	15±4.5	18.1±3.9	38.9±16.2	0±0
	Pentacula	0.84±0.6	36.1±1.6	59±4	24.3±10.3	0±0
24	Auricularia	29.9±3.6	18.2±5.6	9±1.3	14.9±13	1.9±1.3
	Doliolaria	24.1±7	7.8±3.6	11.1±4.9	0.1±.1	0±0
	Pentacula	46±6.5	74±9.1	80±5.2	85±13	98±20.7

Table 9. Mean (±S.E., n=6) percentage of *P. californicus* larvae at each developmental stage at different temperatures.

Treatments	sum of squares	*df*	Mean square	F ratio	P value
Between groups	12804.48	4	3201.12	3.6621	0.0176
Within Groups	21853.192	25	874.13		
Total	34657.672	29			
HSD					
	21				
12	0.0348				
15	0.0348				

Table 10. ANOVA table of the percentage of *P. californicus* auricularia larvae on day 11 at different temperatures with results of Tukey post-hoc HSD test.

Treatments	sum of squares	*df*	Mean square	F ratio	P value
Between groups	30374.181	4	7593.55	15.3509	<.0001
Within Groups	12366.627	25	494.67		
Total	42740.807	29			
HSD					
	15	18	21	24	
12	0.0145	<.0001	0.0001	<.0001	
15				0.0067	

Table 11. ANOVA table of the percentage of *P. californicus* auricularia larvae on day 17 at different temperatures with results of Tukey post-hoc HSD test.

Treatments	sum of squares	*df*	Mean square	F ratio	P value
Between groups	15218.427	4	3804.61	14.157	<.0001
Within Groups	6718.594	25	268.74		
Total	21937.021	29			
HSD					
	12	21	24		
15	0.0081		0.0065		
18	<.0001	0.0011	<.0001		

Table 12. ANOVA table of the percentage of *P. californicus* pentactula larvae on day 17 at different temperatures with results of Tukey post-hoc HSD test.

Treatments	sum of squares	*df*	Mean square	F ratio	P value
Between groups	2356.0536	4	589.014	5.6937	0.0021
Within Groups	2586.2621	25	103.45		
Total	4942.3157	29			
HSD					
	21	24			
12	0.0033	0.0032			

Table 13. ANOVA table of the percentage of *P. californicus* pentactula larvae on day 24 at different temperatures with results of Tukey post-hoc HSD test.

DISCUSSION

Larval development of sea cucumbers has been extensively described in previous studies (Dautov 1997; Hamel and Mercier, 1996; Mashanov and Dolmatov, 2000; McEuen and Chia, 1991; Morgan, 2008a; Smiley, 1986; Strathmann, 1971). The effect of egg source, fertilization, food availability, diets, and environmental conditions on larval survival, growth, and development have been examined for select species globally (Asha and Muthiah 2006; Kashenko 1998, 2002; Martinez and Richmond 1998; Morgan, 2001, 2008, 2009b, 2009c; Ren et al 2016). However, despite all of the available information, there is still little information directly pertaining to sea cucumber aquaculture techniques. Furthermore, most studies have focused tropical sea cucumber species and there is little information regarding ideal environmental parameters for rearing *P. californicus* in hatcheries. Previous studies found that the developmental rates of marine invertebrates generally increase with temperature until a lethal maximum is approached (Strathmann and McEuen, 1987). Echinoderm larvae show increased mortality, delayed development, reduced growth, and reduced metabolic rate in response to temperature stress (Bressen et al., 1995; Przeslawski 2005; Roller and Stickle, 1989, 1993). The optimal thermal range for rearing larvae in a hatchery has been determined for a few other tropical species globally, however, there has not been any studies conducted to determine the optimal thermal range to rear *P. californicus* larvae in hatcheries. It will be imperative to develop

reliable spawning techniques in order for hatcheries to have access to large number of gametes. Developing reliable spawning methods and determining the optimal thermal range for rearing larvae at in a hatchery will further advance the development of aquaculture techniques for *P. californicus*.

Spawning

The first step of developing hatchery techniques for *P. californicus* is to determine reliable methods for inducing spawning in order for hatcheries to have access to large number of gametes and subsequently larvae. Methods for inducing spawning in sea cucumbers have been developed for many species globally, however, spawning methods for *P. californicus* are still in the early development stage and further studies need to be conducted in order to develop more reliable methods.

Spawning became a bottleneck for this experiment. Per our communication with Alutiiq Pride Shellfish Hatchery, Lummi Tribe, and after reviewing published studies conducted by Cameron and Fankboner (1986) in the San Juan Islands and Alaska, we expected our broodstock to spawn much earlier in the season from April to August, with the peak of the spawning occurring May to mid-July. When we attempted to spawn our broodstock collected from Clam Bay, WA, at the hatchery from mid-May to early September we did not have a successful spawn until July 15, 2016, and from that point forward we had success until our last spawn attempt on September 7, 2016, suggesting our broodstock had a later spawning window than observed in previous studies in the San Juan Islands and Alaska. Our broodstock was collected from a different geographical location much further south than the ones observed in previous studies. It is not surprising that our population has a different spawning season window, due to the difference in physical parameters such as location, seawater temperature, salinity, food availability, and seawater chemistry.

The amount of stimulation and spawning methods used did not appear to effect spawning success, as all successful spawns only required thermal shock, indicating that time of season is more important than method and degree of stimulation used for inducing spawning. Alutiiq Pride Shellfish Hatchery only used thermal stimulation for successfully inducing spawning. When we did not have a successful spawn from mid-May to early July, we used other methods in hopes of triggering females to release oocytes. Along with thermal shock, we also tried the addition of algae, gonad extraction, light stimulation, desiccation, water pressure, and using larger individuals without success. The successful spawns on July 15, August 4, and September 7 only required thermal stimulation.

Experiment

Temperature is an important environmental parameter that hatcheries must control during larval rearing because it strongly affects the survival and metamorphosis rate for

each stage of sea cucumber larvae (Ito and Kitamura, 1998; McGurk, 1984). Studies have indicated that thermal stress results in increased mortality, delayed development, and reduction in growth (Bressen et al., 1995; Przeslawski 2005; Roller and Stickle, 1989, 1993). The study by McEdward (1985) suggests that larvae cannot increase their feeding capacity at high temperatures to meet the energy demands of the increased metabolism for the accelerated development that occurs at higher temperatures thus, leading to deformation and mortality.

Survival

In this study the larvae reared at the highest temperature (24°C) had the highest survival of all treatments initially on day 5 (73%), but survival dramatically declined by day 11 (5%), which resulted in decreased survival directly correlating with increased temperature for the remainder of the experiment. The initially high survival in the 24°C treatment could be explained by pathogens initially dying off. Larvae are extremely sensitive to pathogens present in culture tanks. Higher temperatures result in the increased growth of potentially pathogenic ciliates and bacteria in cultures, which leads to the proliferation of invaders and fouling organisms that bring their own microflora and disease problems (Cook et al., 2005; Gruffydd and Beaumont, 1972; His et al, 1989; Olafsen, 2001). Typically, pathogens flourish in heated conditions and have a higher transmission rate between shellfish larvae in heated conditions (Dorfmeier et al. 2011). If the temperature range exceeds the thermal threshold of the pathogen, then larvae wouldn't have to fight off infection. The 24°C treatment could have killed off the pathogens initially, resulting in the highest survival of the temperature treatments. However, the larvae were not able to keep up with the metabolic demands of the heated conditions and quickly declined in survival by day 11. It is important to note that data was not collected on pathogens, and this theory is speculative, but is consistent with the literature and findings from other shellfish researchers.

Larvae reared at 12°C had the highest survival of all the treatments after day 5 with a 14% survival at the end of the experiment on day 24, which was significantly higher than the other treatments, leading us to conclude that hatcheries should rear larvae at a maximum temperature of 12°C for optimal production. This temperature corresponds to the average water temperature of 12.7-13.33°C for July-September, the time of spawning in the Seattle, WA area, as reported by NOAA in the Water Temperature of All Coastal Regions database. This indicates that hatcheries using ambient water pumped in from the sound may be able to rear larvae during the spawning season at 12°C without having to heat or cool the water.

Our findings agree with previous studies, which found that increased thermal stress results in lower sea cucumber larval survival. Too high or too low temperature outside of the threshold range results in low survival and delayed metamorphosis (Asha and Muthia, 2005; Hamel and Mercier, 2005; Kashenko, 1998; Sui, 1990). All previous

temperature studies on larval survival, growth, and development have focused on tropical species and mostly on the early auricularia stage. Li Li et al (2011) found that 21-24°C was optimal for the early development of the tropical species *A. japonicas*. Asha and Muthia (2005) had similar results with the tropical species *H. spinifera* and determined the ideal temperature range that larvae should be reared at is 28-31°C, which is on the higher end of the temperature range of their natural environment (25.5-31.2°C).

Development:

The assessment of larval development during rearing is critical for determining the competence of sea cucumbers to successfully complete the larval cycle and to reach settlement (Morgan 2001, 2008b, 2009b, 2009c). My findings agreed with previous larval studies, which found that, thermal stress affects the metamorphosis and development of sea cucumber larvae (Sui, 1990). Sea cucumber larvae develop rapidly at high temperatures but display slower growth and delayed metamorphosis at lower temperatures as seen in other cultured species (Asha and Muthiah, 2005; Ito et al, 1998; Li Li et al, 2011; Liu et al, 2010; Pan et al., 1997; Sui, 1990). The extension of the larval period requires hatcheries to use more time and resources to care for the delicate life phase.

In this experiment, decreased temperatures resulted in delayed metamorphosis. The lowest temperature treatment (12°C) had the highest mean percentage of auricularia larvae and the lowest mean percentage of pentactula throughout the experiment. The larvae were first observed to metamorphose into the second larval phase of dolioloria on day 11 in the treatments of 18°, 21°, and 24°C. The final larval phase pentactula was first observed on day 17, with the highest occurrence in the 18°C treatment at a mean percentage of 59%. On day 24 there was a statistically significant difference between the coldest treatment (12°C) and the highest treatment (24°C), with the lowest percentage of pentactula occurring in the 12°C. treatment.

Similarly, Hamel and Mercier (1996), Ito and Kitamura (1998), and Li Li et al. (2011) also reported rapid development of *C. frondosa*, *I. japonicas*, and *H. spinifera* larvae, respectively, at higher temperatures than the prevailing natural environmental conditions for the species. Li Li et al (2011) found higher survival, growth, and metamorphosis occurred at 21° and 24°C for *A. japonicas* larvae, and no metamorphosis to doliolaria were observed in the lowest temperature treatment of 18°C. Asha and Muthiah (2015) determined that water temperatures of 28°C would be optimal for the normal growth and development of *H. spinifera* auricularia larvae.

Larval culture of *P. californicus* is currently hindered by poor survival to metamorphosis, and this was evident in this study. The overall survival rates of this study were significantly lower than previous studies conducted on sea cucumber larvae. Previous studies focused on tropical species and only focused on the early auricularia and doliolaria stages. Ren et al (2016) evaluated the effect of microalgal diets on body length,

survival, and metamorphosis of *P. californicus* auricularia larvae. At day 16 the highest survival was 62.4% and the lowest was 38.7% found in the unfed treatment, which was higher than the highest survival of 30% on day 17 of this experiment. Low survival could be due to the gap in knowledge of other parameters such as salinity, stocking density, photoperiod, and the limited genetic variation, as the experiment was inoculated with the cross of five males and a single female.

In conclusion, *P. californicus* only need thermal stimulation to induce spawning when females are ripe. Our broodstock collected from Clam Bay, WA appears to have a spawning season ranging from mid-July to mid-September. The findings from this experiment agree with previous studies that found that temperature stress results in increased mortality and delayed development. Our findings indicate that hatcheries should rear *P. californicus* larvae at a maximum of 12°C for optimal survival and production. However, more studies should be conducted to determine the lower thermal threshold, which may be lower than 12°C. Rearing larvae at 12°C will require hatcheries to use more resources to care for the larvae during the extended larval phase. 12°C is within the ambient temperature range of Seattle area and should be feasible for many small hatcheries to maintain while rearing larvae in July-September.

This experiment suggests that the development of aquaculture of *P. californicus* is possible, but further studies need to be conducted to better understand broodstock conditioning, spawning, larval culturing, and juvenile grow out. Further studies need to focus on determining the optimal conditions for larval rearing such as salinity, stocking density, types of culture tanks, DO, diets, and other physical characteristics to increase the survival rates of larvae in order for aquaculture to be a reasonable option for hatcheries. The development of aquaculture of *P. californicus* would relieve pressure on wild stocks in Washington State and allow PSRF and other restoration work to be carried out in Puget Sound. Once hatcheries are able to produce large numbers of juveniles, then they can out plant them to the populations that have been greatly reduced by overfishing. I would expect broodstock collected in other areas to have varying spawning seasons, as seen with our broodstock. I would also expect that larvae should have similar optimal thermal ranges, but future studies should determine if 12°C is also optimal for larvae spawned from broodstock collected in other areas.

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