Experimental infections of *Orchitophrya stellarum* (Scuticociliata) in American blue crabs (*Callinectes sapidus*) and fiddler crabs (*Uca minax*)

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**A B S T R A C T**

Outbreaks of an unidentified ciliate have occurred on several occasions in blue crabs from Chesapeake Bay held during winter months in flow-through systems. The parasite was initially thought to be *Mesanophrys chesapeakensis*, but molecular analysis identified it as *Orchitophrya stellarum*, a facultative parasite of sea stars (Asteroidea). We investigated the host-parasite association of *O. stellarum* in the blue crab host. Crabs were inoculated with the ciliate, or they were held in bath exposures after experimentally induced autotomy of limbs in order to determine potential mechanisms for infection. Crabs inoculated with the ciliate, or exposed to it after experimentally autotomy, rapidly developed fatal infections. Crabs that were not experimentally injured, but were exposed to the ciliate, rarely developed infections; thus, indicating that the parasite requires a wound or break in the cuticle as a portal of entry. For comparative purposes, fiddler crabs, *Uca minax*, were inoculated with the ciliate in a dose-titration experiment. Low doses of the ciliate (10 per crab) were sometimes able to establish infections, but high intensity infections developed quickly at doses over 500 ciliates per crab. Chemotaxis studies were initiated to determine if the ciliate preferentially selected blue crab serum (BCS) over other nutrient sources. Cultures grown on medium with BCS or fetal bovine serum showed some conditioning in their selection for different media, but the outcome in choice experiments indicated that the ciliate was attracted to BCS and not seawater. Our findings indicate that *O. stellarum* is a facultative parasite of blue crabs. It can cause infections in exposed crabs at 10–15 °C, but it requires a portal of entry for successful host invasion, and it may find injured hosts using chemotaxis.

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1. Introduction

Scuticociliate infections are known to cause significant pathology, mortality, and in some cases castration in a wide range of marine organisms, including molluscs (Bower et al., 1994; Bradbury, 1994; Elston et al., 1999), echinoderms (Cepede, 1907), and teleost fishes (Dickerson, 2006; Smith et al., 2009). The first scuticociliate reported from a crustacean was found in the hemolymph of *Carcinus maenas* by Cattaneo (1888). Since then endoparasitic ciliates, including scuticociliates, have been widely reported in crustaceans, including *Anophryoides haemophilia* in wild-caught and cultured American lobsters, *Homarus americanus* (Ragan et al., 1996; Lavallée et al., 2001; Greenwood et al., 2005), *Collina oregonensis* in euphausiids (Gomez-Gutierrez et al., 2003, 2006), *Parauronema* sp. in the hemocoel of penaeid shrimps (Couch, 1978), *Tetrahymena pyriformis* infections in the freshwater red-clawed crayfish, * Cherax quadricarinatus* (Edgerton et al., 1996) and *Mesanophrys* or *Mesanophrys*-like infections (syns. *Anophrys* and *Paranophrys*) in brachyuran crabs from the Atlantic and Pacific oceans (Bang et al., 1972; Armstrong et al., 1981; Messick and Small, 1996; Messick, 1998; Morado et al., 1999; Wiackowski et al., 1999; Cain and Morado, 2001; Morado, 2011), isopods (Hibbits and Sparks, 1983) and the Norway lobster, *Nephrops norvegicus* (Small et al., 2005a,b).

During winter 2007, systemic ciliate infections caused mortalities in blue crabs, *C. sapidus*, held in large flow-through systems within our facilities at the Virginia Institute of Marine Science. Such mortalities had occurred earlier within our system but they were thought to be restricted to a few crabs injured from dredge operations. The pathogen was putatively identified based on simple morphology and host distribution as *Mesanophrys chesapeakensis*, a parasitic ciliate described from *C. sapidus* by Messick and Small (1996). However, further study and molecular characterization revealed that the ciliate was *O. stellarum* (Small et al. in press). In a previous study, morphological analysis of a *Mesanophrys*-like scuticociliate from *N. norvegicus* indicated that the parasite was...
morphologically consistent with Mesanophrys carcinii, but sequenc-
ing of the internal transcribed spacer and 5.8S regions (ITS1, 5.8S and ITS2) ribosomal DNA revealed an identical match to Orchi-
tophrya stellarum, a closely related scuticociliate (Small et al., 2005a). Small et al. (2005a) hesitated in classifying this ciliate into either genus because of this inconsistency, and because O. stella-
rum had not been previously reported from a crustacean.

O. stellarum has been reported as a parasite of echinoderms (primarily asteroid sea stars) (Cepede, 1907; Vevers, 1951). It in-
fects the gonadal tissues of males and has been observed ingesting sperm cells. Infections can result in decreased reproductive output or castration (Bouland and Jangoux, 1988; Leighton et al., 1991; Byrne et al., 1997; Goggin and Bouland, 1997; Stickle et al., 2007b; Stickle and Kozloff, 2008). Reports of this species from widely separated geographic localities in combination with identi-
cal sequence data from the ITS rDNA region from distinctly located isolates (Japan, Canada, and the Netherlands) suggest that it has a worldwide distribution in temperate and sub-arctic waters (Leighton et al., 1991; Byrne et al., 1997; Goggin and Murphy, 2000; Goggin and Bouland, 1997; Stickle et al., 2001; Stickle and Kozloff, 2008). O. stellarum is considered a facultative parasite be-
cause cultures can survive indefinitely provided there is a bacterial or detrital food source available, but it readily infects sea stars when available (Stickle et al., 2001, 2007a). However, there are no reports of its occurrence as a free-living organism; it has only been isolated from infected hosts. Moreover, blue crabs infected with the ciliate were not feeding on seastars (crabs do not feed during hibernation) nor were they cohabiting with them in the laboratory.

Given the repeated occurrence of the ciliate in our holding facil-
ities during winter months, our objectives were to confirm the pathogenicity of this ciliate in crustacean hosts and examine host injury as a possible route of transmission. We also experimentally exam-
ined features of the host-parasite association such as host speci-
ficity, temperature effects, pathology, minimum infectious dose requirements via dose titration trials and the chemotactic response of O. stellarum to crab sera.

2. Materials and Methods

2.1. Initial Diagnosis and Cultivation of O. stellarum

In January 2007, systemic ciliate infections were discovered causing mortalities in freshly caught blue crabs, C. sapidus, held in flow-through seawater tanks fed from the mouth of the York River, Chesapeake Bay at the Virginia Institute of Marine Science (37°14'49" N, 76°29'57"W). Incoming water was filtered at 30 μm at ambient winter temperature (<6°C). Microscopic examination of hemolymph smears from moribund or freshly dead crabs revealed the presence of large numbers of scuticociliates tentatively identified based on morphological observations and host similarity as M. chesapeakensis. Samples of the ciliate were obtained from infected hemolymph and cultured in a modified Appleton–Vicker-
man medium (MAM) with the addition of 10% fetal bovine serum (FBS) (Appleton and Vickerman, 1998) as described by Small et al. (2007). This medium contained NaCl 19.31 g l⁻¹, KCl 0.65 g l⁻¹, CaCl₂ 1.38 g l⁻¹, MgSO₄ 1.73 g l⁻¹, Na₂SO₄ 0.38 g l⁻¹, HEPES 0.82 g l⁻¹, glucose (1.0 mg ml⁻¹), and the pH adjusted to 7.8. Penicillin (80 U ml⁻¹) and streptomycin (50 mg ml⁻¹) were added to suppress bacterial contamination and the culture medium was filter-sterilized (0.2 μm) prior to use. Ciliates were isolated from infected blue crabs by extracting approximately 1 ml of hemolymph from the base of the fifth pereopod using a sterile disposable 1-ml syringe and 25-ga needle, and adding the infected hemolymph to 10 ml of culture media in sterile 25-cm² tissue culture flasks. Cultures were incubated at 4–5 °C in the dark and the media was refreshed weekly or monthly. Subcultures were propagated using the same technique as above.

2.2. Molecular Identification of O. stellarum

The ciliate was identified as O. stellarum via sequencing and analysis of the ITS rRNA region. Briefly, aliquots of DNA were ex-
tracted from infected hemolymph and culture preparations, and the internal transcribed spacer regions 1 and 2 (ITS1 and ITS2) and the 5.8S ribosomal gene amplified and sequenced following standard procedures given in Small et al. (in press). Comparison with sequences in GenBank indicated that the ciliate causing mortal-
talities in the C. sapidus was O. stellarum and not M. chesapeakensis as originally thought. BLAST analysis of the sequences revealed they were 99–100% similar (100% query coverage) to O. stellarum and only 95% similar to M. chesapeakensis and M. pugetensis (Small et al. in press).

2.3. Ciliate density quantification for chemotaxis, inoculation and exposure trials

The density of ciliates maintained in the culture flasks was quantified by aliquoting 0.9 ml culture medium (containing cili-
ates) into 0.1 ml 5% neutral buffered formalin then counting three to five replicates of 10-μl samples in a hemacytometer (Neubauer Bright-Line) using an Olympus BX51 compound microscope. Appropriate serial dilutions were made in order to obtain desired densities for use in exposure and chemotaxis trials.

2.4. C. sapidus inoculation trial

An initial inoculation trial was performed to fulfill Koch’s postu-
lates and confirm that infections of O. stellarum led to mortality in blue crabs, C. sapidus. Crabs used in this experiment were collected off Wachapreague Creek, (37°37′10″N, 75°40′26″W), King’s Creek (37°18′31″N, 76°25′05″W), or the York River (37°14′50″N, 76°29′57″W), Virginia, in May, June and July, when ciliate infec-
ions are not encountered in blue crabs (Shields unpubl. data). Preliminary culture experiments indicated that the ciliates grew readily at relatively low temperatures (e.g., 4–15 °C) in culture, but begin to show marked mortality at ambient room temperature (~22 °C) and above (to 30 °C). Therefore, two temperatures, 15 °C and 23 °C, were chosen as treatments for the initial infection study. Blue crabs were randomly assigned to each experimental treatment (n = 20 per treatment) or control (n = 10 per treatment) group within each temperature. The crabs were placed into aquaria with filtered York River water (salinity = 20 ± 1 psu) and allowed to acclimate at 15°C or 23°C for 3 days prior to the start of the experiment. Crabs were kept individually in 38L aquaria, but due to space limitations, a few individuals were housed together but separated by a plastic divider in 76 L aquaria.

All crabs were screened for Vibrio spp. at the commencement of the experiment by placing a few drops of hemolymph on thiosul-
fat-citrate-bile salts-sucrose (TCBS) agar. Hemolymph was asepti-
cally obtained by wiping the juncture between the basis and ischium of the fifth walking leg with 70% ethanol, and drawing ~100 μl of hemolymph from that location with a 1-ml syringe equipped with 27-ga needle. Each crab in the experimental treat-
ments was then inoculated with a 100 μl dose containing approxi-
mately 2.0 × 10⁶ ciliates from axenic cultures grown in 10% FBS in MAM. In this and in various exposure trials, ciliate densities were obtained through serial dilution of cultures assessed as described earlier. Crabs serving as controls were inoculated with an equal vol-
ume of 10% FBS in MAM. Crabs were monitored twice daily for signs of morbidity or mortality. Water quality was monitored daily and water changes made when necessary to ensure that parameters...
remained within acceptable limits: ammonia (0–0.3 ppm), nitrite (0–0.6 ppm), pH (7.4–8.2). Crabs in the 23 °C treatment were fed portions of squid 3 times a week and those in the 15 °C treatment fed once a week due to their lowered activity and metabolism. Uneaten food was removed 2–3 h after feeding.

2.5. *O. stellarum* diagnosis and crab tissue processing

Infections with *O. stellarum* in blue and fiddler crabs were diagnosed by direct examination of hemolymph or by histology. In exposure experiments, once an individual exhibited signs of morbidity or mortality, it was removed, the date of death or morbidity recorded, and a hemolymph sample drawn to assess infection status. Hemolymph samples from infected crabs were fixed at a ratio of 1:2 in MAM buffer with 10% neutral buffered formalin and the number of ciliates counted using a hemacytometer with triplicate chamber counts following standard procedures for deriving densities. The carapace was then removed, and tissues (heart, hematopoietic tissue, hepatopancreas, skin, muscle, gonads, gills, eyes and nerve ring) from moribund or freshly dead crabs were fixed in Bouin’s solution or 10% neutral buffered formalin, processed through paraffin, sectioned at 5–6 µm, and stained with Mayer’s hematoxylin and eosin (Wheeler et al., 2007). Due to the small size of the fiddler crabs, the ventral nerve ring was usually left intact in the thorax which was fixed whole for histology. Gill tissue and thoraces were decalcified for 24 h using formic-acid-sodium citrate (Luna, 1968). Standard histological techniques were employed, with sections cut at 5–6 µm and stained with Mayer’s hematoxylin and eosin. Slides were examined for ciliates and histopathology on an Olympus BX51 microscope and photographed using a Nikon DXM1200 digital camera. Control individuals from each treatment were randomly selected (n = 3–5) at the completion of each experiment and processed for histology to confirm the absence of ciliates or other pathogens. The hemolymph was examined from all crabs serving as controls and no control crabs ever possessed ciliate infections.

2.6. Portal-of-entry exposure experiment

The objective of this experiment was to determine whether *O. stellarum* required a temporary portal of entry, such as a wound, to infect *C. sapidus*. Portals of entry were created by inducing natural autotomy of the left and right fourth legs (L4 and R4, respectively) of individual crabs. It was induced by slightly pinching the leg with forceps near the basis and deflecting the leg laterally until released by the crab. Autotomy is a natural, controlled process in crustaceans that may occur during intraspecific aggression or attacks by predators. Moreover, compared to puncturing the carapace, wounds due to autotomy are controlled and clean, with less risk of bacterial infection or incidental mortality. Crabs used in this experiment were collected in early summer as described above when infections are not encountered in nature (Sheilds unpubl. data). Individuals were held in flow-through seawater aquarium containing filtered York River water (salinity = 20 ± 1 ppm) and allowed to acclimate for 7 days prior to the start of the experiment.

The experimental design consisted of bath exposures for two treatment and two control groups. The two treatments included crabs that had two limbs autotomized (i.e., exposed-autotomized treatment, n = 20) and those that were not autotomized (i.e., exposed-unautotomized treatment, n = 20) exposed to a measured dose of ciliates. The controls consisted of an autotomized-unexposed group (n = 11) which was included to determine whether autotomy caused morbidity and mortality independent of infection, and an unautotomized-unexposed group (n = 10) to assess morbidity and mortality due to general handling procedures. A detailed visual inspection of each individual crab was conducted to ensure that there were no signs of damage to the carapace and that all limbs were present and intact before being randomly assigned to the experimental groups. The long and short carapace widths (LCW, with epibranchial spines and SCW, without epibranchial spines, respectively) and the sex of each individual were recorded. Crabs were not screened for pre-existing infections because the needle puncture itself could potentially introduce a portal of entry or cause infection. However, individuals were randomly assigned to treatments, so pre-existing conditions, if present, would occur to individuals in any treatments, and all crabs were assessed for other infections via hemolymph smear post-mortem or at the end of the experiment.

After the initial assessment, crabs were transferred to aquaria containing filtered York River water (salinity = 20 ± 1 ppm) maintained at 10 °C in cold-rooms. Due to space limitations in the cold rooms, individuals within treatment groups were housed two or three each in 38-L aquaria equipped with biological filters with air stones and with plastic dividers to prevent contact. Crabs were allowed to acclimate for 3 days at 10 °C before the experiment commenced. After the acclimation period each crab was processed according to its randomly assigned treatment. Crabs were exposed to ciliates or control medium using bath exposure in small plastic 739-ml containers (Tupperware® Brand, with holes punched in the lids to allow oxygen exchange). Bath exposure was done individually by placing 490 ml of aquarium water (10 °C) into each individual’s exposure container, then adding either 1.0 × 10⁶ ciliates (estimated as above, using 10 ml of culture after appropriate dilutions were made) or an equivalent volume (10 ml) of MAM with 10% FBS culture without ciliates. This dosage (i.e., 1.0 × 10⁶ ciliates in 500 ml of water = 2000 ciliates/ml) was chosen based on the preliminary inoculation trials, in which mortalities were observed in crabs when inoculated with fewer than 2000 ciliates. In the autotomy treatment groups (exposed and unexposed), legs L4 and R4 of the crabs were autotomized as described above and the individuals were then immediately placed into the exposure chambers. Crabs in the unautotomized treatments were similarly handled (i.e., held out of water for the same period of time), but all legs were left intact. Crabs were held in individual exposure chambers for 1 h and then returned to their assigned locations in the aquaria. All four treatment groups were equally represented in both cold rooms to control for any differences between rooms. To reduce the risk of transferring ciliates between tanks, the exposed tanks were put on low shelves to prevent dripping into unexposed tanks, and separate nets were used between exposed and unexposed tanks.

A positive control to show that the ciliate culture was still pathogenic consisted of inoculating two blue crabs at the base of their fifth leg with 2 × 10⁷ ciliates from the same cultures used in the exposure experiment. These were monitored in a separate tank held at 10 °C in a cold room. Water quality parameters were monitored periodically to ensure they remained within acceptable limits as above.

Crabs were monitored twice a day, and once an individual exhibited signs of morbidity or mortality, it was removed, the date of death recorded, and a hemolymph sample drawn to assess infection status. Hemolymph samples from infected crabs were fixed 1:2 in MAM buffer with 10% formalin and the density of ciliates calculated using a hemacytometer. All individuals exposed to ciliates (both autotomized and unautotomized groups) and any controls that died during the experiment were processed for histology. After 25 days the experiment was terminated and surviving individuals were processed for hemolymph smears and for histology.

2.7. Alternate host experiment

The objective of this experiment was to determine whether fiddler crabs, *Uca minax*, can become infected with *O. stellarum,*
U. minax was selected for this study because individuals are easy to collect, they are much smaller and easier to maintain in artificial mesocosms or aquaria than C. sapidus, they are well studied, and they are relatively distant phylogenetically from the Portunidae.

Thirty male U. minax were collected by hand or dip net from near the mouth of King’s Creek (37°18'31"N, 76°25'05"W), Virginia. Six 19-L aquaria were prepared (three experimental and three control) and the habitat simulated by covering the bottom of the tanks with 2 cm of sandy mud obtained from the beach at the Virginia Institute of Marine Science and a small layer of intertidal mud that was collected from the same locality where the specimens were obtained. Filtered York River water (salinity = 20 ± 1 PSU) was added when required to keep the substrate moist and to allow some pooling of water. The six tanks (each containing five individuals of U. minax) were then placed in the cold rooms at 10 °C for 3 days. After this acclimation period, 15 crabs were inoculated using a 27-ga needle at the arthrodial membrane between the fourth and fifth legs with a 50-µl dose containing an estimated 2000 ciliates. The site was wiped with 70% ethanol prior to inoculation. Fifteen crabs were also inoculated with 50 µl of chilled culture medium to serve as negative controls. Two uninfected blue crabs, C. sapidus, were inoculated with 2000 ciliates from the same cultures used in this experiment to serve as positive controls to demonstrate pathogenicity. They were held in adjacent aquaria at 10 °C. Inoculated U. minax were placed in a clean bucket for 5–10 min to let the puncture site clot prior to being returned to their allocated aquaria.

2.8. U. minax dose titration study

After the initial study showing pathogenicity in U. minax, a dose-titration study was undertaken with additional fiddler crabs. Eighteen 19 L tanks were prepared as above, each housing 10 male fiddler crabs. Single doses of 10, 100, 500, 1000, 2000 or 5000 ciliates in 50 µl inoculations were administered to individual crabs assigned to each treatment. Twenty individuals were randomly assigned to each of the dose treatments and crabs from the same treatment were housed together to 10 to a tank due to space constraints (2 replicate tanks total for each treatment). Thirty fiddler crabs were inoculated with 50 µl of 10% FBS in MAM culture medium to serve as controls and three uninfected C. sapidus were inoculated with 5 × 105 ciliates from the same cultures used in this experiment to serve as positive controls to demonstrate pathogenicity. The blue crabs were held individually in adjacent aquaria. The experiment ended after 13 day. A sample of crabs that died during the experiment and exposed crabs that survived to the end of the experiment were processed for histology as above. Hemolymph smears were examined from all crabs serving as controls.

2.9. Statistical analyses

For statistical analyses, survival curves were compared for each treatment using survival analysis with Kaplan–Meier assumptions (Cox and Oakes, 1984). Survival curves for individual treatments were compared with the Tarone–Ware log-rank test using SYSTAT version 11. A Cox proportional hazards test was used to examine the effects of replicate and treatment using R v2.10.1 (http://www.r-project.org). The Kruskal–Wallis test was used to compare frequency of disease-induced autotomy among different treatments. ANOVA was used to analyze main effects in the chemosensory treatments. Post-hoc pairwise analyses performed in JMP v.7.0.1 were used to compare the relative selection of the different nutrient sources.

2.10. Chemotaxis experiment

Chemotaxis was examined to determine if O. stellarum preferred different food sources in the laboratory. Three food sources were included in this study: blue crab serum (BCS), U. minax crab serum (UCS) and fetal bovine serum (FBS). Sterile seawater was included as a control. Crab serum was prepared from hemolymph collected from uninfected adult C. sapidus and U. minax individuals, pooled by species, and allowed to clot in 15 ml centrifuge tubes (BD Falcon™). The clot was then homogenized with a PowerGen 125 electronic homogenizer (Fisher Scientific). The resulting supernatant was removed with a pipette and centrifuged at 1300g for 10 min to remove cellular debris. Crab serum was then filtered through Nalgene® disposable filters (Pore size = 0.45 µm, Nalgene Labware), and stored at −20 °C until use.

Ciliates in these trials were obtained from a single infected C. sapidus individual and cultured in two different media, 10% FBS in MAM or 10% BCS in MAM. Suspensions of the ciliate culture were centrifuged in 15 ml centrifuge tubes (BD Falcon™) three times at 2300 rpm (770 g) for 2 min each at 10 °C with no braking. The supernatant was replaced after each centrifugation with sterile-filtered artificial seawater (ASW) at 27 psu, the same salinity as the culture medium. After washing, ciliate densities were estimated with a hemacytometer, adjusted to 2000/ml using ASW, and then stored at 4 °C for 3 h before commencing the trial. The purpose of the successive replacement of the culture medium with ASW and 3-h settling period was to limit any satiation effect of the ciliates from being held in a particular medium.

The chemotaxis experiments were conducted using microcapillary tubes with procedures modified after Leick and Helle (1983) and Spero (1985). To initiate the experiment, 5 ml of 27 psu ASW (sterile-filtered) at 10 °C was added to four wells of a six-well polystyrene culture plate (BD Falcon™). Into each of these four wells four microcapillary tubes (Drummond Microcaps, 32 mm long) which were filled with one each of the four treatments (ASW control, FBS, UCS, or BCS nutrient sources). The microcapillary tubes were always placed in the same order in the wells. The amount of protein was standardized using a hand-held protein refractometer to 3.6 g/100 ml (Westover Model RHC-200) in the FBS, UCS, and BCS capillary tubes using sterile, nano-pure water. Immediately after the capillary tubes were placed in the well plate, an estimated 10,000 ciliates (2000/ml) were gently pipetted into each well, then the plate was covered and incubated at 4 °C for 10 min. Preliminary trials indicated that 10 min was sufficient for obtaining good differentiation in ciliate counts among treatments. The washed ciliates that were not used were placed in a new culture flask with ASW and returned to 4 °C for replication of the experiment at 24 h after centrifugation and removal from culture medium.

After the 10-min incubation period, the capillary tubes were removed using fine forceps and the outside of the tubes carefully blotted with a Kimwipe® to remove ciliates which may have adhered to the outside of the tubes. No fluid was observed to have left the capillaries during this process. Once the outsides of the capillary tubes were blotted, they were placed individually into a large plastic culture plate with 5% formalin in MAM added to both ends of each microcapillary to kill and fix the ciliates inside. The ciliates were counted using an Olympus IX50 inverted microscope with a Hoffman modulated contrast. Photographs were taken with a Nikon DXM1200 Digital Camera.

Twenty-four hours later the density of ciliates held overnight in ASW was recalculated, readjusted to 2000/ml if needed, and the experiment repeated in another culture plate of four replicates in order to assess possible satiation effects in ciliates used immediately vs. those hold 24 h later in the nutrient chemotaxis treatments (i.e., a satiation effect in the immediate use treatment).
These conditions and the replication of the experiment at 0 h and 24 h were conducted twice, once for ciliates that were grown in culture media supplemented with 10% FBS in MAM, and again for ciliates grown with 10% BCS in MAM.

3. Results

3.1. C. sapidus inoculation trial

Orchitophrya stellarum was highly infectious and pathogenic when inoculated into naïve blue crabs at 2 × 10⁴ ciliates per crab. All crabs in the 15 °C trial died within 13 days post-inoculation, with a mean time-to-death of 9.0 days, and 80% of crabs died in the 23 °C trial, with a mean time-to-death of 10.8 days; all control individuals survived (Fig. 1). Survival was significantly different between the 15 °C treatment and the 23 °C treatment (Tarone-Ware $\chi^2 = 4.424$, df = 1, $p = 0.035$), and both treatments were highly significantly different from the control (Tarone-Ware $\chi^2 = 30.61$, df = 2, $p < 0.001$). Hemolymph examined for each of the infected individuals revealed large numbers of actively motile ciliates and distinct hemocytopenia (i.e., almost no circulating hemocytes remaining). The hemolymph of infected crabs was milky and opaque when drawn into a syringe. The mean density of ciliates remaining). The hemolymph of infected crabs was milky and opaque when drawn into a syringe. The mean density of ciliates in the hemolymph of infected individuals was an estimated $5.5 \times 10^6$ cells ml⁻¹ ($\pm 4.4 \times 10^6$ std). The density of ciliates (log transformed) circulating in the hemolymph was not correlated to the size (LCW) of the individual crab (Pearson's $r^2 = 0.219$, $n = 15$, $p = 0.432$). Ciliate density (log transformed) was not significantly correlated with day of death post-inoculation because of an apparent asymptote with density at approximately 1.0 × 10⁴ ciliates ml⁻¹ (Fig. 2) ($r^2 = 0.219$, $n = 15$, $p = 0.432$; with single outlier removed, $r^2 = 0.360$, $n = 14$, $p = 0.206$). Crabs serving as controls were not infected with the ciliate.

Crabs infected with O. stellarum showed high levels of autotomy to their pereopods. Autotomy was observed in over 40% of the infected individuals in the 15 °C trial, with at least one leg being lost. In the most severe case, all but three legs were autotomized prior to death. Histological analysis of the nerve-rings for these individuals showed penetration of a few ciliates into the neurilemma surrounding the ventral nerve ganglion (see histopathology below), but no distinct pathology or host response was observed.

Temperature played a key role in the development of O. stellarum infections. Ciliates were observed in the hemolymph of inoculated crabs held at 15 °C, but not in inoculated crabs held at 23 °C. Examination of the hemolymph of moribund individuals from the 23 °C treatment revealed marked hemocytopenia, but bacteria rather than ciliates were abundant via microscopy. No mortalities were observed in the control group. Hemolymph samples for all individuals screened for Vibrio spp. on TCBS plates at the beginning of the experiment were negative.

3.2. Portal-of-entry exposure experiment

O. stellarum preferentially infected crabs injured by autotomy. Of the autotomized-exposed individuals, 17 of 20 (85%) died (Fig. 3), and 15 (70%) of these had high density, systemic ciliate infections in their hemolymph at the time of death (Fig. 4). In contrast, only one of the crabs in the unautotomized-exposed group developed an infection with O. stellarum, and it was only detectable as a light infection via histological examination after termination of the experiment. None of the crabs serving as unexposed controls became infected. Two of the 17 individuals in the autotomized-exposed group died in the first few days of the experiment, but did not have observable ciliate infections in their hemolymph or tissues upon histological analysis. The three individuals (15%) from the autotomized-exposed group that survived to the end of the experiment did not have observable pathology or host response.

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**Fig. 1.** Survival analysis (Kaplan–Meier) of blue crabs, Callinectes sapidus, inoculated with Orchitophrya stellarum in 15 °C and 23 °C trials. Only the upper or lower halves of the standard error bars are shown for clarity. Survival in the 15 °C and 23 °C control groups did not differ and is shown as a single group. N = 20 crabs in each experimental treatment, N = 10 crabs in each control treatment.

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**Fig. 2.** Day of death of crabs inoculated with Orchitophrya stellarum in relation to log ciliate density in the hemolymph of blue crabs in the 15 °C inoculation trial. Most deaths occurred at densities over 1 × 10⁴ ciliates ml⁻¹.

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**Fig. 3.** Survival analysis (Kaplan–Meier) for blue crabs in the Portal-of-entry experiment conducted at 10 °C. Bars are standard errors. For clarity, only the top or bottom half of standard error bars are shown for some treatments. Unaut unexp = unautotomized-unexposed control (open square, solid line), Aut unexp = autotomized-unexposed control (open triangle, dashed line), Aut exp = autotomized-exposed treatment (open square, dashed line), Unaut exp = unautotomized-exposed treatment (closed square, solid line), Aut exp = autotomized-exposed treatment (closed triangle, dashed line). N = 20 crabs in each experimental treatment, N = 10–11 crabs in each control treatment.
The experiment had no ciliates in their hemolymph or in histological preparations of their tissues. Three of the autotomized control animals died during the experiment, and in all cases they had bacteria present in the hemolymph at the time of death, but no ciliates were present upon examination. All unautotomized-unexposed individuals (controls) survived and no limbs were naturally autotomized during the trial.

Histologically, ciliates were observed in nearly all of the soft tissues (excluding the hemapoietic tissue) in infected crabs from the autotomized-exposed treatment (Fig. 5). In several cases, ciliates were seen within muscles, nerves and other tissues, indicating the potential for invasiveness directly into protected organ systems. Crabs with high intensity infections exhibited hemocytopenia, edematous change to arterioles, erosion of epithelial layers lining the gill lamellae, and in some cases direct penetration of ciliates through the neurilemma into the neuropyle. The penetration of the neurilemma could explain autotomy in infected crabs.

However, only one crab in the autotomized-exposed treatment experienced additional autotomy during the experiment (right 3rd limb) compared to the >40% that experienced autotomy in the inoculation study.

Survival analysis indicated that autotomized crabs were significantly more likely to acquire infection than unautotomized crabs ($\chi^2 = 15.36, \text{df} = 1, p < 0.0001$). Autotomized-exposed crabs survived a median of 15.75 days; and the unautotomized-exposed crabs survived a median of 21.3 days; the unexposed control treatments experienced too little mortality over the 25 days of the experiment to calculate a mean time to death (Fig. 3). Because mortalities in the two unexposed control treatments were not significantly different (Tarone-Ware $\chi^2 = 3.35, \text{df} = 1, p = 0.067$), they were combined into one treatment (controls) for subsequent comparisons. Mortality was almost significantly different between the unautotomized-exposed treatment and the controls ($\chi^2 = 3.3, \text{df} = 1, p = 0.07$). The survival of the autotomized-exposed treatment was significantly different than the pooled controls ($\chi^2 = 21, \text{df} = 1, p < 0.0001$) and the unautotomized-exposed treatment ($\chi^2 = 8.7, \text{df} = 1, p < 0.005$).

3.3. Alternate host experiment with U. minax

*O. stellarum* was able to infect fiddler crabs exposed via inoculation. All 15 fiddler crabs inoculated with *O. stellarum* died between 8 and 14 days post-exposure (median = 12 day); only one uninfected individual in the control treatment died during the experiment (Fig. 6). In all fiddler crabs inoculated with *O. stellarum*, ciliates were present at high densities in the hemolymph and distinct hemocytopenia was observed in moribund and recently dead crabs. Mortality among the infected crabs was greatest on days 12 and 13. The Cox proportional hazards regression analysis found no significant difference between replicate aquaria ($p > 0.05$) but a significant effect of treatment on mortality (overall log-rank

![Fig. 4. Cumulative number of blue crabs that died from ciliates in their hemolymph in the autotomized and unautotomized treatments exposed to *Orchitophrya stellarum*. Unaut exp = unautotomized-exposed treatment (closed square, dashed line); Aut exp = autotomized-exposed treatment (closed triangle, dashed line).](image)

![Fig. 5. *Orchitophrya stellarum* in tissues from blue crabs in the Portal-of-Entry experiment. (A) Hemal sinus (S) in the muscle (M) of the 5th pereopod with ciliates possibly within the musculature (arrows). (B) Epidermis showing an arteriole filled with large numbers of ciliates and few host hemocytes. Pigment cells (arrow). (C) Ventral nerve ganglion with the neurolemma (L) penetrated by ciliates (arrows) that are inside the neuropyle. (D) Ventral nerve ganglion with a ciliate (arrow) inside the neurolemma (L) between two nerve cells (N). Fibrous connective tissues (F) are outside the nerve region. Mayer’s hematoxylin and eosin (H and E), bars = 50 μm.](image)
Fig. 6. Survival analysis (Kaplan–Meier) for fiddler crabs, *Uca minax*, inoculated with *Orchitophrya stellarum* and held at 10 °C. Bars are standard errors. *N* = 15 crabs in each treatment.

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score = 30.69, df = 2, *p* < 0.0001). Therefore, further analysis combined the replicate aquaria in each treatment. Crabs inoculated with the ciliate had a significantly higher mortality rate than crabs serving as controls (log-rank test, Tarone-Ware *χ²* = 27.2, df = 1, *p* < 0.0001). Blue crabs that served as positive controls at the start of this experiment died with systemic ciliate infections 5 and 10 d post-inoculation, thereby confirming that the inoculum was infectious.

Nine of the fifteen exposed fiddler crabs that died during the experiment were processed for histology. In one individual, no ciliates were visible in tissue sections despite high numbers being present in the hemolymph. In the other eight, ciliates were visible in the tissues (e.g., muscle, hematopoietic tissue, gills) in varying degrees of intensity. Ciliates were also present adjacent to the nerve ring in histological sections (it was unclear whether they had penetrated or damaged the neuromamma) in three individuals, but only one of those crabs had autolysed a limb. There were no differences in the histological presentation of infections between *C. sapidus* and *U. minax*.

### 3.4. Dose titration study with *U. minax*

The dose-titration study with fiddler crabs indicated that the higher doses of the ciliate caused significantly higher intensity infections and rapid mortality than did lower doses (Fig. 7). Fiddler crabs inoculated with 5000 ciliates began dying within a 1–2 days post-exposure. The control and 10-ciliate treatments exhibited too little mortality to generate a median survival time (90% and 87% survived, respectively). There were no significant differences between the two complete replicates of the experiment (Cox proportional hazards, *p* > 0.05) but there was a significant effect of treatment on mortality (log-rank score = 141, df = 2, *p* < 0.0001). Subsequent analysis, therefore, combined replicate aquaria in each treatment. Significant differences were observed between the survival of the control treatment and every exposed treatment except the 10-ciliate inoculation (adjusted *x* = 0.0083 for six post hoc comparisons, Bonferroni correction).

Individual fiddler crabs inoculated with as few as 10 ciliates obtained high-intensity infections in their hemolymph and histologically in tissues. The minimum infectious dose was approximately 10–100 ciliates. Approximately 50% of the fiddler crabs became infected when inoculated with 500 ciliates. Four individuals with infected hemolymph did not have ciliates present upon histological examination. The fourteen exposed crabs that survived the experiment had hemolymph samples drawn at the cessation of the study and their tissues were processed for histology. None of these crabs had ciliates in their hemolymph samples or tissues. No difference was observed between treatments in number of remaining limbs at time of death or the end of the experiment (Kruskal-Wallis, *χ²* = 5.49, df = 6, *p* = 0.48). The blue crabs serving as positive controls died with systemic ciliate infections within 10 day post-inoculation, thereby confirming that the inoculum was infectious.

### 3.5. Chemotaxis experiment

*O. stellarum* was more attracted to various nutrient sources than to seawater controls in each of the chemotaxis trials. However, the medium in which the ciliates were grown markedly affected their chemotaxis. Several tens of ciliates entered capillary tubes loaded with FBS, UCS or BCS when previously grown in 10% FBS in MAM (Fig. 8A), whereas several hundred (approximately ten times more) entered capillary tubes with BCS and FBS when grown in 10% BCS in MAM (Fig. 8B). Because of the large response difference between initial culture media, the responses to attractants were analyzed separately by ANOVA for each initial culture medium.

For ciliates cultured in 10% FBS in MAM, the attractant did have a significant effect, but time (as time in ASW) and the attractant x time interaction were not significant (ANOVA, *F*<sub>7,28</sub> = 5.69, *p* < 0.001, *P<sub>time</sub> = 0.837, *P<sub>attractant x time</sub> = 0.941, *P<sub>attractant</sub> < 0.001). Pair-wise comparisons among adjusted least squares means found differences between all treatments except BCS and UCS (adjusted means ± 3.4 SE; sea water = 4.3 ciliates, UCS = 13.5 ciliates, BCS = 20.5 ciliates, FBS = 32.8 ciliates).

For ciliates cultured in 10% BCS in MAM, the attractant, time and attractant x time interaction had significant effects (ANOVA, *F* = 23.09, *p* < 0.0001, *P<sub>time</sub> < 0.0001, *P<sub>attractant x time</sub> = 0.0008, *P<sub>attractant</sub> < 0.0001). Comparisons between attractants only compared responses from experiments conducted the same number of hours after centrifugation (3 h or 24 h), because time had a significant effect. The adjusted means for each treatment were sea water = 3.1 ciliates, UCS = 106.0 ciliates, BCS = 195.3 ciliates, FBS = 261.2 ciliates (±17.8 SE). Pair-wise comparisons between time-attractant pairs found differences between some attractant-time combinations, but the patterns were highly variable (Fig. 8, Tukey post hoc comparisons). While highly variable, there were significantly more ciliates entering the BCS or FBS treatments than there were entering seawater controls at both times.
and square-root transformations. Carlisle and Morado (2001) found an increase in growth and intensity of infection from *Asterina miniata* and reported a temperature tolerance of 3–27°C (*Echinodermata*) in a wide range of salinities and temperatures, with rates close to those examined here by Cain and Morado (2001). The distinct rapid growth and progression of infection of *O. stellarum* in crustaceans, it is clear that this ciliate can thrive at low temperatures and kill susceptible crab hosts at 10–15°C. Our study is the first to directly test whether a parasitic ciliate enters a crustacean host through wounds such as induced autotomy. The high prevalence in the autotomized-exposed treatment of the blue crab exposure experiment and near absence of infected crabs in the unautotomized-exposed treatment supports the hypothesis that *O. stellarum* requires a portal of entry (e.g., a wound) to initiate infections. Other scuticociliate infections in crustaceans are thought to arise as facultative invasion through the damaged cuticle of the host (Bang et al., 1972); this may be the primary route of infection for *Anophryoides haemophilia* in *H. americanus* (Cawthon 1997, 2011) and *Mesanophrys pugettensis* in *Cancer magister* (Morado et al., 1999). Infections of *M. pugettensis* may arise through abrasions associated with molting (Morado et al., 1999). Other pathogens in crustaceans, including the Gram-positive bacterium *Aerococcus viridans* (Stewart et al., 1969), are known to use wounds or damaged cuticle as a route of infection. We did not investigate other potential mechanisms of infection, such as cannibalism or predation. However, gastric fluids of the lobster, *H. americanus*, are a significant barrier to infection by *Aerococcus viridans* and *Anophryoides haemophilia* (Stewart et al., 1969; Loughlin et al., 1999) and likely inhibit the spread of *Hematodinium perezi* through cannibalism (Li et al., 2011).

Wounds can come from a number of sources and are relatively common in crustacean populations. The frequency of wounded blue crabs in nature supports the viability of wounds as a method for sustaining a chain of transmission in this host species. Numerous surveys have found anywhere from 15% to 40% of blue crabs missing a limb, with a cheliped being the most frequently missing limb (Smith, 1990; Smith and Hines, 1991a,b; Juanes and Smith, 1995). Puncture wounds in species with intraspecific aggression similar to *C. sapidus* may also be relatively common: 40% in the porcelain crab, *Petrolisthes cinctipes* (Rypien and Palmer, 2007) and 25% in male *Uca sericans* (Jones, 1980). The rarity of ciliate infections from crab hosts could indicate that infections are subthreshold and suppressed by an active immune response or that mortality operates very quickly and dead crabs are simply not sampled or are culled from collections. Molting can also increase risk of perforations by temporarily exposing a soft shell to injury (Morado et al., 1999; Sakamoto et al., 2009). Blue crabs can injure each other during agonistic encounters (Jachowski, 1974), and in some instances cannibalism or attempted cannibalism may be an important source of wounds (Juanes and Smith, 1995). Blue crabs may obtain cuts and abrasions or other damage to their cuticle from burying into sediments immediately prior to overwintering hibernation, or from ‘latching behavior’ observed in extremely high-density overwintering sites where individuals are in physical contact (Rome et al., 2005), or possibly from dredge-induced injuries on hibernating, unfished crabs. Such wounds potentially provide a portal of entry for *O. stellarum* but also provide an exit for infected hemolymph (Uhlmann et al., 2009), which could act as an attractant to the parasite as demonstrated in the chemotaxis assay.

4. Discussion

Infections of *O. stellarum* can lead to the rapid death of its crab (*C. sapidus* and *U. minax*) hosts, with significant logarithmic growth of the ciliates in *vivo* (in many cases exceeding 10⁶ ciliates ml⁻¹ in hemolymph). Host death was probably a result of penetration of vital organs or from occlusion of hemal sinuses. Infections with *O. stellarum* progressed rapidly in all of the exposure trials. Similar rapid mortalities (8–12 day) and proliferation of ciliates in the hemolymph were observed in *Mesanophrys pugettensis* (a closely related scuticociliate) infections in *Cancer magister* held at temperatures close to those examined here by Cain and Morado (2001). The distinct rapid growth and progression of infection of *O. stellarum* in crustacean hosts agrees with observations of infections of this species in some of its known echinoderm hosts (Stickle et al. 2007a,b; Bates et al. 2010).

Stickle et al. (2007b) cultured *O. stellarum* from *Leptasterias sp.* (Echinodermata) in a wide range of salinities and temperatures, and reported a temperature tolerance of 3–27°C at 30 psu, with the least growth occurring at either end of the range. Bates et al. (2010) found an increase in growth and intensity of infection from 10°C to 15°C in *O. stellarum* experiments using the sea stars *Asterina miniata* and *Pisaster ochraceus*. Results from these two studies suggest that, at least in sea stars, increasing temperatures may produce more rapid growth and potential pathogenicity of *O. stellarum* in *in vivo* and *in vitro* models. In our system, the parasite seems to thrive at colder temperatures; however, this parasite is not unique in being more virulent in colder water. Some notable parasites causing significant mortalities in cold-water inhabiting crustaceans include the ciliates *A. haemophilia* in *H. americanus* at temperatures around 2–4°C (Athanassopoulou et al., 2004; Greenwood et al., 2005), *Collina* spp. in euphausiid shrimps at <10°C (Gomez-Gutierrez et al. 2006) and *Hematodinium* sp. in snow crabs, *Chionoecetes opilio*, at temperatures near −1–4°C (Shields et al., 2005, 2007). Thus, while further work is certainly needed to characterize the temperature and salinity tolerance range of *O. stellarum* infections in crustaceans, it is clear that this ciliate can thrive at low temperatures and kill susceptible crab hosts at 10–15°C.

Fig. 8. The number of *Orchitophrya stellarum* attracted to each nutrient source in the chemotaxis study at time 0 h and 24 h (±SE). Each bar represents 12 replicates. (A) Ciliates cultured in 10% FBS in MAM prior to their choice in the chemotaxis study. Inset: ciliates inside a capillary tube. (B) Ciliates cultured in 10% BCS in MAM prior to their choice in the chemotaxis study. Note the change in the Y-axis between A and B. Control = artificial seawater, FBS = fetal bovine serum, UCS = *Uca* crab serum, BCS = blue crab serum. N = 4 replicates per treatment per time.

Similar numbers of ciliates entered the seawater control capillaries in all experiments (range 1–7 individuals). The equal attraction of the ciliates to the seawater-filled capillary tubes regardless of what medium they were raised indicated that the medium did not affect their response to the experimental setup. The numbers of ciliates entering capillary tubes were not normally distributed (Shapiro–Wilks’ W test, p < 0.05). Even though the ANOVAs used to analyze these data were robust for normality, the differences between some of the attractants were so great that they skewed the analysis even after log₁₀ and square-root transformations.

<table>
<thead>
<tr>
<th>Capillary contents</th>
<th>Ciliates per capillary tube</th>
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<tbody>
<tr>
<td>Control</td>
<td>5</td>
</tr>
<tr>
<td>FBS</td>
<td>4</td>
</tr>
<tr>
<td>UCS</td>
<td>3</td>
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Infections of *O. stellarum* fulminated rapidly in blue crabs and fiddler crabs. Blue crabs inoculated directly with *O. stellarum* survived a mean of 9.4 days and experienced 100% mortality within 13 days at 15 °C. In bath exposures, autotomized blue crabs survived a mean of 16 days and 85% mortality after 25 days at 10 °C. The dose-titration study with fiddler crabs indicated that infections could arise from inoculation with as few as 10 ciliates but that higher numbers of ciliate were more likely to result in extensive infections that rapidly progressed to death of the host. Inoculation trials of *O. stellarum* in blue crabs and fiddler crabs indicated that infections progressed and manifested similarly in both hosts; thus validating the development of *U. minax* for future use in studying *O. stellarum* infections. Histologically, infections in blue crabs and fiddler crabs were also quite similar, with hemocytopenia, edematous change to the arterioles, and penetration of the muscle and nerve tissues. Given the space and handling requirements for blue crabs, fiddler crabs may make a model host for studying ciliate infections as they are easy to collect, require minimal space to house, cost little to maintain, and are adaptable to many laboratory settings.

4.1. Chemosensory response

Our findings indicate that *O. stellarum* invades susceptible crustacean hosts through wounds, which it probably detects chemotactically. In the chemosensory trials, individual ciliates were more attracted to the BCS and FBS nutrient sources than to artificial seawater regardless of the initial culture medium in which cultures were isolated and grown (i.e., MAM supplemented with 10% FBS or 10% BCS). That is, the scuticociliate actively sought out diverse food sources, further supporting its facultative and opportunistic nature. This apparent attraction to a suitable food source may aid *O. stellarum* in exploiting the brief window when crab hemolymph may be flowing from an open wound, or when mucopolypeptides associated with molting are being released into the environment.

Interestingly, the medium in which the cultures were isolated and maintained prior to the chemosensory trials appeared to condition the response of *O. stellarum* to the attractants. The cultures grown in MAM supplemented with 10% BCS were up to an order of magnitude more attracted to the different nutrient sources than were those isolated and cultured in MAM with 10% FBS. This may be due to differing growth rates or phases between ciliates in the two cultures before commencing the experiments. Ciliates in MAM supplemented with 10% BCS cultures were in logarithmic growth (analogous to growth in blue crab hemolymph) whereas those cultured in MAM with 10% FBS were in stable growth, and rarely grew logarithmically in 10% FBS (Small et al., in press, Shields et al., unpubl. data). This difference is similar to other studies of ciliate chemotaxis, some of which have observed that the response to a given attractant is highly dependent upon the current physiological status of the culture (e.g., Snyder, 1991). Thus, it is conceivable that *O. stellarum* released from infected blue crabs may be highly attracted to hemolymph from other crustacean hosts relative to free-living *O. stellarum* in stable growth.

4.2. *O. stellarum* and winter mortality in *C. sapidus*

Anecdotally, outbreaks of ciliates have occurred in our systems only during winter months. Furthermore, an ongoing survey of the parasite fauna of blue crabs in this region, focusing on the parasitic dinoflagellate *Hematodinium perezi*, has failed to find natural infections of *O. stellarum* (*n = 13,000 crabs*) during spring, summer and fall surveys, nor was it detected in previous surveys (Messick and Shields, 2000). In our challenge experiments and in growth studies, *O. stellarum* proliferated at temperatures below 23 °C, and thrived at 4–15 °C (Small et al., in press). The relationship with temperature and the rapid spread of *O. stellarum* warrants further attention particularly with respect to winter mortalities reported in blue crab stocks.

Winter mortality is a phenomenon in blue crabs subjected to temperatures below 6 °C (Pearson, 1948; Dudley and Judy, 1973; Kennish et al., 1982; Cole, 1998; Khan et al., 1998, for review, see Rome et al., 2005). Significant winter mortalities can occur at temperatures and salinities that normally occur in Chesapeake Bay. However, no studies on winter mortality have examined crabs for the presence of parasitic agents. While our winter dredge survey did not find natural infections of the ciliate (Small et al., in press), it occurred 2–3 years after a moratorium was imposed on the dredge fishery for the blue crab (Virginia Marine Resources Commission, 2013). Given that the parasite can cause rapid mortality in confined systems at ambient winter temperatures, that a portal of entry facilitates its transmission, and that the winter dredge fishery in Chesapeake Bay can cause mechanical injuries to culled crabs, further work may be needed to determine if dredging activities facilitate the transmission and spread of *O. stellarum*.

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