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Wasted and Castrated: Two Diseases Affecting the Ochre Star, Pisaster ochraceus, in North America

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Wasted and Castrated: Two Diseases Affecting the Ochre Star, *Pisaster ochraceus*, in North America

By

Zoë Zilz

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of the Requirements for the Degree
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<u>Master's Thesis</u>

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Zoë Zilz

16 August 2018

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A Thesis
Presented to
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ABSTRACT

Understanding the diseases that plague marine organisms is essential to the management and conservation of coastal ecosystems, especially in the face of a possible sixth mass extinction. An increase in mass-mortality events, often caused by epizootics, is modifying intertidal ecosystems. When predators that have disproportionately large trophic impacts on their community and maintain community structure (i.e., keystone predators) suffer from widespread population declines it destabilizes population dynamics ecosystem-wide, and can have long-term or sometimes permanent effects. This thesis is comprised of two studies that examined two maladies affecting a keystone predator, the ochre star *Pisaster ochraceus,* in Eastern Pacific intertidal zones. A recent massive die-off event affecting sea stars, referred to as sea star wasting disease (SSWD), devastated populations of *P. ochraceus* along the West Coast of North America. *Pisaster ochraceous* also hosts a ciliate parasite, *Orchitophyra stellarum,* that partially castrates males and occupies the epidermis of both sexes, presumably in a commensal association with the sea star. I was interested in using whole-arm removal as a tool for diagnosing *O. stellarum* infections, but whether it had a negative impact on sea star health in concurrence with SSWD needed to be confirmed. I asked whether *P. ochraceus* subjected to surgical arm removal were more susceptible to SSWD and to death from that disease, and designed a three-part experiment to answer that question. I also wanted to understand the potential impact of *O. stellarum* on *P. ochraceus* populations, and needed to start by determining its distribution and prevalence throughout its host range. I surveyed *P. ochraceus* populations for *O. stellarum*, both in the gonads and on the epidermis of its host, along the West Coast of the United States. I explored several factors that could explain variation in the prevalence of this parasite. I also attempted to understand if SSWD contributed to a shift in host-parasite dynamics

by comparing current prevalence to historical reports. *Pisaster ochraceus* does not appear to suffer or die from SSWD after having an arm surgically removed, increasing my confidence in the use of this methodology for this and future studies, although due to my small sample size, I conclude that the potential negative effects of surgical arm removal require further examination. Prevalence of *O. stellarum* infections in the gonads of *P. ochraceus* was very low, but the ciliate was present on the epidermis of 51% of sea stars. I compared current percent prevalence of epidermal association with *O. stellarum* with data from Stickle & Kozloff (2008) and found that, at the three sites they surveyed, percent prevalence has not changed significantly. I did find that smaller *P. ochraceus* populations had a higher prevalence of ciliates. Although this result contradicted my hypothesis and widely accepted epidemiological models, it could be due to parasite-mediated mortality or (more likely) reductions in reproductive output due to castration by *O. stellarum*. I also found that populations with higher percentages of males had a higher prevalence of ciliate association, which is to be expected because *O. stellarum* primarily parasitizes testes. There is high geographic variation in epidermal ciliate prevalence, but there is no clear pattern linking variation in prevalence to sampling region or latitude. This agrees with previous research that reported high variability in both space and time for ciliate infections, although this study is the first to provide information on epidermal *O. stellarum* association at a fine spatial resolution (previous studies focused on infection, or only sampled a few geographically separate sites).

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Chapter 1
Background and Overview

A recent increase in the incidence and extent of marine disease has destabilized community dynamics in some marine populations (Burge *et al.*, 2014). Host-pathogen relationships in marine environments are prone to change as a result of even small shifts in biotic and abiotic conditions and the cascading effects of such shifts. When disease disrupts trophic interactions, it can change the structure of the entire ecosystem, especially in intertidal and nearshore subtidal communities, where trophic interactions regulate population dynamics (Steneck *et al.*, 2004). Trophic regulation by some predatory organisms has a top-down effect on prey communities that is disproportionate to predator population size; these are referred to as keystone predators (Paine, 1966; Power & Mills, 1995; Power *et al.*, 1996). Understanding how diseases can affect the populations of intertidal and subtidal organisms, especially keystone predators, helps researchers predict the stability of marine communities in response to potentially increasing diseases in the ocean (Collinge *et al.*, 2008).

On the whole, infectious diseases are predicted to increase in ocean communities, but the fate of parasites specifically is less clear (Lafferty, 2003). The parasite communities of intertidal and sub-tidal zones of the ocean have recently received attention, especially in the case of parasitic castrators and infectious diseases that regulate host abundance (Mouritsen & Poulin, 2002). Parasite abundance, intensity, and prevalence can also be regulated by host population size (Anderson & May, 1979; 1981), so investigations of intertidal parasite communities should take into account potential changes in host-population dynamics. For example, highly host-specific parasites are likely to go extinct if their host population does (Dobson *et al.*, 2008; Hechinger & Lafferty, 2005). However, we have little understanding of how parasitism changes in host populations that have been greatly reduced, but not driven extinct, due to the inadvisability of experimental tests of such questions. Mass die-off events are increasing in

frequency, contributing to the reduction of many marine populations, and also providing an opportunity to study the ecological consequences of sudden and substantial population declines. Some models have predicted that increasing mortality is due to an increase in infectious diseases, but other research suggests that parasite species richness will decrease as ocean temperature increases; essentially, response of diseases to climate change will depend on existing host-pathogen and host-parasite relationships (Lafferty, 2003; 2009).

Mass-mortality Events

One consequence of both climate change and shifting marine diseases is the recent surge of mass-mortality events (Fey *et al.*, 2015). Mass die-offs usually occur in a short time-span relative to the generation time of the organism, indiscriminately affecting relatively large proportions of affected populations, regardless of age, sex, or size class. Many of these events are attributed to climate change (Coma *et al.*, 2009; Garrabou *et al.*, 2009), both because rising temperature and anthropogenic input are known stressors (Harley *et al.*, 2006a; Harvell *et al.*, 2002; Harvell *et al.*, 2001; Lafferty *et al.*, 2004), and because changing climate can shift the geographical and host range of infectious agents (Harvell *et al.*, 2009; Secord & Kareiva, 1996). Because mass die-offs of marine organisms have intensified in frequency and scope in recent years (Fey *et al.*, 2015; Jurgens *et al.*, 2015), understanding how sudden and catastrophic declines affect host-parasite relationships is essential to address conservation and management techniques for marine populations after mass mortality events.

Mass mortality can further impact host populations by changing host-pathogen dynamics. Often, widespread disease and death result in feedback loops, either facilitating an increase or decrease in future epizootics, depending on host and disease ecology (Collinge *et al.*, 2008). For

example, bleaching in corals often leads to opportunistic secondary infections by bacteria, furthering the extent of mortality (Harvell *et al.*, 1999; Harvell *et al.*, 2001). A change in host density can directly produce positive or negative responses in parasite or pathogen abundance (Lafferty *et al.*, 2004). Depending on the host-specificity of the pathogen in question, host population reductions can lead to reduced opportunity for disease transmission or disrupt parasite life cycles. Alternatively, top-down effects of keystone predator mortality often include population explosions at lower trophic levels, resulting in increased potential for disease outbreaks in prey species (Lafferty, 2004; Lafferty & Kuris, 1993). Additionally, sufficient population reduction limits the genetic diversity of a population, potentially leaving the remaining individuals more susceptible to diseases, but it can also have the opposite effect of selecting for pathogen resistance (Altizer *et al.*, 2003; Collinge *et al.*, 2008).

Sea Star Wasting Disease

On the West Coast of North America, a recent mass-mortality event of stellate echinoderms represents the largest die off of a non-commercially important marine species in recent history (Eisenlord *et al.*, 2016; Miner *et al.*, 2018). This die-off has been attributed to a syndrome known as sea star wasting disease (SSWD). The mortality event began in summer 2013, and quickly devastated multiple species of sea stars on the West Coast, including both subtidal and intertidal taxa. Unprecedented numbers of dead and dying sea stars were first noticed during long-term monitoring surveys in Olympic National Park in Washington. The majority of sea stars with symptoms were *Pisaster ochraceus* and *Pycnopodia helianthoides*, and other species affected included *Orthasterias koehleri*, *Pisaster brevispinus*, *Pisaster giganteus*, *Evasterias troschelii*, *Solaster* spp., *Dermasterias imbricata*, *Mediaster aequalis*, *Leptasterias* spp, and *Patiria miniata*

(MARINe, 2013). Recovery has been slow and intermittent, resulting in abundances that are still

as low as 25% of pre-wasting numbers (Eisenlord *et al.*, 2016; Menge *et al.*, 2016; Miner *et al.*,

2018). Currently, many sea star populations are still exhibiting signs of being afflicted with

SSWD, although prevalence is much lower than during the initial outbreak (Moritsch &

Raimondi, 2018).

The appearance of SSWD is characterized by a suite of pathologies that usually result in

the death of the afflicted individual. The first signs of the disease can include small

(<0.5 cm) lesions indicating loss of epidermal tissue, turgor loss or "deflation" of the sea star,

and the twisting or entwining of rays. These "symptoms" are also indicative of thermal stress and

other health issues in sea stars, and some sea stars recover from these afflictions, especially if

maintained at lower temperatures (Kohl *et al.*, 2016). However, if sea stars develop any of the

later signs of SSWD, including multiple larger lesions, tissue or arm loss, and body

fragmentation (often described as "melting"), they rarely recover and death shortly follows

(Eckert *et al.*, 2000). In fact, onset of SSWD in an individual usually results in the death of the

sea star in a matter of days, which is presumably why it has had such rapid and devastating

effects on wild populations.

Despite the predictable progression of symptoms associated with SSWD, the etiology of

the disease is unclear. The pattern and timing of SSWD emergence across the West Coast does

not mimic that of an infectious disease epidemic "spreading" from population to population.

Recent research suggests that the SSWD epizootic onset occurred in conjunction with pulses of

higher-than-average ocean temperatures, in combination with potential changes in existing

microbial communities (Eisenlord *et al.*, 2016; Hewson *et al.*, 2018; Hewson *et al.*, 2014).

However, in some locations in Oregon, SSWD occurred in concert with decreases in ocean

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temperature (Menge *et al.*, 2016). Immediately following the outbreak, Hewson *et al.* (2014) implicated a virus termed Sea Star Associated Densovirus (SSaDV). Hewson *et al.* (2014) reported results from a series of experimental infections in which SSWD-associated pathology was most often initiated by injection with virus-sized material from symptomatic sea stars, which was then meta-genomically identified as a densovirus. However, the same researchers found the same densovirus present in tissues of apparently healthy sea stars, as well as sea stars in museum collections from before the discovery of SSWD (Hewson *et al.*, 2014).

Population declines due to echinoderm "wasting" are not a new phenomenon. Since 1970, there have been reports of sea stars developing signs similar to SSWD and dying in large numbers. Echinoderms as a whole have been called a "boom-bust" phylum, and some echinoderm taxa consistently experience cyclical, potentially density-dependent population fluctuations (Uthicke *et al.*, 2009). However, the ability of echinoderm populations to recover after a die-off is inconsistent. Ten sea star species (as well as other echinoderm taxa) at the Channel Islands in California experienced a significant wasting event in 1997, and populations of *Pisaster giganteus* and *Patiria miniata* stayed low throughout the next year (Eckert, 2000). Long-term monitoring data for *Pisaster ochraceus* populations presented by Miner *et al.* (2018) indicates that, in the Channel Islands, low numbers in the early 2000s were followed by steady increases in abundance until the abrupt population crash in 2013. *Heliaster kubinjii* in the Gulf of California suffered massive population reductions in the 1980s, and while populations stayed low for several decades, there is evidence that, recently, some populations have recovered (Dungan *et al.*, 1982; Eckert *et al.*, 2000). Considering that even dramatic population fluctuations are relatively normal, and that potential etiological agents (i.e. SSaDV) are found on healthy sea stars, it is likely that SSWD is a phenomenon that is intrinsic to sea star populations. However,

the recent epizootic occurred across a wider geographical, taxonomic, and temporal scale than ever recorded for previous wasting events (Miner *et al.*, 2018).

Wasting related mortality of sea stars is likely to have long-lasting effects on the populations of both sea stars and other intertidal species. The recent wasting event was followed by a large increase in the density of juvenile (< 30 mm) sea stars (Miner *et al.*, 2018). However, estimated juvenile mortality was 90% higher post-SSWD, suggesting that either SSWD or some other variable was impacting survivorship of post-SSWD recruits. Many sea star species affected by SSWD are keystone predators and have a regulatory impact on lower trophic levels that is disproportionate to sea star abundance. In the aftermath of SSWD, intertidal communities have already experienced changes in trophic structure and interspecific competition due to the reduction in these ecologically important taxa. For example, the reduction in *Pycnopodia helianthoides* in British Columbia resulted in increases in many of its prey species, including a grazing sea urchin, and subsequent declines in kelp cover (Schultz *et al.*, 2016). *Tegula* sp. populations shifted their vertical size distribution and intertidal foraging patterns in response to the disappearance of *Leptasterias* spp. from the deeper intertidal zones, and their population also nearly doubled (Gravem & Morgan, 2017). Furthermore, predation pressure by keystone species is often directly related to their population. Because sea star larval and juvenile recruitment was high following SSWD, sea star populations are now skewed towards smaller size classes. For *Pisaster ochraceus* especially, less adult biomass equates to lower predation pressure. Because smaller sea stars are not as effective at consuming their mussel prey, and because they take years to reach adult sizes, a high proportion of juveniles in the population changes the ecological function of *P. ochraceus* on a larger time-scale (Moritsch & Raimondi, 2018).

Pisaster ochraceus

The ochre star, *Pisaster ochraceus*, was one of several species of Asteriid sea stars that were previously very common until heavily impacted by SSWD, and much of the research on SSWD has focused on this sea star's recovery and the effects of its decline on intertidal ecosystems. The ochre star lives in the intertidal and, less commonly, the subtidal zones on the West Coast of North America from Alaska to Baja California. This sea star is easily recognizable because it is a charismatic tide pool animal that was the original "keystone predator". Paine (1966) conducted a series of experiments removing *P. ochraceus* from rocky intertidal areas in Washington state and determined that this predator exerted disproportionate pressure on its community. When the sea stars were removed, their competitively dominant prey, *Mytilus californianus,* would colonize the bare rock in lower intertidal areas normally occupied by *P. ochraceus.* Left unchecked by the predatory sea stars, mussels outcompeted other sessile intertidal invertebrates, often homogenizing the tide pool community. It is easy to see the effect of this keystone predator without conducting experiments as well; in most intertidal areas observers can see a distinct line where *P. ochraceus* meet *M. californianus* on the rocks, preventing the mussels from using lower levels of the intertidal zone. Predation pressure by *P. ochraceus* varies spatially (Menge *et al.*, 1994), with temperature (Sanford, 1999), and in response to prey composition (Robles *et al.*, 1995; Robles *et al.*, 2009). Regardless, declines in this sea star due to SSWD will likely have consistent effects on the structure of Eastern Pacific intertidal communities.

Initially, SSWD reduced populations of *P. ochraceus* dramatically. Some populations completely disappeared, and declines were the most severe in Southern California and the Channel Islands, where over half of surveyed populations decreased by greater than 99%, and in inland water bodies (the Salish Sea and San Francisco Bay) (Miner *et al.*, 2018). Generally, north

of Point Conception, population crashes were slightly less intense, but still resulted in a greater than 75% reduction in sea stars at majority of sites (Miner *et al.*, 2018). As of 2017, Moritsch and Raimondi (2018) estimated that 55% of *P. ochraceus* populations are in recovery, but level of recovery varies depending on the region, and does not necessarily mean that adult sea star density has returned to pre-SSWD levels. Northern Californian populations seem to be recovering, and even exceeding pre-SSWD abundances, but further south some populations are still 0% of pre-SSWD numbers (Moritsch & Raimondi, 2018). Considering it takes an individual *P. ochraceus* 3-5 years to reach adult size under ideal conditions (Feder, 1970), these sea stars are not currently filling their historical ecological role in the intertidal community. Any further constraints on *P. ochraceus* population recovery, reproduction, and survival should be examined in order to adequately predict the long-term effect on this species and on other intertidal organisms.

Orchitophyra stellarum

Pisaster ochraceus have few true parasites, but they do host an opportunistically parasitic scuticociliate, *Orchitophyra stellarum*. *Orchitophyra stellarum* (Scuticociliata) was first identified in 1907, when it was found in extremely low numbers in *Asterias rubens* in France (Cepede, 1907). Since then, it has been reported in many species of asteriid sea stars, including *Asterias forbesi* (Burrows, 1936; Galtsoff & Loosanoff, 1939), *Asterias rubens* (Cepede, 1907; Lowe, 1978; Smith, 1936; Vevers, 1951), *Asterias amurensis* (Byrne *et al.*, 1997; Kuris *et al.*, 1996), *Pisaster ochraceus* (Boom, 1988), *Leptasterias* spp. (Stickle, 2001), *Evasterias troschelii* (Stickle & Kozloff, 2008), and *Sclerasterias richardi* (Febvre et al, 1981). Researchers have also found it in *Patiria miniata* (Sunday *et al.*, 2008)*,* which is in another clade of asteroid

(*Asterinidae*). Outside Echinodermata, researchers have observed *O. stellarum* infecting several tissues in crabs and lobsters, and, while prevalence of infection is low, it is consistently highly pathogenic compared to infections in sea stars (Miller *et al.*, 2013; Small, 2004; Small *et al.*, 2005b). Discovery of *O. stellarum* parasitism outside of asteriid sea stars has been reported fairly recently, suggesting that the host range of this ciliate might be changing (Sunday *et al.*, 2008). However, *O. stellarum* might simply be a fairly ubiquitous opportunistic parasite. Isolates found on different species of sea stars from Japan, British Columbia, Prince Edward Island, and the Netherlands were all genetically identical, according to nucleotide sequencing of the internal transcribed spacers, ITS1 and ITS2, and the 5.8S rRNA gene (Goggin & Murphy, 2000). Sequences from *O. stellarum* found in blue crabs (*Callinectes sapidus*) were also identical, even though those ciliates were morphologically more similar to another parasitic scuticociliate, *Mesanophrys chesapeakensis* (Small *et al.*, 2013). Either this ciliate has a very cosmopolitan distribution, or more refined genetic markers are needed to determine species of scuticociliates.

    Orchitophyra stellarum negatively impacts its host in various ways, but it is most commonly a parasitic castrator (Bouland, 1988; Goggin, 1997; Leighton *et al.*, 1991; Vevers, 1951). The ciliate actively consumes host sperm-cells through phagocytosis. In species of the genus *Asterias*, the parasite also disrupts the germinal epithelium by inserting itself between spermatocyte columns, causing them to separate (Bouland, 1988; Byrne 1997). The host mounts an immune response, increasing the number of amoebocytes in the presence of *O. stellarum*, but this has little effect (Coteur *et al.*, 2004). In fact, presence of the ciliate is associated with milky haemolymph and increased destructive auto-immune activity by the host; the sea star amoebocytes should recognize the ciliates as foreign and target them, but they destroy host sperm cells instead (Byrne *et al.*, 1997). Presence of the ciliate in the gonads is nearly 100%

associated with failure of host amoebocytes to clump properly, suggesting further that *O. stellarum* disrupts immune function (Bang, 1982; Childs, 1970; Taylor & Bang, 1978). Observations of amoebocyte activity in an ammonia solution suggests that ammonia waste secretion from *O. stellarum* contributes to this phenomenon (Taylor & Bang, 1978). Small *et al.* (2005a) found also that in *in vitro* culture (in lobster haemolymph added to culture media), *O. stellarum* secretes metalloproteases, which were found selectively degrading muscular structural proteins (i.e., myosin heavy chain). Those enzymes were isolated from infections in lobsters, but it is likely that *O. stellarum* uses similar mechanisms to break down tissues in sea star hosts. While infecting male sea stars, ciliates multiply within the testes, causing them to harden, shrink, and discolor. Sperm cells lose motility (Vevers, 1951) and, in severe infections, are all consumed by the ciliates, to the point where ciliate abundance eventually decreases (Burrows, 1936). In a series of experiments done by Leighton *et al.* (1991), male *P. ochraceus* infected with *O. stellarum* also showed signs of decreased overall health (e.g., discoloration). Byrne *et al.* (1997) also found evidence of pathology associated with *O. stellarum*, reporting that infected male *Asterias amurensis* were more likely to autotomize arms and die in captivity.

In addition to parasitizing sea star gonads, *O. stellarum* colonize the epidermis of their host, congregating near the gonopores, and can also be free-living. When off-host, *O. stellarum* can survive by feeding on bacteria and detritus found in the marine environment, and are considered facultative, opportunistic parasites (Stickle *et al.*, 2007a). When not in the gonads of male sea stars, *O. stellarum* undergo morphological shifts, most notably a reduction in size and a repositioning of the buccal cavity from the anterior end of the ciliate to the middle of the body (Stickle *et al.*, 2007a; Stickle *et al.*, 2007b). The mechanism of transmission between sea stars is unclear, but the ciliate can exist and replicate in a free-living state for an undetermined period of

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time, suggesting that it could move among individual stars and might enter through the gonopore when its host's gonads are full of sperm (Stickle *et al.*, 2007a).

Incidence and pathogenicity of *O. stellarum* are positively associated with water temperature (Bates *et al.*, 2010). In laboratory experiments, mean doubling time is three times faster at 15° Celsius than at 10°, and sea stars in warmer treatments had more heavily infected testes. In the wild, *O. stellarum* appears to occur on the West Coast of North America until it reaches Alaska, where the temperature is presumably too cold for the parasite to survive (Bates *et al.*, 2010; Stickle & Kozloff, 2008). The southern boundary of the range of *O. stellarum* on the West Coast is unknown. Some sea star species, especially *P. ochraceus,* experience more intense ciliate infections than others. Bates *et al.* (2010) found that shallower living *P. ochraceus* are more burdened by *O. stellarum* than the sub-tidal *Asterias miniata,* and hypothesized that the discrepancy in infection intensity was due to differences in thermal niches between the two species. It is also possible that *P. ochraceus* are more susceptible to intense *O. stellarum* infections because they spawn in the spring and summer, when the ocean is warmer (Stickle *et al.*, 2001a).

Research Summary

The recent mass mortality due to SSWD led me to ask how wasting and other disease processes are synergistically affecting sea stars. Echinoderms are common victims of die-off events (Uthicke *et al.*, 2009), so the recent wasting phenomenon provides researchers with an opportunity to understand how other diseases might respond to population fluctuations. Specifically, I was interested in *O. stellarum* infections in populations of *P. ochraceus* on the west coast of the United States, and the potential of parasite prevalence to shift in response to

smaller host populations. There was a small amount of data on *O. stellarum* infections and

associations in *P. ochraceus* populations in this area that had been collected just over a decade

before the most recent SSWD event, published in Stickle and Kozloff (2008) and Stickle *et al.*

(2001a). This allowed me to make comparisons between pre- and post-SSWD prevalence. My

initial goal was to understand if the recent mass mortality event due to SSWD had any effect on

this host-parasite relationship. I also wanted to create a comprehensive picture of the distribution

and prevalence of *O. stellarum* throughout the West Coast of the United States, since previous

studies had only sampled a few geographically isolated sites in that range. I designed a study that

compared three distinct regions within the range of *P. ochraceus*, to understand if there were

spatial differences in *O. stellarum* prevalence. Because the ciliate parasite lives inside the gonads

of sea stars and requires extracting and inspecting gonads to determine whether an individual is

infected, I explored several methods of diagnosing individual sea stars with *O. stellarum*

infections. It became obvious after my first field season that the most efficient way to do this

would be to remove one arm from each *P. ochraceus* sampled. However, mutilation of these

charismatic tide pool animals caused concern, especially because the causative agent behind the

development of SSWD is as yet unknown. What if injuring the sea star in this way exposed the

animal to pathogens, or induced stress to the point that the sea star was less resilient when

confronted with disease? In my experience with dissecting sea stars in laboratory settings, I had

rarely seen morbidity or mortality in response to arm removal alone, and had always assumed

that this methodology was low impact due to sea stars' ability to shed arms naturally. In response

to this uncertainty, I designed an experiment to test my methods, and confirm if *P. ochraceus*

developed SSWD in response to careful, surgical, removal of arms. Once this experiment

validated my methods, I was able to use surgical arm removal to continue to assess the prevalence of *O. stellarum* infection in *P. ochraceus* throughout my study regions.

This study provides important information relating to the potential of *P. ochraceus* to recover from massive population reductions. I aimed to understand the extent to which *P. ochraceus* populations can currently be sampled using arm removal without increasing the incidence of SSWD. Concurrently, I determined whether they are being differentially affected by *O. stellarum* since the onset of SSWD, throughout their North American distribution. In Chapter 2, I present the results of the arm-removal experiment, and discuss the benefits of using this method for sampling sea stars. In Chapter 3, I compare my current observations on prevalence of *O. stellarum* in populations of *P. ochraceus* to previous estimates of prevalence by Stickle & Kozloff (2008), and examine several ecological and geographic drivers behind patterns of ciliate prevalence. These two chapters were written with the intention of publishing them in separate journals, so there are some redundancies in the information presented.

Chapter 2
Does arm removal from the sea star, *Pisaster ochraceus*, increase its susceptibility to Sea Star
Wasting Disease?

INTRODUCTION

Intentional limb loss, or autotomy, is common throughout the animal kingdom (reviewed by

Maginnis (2006)). It is most likely that autotomy evolved convergently several times due to its

significant adaptive advantage (Bely & Nyberg, 2010; Emberts *et al.*, 2017; Wasson *et al.*,

2002). Animals intentionally sever limbs when the benefits of autotomy presumably outweigh

the cost associated with injury and losing a limb (Wasson *et al.*, 2002). Not surprisingly,

autotomy is most commonly employed by prey organisms escaping predation (and the benefits

of this response are large), but is also used to free the animal from a damaged, injured, trapped,

or infected body part (Byrne, 1985; Emberts *et al.*, 2017; Emberts *et al.*, 2016; Juanes & Smith,

1995; McVean & Findlay, 1979). Although autotomy has been well-documented in vertebrates,

such as lizards, skinks, and amphibians (pertaining to tail loss), this phenomenon occurs broadly

in other phyla, including the phyla Cnidaria, Annelida, Mollusca, Arthropoda, and

Echinodermata (Fleming *et al.*, 2007).

Although benefits of autotomy can outweigh the costs, which explains the frequency of

autotomy in many taxa, the loss of appendages has important repercussions (Lindsay, 2010).

Most notably, the gaping wounds that immediately follow autotomy expose the animal to a

variety of pathogens. When an animal is exposed to disease agents and has a wound, the

combination of stressors can have synergistic effects (Argaez *et al.*, 2018; Davies *et al.*, 2015;

Gignoux-Wolfsohn *et al.*, 2012; Johnson *et al.*, 2006). Injury has been shown to increase

susceptibility to disease and pathogenicity, especially when an animal is near infected

conspecifics (Johnson *et al.*, 2006; Lamb *et al.*, 2015; Mydlarz *et al.*, 2006; Wootton *et al.*,

2012). Organisms that experience autotomy often have reduced ability to fight off infection after

limb loss because energy is being allocated to regeneration of lost body parts (Henry & Hart,

2005; Lamb *et al.*, 2015; Mydlarz *et al.*, 2006). Organisms that autotomize body parts containing digestive tissues, nutrient reserves, feeding implements, or all three, as in some echinoderms, experience an even higher degree of energy loss due to autotomy, which has obvious implications for disease resistance (Lawrence, 2010). Due to all of the energetic consequences of autotomy, many previous researchers have simply assumed that self-induced injury can increase an individual's susceptibility to pathogens. On the other hand, repeated self-injury at a specific location throughout evolutionary time has provided an opportunity for selection towards more rapid wound healing and immune activity at the autotomy site (Emberts *et al.*, 2017). In some taxa, autotomy actually increases survival by reducing infection in an otherwise injured body part, and relocating the healing process to an area where it is more efficient (Emberts *et al.*, 2017). Clearly, the relationship between autotomy and risk of bacterial, parasitic, or viral infection warrants further examination.

Echinoderms with appendages (e.g., sea stars and brittle stars) are well known for being able to autotomize and successfully regenerate limbs. In echinoderms, this process is facilitated by the presence of breakage planes or autotomy zones (Anderson, 1956; Emson & Wilkie, 1980; Wilkie *et al.*, 1990), and usually occurs in response to attempted predation or to injury (summarized by Lawrence (1991b)). Sub-lethal arm loss in sea stars results in reduction in reproductive capacity (Bingham *et al.*, 2000), food storage (Diaz-Guisado *et al.*, 2006; Lawrence & Vasquez, 1996), and feeding (Harrold & Pearse, 1980; Harrold & Pearse, 1987), making regeneration costlier when food is scarce (Lawrence, 2010). Arm loss also exposes the internal organs in the central disk of sea stars. In *Acanthaster planci*, the damaged organisms become more susceptible to micropredation by a scavenging polychaete worm after losing an arm (Glynn, 1981; Glynn, 1984). Bang and Lemma (1962) found that while sea stars normally had

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bacteria-free coelomic fluid, the coelom became contaminated following trauma or injury. Furthermore, when individuals of *Asterias rubens* were bycatch from trawl-fishing, and subsequently lost or autotomized an arm, they were more likely to be infected with bacteria (which presents as lesions on the epidermis), lose additional arms, or die (Bergmann & Moore, 2001).

Most research on autotomy and other injury in echinoderms has focused on energetic or reproductive implications, or on regeneration, and reports of disease or infection are usually anecdotal additions. However, a small body of research indicates that echinoderms are adept at resisting microbial pathogens when self-wounded, likely due to a complex immune response and sophisticated wound healing at the area of injury. Presumably, a long evolutionary history of autotomy has also allowed stellate echinoderms to develop methods for reducing fluid loss and exposure at the autotomy plane after arm loss (Emberts *et al.*, 2017). Ophiuroids have clear adaptations at breakage zones for encouraging healing and preventing haemolymph loss, including a flap of epidermis and lateral plate ligaments that recoil after autotomy to cover exposed tissue (Wilkie, 1978a; Wilkie, 1978b). In studies on histology of the breakage plane of sea stars, researchers found that asteriid sea stars possess a muscular "tourniquet" in the integument that is engaged before and during autotomy. This could facilitate rapid wound closure and prevent invasion by pathogens (Hotchkiss *et al.*, 1991; Wilkie *et al.*, 1990). If pathogens are able to invade after autotomy, they are met with the echinoderm immune response (reviewed by Ramirez-Gomez and Garcia-Arraras (2010)). While Bang and Lemma (1962) found that *Asterias rubens* injured by trawling were initially invaded by bacteria, most sea stars were able to clear these infections within a couple of days. Other investigations have found no signs of bacterial infection in damaged or post-autotomy individuals of the same species

(Ramsay *et al,*. 2001). When subjected to experimental arm-tip removal (to mimic predation)

and, separately, challenged with bacterial injections, this species briefly increased circulating

coelomocytes (amoebocytes), which play a role in capturing and clearing of bacterial invaders

through phagocytosis (Coteur *et al.*, 2002; Pinsino *et al.*, 2007). Little is known, however, about

the response of autotomized or injured sea stars to diseases already present in wild populations.

Sea star wasting disease (SSWD) is an epizootic of recent concern because, from summer

2013 onward, it has caused an ongoing mass-mortality event involving sea stars. Many species of

sea stars suffer from SSWD, which is widespread on the West Coast of North America, often

fatal, and of unknown origin (Bates *et al.*, 2009; DelSesto, 2015). The cascade of symptoms

seen in affected sea stars (loss of turgor, lesions, arm loss, disintegration, and death) is likely

caused by a combination of factors, including environmental shifts, pathogens, or stressors on the

level of the individual. One sign of SSWD is the appearance of lesions similar to those seen in

the studies of *Asterias rubens* that were injured and autotomized arms (as in Bergmann & Moore

2001; Eckert *et al.*, 2000). Hence, if arm loss in an individual sea star somehow exposes that

animal to the causative agents of SSWD, an otherwise relatively benign injury could result in the

mortality of the animal. As already mentioned, arm loss increases stress due to injury and

decreases energetic resource availability, so it is important to clarify whether autotomy is a

potential risk factor for developing SSWD.

The sea star *Pisaster ochraceus*, devastated by SSWD in the recent mass die-off event,

undergoes autotomy like other asteroids, but there has been limited research on how *P.

ochraceus* responds to arm loss and regeneration. Adult *P. ochraceus* are keystone predators,

maintaining trophic structure and species diversity in temperate intertidal ecosystems, but have

few consistent natural predators (Harley *et al.*, 2006b). They are structurally protected against

predation, and employ other defensive mechanisms in addition to autotomy (Lawrence, 1991a; Van Veldhuizen & Oakes, 1981). As a result, autotomy occurs in *P. ochraceus* only when the risk of death is very high. According to the literature, individuals missing limbs are very rare in nature (O'Donoghue, 1926), and it has been hypothesized that this rarity is due to a high cost of regeneration, and potentially food resource limitations (Lawrence, 1991a). However, I have personally observed *P. ochraceus* with missing arms (sometimes multiple) regularly during my fieldwork, and believe that the hypothesis that autotomy is rare for this species needs to be reevaluated. Additionally, while collecting sea stars and forcefully pulling them from rocks, I noticed that the arm sometimes tears free from the body, though this doesn't necessarily equate autotomy.

When studying the morphometrics and health conditions of asteroid echinoderms, researchers often remove arms, assuming the method is low-impact because of the natural inclination of these animals to autotomize limbs. However, considering the potential infectious disease consequences of autotomy, further research is warranted to explore the connection between disease and limb loss in these organisms. Conducting tissue sampling in a way that closely mimics autotomy is likely the most ethical way to collect gonadal and pyloric caeca samples from *P. ochraceus*. While studying the gonadal regulation of *P. ochraceus*, Pearse *et al.* (1986) found that removing the internal organs of *P. ochraceus* through an incision in the epidermis left a persistent wound that took weeks to heal and often resulted in mortality, whereas when a single arm was removed near the oral disk, the sea star would seal the wound and regenerate the limb. The same location-specific reactions to injury were experimentally confirmed by Sanford *et al.* (2009). Such differential responses to injury are likely due to an adaptive response to limb loss near an ancestral autotomy plane—located at the base of the arm,

proximal to the oral disk like in other stellate asteroids—that allows *P. ochraceus* to quickly

close and heal wounds in this area, but not in other places on the body (Wilkie *et al.,* 1990;

Lawrence, 2010). These phenomena were reported before the recent onset of SSWD in summer

2013, and thus did not address the potential for arm removal to affect *P. ochraceus* susceptibility.

Notably, the "stress" signs reported by Pearse et al (1986) as a reaction to incision resemble signs

of SSWD (e.g., loss of turgor). While natural autotomy is not likely playing a significant role in

the prevalence of SSWD in wild *P. ochraceus* populations, these sea stars are often subjects of

intertidal research, so developing non-destructive sampling techniques is necessary to prevent

pressure on sea star populations. Thus, determining if arm amputation increases the risk of

inducing SSWD is extremely important.

In this experiment, I determined whether mimicking autotomy by surgically removing an

arm near the oral disk in *P. ochraceus* increases the incidence of SSWD or the associated

pathology. I was primarily interested in whether removing arms to study sea stars increases an

individual's risk of dying from SSWD. I was secondarily interested in whether surgical autotomy

decreases time to death if individuals do in fact develop signs of SSWD. Because autotomy is a

natural process occasionally used by *P. ochraceus* to escape predators or entrapment, I

hypothesized that arm removal would not increase incidence of sea star wasting-related

symptoms in captive *P. ochraceus*. However, if individuals developed symptoms when exposed

to symptomatic sea stars, I hypothesized that individuals who had an arm removed would have a

shorter time to death than intact individuals. It is important to note that, because the etiology of

SSWD is still unknown, I could not confirm with certainty that individuals appearing to suffer

from SSWD actually had the disease. However, because there is good evidence that a specific

suite of symptoms consistently leads to death in *P. ochraceus*, I hereafter assume, within reason, that those symptoms indicate infection with SSWD.

METHODS

Experimental Design

To understand the role that surgical autotomy plays in the onset of SSWD, I conducted three laboratory experiments in which I experimentally removed arms from adult *P. ochraceus* and observed the sea stars for signs of SSWD. In separate experiments, I addressed whether arm removal would affect susceptibility to and progression of SSWD in apparently healthy stars, in sea stars exposed to infected conspecifics, and in sea stars that were already exhibiting early signs of SSWD.

Arm Removal in Healthy Sea Stars

To address my hypothesis that arm removal did not increase the likelihood of developing SSWD-like symptoms in seemingly healthy stars, I collected healthy stars, with zero visible lesions, turgor loss, or arm-twisting, and did not expose them to any sick sea stars or water contaminated by sick sea stars, and then observed their response to arm removal.

Arm Removal in Sick Sea Stars

In another collection of *P. ochraceus*, I intended to collect only healthy stars, but once those individuals were returned to lab most of the stars, including the individuals intended to replace sick ones, were suffering from what appeared to be early stages of SSWD. I continued the experiment anyway to test the hypothesis that arm removal would increase incidence of infection and decrease time to infection (see definition in "Monitoring of Sea Stars") or death in sea stars exhibiting early signs of SSWD.

Arm Removal and Exposure to SSWD

To address the hypothesis that arm removal does not increase the likelihood of becoming infected with SSWD, I collected healthy *P. ochraceus* and exposed them all to wasting individuals. In this experiment, individuals were exposed to a "wasting" *P. ochraceus* immediately after arm removal (or handling in control sea stars, see "Arm Removal Procedure") by placing a sick individual in each tank for 24 hours.

Sea Star Collection

Each experiment required a separate collection of sea stars, and collection methods were standardized between the three experiments. I collected adult *P. ochraceus* ($\geq$ 20 cm diameter from arm tip to arm tip) from Post Point in Bellingham, WA, immediately prior to each experiment. Sea stars were held for one week prior to the start of each experiment to assess whether they were suffering from SSWD. If any sea stars showed signs of illness (loss of turgor, limb curling, lesions, limb loss) before the beginning of the experiment, they were replaced with other *P. ochraceus* I had collected at the same time. In this case, the wasting individual's tank was also scrubbed and sanitized with a 5% bleach solution, and then rinsed with DI water before being put back into use. Sea stars were held in individual 40 liter tanks with flowing sea water at 10 °C for the entire experiment. To reduce non-experimental stressors, I performed 50% water changes in the tanks once a week. Each tank was filled with sea water from the same source, to control for potential non-experimental exposure to SSWD.

Arm Removal Procedure

During each experiment, the ten sea stars were randomly divided into Arm Removal and Control groups (n = 5 each), and their respective tanks were randomly arranged. I assigned sea stars an identification number before placing them in their tanks. I removed one randomly chosen arm from each sea star in the Arm Removal group using a scalpel and scissors as necessary. Two incisions were made on the aboral side following indentations on the animal's surface that indicate the division between arms, forming a V-shape (Figure 1.1). The arm was then gently pulled until it broke free. This method was chosen after I observed *P. ochraceus* occasionally, but consistently, tearing free from their arms in that location while I was forcefully removing them from rocks, and autotomizing their arms along that "plane" in laboratory studies. Any gonads or pyloric caeca that were still connected were removed from the inside of the oral disk using scissors. Individuals in the control group were removed from their tanks and handled for one minute to control for handling stress. After the sea stars had been subjected to the arm removal treatment, they were returned to their individual tanks and monitored daily for seventeen days.

Monitoring of Sea Stars

Notes were taken on sea star activity level, any signs of healing around the incision, and any signs of SSWD that appeared. Signs of SSWD were classified into four categories, as shown in Table 1.1. Very early afflictions that could indicate SSWD, including singular small lesions, loss of turgor, and twisting of limbs, were recorded as the first potential signs of SSWD, even though I could not determine the exact moment of infection. If multiple small lesions appeared, monitoring was increased to twice a day. Affected individuals were classified as "infected" with

SSWD once they displayed 1-2 small white lesions (Category 1), even though they often recovered from these. I could not determine the exact moment of death in sea stars, so I assumed that in the event of multiple limb loss, the animal was unlikely to recover. If an individual suffered multiple limb loss or showed any signs of SSWD in Category Four, they were euthanized within 24 hours, and were recorded as "dead".
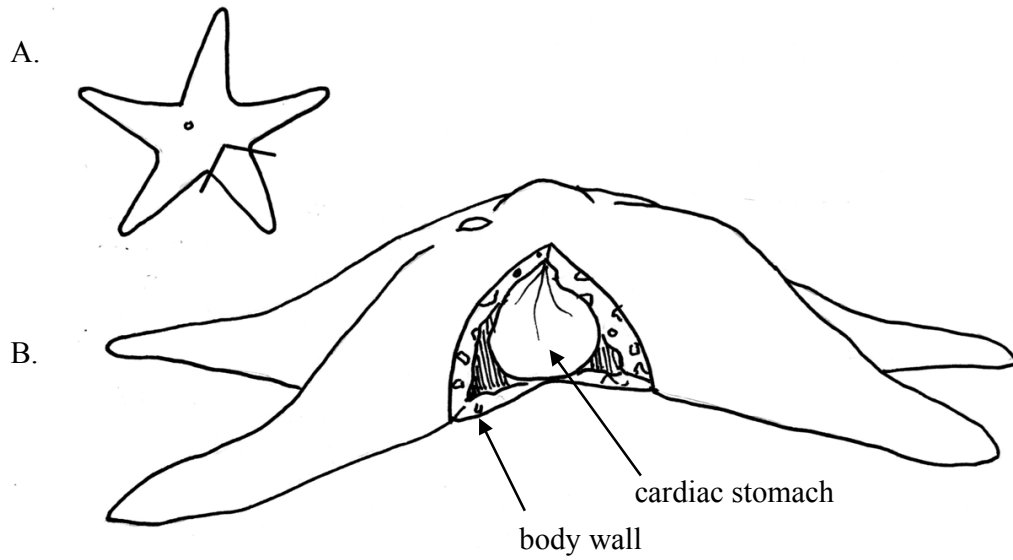
Figure 1.1. Schematic of *P. ochraceus* arm removal, illustrating the surgical autotomy of one arm by making two incisions at the base of the arm where it connects to the oral disk, from above (A) and what the sea star should look like immediately post dissection, side view (B).

Table 1.1. Categorization of signs of SSWD, adapted from the MARINe *Pisaster ochraceus* Symptoms Guide.

Category	Sign	Description
1	Lesions on one arm or body	Tissue degradation limited to one location on arm or body
2	Multiple lesions	Lesions on two arms or arm and body and/or deteriorating arms
3	Lesions on most of body	Lesions on most of body and/or 1-2 missing arms
4	Severe tissue deterioration	Severe tissue loss and/or multiple missing arms and/or death

Statistics

To assess changes in time to death or time to "infection", I conducted a survival analysis by creating right-censored Kaplan-Meier survival curves for each experiment. I analyzed time to two distinct events: "infection," and "death". I compared time to infection between sea stars with limbs removed and those without using a log-rank Mantel-Haenszel test. This test is appropriate for comparing survival curves in which the ratio of hazard functions (events/time) is the same at all time points, and gives equal weight to all time points. All statistics were performed in R, version 3.5.0.

RESULTS

Within all three experiments, *P. ochraceus* subjected to arm removal did not experience faster or slower time to infection compared to control *P. ochraceus*. Overall, mortality was very low; only two sea stars died throughout the entire study, and both were in the same experiment, showing potential early signs of wasting before the experiment began. Arm removal did not decrease the time to death or increase the incidence of death due to SSWD. It is important to note that all previously healthy-seeming *P. ochraceus* subjected to surgical arm removal were able to at least partially close the wound within 24 hours. On occasion, part of the stomach would remain partially outside of the body cavity while the wound healed.

Arm Removal in Healthy Sea Stars

There was no statistical evidence that previously healthy sea stars subjected to arm removal experienced faster times to infection than intact sea stars (Figure 1.2A). On average, sea stars with arms removed showed signs of infection 30.7% faster than control sea stars, but this difference was not significant (Table 1.2). All of the sea stars that had an arm removed showed signs of SSWD, and 90% of control sea stars had signs of SSWD. Only one intact individual remained without signs of SSWD throughout the experiment. None of the sea stars died during the course of the experiment.

Arm Removal in Sick Sea Stars

There was no statistical evidence that sea stars subjected to arm removal developed SSWD infection faster than intact sea stars, even when sea stars had already shown early potential signs of SSWD (Figure 1.2B). After arm removal, sea stars with arms removed showed signs of

SSWD 26.2% sooner than control sea stars on average, but this difference was not significant (Table 1.2). Incidence of infection was the same in both control and experimental treatments; 80% of sea stars showed signs of more advanced SSWD in each treatment. Two sea stars died from SSWD, including one sea star from each treatment. The control sea star died after one day and the experimental sea star died after seven days.

Arm Removal and Exposure to SSWD

There was no statistical evidence that when exposed to infected individuals, sea stars subjected to arm removal showed signs of SSWD faster than control sea stars that were also exposed (Figure 1.2C). On average, control sea stars showed signs of SSWD 10.9% faster than sea stars with their arms removed, but this difference was not significant (Table 1.2). Incidence of infection with SSWD was exactly the same in both treatments; 60% of sea stars showed signs of SSWD in both the arm removal and control treatment. None of the sea stars died during the course of this experiment, but one individual did succumb to SSWD and die shortly after I ended the experiment.

Power Analysis

Because of my small sample size, I presumed that β error might be high in my statistical analyses. I performed a power analysis on the log-rank tests comparing mean time to infection between sea stars with arms removed and intact sea stars (Table 1.3). For all three experiments, the power of these tests were likely not high enough to detect a meaningful difference in time to infection between the two experimental groups. With my sample size, I would have had less than

a 20% chance of even detecting a 50% difference, and less than a 30% chance of detecting a

90% difference, in all three experiments (Table 1.3).

Table 1.2. Mean time to event (infection or death) for *Pisaster ochraceus* after being subjected to arm removal (experimental) or one minute of handling time (control). Means were back-calculated from right censored Kaplan-Meier survival curves, and compared using log-rank Mantel-Haenszel tests (X^2). Significant differences in time to event are denoted by *. Time to death is not shown for Experiments 2 and 3 because no sea stars died during those experiments.

Experiment	Event	Mean time to event (days)		X^2	p-value
		Control	Arms Removed		
Healthy Sea Stars	death	1	7	N/A	N/A
	infection	8.4	6.2	0.2	0.6
Sea Stars With Early Signs of SSWD	infection	7.5	5.2	0.6	0.4
Sea Stars Exposed to SSWD	infection	9.8	11.0	0.3	0.6

Table 1.3. Results of a power analysis of the three Mantel-Haenszel log-rank tests, performed to determine differences between mean survival time for control sea stars and sea stars with arms removed. Power is the probability of detecting a statistically significant difference at the given effect size.

Experiment	Number of Events	Effect Size (% difference)	Power
1	8	50	0.145
		90	0.204
2	9	50	0.158
		90	0.224
3	6	50	0.119
		90	0.164
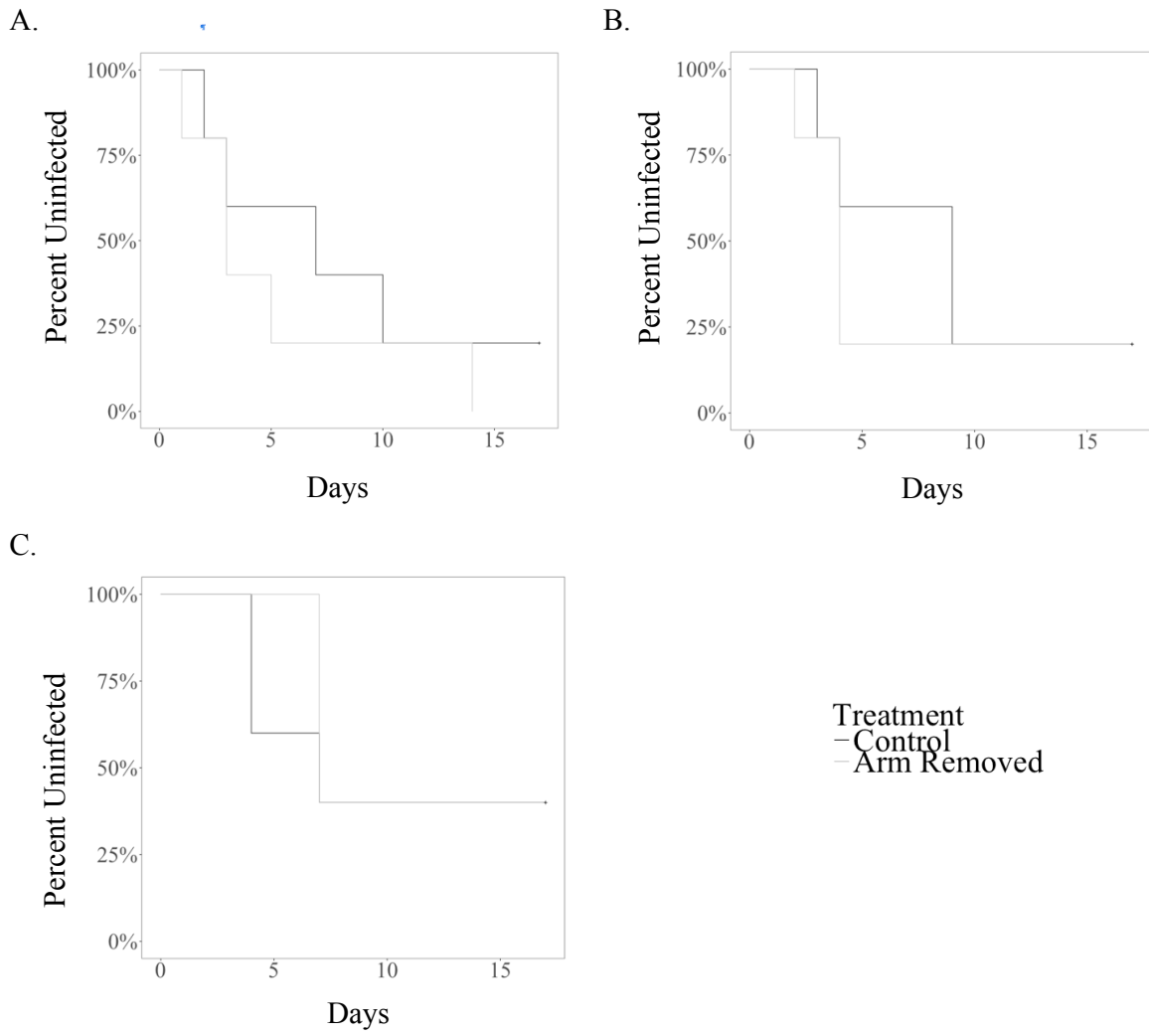
Figure 1.2. Right-censored Kaplan-Meier survival curve estimates, depicting time to infection for sea stars with arms removed and without arms removed (Control) in (A) previously healthy sea stars, (B) sea stars previously showing early signs of SSWD, and (C) healthy sea stars exposed to wasting sea stars. N=10 for each experiment.

DISCUSSION

Although injury typically increases susceptibility to disease in many other, non-asteroid taxa, I found no evidence for this in *P. ochraceus* exposed to SSWD. My results suggest that for *P. ochraceus,* arm loss, which is arguably a stress-inducing condition, has little or no impact on their disease status. In all three experiments, surgical removal of arms along the autotomy plane did not increase the incidence of SSWD, even when individuals were exposed to other infected stars, or when individuals were showing signs of SSWD before the experiment started.

This finding is consistent with previous research that demonstrates that, in both *P. ochraceus* and other species of asteroids, injury does not necessarily initiate infection or invasion by pathogens. In *Asterias rubens* injured by trawling, sea stars with damaged arms showed no signs of bacterial infection, even after autotomizing those arms (Ramsay *et al.*, 2001). In addition, *P. ochraceus* show no signs of morbidity when an arm is removed at the junction with the oral disk, but experience pathology similar to SSWD in response to more distal injuries (Pearse *et al.*, 1986; Sanford *et al.*, 2009). Presumably this has to do with the ability of the sea star to close wounds due to entire arm loss, perhaps as part of an evolved response to autotomy (Wilkie *et al.*, 1990). Although *P. ochraceus* reportedly don't readily autotomize in natural settings, and have almost no predators that would necessitate this response, I noticed individuals missing arms regularly in the field. This suggests that *P. ochraceus* could be utilizing autotomy more frequently than previously assumed. Regardless, they seem to be able to heal after an autotomy-like injury. I found that *P. ochraceus* individuals appear to heal best following a removal of tissue that extends into the oral disk slightly (Figure 1.1), instead of at the base of the arm in the previously defined autotomy zone for asteroid taxa that autotomize frequently (Lawrence, 2010; Wilkie *et al.,* 1990). In my experiment, all sea stars closed the wound left by

surgical arm removal by squeezing the two arms adjacent to the wound together, and this process happened fairly quickly (within 24 hours). This could be evidence for a new location for the autotomy zone or breakage plane in stellate echinoderms, and at the very least is a new observation regarding wound healing in sea stars. Understanding wound healing and disease susceptibility in echinoderms is especially important because the frequency of both injury and pathogenic infection are likely to increase as climate change contributes to increased storm action and disease prevalence (Burge *et al.*, 2014; Harley *et al.*, 2006a; Lafferty *et al.*, 2004).

Arm removal seemed to have no effect on the time to death of sea stars that were already showing signs of SSWD. However, I only observed death due to SSWD in two individuals, one in each treatment, and the intact sea star died before the individual that had an arm removed. This could have simply been because the intact sea star was experiencing SSWD that had progressed further than the other sea stars before the experiment began. It is also possible that, once afflicted by SSWD, disease progression is so swift that additional injury has no effect on the eventual mortality of the sea star. In fact, arm autotomy is one of the commonly noted symptoms of late stage SSWD (Hewson *et al.*, 2014).

Individual susceptibility to SSWD and eventual death does not seem related to prior arm loss, but because this was a laboratory-based experiment, whether sea stars survive arm removal in the field is still in question. Temperature and water quality were very tightly controlled, so the sea stars in this experiment had no exposure to temperature stressors that would likely affect their ability to resist infection in a more natural setting (Bates *et al.*, 2009; Eisenlord *et al.*, 2016). Almost all of my the sea stars in the three experiments developed lesions indicating SSWD infections, but arm removal had no effect on the incidence of these lesions. The lesions could have appeared due to stress from being in aquaria, however, as most of the sea stars did not

progress to more advanced stages of SSWD. Additionally, because all three of my experiments had very low power, it is unlikely that I would have detected a statistically significant difference in time to infection or time to death between intact sea stars and those with arms removed. Regardless, based on the low observed incidence of mortality associated with removing arms along the autotomy plane of these sea stars, I would recommend use of this methodology for research on both sick and healthy *P. ochraceus* populations, especially over whole-animal sampling or removal of organs by opening a "flap" in the epidermis, supporting the conclusions of Sanford *et al.* (2009). *Pisaster ochraceus* have evolved to respond better to whole-arm loss, originating as close to the oral disk as possible, than to other types of injuries, even in the face of population-wide disease. My results support the hypothesis that arm removal closely mimicking autotomy does not increase *P. ochraceus* susceptibility to SSWD, but future research should test this hypothesis more rigorously in the laboratory, and determine if sea stars survive in the field following arm removal.

Chapter 3
Prevalence of the sperm-feeding ciliate, *Orchitophyra stellarum*, in *Pisaster ochraceus*
populations on the west coast of North America

INTRODUCTION

Mass die-offs of marine organisms have intensified in frequency and scope in recent years (Fey *et al.*, 2015; Jurgens *et al.*, 2015), presumably due to climate change and anthropogenic impacts (Harley *et al.*, 2006a). Most research concerning the cascading effects of die-offs has focused on free-living taxa, but mass-mortality events also represent a real threat to parasite biodiversity (Dunn *et al.*, 2009). Disease mediated mass-mortality events are problematic for highly host-specific parasites that are more likely to go extinct if their host population is greatly reduced (Dobson *et al.*, 2008; Hechinger & Lafferty, 2005), but the effect of extreme host-population reduction on multi-host pathogens is less clear (Dobson, 2004; Woolhouse *et al.*, 2001). Understanding how sudden and catastrophic declines affect host-parasite relationships is essential to address conservation in the face of such phenomena.

A recent marine mass-mortality event caused by sea star wasting disease (SSWD) has dramatically reduced the numbers of sea stars along the west coast of North America. Several species of sea stars have been affected, and populations have been reduced by up to 100% in some locations (Moritsch & Raimondi, 2018). Researchers are still uncovering the exact cause of SSWD, but signs of the disease are associated with increases in water temperature (Bates *et al.*, 2009; Eisenlord *et al.*, 2016; Hewson *et al.*, 2014; Staehli *et al.*, 2009) and the presence of a densovirus (Hewson *et al.*, 2014), although neither are causally linked. Because sea star species are charismatic tide pool animals, as well as important keystone predators in many intertidal ecosystems (Paine, 1966; 1969), there has been much concern about the ability of sea star populations to recover.

In addition to suffering from SSWD, several species of sea stars host a sperm-feeding ciliate, *Orchitophrya stellarum,* Subclass Scuticociliata, that is a generalist parasitic castrator of

sea stars (Cepede, 1907; Lowe, 1978; Smith, 1936; Vevers, 1951). This ciliate parasite was first

noted in sea stars in the eastern Pacific in 1988 (Boom, 1988). *Orchitophyra stellarum* castrates

its host in various ways, most notably by actively consuming host sperm cells through

phagocytosis, degenerating germinal epithelium, and disrupting host immune function (Byrne *et

al.*, 1997; Bang, 1982; Childs, 1970; Taylor & Bang, 1978; Bouland, 1988; Goggin, 1997;

Leighton *et al.*, 1991; Vevers, 1951). As the disease progresses, the testes harden and discolor,

becoming completely devoid of sperm, but packed with ciliates. Eventually, as the supply of

germ cells dwindles, so do the ciliates (Burrows, 1936; Vevers, 1951). Incidence, infection

intensity, and pathogenicity of *O. stellarum* are positively associated with temperature increases,

according to laboratory experiments (Bates *et al.*, 2010). On the West Coast, the range of *O.

stellarum* extends to Alaska, beyond which the temperature is presumably too cold for the

parasite to survive (Bates *et al.*, 2010; Stickle & Kozloff, 2008).

   Infection by *O. stellarum* facilitates such a profound impact on host health and fecundity

that researchers have suggested it for use in biological control of invasive and destructive sea

stars in other parts of the world (Galtsoff & Loosanoff, 1939; Kuris *et al.*, 1996). The presence of

O. stellarum is associated with significant reductions in male:female ratios of adult sea stars

(Claereboudt & Bouland, 1994; Leighton *et al.*, 1991). Furthermore, Byrne (1997) showed that

populations of *Asterias amurensis* that had equal (1:1) male:female ratios at the beginning of the

breeding season were heavily female biased by the time spawning had ended. Anecdotal

evidence shows that the parasite is associated with decreased overall health of male *Pisaster

ochraceus* (Leighton *et al.*, 1991). Infected male sea stars in aquaria were more likely to

autotomize arms and eventually die (Byrne 1997). If the two aforementioned patterns are

widespread, that could indicate that the ciliate is somehow facilitating the death of infected male sea stars.

Orchitophyra stellarum is a facultative parasite that survives commensally on the epidermis of sea stars and as a free-living organism, suggesting that it could move among individual stars (Stickle *et al.*, 2007a). However, this parasite's response to changes in host population is unstudied. Stickle & Kozloff (2008) found that, in one ciliate-associated population of *P. ochraceus*, males were rare, indicating male mortality. However, only 10% of those remaining were infected with *O. stellarum*, suggesting that a large reduction in host population could be associated with reduced parasite prevalence, potentially because the parasite consumes host reproductive resources and then leaves the population. However, to date, there has been no research on how *O. stellarum* responds to changes in its host population. Anderson and May (1981) suggest that microbial pathogens should increase in prevalence in denser host populations, but very little is known about the population dynamics of generalist, non-obligate parasites (Woolhouse, 2001). My study aimed, in part, to address whether the prevalence of *O. stellarum* has increased or decreased in declining populations of the ochre sea star, *Pisaster ochraceus*.

Pisaster ochraceus has the highest incidence of *O. stellarum*, compared to other Eastern Pacific sea stars, and experiences the most pathogenicity associated with *O. stellarum* (Leighton et al, 1991, Stickle & Kozloff, 2008). Intensity and pathogenicity of *O. stellarum* infections increase with temperature; because *P. ochraceus* lives in the shallower, warmer part of the intertidal zone, and has fully developed gonads in warm spring and summer months, it is an ideal host for this ciliate (Bates *et al.*, 2010; Stickle & Kozloff, 2008). Additionally, wasting disease

has negatively impacted *P. ochraceus* populations, which provides an opportunity to study the impacts of a mass die-off event on this host-parasite relationship.

In this study, I quantified the current prevalence of *O. stellarum*, both infecting the gonads and associated with the epidermis of *P. ochraceus* between Puget Sound, WA, and Santa Cruz, CA, comparing prevalence of *O. stellarum* to available pre-SSWD data. Stickle and Kozloff (2008) presented prevalence estimates for *O. stellarum* epidermal associations from several sites in the Eastern Pacific, sampled while sea stars were reproductive between 1999 and 2001, and I used data from their three sites within the United States as my pre-SSWD baseline (Figure 2.1). I tested whether current *P. ochraceus* populations have a higher or lower prevalence of *O. stellarum* than in the past. I hypothesized that variability in *O. stellarum* prevalence would be be significantly different between three regions I studied because of abiotic differences in the intertidal ecosystems in those regions. To address a potential mechanism behind the previous hypothesis, I also examined the relationship between latitude and *O. stellarum* prevalence. I predicted that as latitude decreased, prevalence of the ciliate would increase, and that *P. ochraceus* populations in Puget Sound would be more heavily infected because latitude can be used as a proxy for temperature (De Frenne *et al.*, 2013). Furthermore, I hypothesized that increases in parasite prevalence would be explained by increases in host population density. I also hypothesized that populations with higher prevalence of *O. stellarum* would have lower male:female ratios. Finally, I hypothesized that there would be a difference in prevalence of *O. stellarum* epidermal association when sea stars were reproductive (spring and summer) and when they were not (winter). However, I was unsure if prevalence would be higher due to gonad infections resulting in more ciliates in the host population, or if all the ciliates would move off of host epidermal tissues and into host gonads, reducing epidermal association prevalence.

METHODS

Site Selection

To understand the prevalence, distribution, and host-parasite relationship of *P. ochraceus* and *O. stellarum*, I surveyed *P. ochraceus* populations on the West Coast of the United States in search of this ciliate parasite. To address my hypothesis about regional variability in parasite prevalence, I collected sea stars from three regions. I sampled *P. ochraceus* from Washington State (including Washington Outer Coast, Salish Sea, and Puget Sound), the Oregon Outer Coast, and Northern California (Figure 2.1). I hypothesized that *O. stellarum* infection and epidermal association prevalence had changed in response to SSWD-related host population declines, so I selected sites that had demonstrated declines in *P. ochraceus* populations since the onset of SSWD, using survey data from MARINe (presented in Miner *et al.* (2018)). I also included sites that were surveyed by Stickle and Kozloff (2008) in the same regions for associations between sea stars and *O. stellarum* before the most recent mass mortality event. I selected sites that were relatively evenly spaced throughout each region to ensure adequate spatial coverage. I sampled five sites in Northern California, five sites in Oregon, and six sites in Washington State to account for the Salish Sea and Puget Sound (Figure 2.1).

Field Survey Protocol

Sea star collection followed the same protocol at every site. I collected my samples by hand during negative low tides. I arrived at each site at least three hours prior to low tide, and generally worked about 1-2 hours after low tide. I haphazardly selected three 30 by 30-meter plots that spanned upper and lower intertidal zones. If there was insufficient habitat for *P. ochraceus* to make a 30 by 30-meter square, plot dimensions were adjusted as necessary. I then

searched the plot for all *P. ochraceus* individuals, and marked each sea star's position with a flag, while assigning each individual a number. I randomly chose five of the sea stars to examine for ciliate association and infection. If three plots were not sufficient to find fifteen sea stars, I searched the area bordered by my first and last plots, flagged and numbered all sea stars in that area, and randomly selected the remainder of my sample. Rarely, there were fewer than fifteen sea stars present at a site, in which case I examined all available individuals for ciliates. I was testing hypotheses about the relationship between population density of sea stars and prevalence of ciliate association, so I recorded the density of sea stars (individuals/meter^2) in each plot.

Gonadal Infections

To understand the prevalence of *O. stellarum* infections in the gonads, I needed to collect samples when sea stars had fully ripe gonads and were ready to spawn. The annual spawning cycle for *P. ochraceus* begins in early March and ends between late May and late June, depending on location (Table 2.1). This sea star undergoes slightly differential reproductive cycles depending on whether they live in northern or southern regions (Mauzey, 1966), but generally gametogenesis starts in September, and gonadal indices increase steadily until spawning in the late spring or early summer, when they drop dramatically after spawning occurs (Farmanfarmaian *et al.*, 1958; Giese, 1959; Sanford & Menge, 2007).

I obtained gonadal material one of two ways: by inducing spawning or surgical autotomy. I only induced spawning during my first field season, during which I surveyed the Washington State region. I induced spawning in collected sea stars by injecting each arm and the central disk near the base of the arms with at least one milliliter of 100 micromolar 1-methyladenine (Fraser *et al.*, 1981). The chemical 1-methyladenine is naturally produced and isolated from sea stars,

and its injection induces both males and females to spawn (Kanatani, 1969; Strathmann, 1987). Sea stars were then placed in individual watch-glasses with filtered sea water for 1.5 hours to collect sperm and eggs. If this method failed to induce spawning, I surgically removed arms to reach the gonads. Because 1-methyladenine injection was unreliable, and because experimental data suggested that surgical autotomy was a low impact alternative, I switched to solely using this method while sampling Northern California and Oregon. Surgical autotomy of one arm from each sea star was completed by making two incisions on the aboral side of the individual in a V-shape, following indentations on the central disk that indicate separations between arms. The arm was then gently pulled until it broke free. The gonads were then excised from the body cavity wall, placed in labeled bags, and stored on ice for later examination. Infection prevalence of *O. stellarum* was determined by examining the spawn or the gonads of the male sea stars under a dissecting microscope. Presence or absence of ciliates was recorded. I hypothesized that low male:female ratios would be associated with higher prevalence of ciliate infection, so I recorded the sex of each sea star that I collected. In several cases, sea stars were either already spawned out or otherwise missing sexually dimorphic gonads, and their sex was recorded as unknown.

Epidermal Ciliate Association

Each collected individual was analyzed for epidermal association of the ciliate parasite in accordance with Stickle and Kozloff (2008). To address the association of ciliates with the epidermis of the sea star, the aboral central disk of each individual was flushed with filtered sea-water, the water was carefully collected, and then stored at 8° C for later analysis. Within one week, I examined the flush fluid for the presence of *O. stellarum* using a compound microscope. Early observations confirmed that, when kept refrigerated, ciliates stayed alive in the tubes for up

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to two weeks. I determined an association of *O. stellarum* with the outer tissues of *P. ochraceus* by emptying the flush fluid into a glass petri dish and examining it under a dissecting scope for the presence of ciliates. If ciliates were found, they were pipetted onto a slide, and examined under a compound scope at 400x magnification to confirm that they were *O. stellarum*. I recorded presence or absence of *O. stellarum*.

Seasonality

To test my hypothesis that the relationship between *P. ochraceus* and epidermal *O. stellarum* changes depending on the reproductive cycle of the host, I collected sea stars from sites while sea stars were reproductive and while they were not. I conducted "Spawning Season" surveys throughout the spring and summer, completing surveys of Washington State in June and July of 2016, surveys of Northern California in April of 2017, and surveys of the Oregon Coast in May of 2017. In Washington State, I did not find any evidence of parasitism during spawning season, so I elected not to return for a second survey. I was unable to sample sites in Oregon during the winter months. I surveyed sites in Northern California during the "Non-Spawning Season" in December 2016.

Ciliate Identification and PCR-RFLP

I visually inspected all fluid, spawn, and gonad samples for ciliates, identifying them as *O. stellarum* using a series of morphological characteristics. Individual *O. stellarum* are morphologically plastic, depending on their location on the host, or whether they are free living. They have been reported to be between 15 and 65 μm in length and 5-17 μm wide, but ciliates on the lower end of that range are usually only found when starving (Stickle et al 2007a). Ciliates

have an oblate spheroid shape with one pointed end, and have 10-20 somatic kineties, 3 oral polykinetids, and one oral dikinetid. They also have an easily distinguishable macronucleus and micronucleus. Usually, several food vacuoles and a prominent contractile vacuole are visible. Typically, if ciliates found in samples were oblate in shape, fell within the aforementioned size range, and had between 10 and 20 kineties and a visible contractile vacuole, macronucleus, and micronucleus, I recorded a positive presence of *O. stellarum* (Figure 2.2). However, after observing morphological variability in my samples, I wanted to further confirm that the ciliates I was seeing were indeed *O. stellarum* and not another ubiquitous marine scuticociliate. I elected to confirm my morphological identification of *O. stellarum* using molecular methods. Ideally, I would have extracted DNA from all of my samples to confirm that each one with ciliates contained *O. stellarum* specifically. However, I did not originally plan to do molecular work, so my samples were not appropriately preserved and I was not able to analyze each sample of ciliates molecularly. I used a sample of fresh ciliates isolated from the epidermis of a captive *P. ochraceus* that I visually identified as *O. stellarum* using the methods described above. The ciliates were immediately centrifuged at 1000 x g for 5 minutes at 4°C.

I extracted DNA from each sample of ciliates using a Quick gDNA Blood MiniPrep Kit (Zymo Research) according to manufacturer instructions. I used a PCR-RFLP (polymerase chain reaction-restriction fragment length polymorphism) assay developed by Small *et al.* (2013) to rule out other ciliate species. I used forward and reverse primers designed by Small et. al. (2013) to amplify the ITS1 region of rRNA in *O. stellarum* and three other scuticociliate species (*Mesanophrys chesapeakensis, Mesanophrys pugettensis,* and *Uronema marinum*), which resulted in a 238 base pair reaction product. Each 50 μl reaction contained 34 ng of DNA, 200 μm of each primer, 800 μm of dNTPs, 2.5 mM $MgCl_2$, 1x Gold PCR Buffer (ThermoFisher

Scientific), and 1.25 units of AmpliTaq Gold (ThermoFisher Scientific). To determine that amplified rDNA fragments were from *O. stellarum* and not another marine scuticociliate, the PCR product was digested using both HpyCH4III and HpyCH4IV restriction enzymes, separately. HpyCH411 would have cut the gene fragment if it belonged to *U. marinum*, and HpyCH4IV would have cut the fragment if it belonged to *M. chesapeakensis* or *M. pugettensis,* but both enzymes would have left the *O. stellarum* rDNA amplicon intact. Each digestion reaction using HpyCh4III included 10 μl amplified DNA, 10 units HpyCH4III, and 1x Tango Buffer (10 mM Tris-HCl, pH 8.5, 10 mM $MgCl_2$, 100 mM KCl, 0.1 mg/mL bovine serum albumin) for a total of 20 μl, which was incubated at 65°C for one hour. Each digestion reaction using HpyCH4IV included 10 μl amplified DNA, 10 units HpyCH4IV, and 1x Buffer R (33 mM Tris-acetate, pH 7.9, 10 mM magnesium acetate, 66 mM potassium acetate, 0.1 mg/mL bovine serum albumin) was incubated at 65°C for 1 hour. Gel electrophoresis (2% agarose gel stained with ethidium bromide to a 0.5 μl/ml final concentration) allowed me to use the products of the PCR-RFLP analysis to confirm that the ciliates were in fact *O. stellarum*, and not one of the other three species. if bands were shorter than 238 base pairs, then the sample would have been one of the other closely related and morphologically similar scuticociliates. The sample of ciliates contained only *O. stellarum*, indicating good agreement between morphological and molecular identification methods (see Chapter 1 -*Orchitophyra stellarum*).

Statistics

All statistical analyses were completed in R, version 3.5.0. To address the hypothesis that

prevalence of *O. stellarum* infections and epidermal associations had changed since SSWD, I

graphically compared current prevalence estimates with historical estimates published by Stickle

and Kozloff (2008). However, I was unable to obtain the raw historical data needed to calculate

confidence intervals on the percent association estimates. Instead, I calculated 95% confidence

intervals on my prevalence estimates, and if the estimate from Stickle and Kozloff (2008) fell

within those intervals, the two estimates were not considered statistically different. To determine

whether there was a difference in *O. stellarum* prevalence between the three regions I sampled, I

ran a generalized linear mixed model, with binomial error structure and a logit link function,

including region as a predictor variable and site as a random variable, and compared that to a null

model with just site as a random variable. I used Akaike information criterion (AIC) comparison

to determine relative model quality. When testing my hypotheses about variance in *O. stellarum*

association or infection prevalence, I intended to run a single full model including all of my

predictor variables, but did not have adequate degrees of freedom to address each factor. Instead,

I ran separate generalized linear models. I used a binomial error distribution with a logit link

function because I collected binary presence/absence data for ciliate infection and association

(i.e., prevalence). In all of the following analyses, the response variable was blocked by sampling

site, and all of the factors were fixed variables. To address the hypothesis that *O. stellarum*

prevalence would increase with latitude, I ran a logistic regression testing for a significant

relationship. To address the hypothesis that host density would explain variation in *O. stellarum*

prevalence, I ran a logistic regression testing for a significant relationship between *O. stellarum*

prevalence and host population density. To test the hypothesis that male:female ratios would

have a positive relationship with *O. stellarum* prevalence, I ran a logistic regression. Finally, to determine if prevalence of external ciliates was different when sea stars were reproductive or not, I ran a logistic regression testing for a significant relationship between epidermal *O. stellarum* prevalence and sampling season.

Although not included as one of my original hypotheses, the position of sampling site within region (southness) could influence the prevalence of epidermal *O. stellarum*. To address this relationship, I gave each site in a region a ranking between 1 and 6, with 1 being the southernmost site and six being the northernmost. This gave me a continuous scale of "southness" that was standardized between regions to use as a predictor variable. I then ran and compared a series of generalized linear models including southness, region, and the interaction between the two as predictor variables for *O. stellarum* prevalence, using AIC to determine relative model quality.
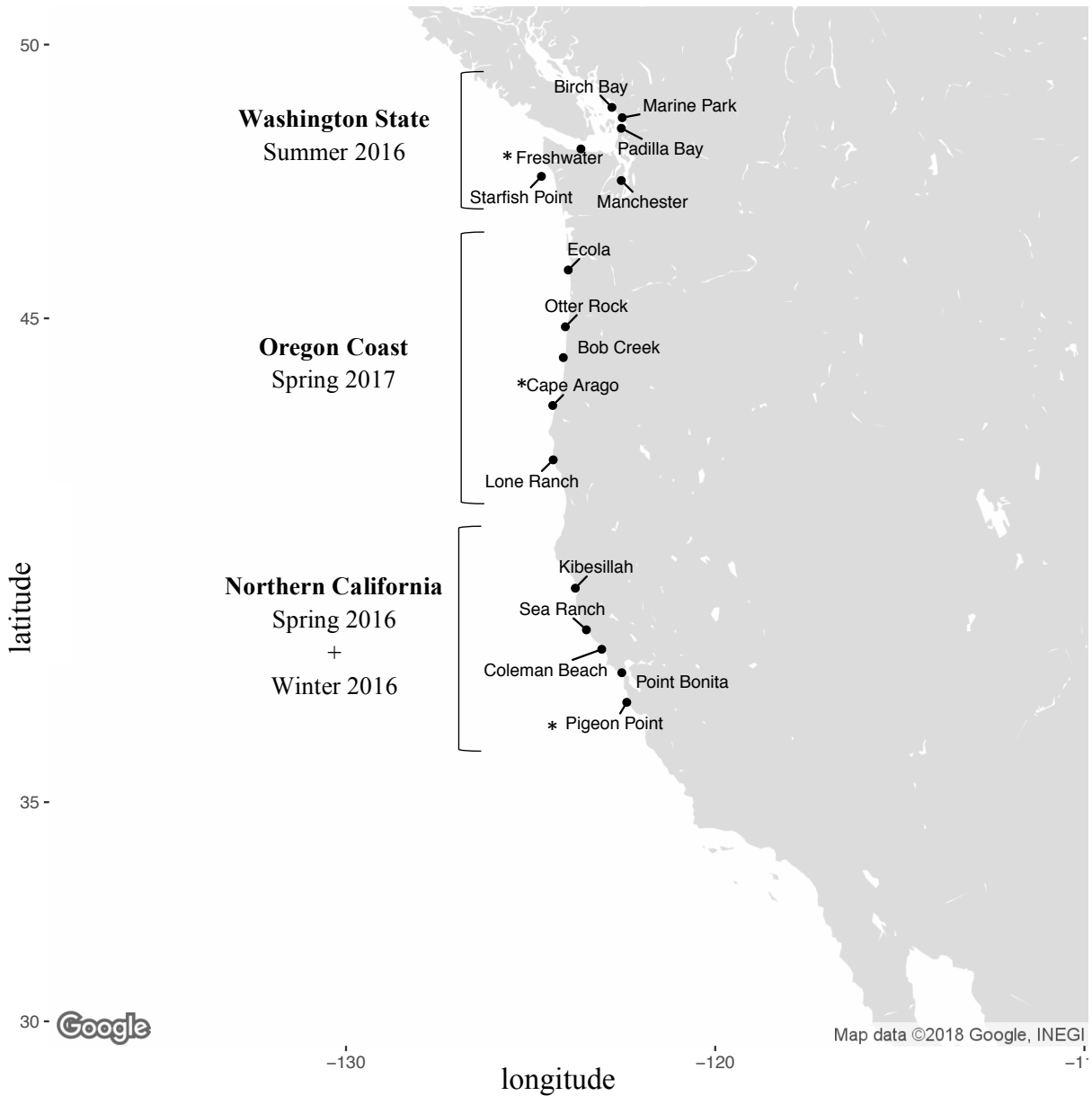
Figure 2.1. Map of the three regions surveyed by this study and individual study sites within those regions. The northernmost sites are in the Pacific Northwest region, the Oregon Coast region extends from Ecola, OR, to Burnt Hill, OR, and the Northern California region extends from Cape Mendocino, CA, to Pigeon Point, CA. Sites indicated with a "*" were sampled from 1999-2001 by Stickle and Kozloff (2008).
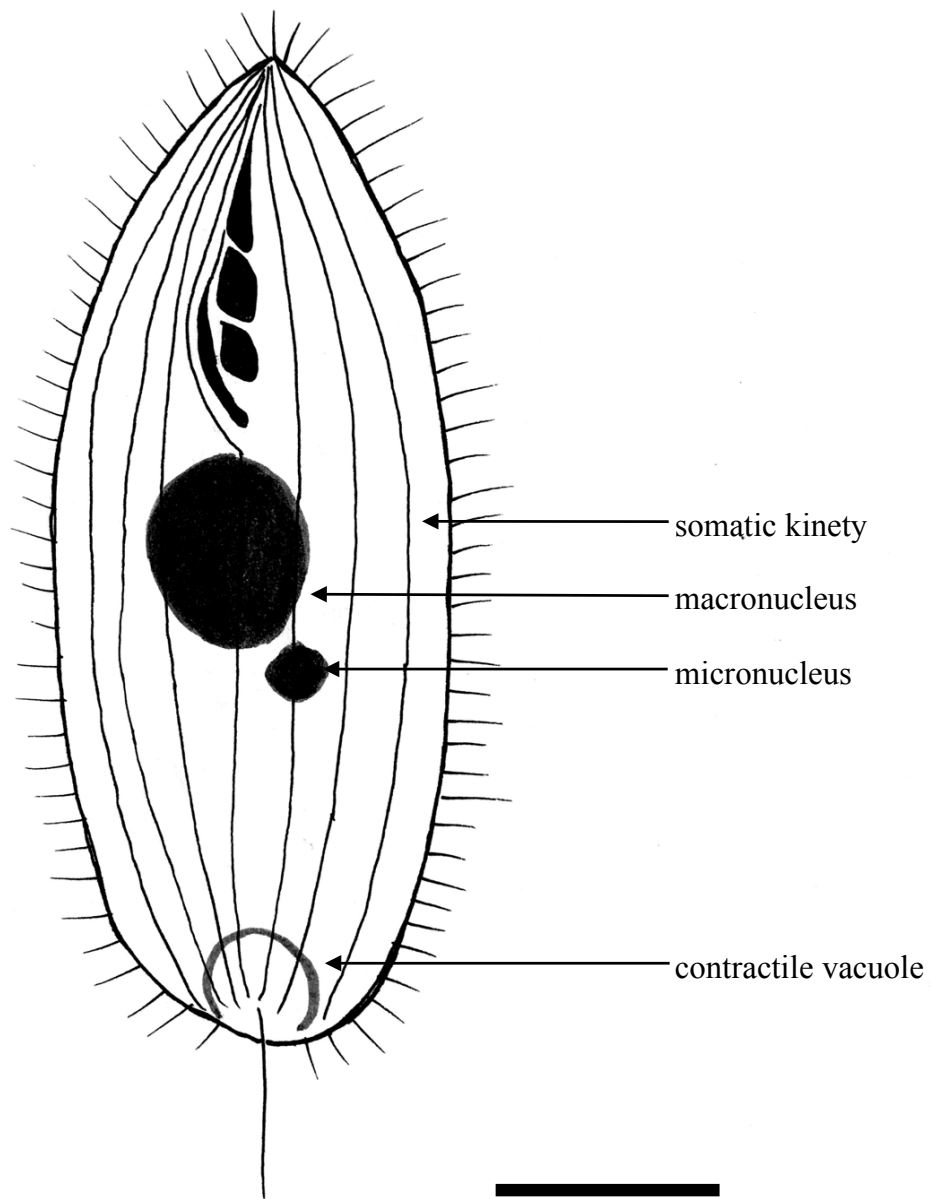
Figure 2.2. Morphology of *O. stellarum* and diagnostic characteristics used to identify the ciliate in gonad, spawn, and fluid samples. Scale bar = 10 μm.

Table 2.1. Annual spawning dates for some *P. ochraceus* populations in California, Oregon, and Washington State, reported from the literature.

State	County	Location	Spawning begins	Spawning ends	Paper
CA	Marin	Duxbury Reef	May 1965. March and June 1966	June	(Nimitz, 1971)
OR	Lane/ Lincoln	Strawberry Hill & South Jetty, Newport	early May	mid June	(Sanford & Menge, 2007)
WA	San Juan	Lonesome Cove	early May and mid June	late June	(Mauzey, 1966)
CA	Santa Cruz	Santa Cruz	March	late June	(Pearse & Eernisse, 1982)

RESULTS

Pisaster ochraceus were rarely infected by *Orchitophyra stellarum* in the three regions I sampled. Across all sites, I found 2.8% of individuals infected with *O. stellarum*. Only six sea stars from three sites (two in Oregon and one in Puget Sound) showed evidence of the ciliate in their gonads, out of a total of 212 sea stars collected. By contrast, 51.4% of *P. ochraceus* sampled had *O. stellarum* on their epidermal tissues. Every site had sea stars associated with epidermal ciliates, including 47 individuals in Washington (n = 92), 45 individuals in Oregon (n = 76), and 17 individuals in California (n = 44). Infected sea stars were all male, while sea stars with epidermal associations with *O. stellarum* included males, females, and sea stars of unknown sex. The following results pertain only to prevalence of epidermal association with *O. stellarum*, since there were not enough infected *P. ochraceus* for statistical analyses.

Differences in Association Prevalence Since SSWD

Rates of association with *O. stellarum* have not shifted since the onset of SSWD on the west coast. At the three sites previously sampled by Stickle and Kozloff (2008) between 1999 and 2000, I found that there has not been a consistent directional shift in the percent of *P. ochraceus* associated with *O. stellarum* since SSWD related die offs. My sample sizes for each gender of *P. ochraceus* at these three sites were likely lower than those used historically; we sampled fifteen stars from each site, while Stickle and Kozloff collected all sea stars present at a site. However, the estimates of historical percent prevalence fell well within the 95% confidence intervals for the estimates of current percent prevalence at all three sites. The one exception was that females at Pigeon Point, CA, experience significantly higher epidermal association rates now than they did before SSWD. However, overall, there was not a significant difference between the past and present prevalence of association with *O. stellarum* (Figure 2.3).

Spatial Variation in Association Prevalence

Variance of *O. stellarum* did not follow clear spatial patterns. I found that variation in *O. stellarum* prevalence was low between the three regions sampled, but was high amongst sites (Figure 2.4). The generalized linear mixed model including region as a predictor did not perform better than the null model when comparing Akaike information criterion (AIC) values (Table 2.2), indicating that region does not explain the variance in *O. stellarum* association prevalence. I did not find a significant relationship between latitude and the prevalence of individuals associated with *O. stellarum* ($P = 0.139$, $n = 15$, $z = 1.478$). My original hypotheses did not address the correlation between site position within a region and parasite prevalence. However, because I found little variation between regions, but high variation within them, I elected to follow up by exploring this relationship. I ran several generalized linear models explaining the relationship between southness within a region and prevalence of association (Table 2.3). The model with the lowest AIC included southness within a region as an important variable affecting the prevalence of association, though this effect differed among regions (Figure 2.5). In Northern California and Washington, I found that the prevalence of association with *O. stellarum* increased from south to north, which was the opposite of what I predicted, but in Oregon the prevalence of association decreased from south to north (Figure 2.5).

Host-Related Variation in Association Prevalence

Generally, host population characteristics affected the association with *O. stellarum*. Increases in host population density (sea stars per square meter) was associated with a reduction in the prevalence of *O. stellarum* on host epidermal tissues, which is the opposite of what I predicted ($P = 0.039$, $n = 15$, $z = -2.064$) (Figure 2.6). Two sites had relatively large mean host densities (0.37

sea stars per m^2 at Starfish Beach, WA, and 0.29 sea stars per m^2 at Bob Creek, OR) and low prevalence of epidermal ciliates, which resulted in high leverage and a large effect on the model's estimation of this relationship (see Discussion). Additionally, prevalence of *O. stellarum* was positively associated with the percentage of males in a population ($P = 0.039$, $n = 15$, $z = 2.602$) (Figure 2.7). Association of *O. stellarum* with *P. ochraceus* did not differ between the two seasons I sampled. At sites sampled twice in California, I found that the season sampled (spring, when host sea stars are reproductive, versus winter) had no significant effect on the prevalence of *P. ochraceus* associated with the ciliate ($P = 0.202$, $n = 3$, $z = -1.275$).

Table 2.2. Generalized linear mixed models, with binomial error distribution and logit link-function, explaining the geographical variability in prevalence of association with *O. stellarum* in populations of *P. ochraceus* by the three coastal regions. Akaike information criterion (AIC) is included for relative comparison of model fit.

Formula	Degrees of Freedom	AIC
Ciliates ~ (1 | Site)	2	80.13
Ciliates ~ Region + (1 | Site)	4	82.42

Table 2.3. Generalized linear models, with binomial error distribution with a logit link-function, explaining the relationship between southness of a site within one of three coastal regions and the prevalence of individual *P. ochraceus* associated with *O. stellarum*. Sites in each region were ranked between 1 and 6, with 1 being the southernmost site. Akaike information criterion (AIC) is included for relative comparison of model fit.

Formula	Degrees of Freedom	AIC
Ciliates ~ Southness * Region	6	87.069
Ciliates ~ Southness + Region	4	96.005
Ciliates ~ Southness	2	96.979
Ciliates ~ -1	15	102.38
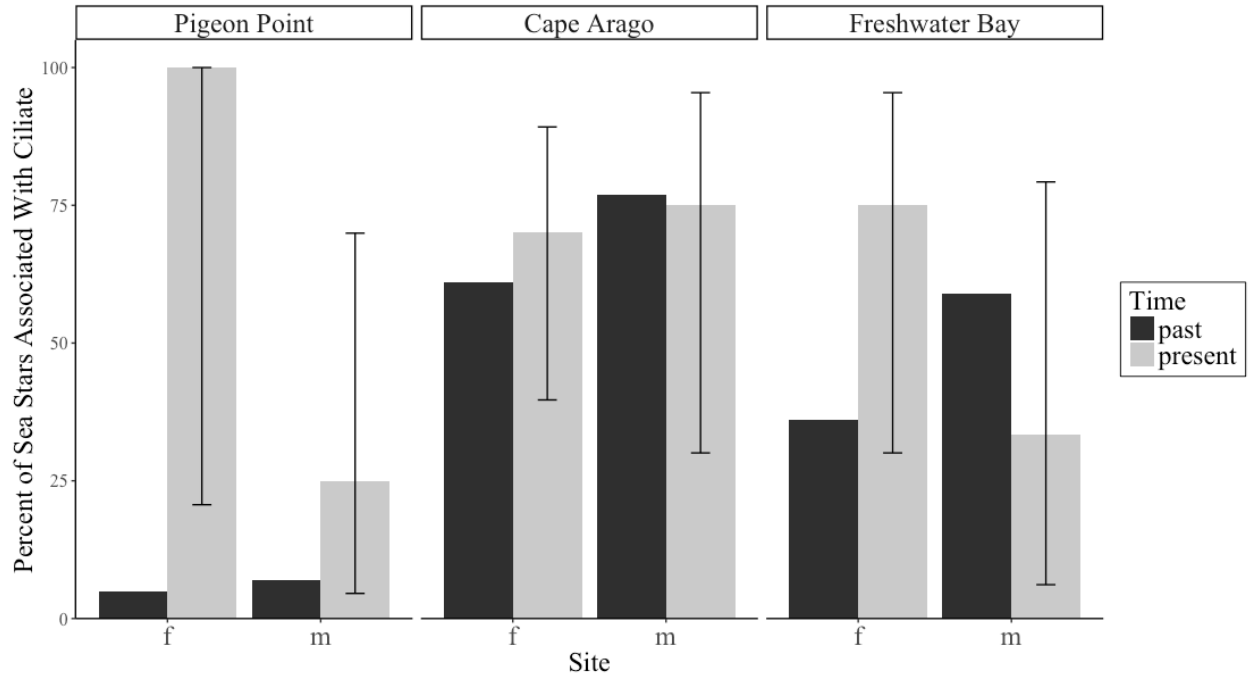
Figure 2.3. Current and historical percent prevalence of *P. ochraceus* associated with *O. stellarum*, as reported by Stickle and Kozloff (2008). Historical percentages were calculated from data collected from 1999-2000, and the sample size is unknown for these estimates. Current estimates are accompanied by 95% confidence intervals.
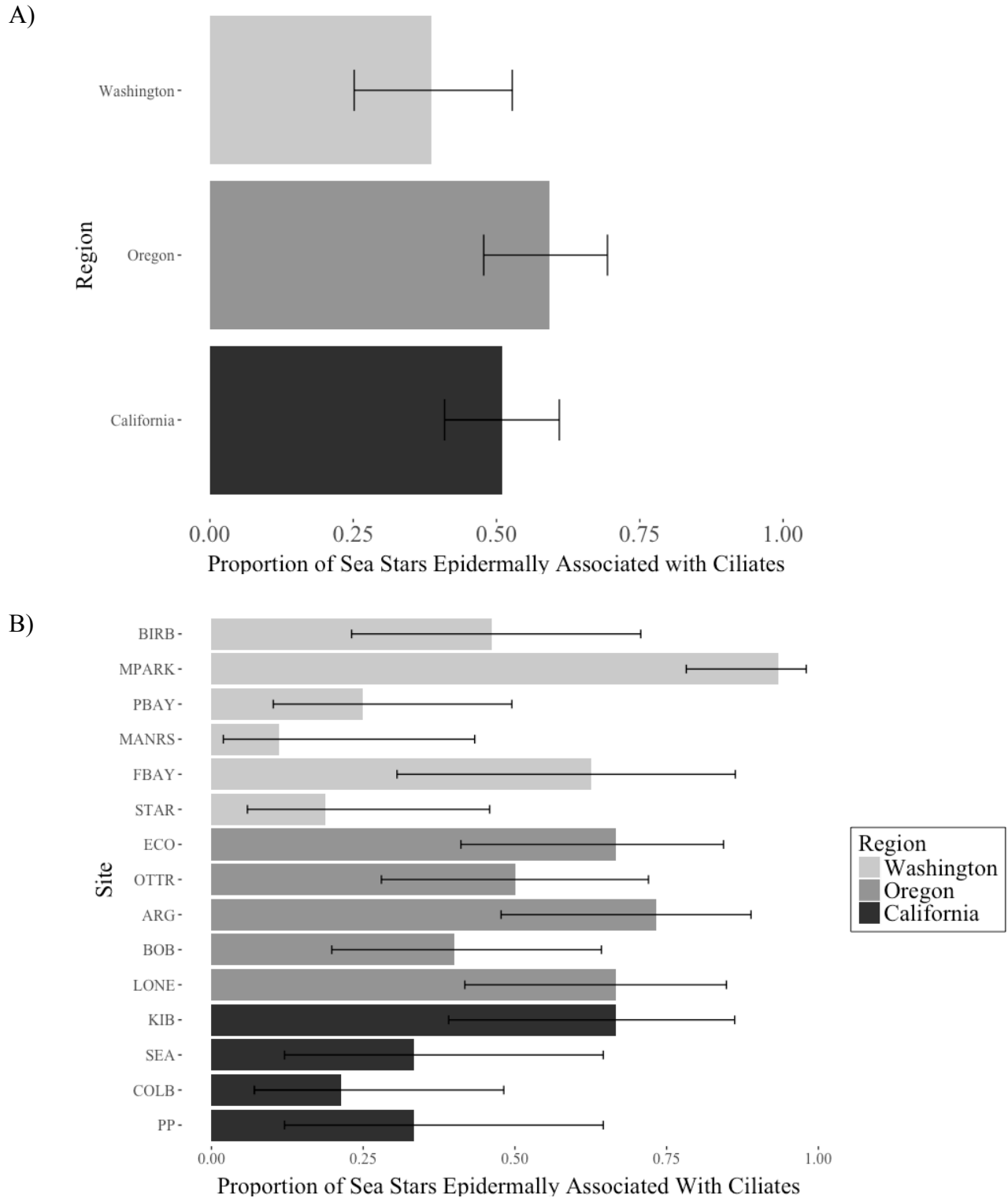
Figure 2.4. The proportion of the population of *P. ochraceus* associated with *O. stellarum* by (A) region sampled and (B) site sampled, with 95% confidence intervals. Regions and sites on the vertical axis run North to South.
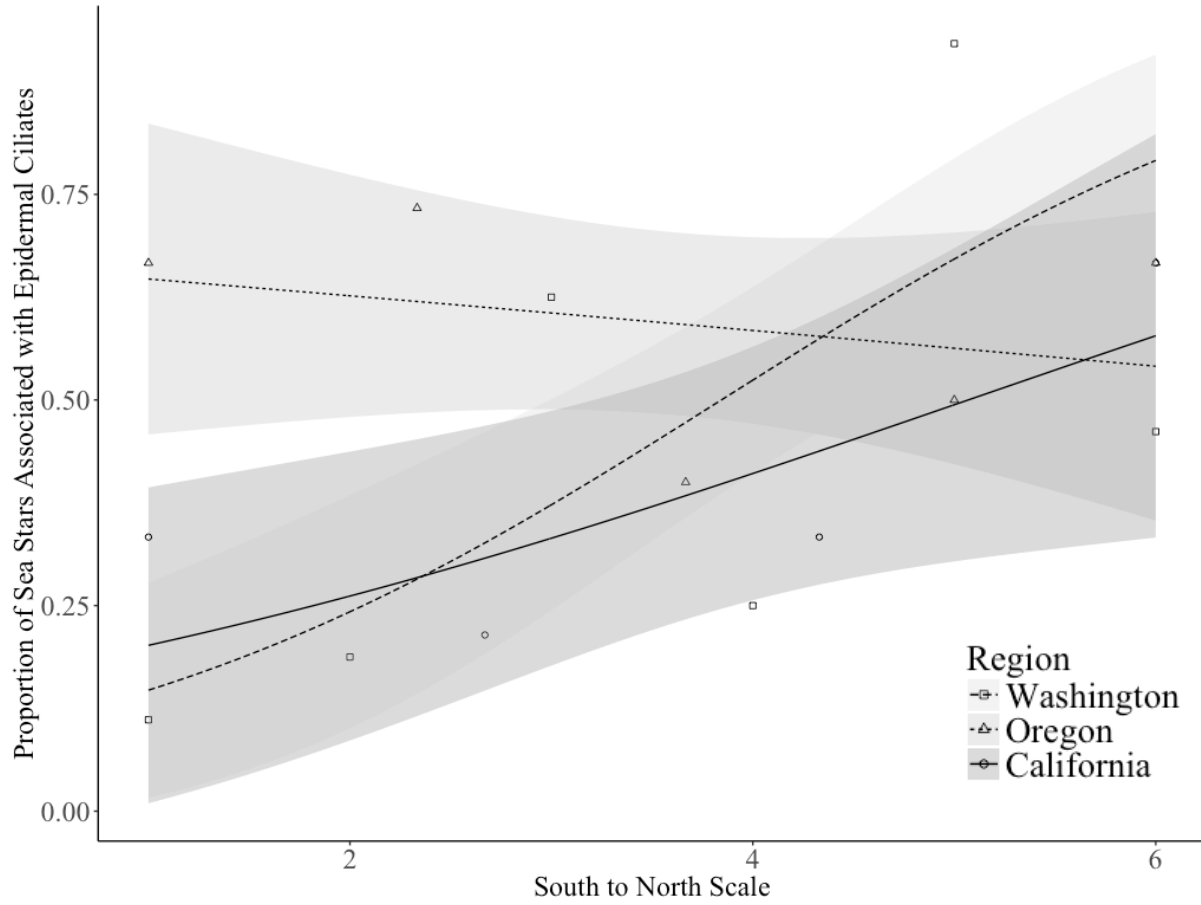
Figure 2.5. Generalized linear model-predicted relationship between southness of a site within a region and prevalence of *P. ochraceus* epidermally associated with *O. stellarum*, with 95% confidence interval. Sites in each region were ranked on a continuous scale between 1 and 6, with 1 being the furthest South in a region.
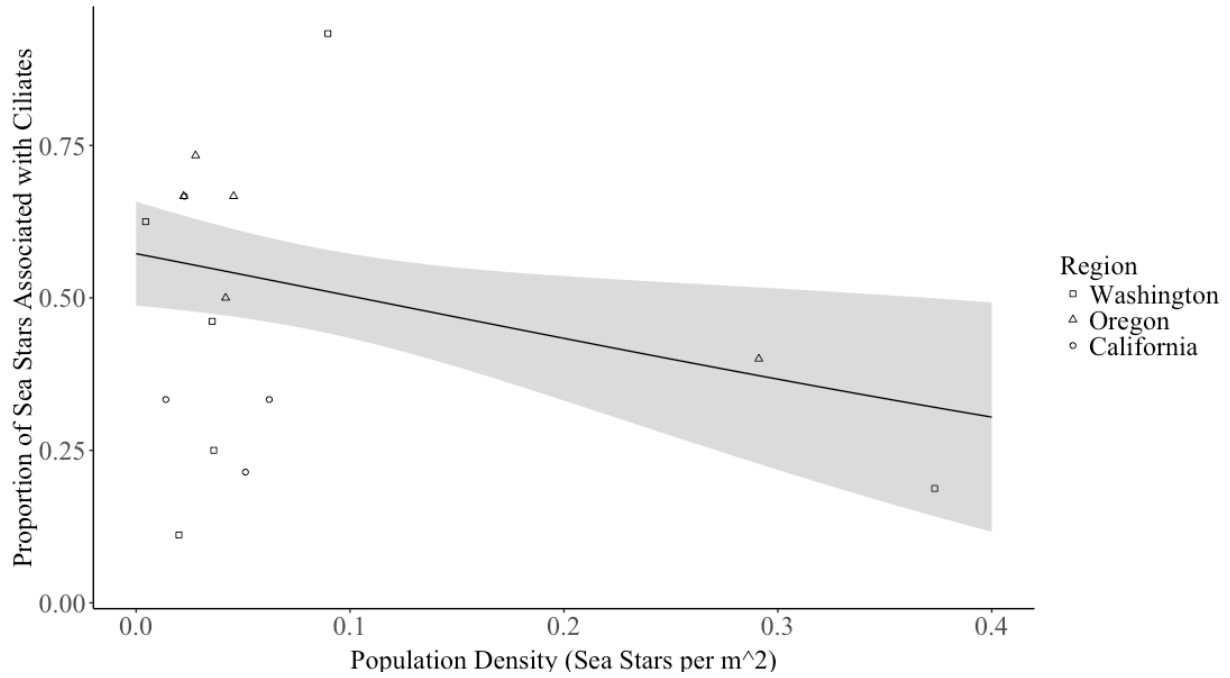
Figure 2.6. Generalized linear model-predicted relationship between *P. ochraceus* population density and the prevalence of *P. ochraceus* epidermally associated with *O. stellarum,* with 95% confidence interval. Points represent actual proportion of association by the mean population density at each site sampled.
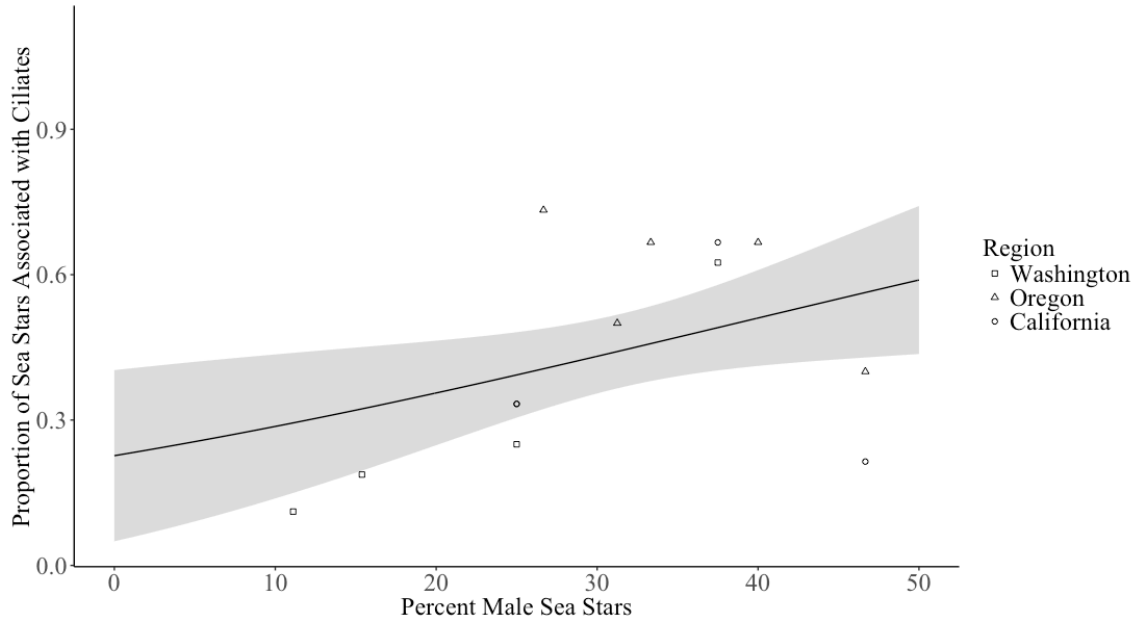
Figure 2.7. Generalized linear model-predicted relationship between the percentage of males in *P. ochraceus* populations and the prevalence of individuals of either sex epidermally associated with *O. stellarum,* with 95% confidence interval. Points represent actual prevalence of infection by the percent of males at each site sampled.

DISCUSSION

Temporal Changes in parasite distribution

There is evidence that temporal variation is common in the host-parasite dynamics of *O. stellarum* and *P. ochraceus*. Male gonad infection by *O. stellarum* was only present in three populations of *P. ochraceus* surveyed in this study, and prevalence was very low in these populations. Frequent infections were first noted in *P. ochraceus* in 1987, but previous examination of the same population in 1985 showed no signs of *O. stellarum* or associated pathogenicity (Boom, 1988). At Manchester Research Station in Puget Sound, WA, in 2001, 32% of males were infected with *O. stellarum* (Stickle & Kozloff, 2008), but in 1999, no individuals were infected (Stickle *et al.*, 2001a). Similarly, 100% of males in Clallam Bay were infected in June 1999, but prevalence was only 17% the previous month (Stickle *et al.*, 2001a). In June 2016 when I resampled these sites, I found no infected individuals in either the Manchester or Clallam Bay populations. In addition, previous studies on *O. stellarum* in other sea star species have reported vastly different rates of infection, but the first time the parasite was discovered in *Asterias rubens* in 1907, infection rates were extremely low as well (Cepede, 1907). Infection prevalence in ochre stars seems to vary greatly among host populations, locations, years, months, and seasons (Bates *et al.*, 2010; Leighton *et al.*, 1991; Stickle & Kozloff, 2008; Stickle *et al.*, 2001a), so I could have sampled during a period of low infection. However, it is interesting that infections were low across so much of the host's range, because most other studies have found high spatial variability in infection rates (e.g. Bates *et al* 2010).

Temporal variation in rates of parasitism could be driven by the population of the parasite itself fluctuating in time due to inherent seasonal variation. Additionally, in the case of non-host-

specific facultative parasites like *O. stellarum*, the parasites could simply be somewhere else (another host, free living, etc.). Evidence shows that generalist pathogens have an inherent ability to capitalize on new host taxa, and often the parasites with the widest host range are the most likely to expand this range (Dobson & Foufopoulos, 2001; Poisot *et al.*, 2013; Woolhouse & Gowtage-Sequeria, 2005). In the past two decades, two surveys of decapod crustaceans (*Nephrops norvegicus* (Small, 2004; Small *et al.*, 2005b), *Callinectus sapidus*, and *Uca minax*, (Miller *et al.*, 2013)) revealed low, but notable and highly pathogenic *O. stellarum* infections. Often, host encounter rate determines rate of alternative host use for generalist parasites (Cooper, 2012; Kuris *et al.*, 2007; Loot, 2006), so if sea star populations are low but other compatible hosts are present, *O. stellarum* could exhibit preferential parasitism for other host species (Lootvoet *et al.*, 2013). Additionally, *O. stellarum* can and will migrate from the gonads to the epidermal tissues of its sea star host if conditions there are more favorable, although the specific drivers behind this transition are currently unclear (Stickle *et al.*, 2007a; Stickle *et al.*, 2007b). Furthermore, seasonal progression of *O. stellarum* infections, as the ciliate enters the gonads, consumes host sperm, and leaves once the host is spawned out, or partially or fully castrated, could result in different estimations of parasitism from week to week. The inherent variability in ciliate prevalence could have resulted in my very low observations of infection (in my limited sampling, I could have simply missed infected sea stars) and justifies a more comprehensive look at this host parasite system.

Temperature plays a role in the biology of *O. stellarum*, and could contribute to temporal variation in ciliate prevalence. Doubling time of *O. stellarum* populations, and corresponding pathogenicity in male *P. ochraceus*, decreases with increasing temperature, which could further explain seasonal variability (Bates *et al.*, 2010), but would also imply that ciliate infections

should be increasing with rising ocean temperatures. Some species of parasitic ciliates exhibit seasonal changes in host preference and specificity, presumably due to temperature changes as well as both the hosts' and parasite's reproductive cycle (Ohtsuka *et al.*, 2004). Stickle *et al.* (2001b) proposed that *O. stellarum* favors winter brooding *Leptasterias* sp. in the colder winter months, and spring spawning *P. ochraceus* in the spring and summer, which could indicate a response of gonadal infection intensity to seasonal temperature changes. I could have sampled before or after the gonads of *P. ochraceus* were invaded by *O. stellarum*, resulting in low estimates of infection prevalence. Because of the fluid nature of this parasite, I propose that multiple mechanisms are interacting to influence the population dynamics of *O. stellarum*.

My sampling design yielded fewer males than expected (mean percentage of males: 30.8%), so it is also possible that I missed infections or that infected males had already suffered mortality, as is suggested by several previous studies (Byrne *et al.*, 1997; Byrne *et al.*, 1998; Leighton *et al.*, 1991). I found low percent association with *O. stellarum* (ciliates on the epidermis) in conjunction with low percentages of male *P. ochraceus*. Previous studies have reported the opposite relationship when true infections were concerned; typically, a high prevalence of infection is found in conjunction with low numbers of male sea stars. Ciliates could either be preferentially selecting against populations with a previously low number of males, or the parasite could be facilitating male mortality and then leaving the population once its supply of sperm-rich hosts has dwindled. However, if males were negatively impacted by *O. stellarum* outside of castration, I would expect to have seen more males with evidence of castration, and I did not. Furthermore, the only males I found with deformed or shrunken testes still had ciliates in their body cavity and gonads. At this time, it is unclear if populations of *P.*

ochraceus recovering from sea star wasting-related declines are reproductively constrained by this parasite.

Despite the low incidence of true infection by *O. stellarum*, the ciliate was still present on the epidermis of sea stars along the west coast. Roughly half of the *P. ochraceus* between Birch Bay, WA, and Pigeon Point, CA, were associated with epidermal *O. stellarum*, and every population that I surveyed had sea stars with the ciliates on their epidermis. It was previously assumed that epidermal ciliates were diagnostic of a gonadal infection (e.g., Stickle and Kozloff (2008)), but as I found extremely low rates of actual infection, it is now clear that epidermal association is not necessarily linked to pathogenicity, although it could be a precursor to infection (Stickle *et al.,* 2007a). Whether epidermal association represents a functional relationship with the host is still unclear; *O. stellarum* could be commensal, or simply an opportunistic part of the epibiota of the sea star. However, the three populations with the largest proportion of ciliate-associated sea stars were the only populations I found to have infected individuals, suggesting that high rates of epidermal association might increase the probability of individual sea stars becoming invaded by these facultative parasites. Bates *et al.,* (2010) experimentally demonstrated that when ciliate densities are high in a host, the probability of infection in nearby testes of both the same and different sea stars increases.

Of the three populations that were previously examined for epidermal association with *O. stellarum* in 2001, only female sea stars from Pigeon Point, CA, experienced significant change in epidermal association prevalence as of spring 2016. This suggests that, unlike infection rates, epidermal association with this ciliate is relatively stable over time. Ciliate association prevalence was also consistent on a more acute time scale, considering that *O. stellarum* prevalence on external tissues did not change significantly between the winter of 2016 and the

following spring, which contradicted my hypothesis. It is unclear why *O. stellarum* association with females' epidermis would increase, but because this study did not include equal sample sizes of males and females, further research is warranted to explore the relationship between epidermal association with ciliates and host sex.

I found that epidermal ciliate prevalence decreases with increases in host population density, although estimation of this relationship was primarily driven by two very high density populations with correspondingly low *O. stellarum* associations. This result is contrary to both my hypothesis and most accepted epidemiological models, which state that disease transmission and prevalence is positively linked to host density (Anderson & May, 1981). Further research on *O. stellarum* should sample host populations in a range of host densities, to test whether or not high host density truly leads to reduced *O. stellarum* association. However, there are a number of potential explanations for a negative correlation between *O. stellarum* prevalence and population size. First, the *P. ochraceus* populations with the highest prevalence of *O. stellarum* could be small because of mortality due to potential pathogenic impacts of *O. stellarum.* Anderson & May (1979) suggest that in host species with a high influx of new individuals susceptible to infection (high birth rate, for example), parasites will persist and cause severe reductions in host populations. In my study, evidence for *O. stellarum*-related deaths is weak because no morbidity was observed in infected or associated *P. ochraceus* while sampling in the field. Unpublished data referred to by Vevers (1951) lead him to hypothesize that *O. stellarum* reproductively constrains host populations, resulting in smaller future generations. Initially, in 1947, a dense population of *Asterias rubens* in Plymouth Sound, England, was over 20% infected with *O. stellarum*, and the host population decreased in the three subsequent years. However, *P. ochraceus* broadcast spawn, and their planktonic larvae disperse widely from their source

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populations (Strathmann, 1974; Strathmann, 1978). While reductions in male sperm output could be a consequence of *O. stellarum* association, it is unlikely that a population with a high prevalence of ciliate association would experience declines due to reproductive constraint; recruitment of juveniles is likely influx from other *P. ochraceus* populations. Regardless, future studies should explore the degree to which *O. stellarum* presence in a population is related to host reproductive output, especially because SSWD consistently reduced populations of *P. ochraceus* along the West Coast, and reduction in reproductive success would impact recovery coast-wide. Future research on *O. stellarum* should also consider that low host density might be a good predictor for high parasite prevalence simply because it correlates with an unknown abiotic variable that determines *O. stellarum* intensity. Finally, if there are density-dependent changes in *O. stellarum* prevalence, and if the three populations that I compared over time have recovered to pre-SSWD densities, that would explain why I have not seen any changes in association from the estimates presented in Stickle & Kozloff (2008).

Spatial changes in parasite distribution

The distribution of *O. stellarum* in *P. ochraceus* populations is generally consistent along the west coast of North America. Prevalence of epidermal *O. stellarum* association did not change from region to region, but prevalence did vary significantly from site to site, within regions. Stickle and Kozloff (2008) found that rates of association varied between coastal Washington, Oregon, and especially Northern California, but only sampled one site in each of those areas. Because I found that prevalence varies significantly on a smaller scale, they most likely missed intraregional variation due to their limited sampling regime. Although not one of my original questions, I elected to further determine if southness within regions, and potentially a relative

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change in mean seawater temperature, impacted ciliate prevalence. I included sampling region as a covariate in this analysis because in Washington state the relationship is muddled by the presence of the warmer, highly tidal, Puget Sound. There was no clear relationship between latitude and prevalence across the entire sampling range, but in Oregon, association increased in southern *P. ochraceus* populations, initially leading me to believe that abiotic variables in lower latitudes in Oregon might play some role in host-ciliate interactions. In California and Washington State, association prevalence increased in populations that were further North. I hypothesize that, due to the convergence of high ciliate prevalence on the border between California and Oregon, that there might be hotspots where *O. stellarum* growth is maximized. Additionally, the pattern observed in Washington state suggests that the Puget Sound, because of its low wave action and warmer mean seawater temperatures, could also be a hotspot for this ciliate. For ectoparasites, other support has been found for a "center of abundance" hypothesis, which states that species are the most abundant in the locality with the most favorable conditions for that species, and abundance decreases proportionally with the distance from that locality (Krasnov & Poulin, 2010; Krasnov *et al.*, 2008). When the ciliate is externally associated with *P. ochraceus*, it is exposed to the environment in the same way ectoparasites are, so distribution of *O. stellarum* ecto-association likely follows similar rules.

Orchitophyra stellarum can withstand temperatures between 3°C and 27°C, and its growth is positively correlated with water temperature within these limits (Bates *et al.*, 2010; Stickle *et al.*, 2001a). While the seasonal temperature fluctuations in the area I sampled fall well within this range, small-scale differences in intertidal water temperature due to the topography or substrate composition of a specific location could influence transmission and proliferation of this ciliate within host populations. However, differences in intertidal ecosystems between regions do

not seem to play a role in the ecology of this ciliate. Bates (2010) reported high prevalence of infection in populations of *P. ochraceus*, and postulated that high prevalence of *O. stellarum* was found because the thermal niche of *P. ochraceus*, which forages intertidally, was warmer than that of *Asterias miniata*. He further supported this conclusion by reporting lower rates of infection in *A. miniata*. There is a clear relationship between ciliate populations and ambient sea water temperature, with *O. stellarum* increasing in association, infection, and pathogenicity with an increase in temperature (Bates *et al.*, 2010, Stickle *et al.*, 2001a). In addition, *P. ochraceus* (and other asteroid sea stars') overall health tends to decline with an increase in temperature, which caused researchers to hypothesize that rising ocean temperature might play a role in the appearance of SSWD (Eisenlord *et al.*, 2016; Kohl *et al.*, 2016; Staehli *et al.*, 2009). The relationship between ciliate intensity and temperature, as well as wasting disease and temperature, in *P. ochraceus* could be linked to reduced host immune performance (Mydlarz *et al.*, 2006). Regardless of micro-thermal regime, the whole ocean is projected to warm due to climate change, which will inevitably change the dynamics of this parasite. To fully understand spatial changes in this host-parasite relationship, future studies should examine the relationship between intertidal habitat temperature and association with epidermal *O. stellarum.*

Conclusion

In the last two decades, *P. ochraceus* infection by *O. stellarum* has declined, while seemingly non-pathogenic epidermal association with the ciliate has stayed relatively stable over time and throughout the host range. Shifts in host-parasite dynamics seem to be more due to inherent stochasticity in the system than because of SSWD-mediated die-offs. That being said, there is weak evidence for *O. stellarum*-mediated population reductions, due to the presence of small

populations of *P. ochraceus* with high rates of ciliate association. The mechanism behind the link between small populations and high prevalence of ciliates needs to be clarified, especially because in the summers after the initial SSWD outbreak, *P. ochraceus* populations continue to be seasonally plagued by wasting (Benjamin Miner, pers. communication). The questions raised by this study reinforce the notion that we understand very little about the population dynamics of multi-host parasites like *O. stellarum* (Woolhouse *et al.*, 2001); generalist parasites like these can sometimes be the most pathogenic because their survival is not necessarily linked to that of their host (Leggett *et al.*, 2013). Further research on this host-parasite system should include investigation of the abiotic site-specific drivers behind both infection and association rates, for example, small scale temperature fluctuations, wave exposure, and substrata composition.

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Table S1. Summary data on the number of male and female *P. ochraceus,* and sea stars of unknown sex, presenting with epidermal association with *O. stellarum* in each region and sampling season. Numbers of male sea stars with *O. stellarum* infections in their gonads are also included. Non-reproductive sampling in California occurred in the winter, when it was impossible to determine sea star sex, and when there were no gonad infections. Sample size refers to the number of sea stars collected for that sex in that region or sampling season.

	Washington			Oregon			California			California (non-reproductive sampling)
	Males	Females	Unknown sex	Males	Females	Unknown sex	Males	Females	Unknown sex	Unknown sex
Infected with *O. stellarum* in gonads	1	N/A	N/A	2	N/A	N/A	0	N/A	N/A	N/A
Associated with *O. stellarum* on epidermis	4	11	32	12	21	12	6	5	6	39
Sample size	10	27	55	27	30	19	16	14	14	81