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Claire K. Howell for the degree of Master of Science in Microbiology presented on December 12, 2017.

Title: Molecular Analysis of Environmental Water Samples as a Monitoring Method for the Fish Parasite *Ichthyophthirius multifiliis*

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ABSTRACT

Parasites are ubiquitous members of ecological communities, capable of contributing to the decline of vulnerable populations. Therefore, monitoring parasite level is a critical component for host management. Molecular tools, such as quantitative polymerase chain reaction (qPCR), can be valuable additions to monitoring protocols that assess parasitic disease risk to hosts. To be successful, monitoring protocols must capture biologically relevant environmental data, and relate that data to disease risk. This project assesses the application of molecular analysis of environmental water samples for monitoring the ciliate protozoan *Ichthyophthirius multifiliis* (Ich) in the Klamath River, CA. Ich is an economically and ecologically significant parasite of freshwater fishes, and has contributed to mortality of pre-spawning adult salmon throughout the Pacific Northwest, including in the Klamath River. Chapter 1 introduces the study site (the lower Klamath River, CA), host population of concern (Klamath River salmonids), parasite of interest (Ich), and primary molecular technique (qPCR). Chapter 2 describes the development and validation of a qPCR assay targeting the SSU rDNA of Ich, as well as the initial application of the assay to environmental water samples. Chapter 3 presents a field application of the sampling method. Chapters 2 and 3 demonstrate the assay's potential to inform the management of Ich

infections in the Klamath River. Chapter 4 compares the genetic variation of Ich isolates published in GenBank, and those collected from pet shops (OR) and natural rivers (CA) (primarily the Klamath River) at the *cox1* and SSU gene loci. This research indicates that qPCR analysis of environmental water samples could be a valuable monitoring tool for waterborne Ich in the Klamath River.

Molecular Analysis of Environmental Water Samples as a Monitoring Method for the Fish Parasite *Ichthyophthirius multifiliis*

by

Claire K. Howell

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I understand that my thesis will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my thesis to any reader upon request.

Claire K. Howell, Author

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CONTRIBUTION OF AUTHORS

Contributing authors included Sascha L. Hallett, Stephen D. Atkinson and Jerri L. Bartholomew of Oregon State University, and Michael Belchik and Barry McCovey Jr, of the Yurok Tribal Fisheries Program. Sascha Hallett contributed on all levels of the research, including project design, implementation and interpretation for chapters 2 and 3. Stephen Atkinson provided training and advice on molecular techniques, and primarily contributed to the development of the qPCR assay in chapter 2, and the genotyping of Ich isolates in chapter 4. Jerri Bartholomew served as the major advisor for this research, and contributed to project design and interpretation. Michael Belchik and Barry McCovey informed project design for chapter 3, and assisted with the collection of water samples from the Klamath River. All authors contributed to the writing of the manuscript.

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CHAPTER 1: INTRODUCTION

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RISING WATER TEMPERATURES AND PARASITIC DISEASES OF SALMON IN THE PACIFIC NORTHWEST

Parasites, by definition, cause harm to their hosts. By living on and feeding upon hosts, parasites appropriate nutrients, impeding growth, survival and reproduction. To combat this, hosts evolve physiological and behavioral strategies for resistance to and tolerance of parasitic infections (Blanchet et al. 2010). These efforts are energetically costly, and their success is diminished by additional environmental stressors (Paull and Johnson 2011; DietrIch et al. 2014). Anthropogenic activities modify host environments, introducing stressors such as habitat loss (Gillis et al. 2017), pollutants (Arkoosh et al. 2010) and alterations associated with climate change (Studer et al. 2010). Of the environmental parameters impacted, temperature is among the most relevant to parasite-host dynamics. Temperature influences the timing and progression of development, metabolism, fecundity and key behaviors of both parasites and hosts (Gallana et al. 2013), and therefore has the potential to increase the severity and distribution of parasitic diseases (Karvonen et al. 2010). The scale of this is contingent on the thermal tolerances and adaptability of all species involved (Altizer et al. 2013).

Water temperatures in Pacific Northwest rivers and streams have increased steadily due to changes in air temperature, water flow, snowpack and riparian vegetation (Sawaske and Freyberg 2014). Snowpack is the primary contributor to rivers and streams during typical low-precipitation summer months; thus a reduction in snowpack typically results in a reduction in water flow (Sawaske and Freyberg 2014), and an increase in water temperature (Cristea and Burges 2010). Anthropogenic modifications to river and stream morphology such as, water diversion for irrigation or consumption, livestock grazing, construction of hydraulic infrastructure, and the harvest of riparian vegetation, can increase water temperatures (Poole and Berman 2001). As water temperatures rise, migrating salmon are exposed to higher temperatures for longer periods; increasing the incidence and intensity of parasitic infections (Martins et al. 2012). These infections are believed to be among the key factors

limiting salmon recovery efforts throughout the Pacific Northwest (Hinch et al. 2012; Miller et al. 2014; Bass et al. 2017).

Elevated temperatures (19°C or above) trigger physiological and behavioral changes to salmon that increase disease risk (Hinch et al. 2012). Temperatures at the upper limit of thermal tolerances reduce immune function (Martins et al. 2012; Bailey et al. 2017). Migration behaviors such as run timing (Crossin et al. 2007) and the use of thermal refugia (Benda et al. 2015) can impact disease susceptibility by altering the duration and intensity of water temperatures experienced by migrating adults. While some runs adjust their migration timing to avoid temperature peaks (Strange 2012), not all are able to do this, possibly because of a tightly constricted spawning window, and they will migrate despite warm water conditions. Thermal refugia can help salmon recover from and resist disease (Benda et al. 2015; Chiaramonte et al. 2016); however, holding in dense concentrations within refugia may increase exposure to density dependent pathogens such as *Ichthyophthirius multifiliis* (Ich) and *Flavobacterium columnare*. Thermal refugia use has been positively correlated with pre-spawn mortality (Keefer et al. 2009), although it has not been established as the cause of that mortality.

While salmon are negatively impacted by raising water temperatures, these conditions are favorable for some parasites. Temperatures at a parasite's upper thermal limit can facilitate proliferation by accelerating parasite metabolism (Woo and Buchmann 2012). This can have deleterious impacts on fish hosts, as parasite load is positively correlated with host disease and mortality (Ferguson et al. 2011). Elevated water temperatures concurrent with adult salmon migration is associated with high pre-spawn mortality rates (Richter and Kolmes 2005), and have contributed to an increase in the incidence of parasitic epizootics among pre-spawning adult salmon from British Columbia (Traxler et al. 1998), to California (Belchik et al. 2004). Common pathogens associated with these epizootics include the ciliate *Ichthyophthirius multifiliis* (Ich), the myxozoan *Parvicapsula minibicornis* and the bacterium *Flavobacterium columnare* (Traxler et al. 1998; Raverty et al. 2000; Belchik et al. 2004). While rising

water temperatures favor these parasites, increases in water temperatures do not affect all parasites equally. Higher temperatures can challenge parasite physiology as well, and sometimes no effect is observed between increases in temperature and salmon mortality associated with parasite infection (Bentley and Burgner 2011).

KLAMATH RIVER SALMON POPULATIONS AND PARASITE INDUCED MORTALITY

The Klamath River flows from Klamath lake in Oregon, to the Pacific Ocean of the northern California coast (Figure 1.1). Historically, the Klamath River basin supported robust populations of Chinook (*Oncorhynchus tshawytscha*), coho (Oncorhynchus kisutch), chum (Oncorhynchus keta) and pink (Oncorhynchus gorbuscha) salmon, as well as steelhead/rainbow trout (*Oncorhynchus mykiss*) (Jenkins 2011). However, these populations are now either in decline or extinct. By the 1990s, Klamath River spring run Chinook salmon had declined by 95% (Nehlsen et al. 1991), and Klamath coho salmon (Southern Oregon and Northern California Coasts Evolutionarily Significant Unit) were listed as threatened under the federal and state Endangered Species Acts (Wainwright and Weitkamp 2013). Salmonids in the Klamath River experience natural and anthropogenically generated stressors typical to those throughout the Pacific Northwest. Currently, tribal communities along with state and federal environmental agencies are working to combat these challenges and encourage the recovery of Klamath salmon populations.

Recovery efforts are complicated by the competing demands for limited water resources in the basin (Boehlert and Jaeger 2010). Hydroelectric dams were installed in the Klamath River as part of the national reclamation act of 1902 to divert water from the river for agriculture (Powers et al. 2005), and modern agricultural communities in the upper Klamath basin rely on this water for irrigation. Water allocation policies have direct implications for Klamath River salmon populations. A large-scale die-off of pre-spawning adult salmon occurred in 2002 when the bulk of river water was diverted for agriculture

(Jenkins 2011). Low water flows coupled with elevated mainstem temperatures and high fish density resulted in an epizootic that was traced back to joint infections of Ich and *F. columnare*. An estimated 35,000-70,00 fish, predominately pre-spawning fall run Chinook salmon perished (Belchik et al. 2004).

Initiated by the 2002 fish kill, Yurok tribal biologists have conducted yearly monitoring for elevated Ich levels in the lower Klamath River. Threshold levels for Ich were established at 30 feeding trophonts/first gill arch. When this metric is detected, it can trigger the release of water from upriver dams. Ich levels in the Klamath River remained below serious levels until the summer and fall of 2014 (Belchik 2015). These detections triggered emergency flow releases from the Trinity reservoir and the Iron Gate dam. Tribal biologists believe that the emergency flow releases, coupled with natural rainfall, helped lower the water temperature and disperse migrating fish, ultimately preventing another outbreak of Ich (Belchik 2015).

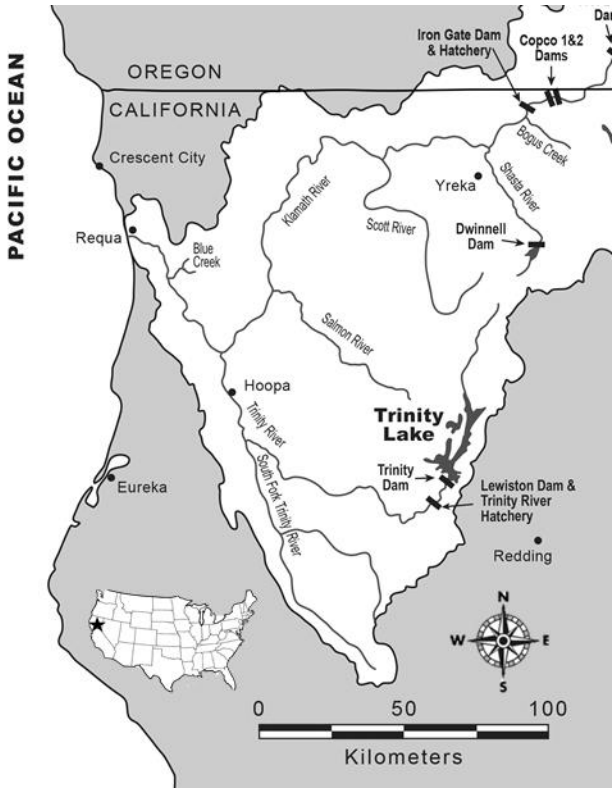

Figure 1.1 Lower Klamath River basin. Figure adapted from Quiñones et al. 2014. Water flows out to the Pacific Ocean by Requa, CA. Star (inset) shows the location of the river in the contiguous United States.

BIOLOGY OF *ICHTHYOPHTHIRIUS MULTIFILIIS* (ICH)

Ichthyophthirius multifiliis Fouquet 1876 is a ubiquitous parasite of freshwater fishes. It accounts for significant losses of aquarium and aquaculture fish worldwide (Matthews 2005a), while infections in wild populations can result in epizootics (Traxler et al. 1998). Disease (Ichthyophthiriosis/white-spot disease) develops on fish hosts when feeding parasites establish in the skin and gills, causing epithelial hyperplasia, disrupted osmoregulation, anemia, and increased vulnerability to secondary infections (Dickerson and Findly 2014; Abdel-Hafez et al. 2014). Much of the fundamental research conducted on Ich biology has been focused on strains isolated from cultured fishes such as channel catfish (Ictalurus punctatus), carp (Cyprinus sp.), and rainbow trout in an aquaculture context (Matthews 2005). This bias may obscure important variations in Ich biology. Described biological differences between isolates are primarily due to variations in environmental conditions such as temperature and/or salinity (Aihua and Buchmann 2001; Matthews 2005), rather than host factors such as species or age. Ich strains have extremely low host specificity (Matthews 2005), therefore biological research on an isolate collected from one fish species is considered to be applicable to isolates from another fish species (Woo and Buchmann 2012).

Ich is a member of the phylum Cilophora, a diverse group of eukaryotes with free living, commensal, facultative and obligate parasite members. These organisms are physiologically distinguished by the presence of cilia, macro and micronuclei, and a cytosome (Dickerson 2006). Ich is classified into class Oligohymenophorea, subclass Hemenostomata, order Hymenostomatida, suborder Ophryoglenina and family Ichthyophthiriidae. Suborder Ophyroglenina includes the families Ichthyophthiriidae and Ophyroglenidae (Wright and Lynn 1995). Similarly to Ich, Ophyroglenidae species are globally distributed throughout freshwater environments, and have been recorded parasitizing freshwater fishes (Hoffman 1967), although they are more frequently detected in invertebrates (Karatayev et al. 2002). The ciliate *Cryptocaryon irritans*, the etiological agent of marine white-spot

disease, was initially placed in the suborder Ophrioglenina, but has since been re-classified into the family Protostomatea (Lynn 2008). The remaining families in the order Hymenostomatida are classified into the suborder Tetrahymenida and include; Curimostomatidae, Claucomidae, Spirozonidae, Tetrahymenidae, TrIchospiridae and Turaniellidae (Lynn 2008). Of these, species in the family Tetrahymenidae are the most thoroughly described, as *Tetrahymena* sp. have been used in research as model ciliates (Lynn 2008). Hymenostomes share many biological similarities, including a multistage life cycle consisting of dividing tomonts, dispersing theronts and feeding trophonts (Figure 1.2). Genetic analysis of Ich isolates indicate a likely component of sexual reproduction in the life cycle (McColl et al. 2015), however this has not yet been described and the life stage at which this occurs is unknown.

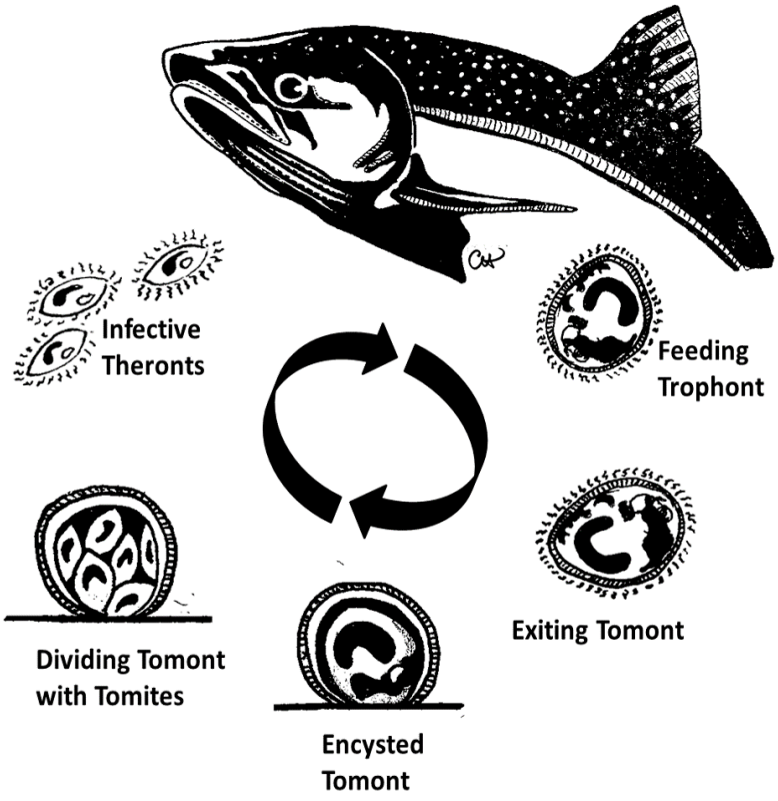

Figure 1.2 life cycle of Ich. The life cycle progresses from the feeding parasitic trophont, to the exiting tomont that encysts onto the substrate and divides, releasing hundreds of infective theronts.

Theronts are the waterborne infective stage of the parasite (Matthews 2005). Theront size varies in response to tomont size (Ewing et al. 1986) and environmental conditions (Aihua and

Buchmann 2001), and averages approximately 30x50 µm. Environmental conditions influence the period of infectivity, which has not been clearly defined. Theronts have been recorded swimming for up to 4 days (Dickerson 2006), with waning infectivity across this period. McCallum recorded a decline in viability after 12 hours at 20˚C, and mortality by 22.5 hours (McCallum 1982). Theronts demonstrate chemotactic responses (Buchmann and Nielsen 1999), and phototactic orientation (Wahli et al. 1991). Phototaxis is likely accomplished with the organelle of Lieberkuhün, a light sensing organelle unique to the suborder Ophryoglenina (Lynn 2008). It has been proposed that theronts move towards light to congregate at the surface and in shallow waters, infecting fish during the day when fish break the surface to feed (Matthews 2005a). This hypothesis is supported by observations that the dorsal region of the fish often has higher parasite loads than the ventral region (Mines and Spira 1973; Forwood et al. 2015), and that this trend disappears when fish are infected in darkness (Matthews 2005a). This strategy could be necessary in rivers and streams, where theronts can't navigate flowing water (MacLennan 1937). Once contact with a fish host is established, theronts utilize the apical perforatorium organelle to burrow into the basal layer of the epidermis (Dickerson 2006), where the theront differentiates into the feeding trophont stage.

The trophont (Figure 1.3) rotates within the epidermis, scraping cell debris and mucus into feeding vacuoles (Dickerson 2006). As trophonts feed, they clear a space in the epidermis that becomes visible as a raised white nodule, thus the common name of the infection; "white spot disease". Trophonts have a characteristic horse-shoe shaped macronucleus, and up to four micronuclei. One white nodule typically corresponds to one feeding trophont, however the division of feeding trophonts within the fish has been documented (Ewing et al. 1988). The time to maturation is dependent on temperature, averaging 5-7 days at 25°C (Woo and Buchmann 2012). Trophonts exit the fish when mature, when the host dies, or when expelled by the host's immune system (Matthews 2005). In the case of a forced exit, future viability is proportional to trophont size (Ewing et al. 1986). Ich typically

reproduces via palintomy of the encysted tomont phase, however binary fission of the feeding trophont has been documented (Ewing et al., 1988). It has been hypothesized that sexual reproduction via conjugation occurs on the fish host as well (Matthews et al., 1996) although sexual reproduction has not yet been described (MacColl et al., 2015).

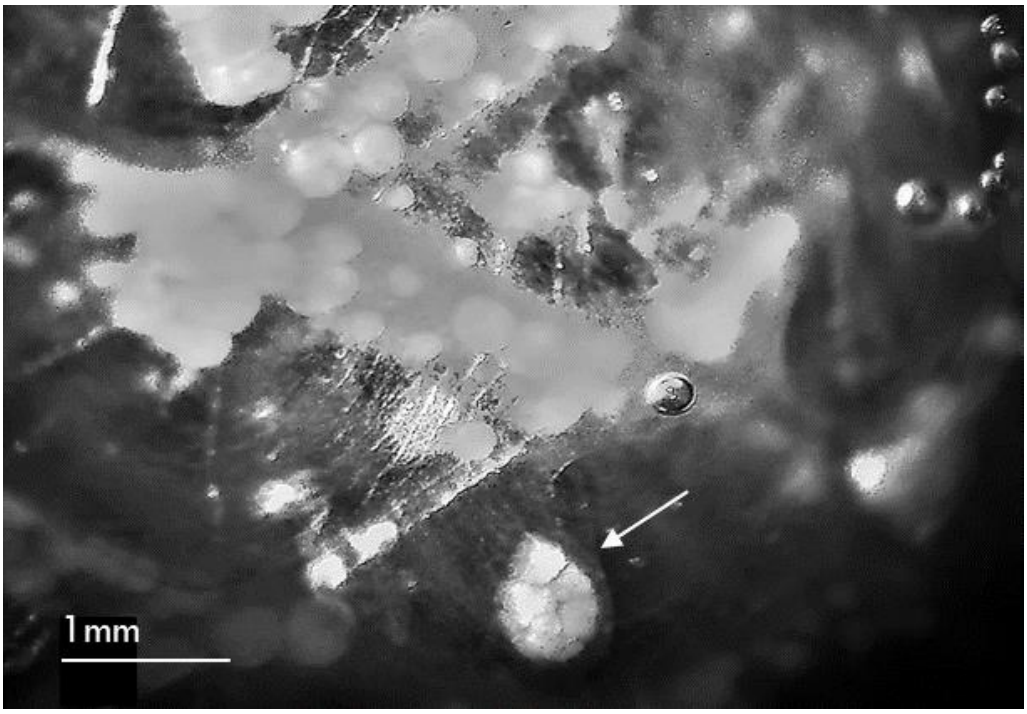

Figure 1.3 lethal infection of Ich on a goldfish infected at the Aquatic Animal Health Lab, OSU, Oregon State University. Parasites were collected from an infected tiger barb sold from a pet shop in Corvallis, OR. White circular trophonts are congregated along the scale margins. Some trophonts appear to be dividing on the host (white arrow).

The tomont (Figure 1.4) is motile in the water column for approximately 1 hr at 21-23°C, and then settles onto the substrate (sediment, debris, aquatic plants) to form a protective cyst (Lynn 2008). Light-colored substrates are significantly preferred over dark (Nickell 1989). The cyst wall is composed of protein rich mucus and environmental debris that protect the tomites (daughter cells) from bacteria, fungus and micro-predators (Dickerson 2006). The tomont undergoes a series of palintomic divisions,

producing 200-800 theronts (Dickerson 2006). The number and size of theronts produced per cyst, as well as duration of division varies in response to environmental conditions such as temperature, and size of the tomont (Ewing et al. 1986; Aihua and Buchmann 2001).

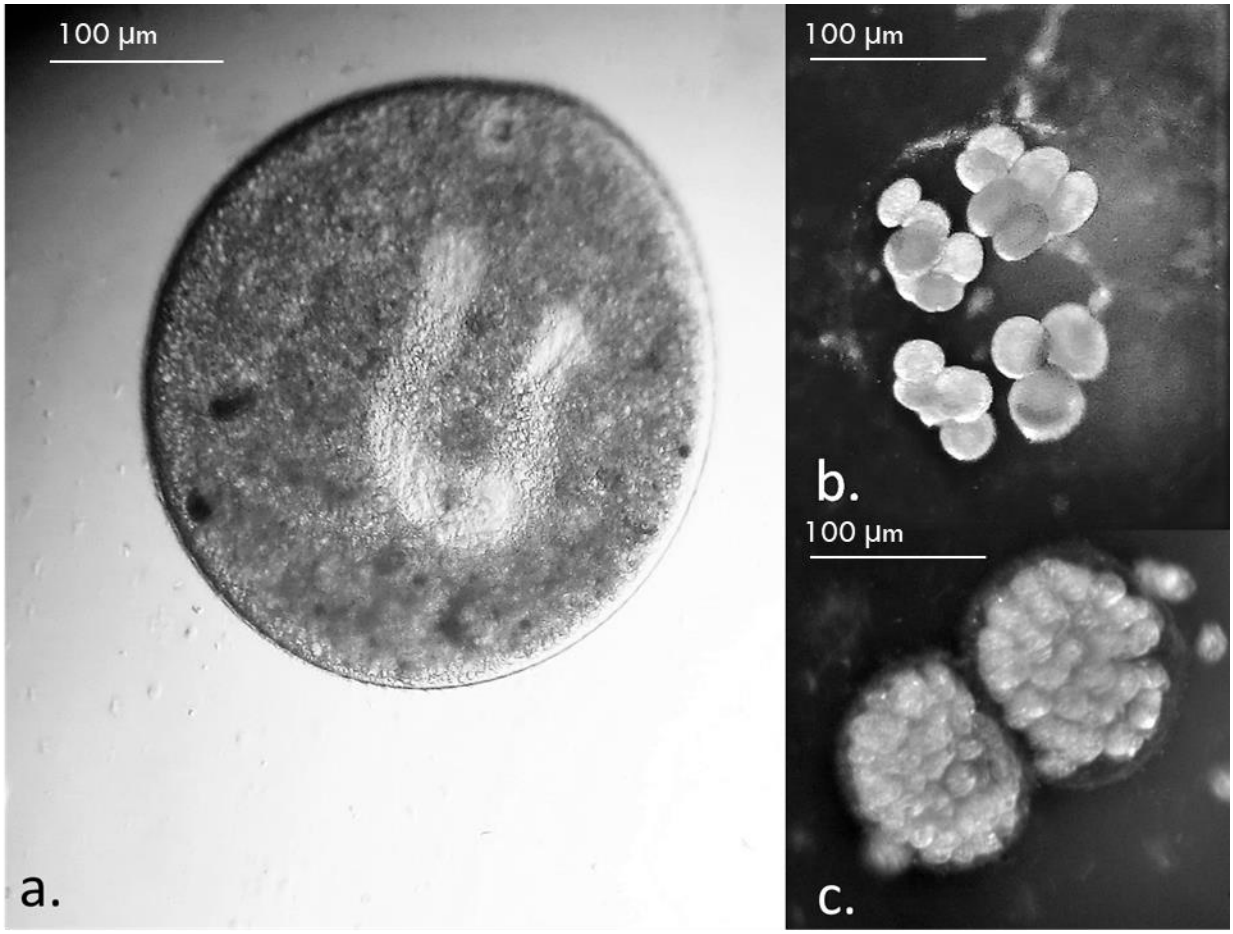

Figure 1.4 photos of a dividing tomont with tomoites. Photo taken at the Aquatic Animal Health Lab, Corvallis OR. Ich at the tomont stage prior to encysting (a), during early division (b), and late division as theronts are exiting the cyst (c). The double cyst shown in c) was likely produced by an early division that separated the daughter cells within the inner layer of the cyst wall (Ewing, 1983).

The timing of this life cycle is primarily dictated by temperature. Ich infections have been described at temperatures ranging from 5-30 °C , across a wide host range (e.g. cultured rainbow trout in Norway (Aihua and Buchmann 2001), to wild Killifish in Peru-Bolivia, (Wurstbaugh and Tapia 1988).

Outbreaks typically occur at the upper end of this thermal range (Karvonen et al. 2010). Trophonts remain on the fish host at low temperatures; this is likely the mechanism by which Ich overwinters. Trophonts infecting channel catfish at 9 ˚C remained on the fish for 204 days before exiting and producing viable theronts (Noe and Dickerson 1995). Temperatures above 30 °C are lethal for theronts (Matthews 2005). Temperatures nearing the upper thermal limit increase the parasite's metabolism, and are associated with faster division rates and the production of more theronts per tomont (Aihua and Buchmann 2001). A life cycle that takes over 5 weeks to complete at 10°C can accelerate to 3-4 days at 21-24°C (Warren 1991). These temperatures facilitate rapid development and reproduction, increasing parasite numbers and disease risk among fish hosts (Xu et al. 2011).

Numerous studies have characterized isolates based on physiology, environmental tolerances and genetic composition (Nigrelli et al. 1976; Dickerson et al. 1993; Aihua and Buchmann 2001; Forwood et al. 2015; MacColl et al. 2015). Currently, the geographic range and host distribution of Ich strains is not well understood. Traditionally, strains have been described by serotype, which is defined by the composition of the i-antigen on the surface of the cell (Clark et al. 1995). This method is limited because it does not reveal genetic relationships, or provide insight into physiology or behavior of the parasite. Genetic sequencing has confirmed the phylogeny of Ich in the suborder Ophrioglenina, and is beginning to reveal the genetic variation between isolates (MacColl et al. 2015).

Ich infections in cultured fish populations are primarily managed through a combination of husbandry techniques and chemical agents. Infections can be controlled by manipulating the environment to favor the host over the parasite by increasing salinity, raising pH, and adjusting temperature (Picón-Camacho et al. 2012). Environmental manipulations such as adjusting water velocity and turnover rate are have also been used to reduce and even eliminate the parasite in raceways (Bodensteiner et al. 2000). Additionally, standard husbandry practices to reduce stressors and promote host immune health and infection resistance can reduce the severity of an outbreak. Chemical agents

such as formalin, potassium permanganate and chloramine-T can be effective when added to the water, targeting the waterborne stages of the parasite. Chemical application is controlled by governmental agencies, particularly in the case of food fish, and limit the application of these treatments. Chemicals can also be lethal to the fish host, and so must be administered carefully (Matthews 2005). Although hosts acquire immunity post infection, a commercially available vaccine has not been developed. The primary challenge to the development of a vaccine is production. Ich is an obligate parasite and therefore cannot be grown in culture. To address this, researchers are attempting to develop a transgenic *Tetrahymena thermophila* that expresses Ich i-antigens (Woo and Buchmann 2012).

MOLECULAR TECHNIQUES AS MONITORING TOOLS FOR WATERBORNE PARASITES

Increases in parasitic disease rates and severity have serious implications for vulnerable host populations (Marcogliese 2008), as elevated disease rates can result in extinction (Smith et al. 2009). Therefore, monitoring and managing parasite levels is necessary to protect populations of concern (Miller et al. 2014). Monitoring protocols are defined by the species, environments, and human stakeholders involved. These protocols collect information on parasite impact, establish biological thresholds, and initiate management actions (Beeden et al. 2012). Metrics can include population impacts of parasite-associated mortality, parasite incidence and load, and environmental parasite levels (Mörner et al. 2002; Hallett et al. 2012; Stringer and Linklater 2014). Host-dependent measures are difficult to assess in wild systems, due to confounding factors such as multiple infections and the challenge of observing moribund or deceased hosts (Miller et al. 2014). Traditional assessment methods rely on direct sampling of hosts, followed by direct visual examination and histology to assess pathology, and molecular methods such as ELISA and PCR assays to screen for pathogens of concern (Woo and Buchmann 2012). Medical treatments are not typically feasible in wild systems, so management responses rely on altering the environment to reduce stress on the host (e.g., restore riparian foliage to

lower water temperatures in an attempt to improve fish health, Pierce et al. 2014), or reduce parasite success (e.g., disrupt a parasite's life cycle by removing/reducing alternate hosts, Clausen et al. 2012).

Advancing molecular technology is providing new tools for ecological research and conservation. These methods include genetic assessments, gene expression profiling and molecular monitoring (Miller et al. 2014). Molecular monitoring based on quantitative polymerase chain reaction (qPCR) has the potential to decrease monitoring effort and/or increase detection sensitivity (Huver et al. 2015). When parasites are transferred through the environment to infect hosts, the quantification of environmental parasite levels can be used to predict population level impacts of that parasite (Hallett et al. 2012). This reduces monitoring effort by collecting environmental samples instead of capturing hosts, and increases detection sensitivity beyond observations of disease. Ideally, this monitoring method should be able to detect harmful parasite levels and trigger management responses prior to negative impacts on the host population of concern (Bastos Gomes et al. 2017). The interpretation of environmental qPCR results is limited by the complications to the assumption that the parasite level detected in the environment corresponds to the host's parasite abundance and infection risk. This is not always reasonable because environments are heterogenous, and the overlap of parasites and hosts can be unpredictable (Marcogliese 2008). Homogenous aquatic environments are the best suited for this monitoring method. Even so, these limits must be assessed and accounted for.

Quantitative PCR (qPCR) analysis of water samples has been successfully implemented as a monitoring tool for aquatic parasites, including the protozoan *Chilodonella hexasticha* (Bastos Gomes et al. 2017), the trematode *Nanophyetus salmincola* (Purcell et al. 2016), and the myxozoan *Ceratonova shasta* (Hallett and Bartholomew 2006). In the case of *C. shasta*, the qPCR assay developed serves as the foundation for routine monitoring of *C. shasta* levels in the Klamath River. qPCR analysis of water samples collected year-round from index sites along the Klamath River enables the rapid detection of elevated environmental levels of *C. shasta* (Hallett et al. 2012). The primary management response is to

release water from upriver dams, which can flush the parasite from the system (Javaheri et al. 2016). The relationship between environmental levels and disease risk have been studied extensively in the laboratory and field to ensure that the environmental levels captured in water sampling are biologically relevant and accurate (Hallett et al. 2012; Ray et al. 2012; Bartholomew and Ray 2013). Additionally, monitoring efforts incorporate traditional methods such as sentinel fish exposures (Hallett and Bartholomew 2006).

QUANTITATIVE PCR

Quantitative PCR (qPCR) is an extension of presence/absence PCR to include a quantification component (Bustin 2004). A fluorescent doubles stranded DNA binding dye is included in each reaction, enabling the quantification of the DNA amplicon produced after each thermo-cycle. The cycle quantity (Cq), or cycle threshold (Ct), is the reported value given by the qPCR instrument at the end of the reaction (Cq is the recommended abbreviation; Bustin et al. 2009). To determine this value, the qPCR instrument compares the change in relative fluorescence of reacting against non-reacting samples. The instrument establishes a threshold fluorescence level that is met during the exponential phase of all amplifying reactions. The value reported (Cq) is the cycle at which the sample met the threshold amount of fluorescence (Bustin 2004). There are two basic fluorescence chemistries used in qPCR reactions- non-specific intercalating dyes that bind non-discriminately to double stranded DNA, and probe-bound dyes that bind and fluoresce only with the target of interest (Bustin 2004). Non-specific binding assays are cheaper and simpler to design; however, they produce fluorescence with non-target products. Therefore, assays using this detection chemistry often include a melt curve analysis of the final amplicon to ensure that a single amplicon, of the expected melting temperature, was generated (Bustin 2004).

Cq values from separate runs are not directly comparable because the threshold value is unique for each run. Comparisons require standards-samples that are run on each plate, ideally representing a

biologically significant unit. Additionally, a change in reaction efficiencies between plates can skew comparisons. Therefore, a dilution series is typically included with each run to assess the reaction efficiency (Bustin 2004). In an ideal reaction with 100% efficiency, samples that differ in original target DNA quantity by 1 order of magnitude should be 3.3 cycles apart. Efficiency can be impacted by inhibition within samples or by non-optimal reaction conditions. A reliable assay should have stable efficiencies between plates, however it is necessary to confirm that the assay performed as expected so that Cq values can be accurately interpreted (Bustin et al. 2009).

The analysis of environmental samples introduces an additional variable, inhibition. Environmental inhibitors include organic and inorganic compounds present in environmental samples that can interfere with qPCR reaction components such as polymerase or magnesium, resulting in false negatives or reduced detections (Wilson 1997). A common strategy to assess and account for inhibition is the inclusion of an internal positive control (IPC) reaction. IPC reactions have a probe-based chemistry. The IPC DNA, primer and probe are added to the sample reaction, and the resulting IPC Cq is compared to that of the negative control. If the sample IPC has a higher Cq than the negative control, it is inhibited. This inhibition can be accounted for by directly subtracting the inhibition value from the sample's target DNA Cq, or by further diluting the sample and assaying it again. Diluting the sample further is preferable when inhibition values are high (exceeding a Cq of 2) because the relationship between inhibitors and sample targets can be non-linear. IPC assays can be run simultaneously with other probe based assays (e.g. (Hallett and Bartholomew 2009)) however they must be assayed separately for non-specific fluorescent assays that will bind to, fluoresce, and interfere with the IPC DNA (Bustin 2004).

Additional factors influencing Cq values include, sample processing and storage techniques, sample template, assay reagent combinations, thermal cycling conditions and instrument performance (Brankatschk et al. 2012). As a result, qPCR-based research can be inconsistent and difficult to reproduce. In 2009, the Minimum Information for Publication of Quantitative Real-Time PCR

Experiments (MIQE) guidelines were established to address this issue. These guidelines are intended to promote consistency, reproducibility and reliability in experiments utilizing qPCR (Bustin et al. 2009).

PROJECT OBJECTIVES

The purpose of this study is to determine the feasibility of qPCR analysis of water samples as a monitoring method for Ich in the Klamath River, CA.

Chapter 2 describes qPCR assay development, optimization, and initial application to environmental water samples. This chapter will be submitted to the journal *Diseases of Aquatic Organisms* for publication.

Chapter 3 presents a field application of the sampling method, demonstrating its potential to inform management of Ich infections in the Klamath River. This chapter will be submitted to the journal *Transactions of the American Fisheries Society*.

Chapter 4 explores the genotypic variation of cox1 and SSU rDNA genes of Ich isolates collected in the Klamath River. This work informed the development of the qPCR assay, and presents a foundation for future work on Ich isolate genetic diversity. At this time, it is not adequately comprehensive to be submitted for publication.

Appendix 1 provides supplemental information on the presistance of Ich DNA after parasite mortality.

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CHAPTER 2: DEVELOPMENT AND APPLICATION OF A QPCR ASSAY TARGETING *ICHTHYOPHTHIRIUS MULTIFILIIS* IN ENVIRONMENTAL WATER SAMPLES

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ABSTRACT: *Ichthyophthirius multifiliis* (Ich) is a globally distributed freshwater parasite that infects wild and cultured fishes. Its direct, temperature dependent life cycle enables rapid multiplication when hosts are plentiful and environmental conditions are favorable. Early detection is central to the control of infections and prevention of mortality, particularly in wild systems where chemical treatments are not feasible. In the Klamath River, CA, Ich infections threaten pre-spawning adult salmon. Currently, Ich is monitored via lethal sampling of fish hosts and visual quantification of parasite load. This method is time intensive, imprecise and light infections are easily missed. We developed and validated a qPCR assay that targets Ich small-subunit ribosomal DNA, demonstrating strong linearity, efficiency and repeatability. Levels in environmental water samples collected from the lower Klamath River from July to October in 2014 through 2016 relate to observed parasite load on salmon sampled concurrently, indicating that the qPCR assay could be a useful monitoring tool for Ich in the Klamath River, with application beyond the region.

KEY WORDS: *Ichthyophthirius multifiliis*, qPCR, SYTO9, water sample, theront, salmon, infection monitoring, Klamath River, adaptive management

INTRODUCTION

The freshwater fish parasite *Ichthyophthirius multifiliis* (Ich) causes significant losses of aquaculture, aquarium and wild fishes worldwide (Matthews 2005). Ich has a direct life cycle that progresses from a fish-associated parasitic trophont, to a substrate-associated tomont, which divides to release infective theronts into the water column (Figure 2.1). Ich infections can result in disease (Ichthyophthiriasis, or white-spot disease), and mortality due to tissue damage, anemia, disrupted osmoregulation and secondary infections (Abdel-Hafez et al. 2014). Infections progress quickly when fish hosts are in a contained area, and become more severe as the number of feeding trophonts per host increases (Xu et al. 2011). Therefore, rapid detection of Ich is necessary to reduce fish mortality in the

event of an outbreak. Ich infections are typically detected by visual observations of parasites, as well as fish behaviors such as flashing (Woo and Buchmann 2012). Diagnosis is confirmed by presence of the ciliated trophont on a skin or gill scraping (Ewing 2002). Using these methods, infection typically remains below detection until the development of disease (Traxler et al. 1998), at which point treatment may be inadequate to prevent mortality.

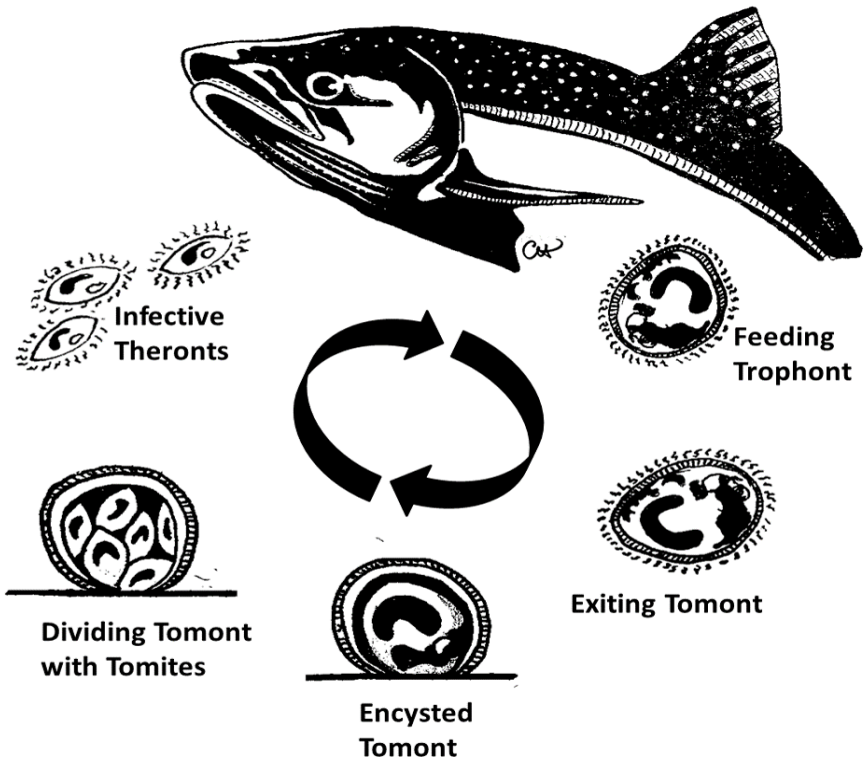

Figure 2.1 life cycle of *Ichthyophthirius multifiliis* (Ich). The life cycle progresses from the feeding parasitic trophont, to the exiting tomont that encysts onto the substrate and divides, releasing hundreds of infective theronts.

Ich infections have been associated with wild freshwater fish mortality globally, including redtail barbs (*Barbus haasi)* in Spain (Maceda-Veiga et al. 2009), killifish (*Orestias* sp.) in lake Titicaca in Peru-Bolivia (Wurstbaugh and Tapia 1988), and Chinook salmon (*Oncorhynchus tshawytscha*) in California (Belchik, 2002). In the Klamath River, CA, the parasite has contributed to die-offs of pre-spawning

Chinook salmon adults soon after they enter the river from the ocean (Belchik 2015). Annual monitoring for Ich, conducted by Yurok tribal biologists, commenced after a mass mortality of Chinook in 2002. This method is reliant on the capture, lethal sampling, and visual assessment of migrating adult salmon. The approach contributes to pre-spawn mortality, is time and labor intensive, and Ich detection is typically limited to advanced infections. Molecular methods such as quantitative PCR (qPCR) have been developed to quantify the level of waterborne pathogens in aquatic environments including; the protozoan *Chilodonella hexasticha* (Bastos Gomes et al. 2017), the trematode *Nanophyetus salmincola* (Purcell et al. 2016), and the myxozoan *Ceratonova shasta* (Hallett and Bartholomew 2006). This method has been applied as a non-invasive early warning system for developing outbreaks (e.g. Bastos Gomes et al. 2017). Water sampling and qPCR analysis to target Ich has the potential to increase monitoring efficiency and sensitivity, as well as provide information on the spatial and temporal distribution of Ich in the Klamath River to inform adaptive management strategies.

River water flow is the only easily manipulated parameter in the Klamath River, and the sole option for mitigating salmonid disease (Turek et al. 2004). The Klamath River is a hydrologically managed system with dams on the mainstem and principal lower basin tributary, the Trinity River. In response to disease triggers such as the detection of serious (over 30 Ich parasites / first gill arch) parasite levels on salmon, emergency flows have been released from the Lewiston dam on the Trinity River to reduce infection levels (Belchik 2015). A more sensitive detection method could enable earlier release, preventing parasite levels from reaching levels associated with pathology and disease. Waterborne parasite monitoring has been applied to manage another parasite in the Klamath River, *Ceratonova shasta* (Hallett et al. 2012). Pulse/dilution flows from Iron Gate Dam have been implemented in response to thresholds of the waterborne parasite during salmonid outmigration to mitigate disease and population level impacts. Ideally, this method could be applied to monitor and manage Ich as well.

To this end, we assessed the potential of qPCR-analysis of water samples as a monitoring tool for Ich in the Klamath River. We developed a novel qPCR assay that targeted the small subunit ribosomal DNA (SSU) of Ich, and applied the assay to environmental water samples collected from 3 years in which Ich was observed on salmon in the lower Klamath River. We described the parameters of specificity and sensitivity for the assay, and compared results from water sample analysis to concurrent observations of Ich levels on migrating adult salmon.

MATERIALS AND METHODS

Ich Samples

Lab-prepared samples

A range of samples with varying numbers and developmental stages of Ich were prepared to inform assay performance, calibrate field sample data and inform differences in DNA content of the target gene among parasite stages. Ich-infected fish were obtained from local pet shops (OR) and the Klamath River (CA), and transported to the J.L. Fryer Aquatic Animal Health Laboratory, OSU, where parasites were then raised on goldfish (*Carassius auratus* L.) and maintained in the laboratory for several months (in compliance with OSU ACUP # 4794). Infected fish included; tiger barb (*Puntigrus tetrazona*), goldfish, and Chinook salmon. To collect Ich trophonts, theronts and tomonts, an infected goldfish was euthanized with an overdose of MS222, and placed in a petri dish with UV sterilized river water. Individual trophonts were removed from the fish surface with a pipette and either placed directly into individual 1.7 ml collection tubes (N = 6) or on 5 µm nitrocellulose membrane filters (to mimic a filtered water sample; N=6). Filters were then folded and placed in individual 1.7ml collection tubes. The remaining fish were left at room temperature (~21°C) to allow the trophonts to differentiate into tomonts and exit the fish. After ~20 min, the tomonts were visualized in the holding water with a dissecting microscope, collected with a pipette, and transferred to a clean dish with UV-sterilized river

water. Water source was the Willamette River, Corvallis, OR. Individual tomonts were each placed into 1.7 ml collection tubes (*N*=4). Parasites were further incubated in the dish at room temperature for ~24 h. The tomonts encysted, divided, and released theronts into the dish within this time. Theronts were observed with a dissecting microscope, and pipetted individually either into 1.7 ml collection tubes (*N*=5) or transferred onto a 5 µm nitrocellulose membrane filters (*N*=8) prior to placement into a collection tube. 15 additional theronts were grouped into 3 sets of 5, and transferred onto a 5 µm nitrocellulose membrane filters (*N*=8). All specimens were held at -20 °C until processing.

Environmental water samples

Water samples were collected from the mainstem of the Klamath River, CA, USA, downriver of the Tully Creek inflow (rkm 62, Figure 2.2). 4 x 1-liter water samples were collected as part of a multi-year monitoring program for parasite *Ceratanova shasta*. These samples were collected manually as grab samples (biological replicates), or with an ISCO machine (technical replicates), as is outlined in the methods of Hallett et al. 2012. Environmental samples re-analyzed for Ich were collected weekly from July-October in 2014, 2015, and 2016. Samples were stored on ice and filtered within 24 h through a 5 µm nitrocellulose membrane filter, and stored at -20 °C until processing. These samples were selected based on Chinook salmon migration timing, and Ich infection observations by Yurok tribal biologists. Ich was not detected on salmon from 2003 to 2013, but severe infections were recorded on migrating adult salmon downriver of Tully Creek in 2014 and 2016, and moderate infections were observed in 2015. In 2014 the parasite appeared to be well established in the mainstem (Belchik 2015). However, in 2016 these infections were contained within the cool water refugium 'Blue Hole', ~40 rkm downriver from Tully Creek (Yurok Tribal Fisheries Program, 2016) To investigate the relationship between waterborne Ich levels and Ich infections on adult salmon, correlations between Ich detections on fish and in water samples were analyzed for 2014 and 2015. Information on Ich detections on migrating salmon was obtained from the Yurok Tribal Fisheries Program. Environmental river conditions, temperature and

water flow data from the sampling period were downloaded from the U.S. Geological Survey water gauge number 11530500 near Klamath, CA, and compared to waterborne Ich levels.

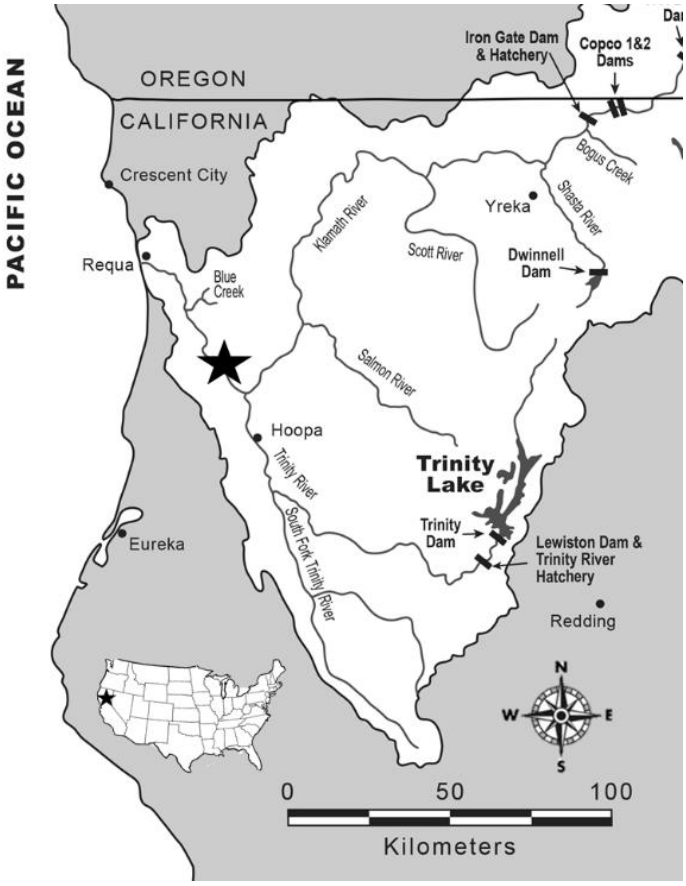

Figure 2.2. Environmental water sample collection site in the lower Klamath River basin. The Tully Creek water sample collection point in the mainstem of the Klamath River, downriver of the Trinity River inflow (star). Inset, location in the USA (star). Figure adapted from Quiñones et al. 2014.

DNA extraction and purification

All samples on filter membranes (i.e. lab-prepared and environmental water samples) were pre-treated by dissolving the filter in a series of acetone and ethanol washes (Hallett et al. 2012). DNA was extracted and purified from all samples with a DNeasy Blood and Tissue Kit (Qiagen), according to the modifications outlined in Hallett et al. (2012). The final elution volume was 120 µl in buffer AE (Qiagen).

Lab prepared specimens without the nitrocellulose filter did not require pre-treatment and were processed directly for DNA extraction and purification, with a final elution of 50 µl AE.

Primer design

Our initial qPCR assay was based on the Ich small subunit ribosomal DNA (SSU rDNA) assay developed by Jousson et al. (2005), modified by substituting the intercalating fluorescent dye SYTO9 for SYBR Green (Monis et al. 2005). Application of the assay to environmental water samples revealed issues with target specificity and consistent detection (data not shown). We therefore used the software program BioEdit (Hall 1999), to align all available Ich SSU rDNA sequences, and available sequences from ciliates in the order Hymenostomatida to assess the Jousson primers (which were developed prior to the sequencing of many of these isolates). The alignment included SSU rDNA Ich sequences NCBI GenBank accession numbers U17354, KJ690565- KJ690572, EF469202, KM870913, KM822612, KC512768, plus closely-related species including *Ophryoglena catenula* (U17355), *Lambornella* sp. (JQ723973), *Colpidium colpoda* (EF070266.1) and *Tetrahymena* sp. (M98015, KJ028516, KJ028505, JX12937). We observed that the taxon *I. multifillis* included a range of SSU genetic variants (Figure 2.3). Therefore, the issues we encountered with the Jousson assay were likely related to base pair differences (SNPs) in the primer regions. To overcome this problem, we designed six primer combinations based on portions of the alignment that were inclusive for all Ich isolates, exclusive to closely related organisms, and with consideration of the primer and amplicon guidelines from the Applied Biosystems qPCR manual. Prior to application, primer melting temperature, primer-dimer formation, sensitivity and specificity were assessed *in silico* using BioEdit and ThermoFisher multi-oligo analyzer (https://www.thermofisher.com). Primers specificity was evaluated empirically via gel electrophoresis, a qPCR melt curve analysis, and Sanger sequencing of qPCR amplicons at OSU's Center for Genome Research and Biocomputing Core Laboratory using previously reported methods (Atkinson and Banner 2016).

```
                                  450       460       470       480       490         630       640       650
U17354.1    Ichthyophth.CTAATTCAGGGAGGTAGTGACAAGAAATAGCAAGCCAGGAGATTC~~TTCTCTACG   GAACTTCTGCCTATTT-AGCTGGGTTCT
KJ690565.1  Ichthyophth.....................................G........~~.........   ................T...........
KJ690566.1  Ichthyophth.....................................G........~~.........   ................T...........
KJ690567.1  Ichthyophth.....................................G........~~.........   ................T...........
KJ690568.1  Ichthyophth.....................................G........~~.........   ................T...........
KJ690569.1  Ichthyophth.....................................G........~~.........   ................T...........
KJ690570.1  Ichthyophth.....................................G........~~.........   ................T...........
KJ690571.1  Ichthyophth.....................................G........~~.........   ................T...........
KJ690572.1  Ichthyophth.....................................G........~~.........   ................T...........
KM870913.1  Ichthyophth                  CA.GAG...................~~.........   ................T...........
KM822612.1  Ichthyophth           TA...............................~~.........   ................-...........
KC512768.1  Ichthyophth.....................................G........~~G........   ...........C....T..A.A......
Primer-IMRf1 (Jousson)                AGTGACAAGAAATAGCAAGCCAGGAG
Primer-IMRr1 (Jousson)                                                                 TCTGCCTATTT-AGCTGGGT
U17355.1    Ophryoglena...................................T.G........~~G........   .........GT..C..TG.A.A..C..G
JQ723973.1  Lambornella.............CC....................TG...A.C.TA~G.T......   .........TTC.GA.TCATAAC.ACTC
EF070266.1  Colpidium c.............CC.....................G..GAGCAATTGCT......   .........TTC.GGGCTA.CT~.A.TC
JX129387.1  Tetrahymena.............CC....................TG...A.CCTA~G.T......   .........TTC.GG.TTATAAC.ACTC
KJ028516.1  Tetrahymena............................T....T.G...AGA.TT~.CT......   ...T......TCGCC.AT.ACTAC...G
KJ028505.1  Tetrahymena.............CC....................TG...A.C.TA~CGT......   .........TTC.GG.TCAT.TC.A.TC
```

Figure 2.3 BioEdit alignment of Ich isolates downloaded from GenBank (n=12), Jousson et al. 2005 primers, and closely related organisms *Ophryoglena catenula*, *Lambornella* sp., *Colpidium colpoda* and *Tetrahymena* sp (n=6). The forward primer includes an A/G SNP, and the reverse primer includes a T insert when compared to isolates of Ich.

qPCR amplification conditions

Ich samples were analyzed twice, with 2 separate qPCR assays: 1) the assay that we developed for Ich, and 2) an internal positive control (IPC) assay to assess inhibition. Both qPCR assays used 10 µl reactions with a StepOnePlus Real-Time PCR System (Applied Biosystems) and MicroAmp Fast Optical 96-well reaction plates (Applied Biosystems). The optimal primer concentration for the Ich assay was determined experimentally (primers were assessed at concentrations of 200, 400, 800 and 1600 nM per reaction). All samples were diluted 1:5 in buffer AE to reduce environmental inhibition, and then diluted further if needed. Each Ich qPCR reaction consisted of 200 nM of each primer (Applied Biosystems), 5 µl TaqMan Universal PCR Master Mix (contains AmpliTaq gold DNA polymerase, AmpErase UNG, dNTPs with dUTP, passive reference 1 and optimized buffer components) (Applied Biosystems), 0.25 ng bovine serum albumin (ThermoFisher Scientific), 0.15 mM SYTO9 (ThermoFisher Scientific), 2.05 µl of molecular grade water, and 2 µl of diluted sample DNA. The 10 µl IPC qPCR reactions utilized the TaqMan Exogenous IPC Reagents kit (ABI), and consisted of: 5 µl TaqMan Universal PCR Master Mix, 0.25 ng BSA, 1.0 µl IPC probe/primer mix (0.5×), 0.2 µl IPC DNA (0.5×), and 2 µl diluted DNA template. IPC cycling

conditions were the same as the Ich SYTO9 assay, but did not include the melt curve analysis (see below). Cycling conditions for all assays were: 2 min at 50°C, 10 min at 95°C, followed by 40 cycles of 15 s at 95°C and 1 min at 60°C. For the Ich qPCR only, a melt curve analysis (64°C to 88°C in increments of 0.3°C in 15 s steps) was performed to verify that a single amplicon, with the correct melting temperature, was generated in each positive reaction. Each qPCR included a standard curve spanning 4 orders of magnitude, a negative control (molecular grade water), and a positive reference sample to compare inter-plate Cq values. Reference samples were composed of a pool of 5 theronts extracted with a filter paper. Test samples were assayed in duplicate and then averaged. If duplicate wells fluoresced more than 1 cycle apart and were detected at least 0.5 cycles sooner than the limit of quantification, we re-assayed the sample. The negative control, reference samples and standard curve samples were assayed in triplicate. Any sample that did not fluoresce, or had a Cq above 37, was regarded as negative. Inhibition levels were assessed for each sample by subtracting the sample IPC value from the IPC of the negative control; IPC values that differed by 0-2 cycles were simply subtracted from the Ich assay Cq value before any further calculation; any samples that had an IPC difference >2 cycles from the negative control were diluted further (concentration reduced 50%) and re-assayed.

qPCR validation

We determined assay efficiency, linearity, and dynamic range using 10-fold and 2-fold serial dilutions of Ich DNA, obtained by extracting 10 mature feeding trophonts. Intra- and inter-plate assay reproducibility was determined by comparing the Cq values, efficiency, and linearity of the standards and reference sample run in triplicate on each plate. Assay sensitivity was determined for DNA extracted from individual parasites at each life stage, with or without our acetone pre-treatment, to determine; a) if a single target life stage (theront) could be detected, b) the biological variation between detections from an individual theront, c) how the SSU copy number compared between all life stages of the

parasite, and d) the efficacy of sample preparation methods. The sets of 5 theronts were assayed to confirm the linear relationship between individual and multiple theronts.

Assay specificity

Assay specificity was determined by visual inspection of the melt curve of each qPCR product (differences in melting temperature, additional/ abnormal peak formation). We also visualized qPCR products from an Ich isolate (n=1) and environmental water samples (n=6) on a 1.5% agarose gel, to confirm that the amplicon consisted of a single 118 bp product. These products were sequenced to confirm amplicon identity. Environmental water samples selected for sequencing included 5 samples from the Tully Creek water sample collection site (2 samples from 2014, 1 sample from 2015 and 2 samples from 2016), and 1 sample from ~20 rkm downriver at the Blue Hole refugium. Additionally, samples (n=6) of 2 closely-related organisms (*Tetrahymena* sp. and *Colpidium* sp.) were assayed to confirm that no amplification occurred. These organisms were obtained as live cultures from Carolina Biological, and each sample consisted of approximately 500 individuals. DNA was extracted and purified using the Qiagen Blood and Tissue kit, and confirmed by PCR using universal SSU primers ERIB1 (Barta et al. 1997) and ALL1r (Hallett et al. 2002), then Sanger sequencing. The samples were assayed by qPCR at dilutions of 1:1, 1:10, 1:100 and 1:1000 to account for any inhibitory effect of DNA concentration.

Converting Cq to copy number

To determine the relationship between Cq and SSU DNA copy number, we used PCR to generate a 792 bp amplicon spanning the qPCR primer binding sites. The PCR reaction included the forward primer 415f (5’ -GCA GCA GGG ACG TAA ATT ACC- 3’) (present study) the reverse primer ALL1r (5’ -AAC TAA GAA CGG CCA TGC ACC AC- 3’) (Hallett et al. 2002) . PCR reaction components and cycling conditions were the same as stated for these primers in the primer design section. Template DNA was from a sample of 10 infective theronts. The amplicon was visualized on a gel, excised, placed into a 2 ml

tube, submerged in 100 µl of buffer AE, and then incubated at 4°C for 24 h. All liquid was transferred into a new microfuge tube and the concentration of the amplicon was determined using a Qubit spectrophotometer. The amplicon copy number present in 1 µl was calculated using the concentration and the molecular weight of the 792 bp amplicon of double stranded DNA. The amplicon was used to generate a 1:10 serial dilution spanning the full linear dynamic range of the assay. The linear relationship between the log of copy number/reaction and the Cq value reported was used to convert sample Cq values into copy number. To determine the copy number/liter of water samples, the number of copies in 1 µl was calculated, then this value was multiplied by the dilution factor for the reaction, and the final elution volume of the sample.

RESULTS

Validation of qPCR primer specificity

Ultimately, we selected the forward primer 667f (5´-AAT GGG CAT ACG TTT GCA AAC C-3´), and reverse primer 765r (5´-ATA GCC CGG ATA CAT CAG CAT G-3´). These primers generated a 118 bp SSU amplicon that spanned a region conserved among Ich isolates, but which varied from related organisms (Figure 2.4 A). Gel visualization of the qPCR products generated from an Ich isolate and an environmental water sample confirmed that the primers generated a single amplicon with identical melt curves (Figure 2.4 B, C,), and the 6 environmental qPCR amplicons analyzed by Sanger sequencing had 100% similarity to Ich. Ich standards (*N* = 39) and environmental samples (*N* = 53) both had an average melting temperature of 78.1 ˚C. Of the 53 environmental reactions that amplified, only 2 reactions had melt curves that differed. One reaction from the 2015 data had a melting temperature of 77.4 ˚C at Cq 39. The other irregular reaction (in only 1 of the paired wells) had 2 peaks – at 78.1 and 71.1˚C. On an agarose gel, the sample with 2 peaks produced a wider band than single peak environmental amplicons,

which we interpreted as at least 2 amplicons (data not shown). The primers did not amplify the genetically similar *Tetrahymena* or *Colpidium* samples (data not shown).

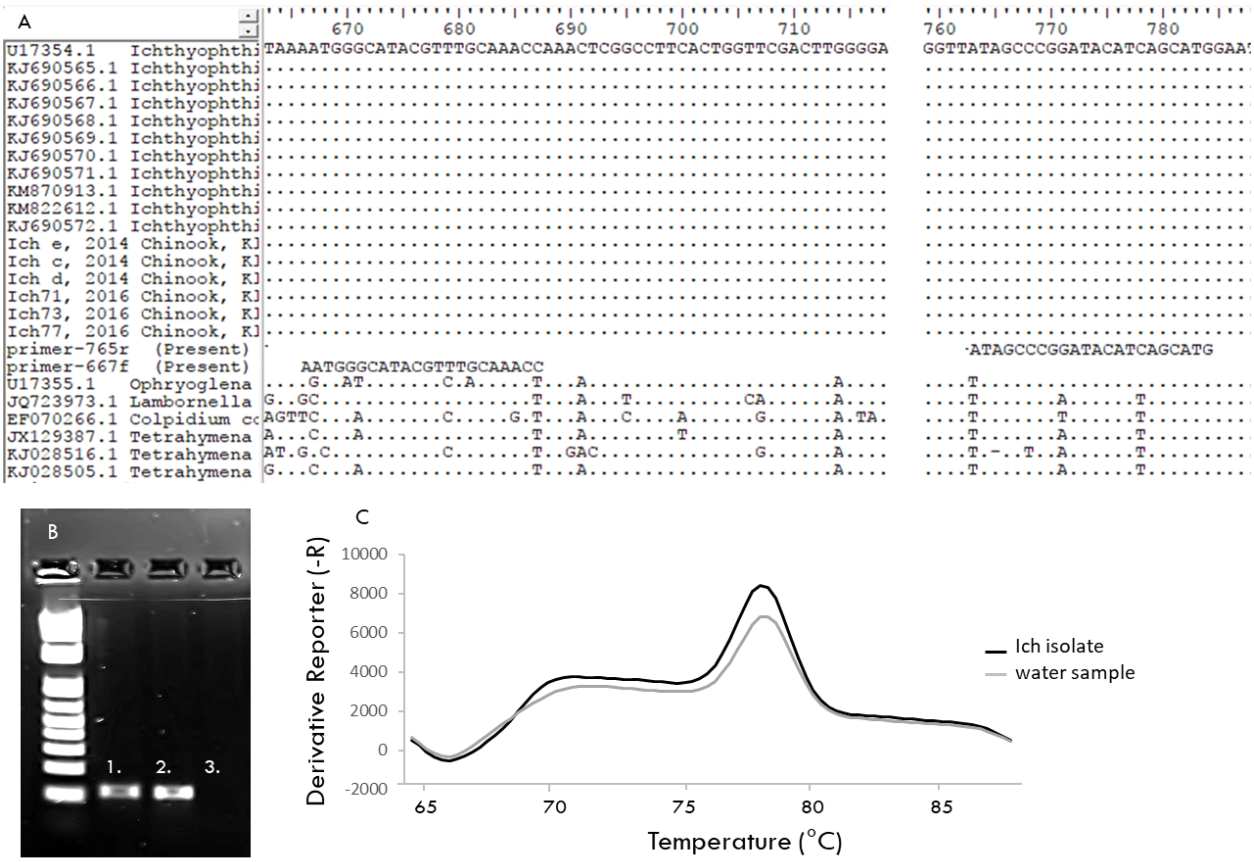

Figure 2.4 Primer specificity. A) BioEdit alignment including Ich isolates from GenBank (n=11), Ich isolates sequenced from Chinook salmon in the Klamath River (n=6), newly designed primers (n=2), and a representative selection of closely related organisms (n=6). B) 118 base pair qPCR amplicon from an Ich isolate (1) and an environmental water sample collected from the Klamath River in 2016 (2), also shown is the negative control (3). C) Melt curves from the Ich isolate (black), and environmental water sample (grey) shown in B.

qPCR assay validation

The assay performed linearly over at least 7 orders of magnitude, a Cq range of 10 - 36, with R^2 0.99 (95% CI: 0.99-0.99, *N*=10), and slope 3.3 (95% CI: 3.27-3.36, *N*=10) (Figure 2.5 A). The limit of detection (the point at which 95% or fewer of reactions with template fluoresced (Bustin et al. 2009)

was Cq 34, which corresponded to ~50 copies of SSU per reaction. Under our reaction conditions, a 1 liter water sample generating a Cq of 34 would consist of ~2,800 SSU copies / liter, equivalent to ~3 theronts (Figure 2.4). The intra-plate coefficient of variation for samples below the limit of detection is 0.7% (95% CI: 0.4%, 0.9%, *N*=8), this increases to 2.0% (95% CI: 1.4%, 2.68%, *N*=6) for samples at the limit of detection. The inter-plate coefficient of variation is 2.2% (95% CI: 1.9%, 2.6%, *N*=4, across 10 plates).

The assay was sensitive to all life stages of Ich. The average copy number in an individual parasite differs between each life stage, and is influenced by loss of DNA due to the acetone pre-treatment necessary for samples on 5 µm nitrocellulose filters. Theronts extracted under both conditions had the fewest and most consistent copy numbers per individual of the three life stages (Figure 2.5 B). Pre-treatment decreased the copy number for both theronts and trophonts. The assay could detect an individual infective theront extracted on a filter, although the average Cq, Cq 35.5 (34.8-36.1, *N*=7), was beyond the limit of detection (the point at which 5% or more of reactions with template DNA do not fluoresce) for the assay. Of the 7 individual theronts assayed, all were detected in at least 1 of 3 wells. We confirmed that although the Cq value for a single theront was beyond the limit of detection, the assay still performed linearly to this point (Figure 2.5 C).

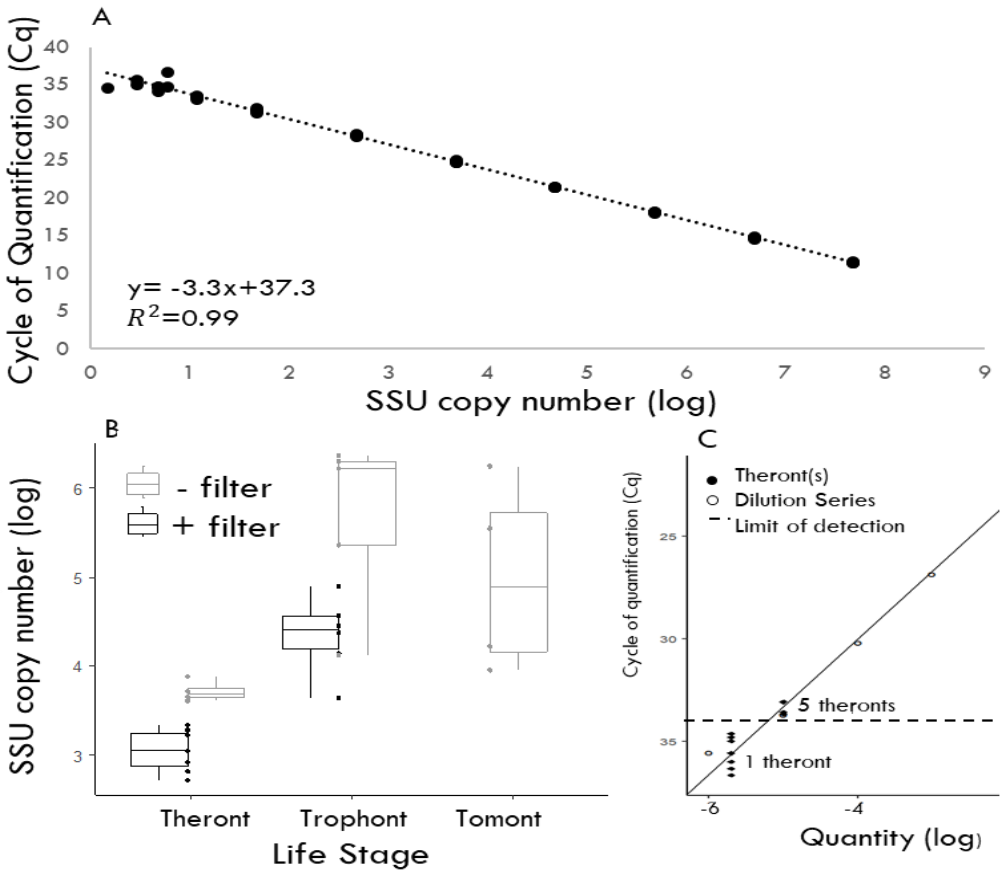

Figure 2.5 Assay validation. A) Dilution series demonstrating assay linearity, efficiency and limit of detection. Template DNA consisted of PCR product with known copy numbers. Each dilution step was assayed in triplicate. B) Log copy number of each life stage of Ich. Theronts and trophonts were analyzed with (black) and without (gray) a filter dissolving step in the DNA extraction protocol. C) Linear relationship between 1 and 5 theronts, in relation to the limit of quantification (Cq 34, dashed line).

qPCR detection of Ich in environmental water samples

Detections of Ich in environmental water samples from the Klamath river mainstem were consistent with the severity and timing of Infections observed on migrating salmon in the area (Table 1). 2014 was the only year that serious Ich infections were observed on salmon in the mainstem, and waterborne Ich was detected more frequently, and at higher levels in 2014 than in 2015 or 2016 (Figure 2.6 A). The average SSU copy number / liter in 2014 corresponded to ~90 theronts / liter, compared to an average of ~2 theronts / liter in 2015 and 2016 (Table 1). In August-September of 2014, migrating

Chinook salmon were captured within 5 rkm of the water sampling site at Tully Creek, and assessed for Ich. Water sampling detected Ich ~1 week prior to the observation of serious Ich infections on these salmon (Figure 2.7). In all years, water sampling detected Ich 1-3 weeks after the parasite was detected on Chinook salmon downriver There was a moderate positive correlation between the percentage of positive water samples detected at 2-week intervals July-October in 2014, and the percentage of positive fish that were detected throughout the reach in the same period (r= 0.9, p=0.05). This correlation was not observed in 2015 (r=0.1, p=0.9), and data on Ich infections on fish was not available for 2016.

Table 2.1. Comparison of Ich detections in water samples and on salmon in 2014, 2015 and 2016. Chinook salmon run size was reported by the Pacific Fishery Management Council (Pacific Fishery Management Council, 2017), and the information on the presence and location of serious Ich infections on migrating salmon were provided by the Yurok tribal fisheries biologists (Belchik 2015).

Year	Initial detection on salmon	Initial detection in water	Chinook run size	Severe Ich infections	Severe infections observed in mainstem	Average SSU copies / liter	Average theront /liter equivalent
2014	21-Aug	26-Aug	160,396	Yes	Yes	90,856 (n=13)	~ 91
2015	28-Jul	18-Aug	77,821	No	No	2,383 (n=8)	~ 2
2016	18-Aug	7-Sep	24,567	Yes	No	2,309 (n=8)	~ 2

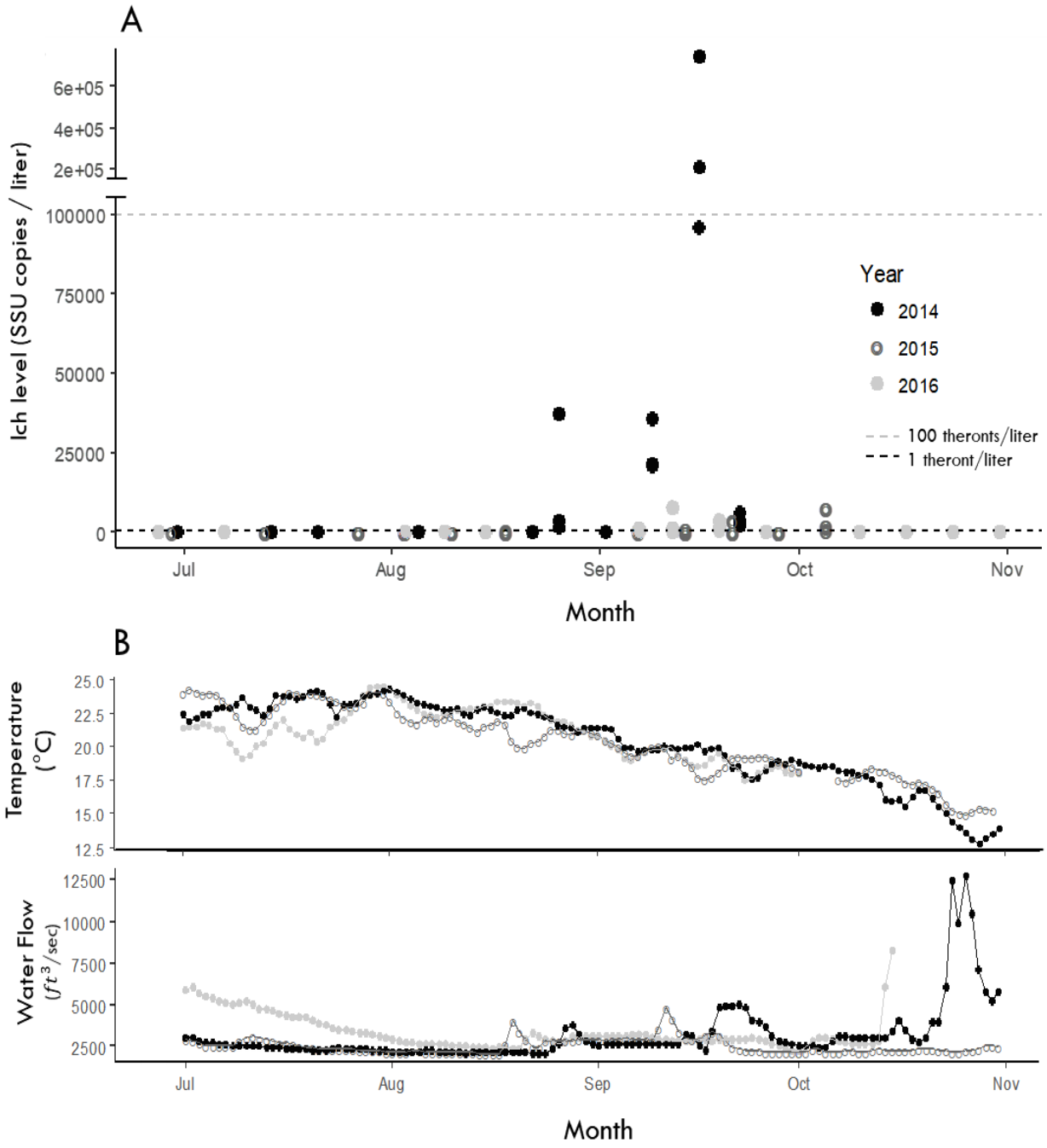

Figure 2.6 Ich detections in water samples from 2014, 2015 and 2016 (A). Dashed lines represent the average copy number for 1 theront (black), and 100 theronts (gray) median daily temperature and mean daily water flow across the sampling period from the U.S. Geological Survey water gauge number 11530500 near Klamath, CA (B).

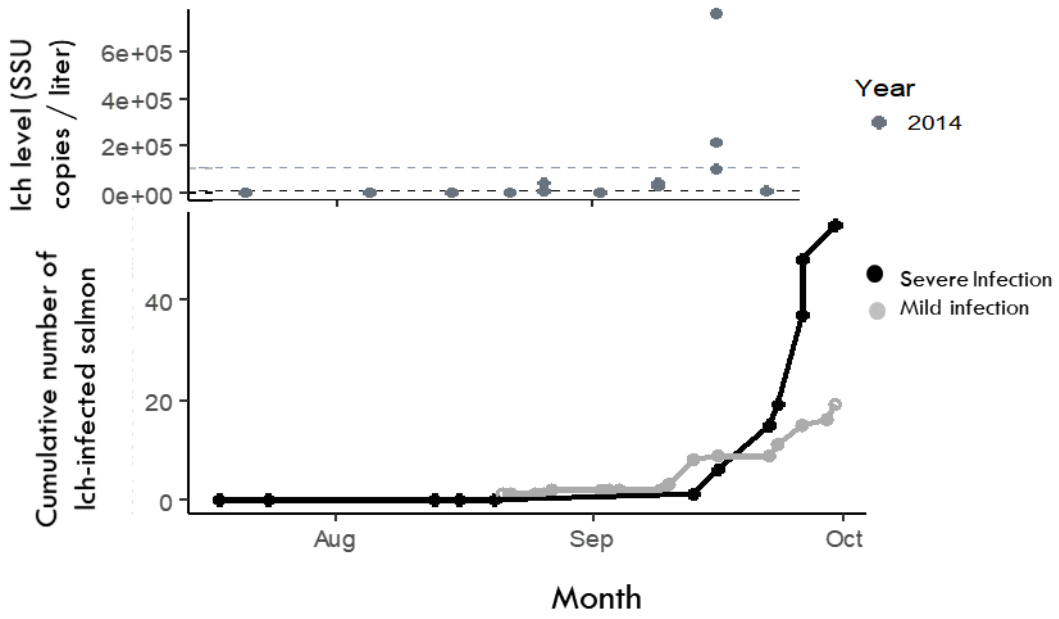

Figure 2.7 Ich detections in water samples, and salmon in 2014. Salmon data reflects the cumulative number of severe (black), and mild (grey) Ich-positive salmon. Salmon data prior to September was collected ~40 rkm downriver of the water collection site, and data collected after September was collected within a 5 rkm radius from the water sample collection site.

DISCUSSION

We successfully detected and quantified Ich in environmental water samples using the novel SYTO9 qPCR assay. The assay demonstrated good specificity and sensitivity when applied to standards and environmental samples. The infective stage of the parasite (theront) had a consistent SSU copy number, and the assay could detect an individual theront. Environmental detections were consistent with the timing and severity of infections observed on migrating salmon, indicating that the method can describe patterns in waterborne Ich levels that are relevant to fish health. Overall, this research demonstrates the promise of water sampling coupled with molecular quantification as a monitoring tool for waterborne Ich levels, and a potential replacement for lethal sampling of migrating adult salmon.

We confirmed specificity of the assay by melt curve analysis and Sanger sequencing of 6 environmental amplicons, plus tested it against two closely related organisms. Those few (less than 0.03%, n=87) environmental water sample reactions that showed non-target amplification had Cq values beyond the limit of detection. The assay detected all life stages of the parasite (Figure 2.5 B), and was capable of detecting 1 infective theront / liter. Cq values for an infective theront were beyond the limit of 95% detection for the assay, therefore we recommend that water sample analysis requiring the detection of individual theronts assay samples in triplicate, at minimum.

We observed variation in SSU DNA content between individual waterborne Ich parasites and between life stages. This variation was lower for theronts than for trophont and tomont stages (Figure 2.5 B). Theronts had fewer SSU copies on average (~5,000) than either trophonts (~1,000,000) or tomonts (~500,000). These copy numbers were consistent with previously described ciliates and known aspects of ciliate biology. *Cryptocaryon irritans* theronts filtered with a 10 um filter average ~3,000 SSU copies per organism (Taniguchi et al. 2011), and the ciliate *Stylonychia lemnae averaged* ~200,000 SSU copies / organism (Heyse et al. 2010). Ich possesses a diploid germ-line micronuclei and polyploid somatic macronuclei, both of which have variable DNA content. The number of micronuclei present in a cell ranges from 1 - 4 (Matthews et al. 1996), and the polyploid macronucleus averages a ploidy of 12,000 (Coyne et al. 2011), to 12,600 (Khanna 2004). The macronucleus of Ich increases in size as the trophont feeds and grows (Matthews 2005), and its genetic content may increase accordingly (Thorp and Covich 2010). Additionally, trophont division while on the fish host has been described (Ewing et al. 1988), and a sexual reproductive component of the life cycle is considered likely, although it has not been confirmed (MacColl et al. 2015). The sum of this underlying biological variation likely accounts for the differences in copy number that we observed between life stages, and the variation within the trophont and tomont phases.

The assay was successfully applied to environmental water samples collected from the Klamath River mainstem in 2014, 2015 and 2016. The degree and timing of Ich detections in water corresponded to Ich levels on migrating salmon. Ich infections in salmon were most severe in 2014 (Belchik 2015), and Ich detections in water samples were over 40-fold higher that year. In each year, Ich was detected from fish before it was detected in water samples, but the timing of detections was likely influenced by the distance between water sampling and fish collection sites. As this was a retrospective comparison, the location of water and fish monitoring sites were not coincident. Fish monitoring followed the upriver migration of the salmon. Therefore, most salmon captured prior to mid-August or September were captured in and around a confluence of cool water refugia, ~ 40 rkm downriver of the water collection site, well below the water sample collection point upriver. In 2014 salmon monitoring included several sites ~5 rkm from the water collection point. Salmon monitoring in this area began in September, and first detected Ich September 10th, ~ 2 weeks after the initial detection of Ich in water samples (Figure 2.7). To better establish the relationship between Ich levels in water and on fish, these monitoring efforts should occur at the same location and time, and should quantify fish presence and density at the site. Additionally, the simultaneous exposure of sentinel fish and the collection of water samples could inform the relationship between waterborne Ich levels, and the development of disease.

Previous research has established variables that influence the severity of Ich outbreaks: water temperature, water flow, host availability and host disease resistance (Dickerson 2006). To investigate the influence of these factors in the Klamath River, we compared the temperature, waterflow, and available information on salmon migratory patterns between the three years. Interestingly, we observed a 2 to 3 week lag between maximum temperatures/minimum water flow and the initial detection of Ich in the mainstem. This pattern was consistent for all 3 years, and was unexpected due to the acceleratory impact of elevated temperatures on Ich's life cycle (Aihua and Buchmann 2001). In the Klamath River, the observed relationship could be a result of the over-riding effects on the parasite of the migratory

behavior of the salmonid hosts. When mainstem temperatures exceed ~20°C, migrating salmon are observed to retreat from the mainstem into cool water refugia (Magoulick and Kobza 2003). We observed that in each year, we could not detect Ich until mainstem temperatures fell below 20°C, at which point salmon were likely moving from refugia back into the mainstem, with a corresponding dispersal of their parasites.

The goal of this research is to produce a monitoring protocol capable of rapidly detecting an increase of the abundance of Ich, and informing disease management decisions in the Klamath River. Flow augmentation is the primary management response available for controlling Ich levels in the Klamath River (Strange, 2005). Water is a scarce resource in late summer and its prudent assignment is essential, particularly in drought years. Earlier detection of the parasite would enable a preventative flow release (prior to a disease outbreak) rather than an emergency flow release (in response to a disease outbreak).

In summary, we developed a SYTO9 qPCR assay targeting the SSU DNA of Ich. The qPCR was efficient, performed linearly over several magnitudes of template DNA, and was repeatable, specific and sensitive. We demonstrated that the qPCR assay performed well with environmental water samples, i.e. had adequate sensitivity to detect low levels of Ich, despite environmental inhibitors, and excluded closely-related organisms likely to be present in the same water samples. Ich detections in water samples related to the severity and timing of Ich infections observed on migrating adult salmon, indicating that this method is a promising alternative to lethal sampling. Prior to the implementation of this strategy, further work is needed to inform management actions. Host/parasite relationships are complex, and it may not be appropriate to consider abundance of waterborne parasite stages alone. Management actions may only be needed if several thresholds are surpassed, such as fish densities, residence time, and/or temperature. Necessary future actions include: 1) the establishment of relevant index sites for regular water sampling during salmon in-migration (July - October), 2) a comparison of

parasite to levels on migratory salmon, resident fishes and in water samples collected at established index sites, and 3) an analysis of these data with other parameters, such as water temperature, fish density and exposure duration, to determine disease thresholds and enable adaptive management.

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CHAPTER 3: ELEVATED LEVELS OF THE AQUATIC PATHOGEN *ICHTHYOPHTHIRIUS MULTIFILIIS* DETECTED IN WATER FROM A THERMAL REFUGIUM USED BY ADULT CHINOOK SALMON (*ONCORHYNCHUS TSCHAWYTSCHA*)

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ABSTRACT

In 2002, a large mortality event of pre-spawn adult Chinook salmon occurred in the Klamath River, CA, in part due to severe infections with the parasite Ich. Following this, a monitoring program was established to detect Ich outbreaks on migrating salmon, prior to the development of lethal infections. In the Klamath River, the detection of elevated Ich levels can trigger the release of water from upriver dams, a method intended to facilitate upriver migration and reduce disease. These monitoring methods rely on lethal sampling of fish hosts, and detection is often limited to advanced infections. In contrast, direct detection of Ich DNA in river water samples, using a specific quantitative polymerase chain reaction (qPCR), has the potential to identify areas with elevated infection risk to fish, and serve as a less labor-intensive method for routine monitoring. To investigate these applications, we analyzed water samples collected in and around a partially isolated, cool water refugium in the lower Klamath River. At the time of collection, nearly 200 adult Chinook salmon (*Oncorhynchus tshawytscha*) were congregated in the refugium. We sought to determine a) if levels of Ich were elevated in the refugium compared to mainstem sites, and b) how Ich levels were distributed horizontally and vertically within the refugium. We found that Ich levels were significantly elevated in the refugium compared with both upriver and adjacent mainstem sites. This indicates that conditions in the refugium can promote higher densities of waterborne stages, an important consideration for managing Ich in the Klamath River. Additionally, we detected horizontal and vertical stratification of Ich within the refugium. These patterns were more consistent with passive parasite distribution due to water flow than with active distribution by motility of the phototactic parasites. Future work is needed to determine the relationship between the levels of Ich detected in water samples and disease risk to migrating salmon in the Klamath River.

INTRODUCTION

Ichthyophthirius multifiliis (Ich) is a globally-distributed, ciliate protozoan parasite of freshwater teleost fishes. Ich infections result in significant losses in aquaculture and ornamental fish industries worldwide, and generate epizootics in wild populations (Matthews 2005). In the Pacific Northwest, Ich infections have accounted for mortality of prespawn migrating adult salmon (Traxler et al. 1998; Belchik et al. 2004). The parasite infects the gills and epithelia, resulting in tissue damage, disrupted osmoregulation, anemia, and increased vulnerability to secondary infections (Abdel-Hafez et al. 2014). Infection severity is dependent on the number of parasites that establish themselves on the fish (Xu et al. 2011), which is determined by the dose of waterborne infective stages and the degree of host resistance (Dickerson and Findly 2014). River conditions such as water temperature and water flow impact both factors. Warmer water temperatures accelerate the parasite's rate of maturation and production of waterborne stages (Aihua and Buchmann 2001)**,** and reduce host resistance to disease (Fryer and Pilcher 1974). Lower water flow concentrates the parasite and facilitates host contact (Traxler et al. 1998; Bodensteiner et al. 2000).

Additionally, water temperatures can influence salmon migratory behavior. When water temperatures in river mainstems become elevated (19 – 23 °C), migrating salmon may retreat to thermal refugia (Richter and Kolmes 2005; Strange 2012). These cooler portions of the river are formed by the inflow of water from tributaries or by upwelling of ground water along the hyporheic zone (Nichols et al. 2014). Access to thermal refugia is considered critical for cool water fishes such as salmon (Goniea et al. 2006; Kurylyk et al. 2015), and the utilization of these refugia has been demonstrated to reduce the risk of disease caused by the parasite *Ceratomyxa shasta* among juvenile Chinook and Coho salmon in the Klamath River, CA (Chairmonte et al. 2016). However, the use of thermal refugia is not without risk, as it can result in large numbers of fish congregating in an otherwise sub-optimal

environment. For example, migrating adult steelhead trout (*Oncorhynchus mykiss)* that utilize cool water refugia in the Columbia River, WA were approximately 8% less likely to complete migration to natal streams than those that did not (Keefer et al. 2009). This increase in mortality was largely attributed to increased harvest of fishes congregating in refugia. Additionally, it has been suggested that the use of refugia could increase disease risk by facilitating host contact for horizontally transmitted pathogens (Chairmonte et al. 2016).

In the Klamath River, the development of density-dependent disease is a concern for adult fall run Chinook salmon. In 2002, Chinook salmon migration was stalled low water flow and high-water temperatures, causing salmon to congregate in the lower reach of the river (Belchik et al. 2004). These conditions contributed to pre-spawn mortality of an estimated 35,000 - 78,000 salmonids (97% of which were fall run Chinook), primarily due to concomitant infections from Ich and the bacterium *Flavobacterium columnare* (Turek et al. 2004) (Figure 3.1 A). In 2014, and again in 2016, migrating Chinook salmon congregating in a cool water refugium presented with serious infections of Ich (30 parasites / first gill arch) (Belchik 2015). Unlike 2002, the infections did not result in observable disease or salmon mortality. The difference in outcome has been attributed to emergency summer flow releases from upriver dams and rainfall, which reduced water temperatures, facilitating fish migration and reducing fish densities (Belchik 2015).

Following the 2002 mortality event, Yurok Tribal Fisheries Program (YTFP) biologists began annual monitoring of Ich levels on returning adult salmon in the lower Klamath River (Belchik 2015). Although monitoring can detect elevated Ich infections (Belchik 2015), this method does not provide information on the distribution of waterborne parasites in relation to fish, information that could inform disease management in the Klamath River. Additionally, lethal sampling is time and labor intensive, and results in additional mortalities of migrating salmon. To supplement direct examination of fish, we developed a quantitative PCR (qPCR) assay that can detect and quantify Ich levels in environmental

water samples (Chapter 2). This assay could provide information on parasite distribution and environmental levels, and may be utilized as an alternative monitoring tool to lethal sampling.

River and fish conditions in 2016 provided an opportunity to apply our molecular detection method. August temperatures in the mainstem exceeded 22 °C, forcing migrating fall-run Chinook salmon to congregate in the Blue Hole, a refugium created by hyporheic flows in a side channel of the lower Klamath River. YTFP biologists sampled these fish August 18th, and found severe Ich infections (over 1,000 Ich/first gill arch) (YTFP Technical Memorandum 2016). Tribal biologists suspected that the Blue Hole was acting as a hotspot for Ich infections due to high fish density in the enclosed low flow waters of the refugium. We collected water samples in and around the refugium to describe parasite distribution and environmental levels, and to assess the method's promise as a supplement or substitution for lethal sampling.

METHODS

Study site. The Klamath River is 423 km long and flows westward through Oregon and Northern California, in the Pacific Northwest of the United States (Figure 3.1). Blue Hole is part of a confluence of cool water refugia at river kilometer 25 (Figure 3.1 A). The refugia include a mainstem refugium, created by the inflow of Blue Creek, and the Blue Hole refugium, an ephemeral geomorphic feature fed by cold (10 – 15 ˚C) groundwater. The configuration of the Blue Hole refugium changes from year to year with bed sediment movement, as does the degree of accessibility to migrating salmon. At the time of sample collection, it was partially connected to the river mainstem, and had approximate dimensions of 270 m long x 45 m wide x 6 m deep. The refugium has very little current, and the turnover rate (inflow volume divided by refugium volume) is unknown.

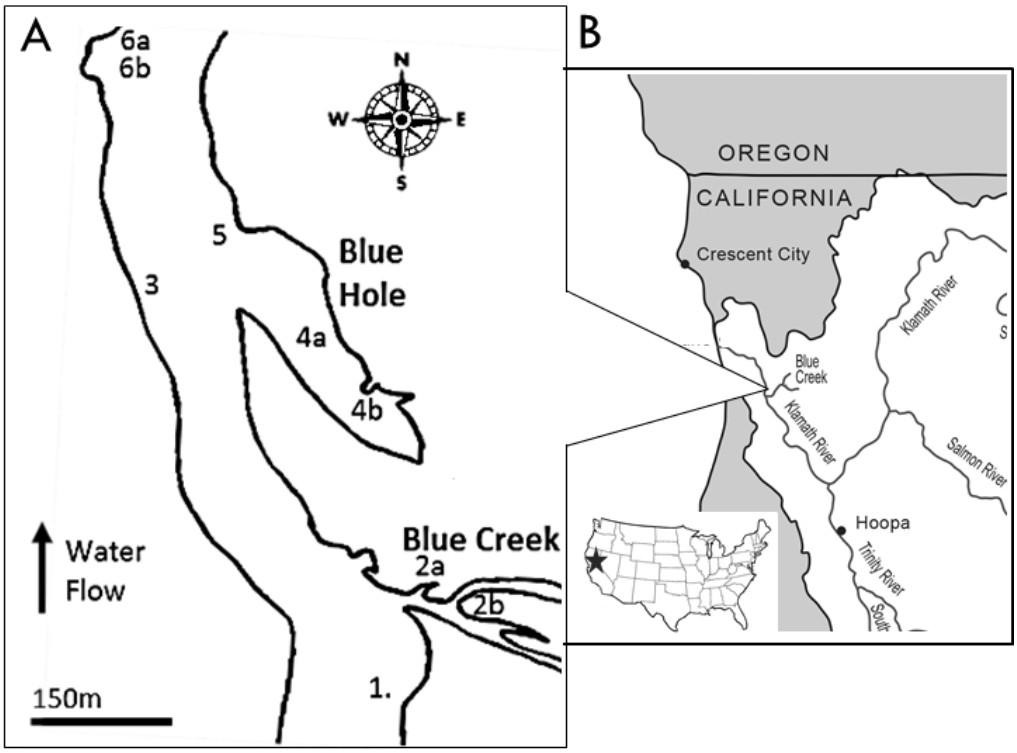

Figure 3.1 Sampling locations in and around the cool water refugium Blue Hole in the Klamath River basin. A) Configuration of the Blue Hole refugium on August 2016, andsampling locations in and around the refugium (1-6). B) Lower Klamath basin, figure adapted from Quiñones et al., 2012. Inset. Location in the USA.

Water sample collection. Water samples were collected on August 25th, 2016, in and around the refugium. 18 x 1-liter water samples were collected in triplicate from the surface water of 6 locations, 12 x 1-liter water samples were collected in a grid pattern across the surface waters of Blue Hole, and 2 sets of triplicate water samples were collected at multiple depths within Blue Hole and the mainstem (Figure 3.1 A). Surface water samples were collected as hand 'grab' samples off the side of a boat. We used a Niskan sampling bottle for vertical samples: at 0, 3 and 6 m within Blue Hole, and at 0 and 3 m downriver of the refugium. Water temperature data were collected at each locality. Samples were held on ice and transported to the J.L. Fryer Aquatic Animal Health Lab (AAHL; Oregon State University,

Corvallis, OR) within 24 hr after collection. There they were filtered onto a 5 µm nitrocellulose membrane filter, and stored at -20 °C for later processing (DNA extraction and qPCR).

DNA extraction. Filters were dissolved using acetone and ethanol washes, as described in Hallett et al. (2012). DNA was extracted and purified using a DNeasy Blood and Tissue Kit (Qiagen), along with the modifications outlined in Hallett et al. (2012). Purified DNA was eluted into 120 µl buffer AE.

qPCR assay. The quantity of Ich DNA in each 1-liter water sample was determined as described by Chapter 2. A subsample of extracted DNA was diluted 1 part DNA to 4 parts buffer AE to reduce inhibition. Then 2 µl of diluted DNA was assayed in 10 µl reactions, followed by a melt curve analysis. Each plate included a set of standards (a pool of DNA extracted from 5 Ich trophonts collected from Klamath River Chinook salmon) which were diluted to span 4 orders of magnitude. Standards, reference samples, and a negative control were assayed in triplicate reactions, and environmental samples were assayed in duplicate. Standards were used to assess assay performance between plates and reference samples were used to assess assay sensitivity on every plate. Unknown sample values were averaged across the two duplicate wells. Duplicate wells that differed by more than one Cq value were re-analyzed if they included a detection below the limit of quantification (Cq 34). Additionally, samples were assessed for inhibition using an IPC assay, and Cq values were adjusted accordingly, as described in Hallett et al. (2012). Net Cq values of samples were converted to copy numbers of SSU rDNA based on calculations from a purified, lab-generated PCR amplicon (see Chapter 2). Statistical analysis of waterborne Ich levels included an ANOVA, followed by post-hoc analysis with Tukey's honest significant difference test.

RESULTS

Yurok tribal biologists examined 8 Chinook salmon from the Blue Hole ~3 hr after water sample collection and identified 7 with severe infections (over 30 trophonts/gill arch). Of these 8 salmon, 3 had

“super infections” (over 1,000 trophonts/gill arch) and 1 had a mild infection (fewer than 30 trophonts/gill arch). Two steelhead trout were captured from Blue Creek on the previous day, 1 of which had a mild Ich infection and the other no observable infection. Despite attempts, no fish were captured from the mainstem (YTFP Technical Memorandum 2016).

Surface water temperature was 23.6°C at all mainstem sites, except at the mouth of the refugium, which was 21.5°C. Water temperatures in Blue Creek and Blue Hole were lower, with surface temperatures averaging 19.3°C and 19.4°C, respectively. There was no vertical stratification of water temperature in the mainstem; top and bottom temperatures were 23.6 °C. Water temperatures in Blue Hole refugium were stratified horizontally and vertically. Surface water temperatures ranged from 19.0°C to 19.7°C, with warmer values towards the mouth of the refugium. For vertically sampled water within Blue Hole, temperatures were 19.2°C at the surface, 15.4°C at 3 m and 13.2°C at 6 m.

There was a significant difference in Ich levels among the sample sites (ANOVA, $p<0.00001$). Surface waters in Blue Hole were elevated compared with unconnected mainstem and Blue Creek sampling locations (Tukey’s HSD, $p<0.05$; Figure 3.2). Ich levels from samples collected upriver and adjacent to Blue Hole, and from Blue Creek were detected at or below levels consistent with 1 infective stage/liter. There was no significant difference between Ich levels at these sites. Mean Ich levels from within the Blue Hole refugium and at the mouth of the refugium were significantly elevated compared to all other sites (Tukey’s HSD, $p<0.05$). Ich levels in the Blue Hole were nearly 100 times higher than levels detected upriver and adjacent to the refugium (Figure 3.2 A, 3.2 B). Samples from the refugia mouth were significantly higher than other samples collected in the mainstem, however these samples were more variable than those collected downriver (Figure 3.2 B).

Ich levels across the surface waters of Blue Hole were stratified horizontally (Figure 3.2 A). Surface water detections were higher in the refugium nearer to the mouth, with the maximum

detection 100-fold higher than the lowest (Figure 3.2 B). Vertical stratification was observed in Blue Hole, but not in the mainstem (Figure 3.2 C). Ich levels from the surface and midwater of the Blue Hole were significantly lower than the bottom samples (Tukey's HSD, p<0.001). Conversely, Ich levels from the top and bottom of the water column in the mainstem sampling location did not show any significant difference.

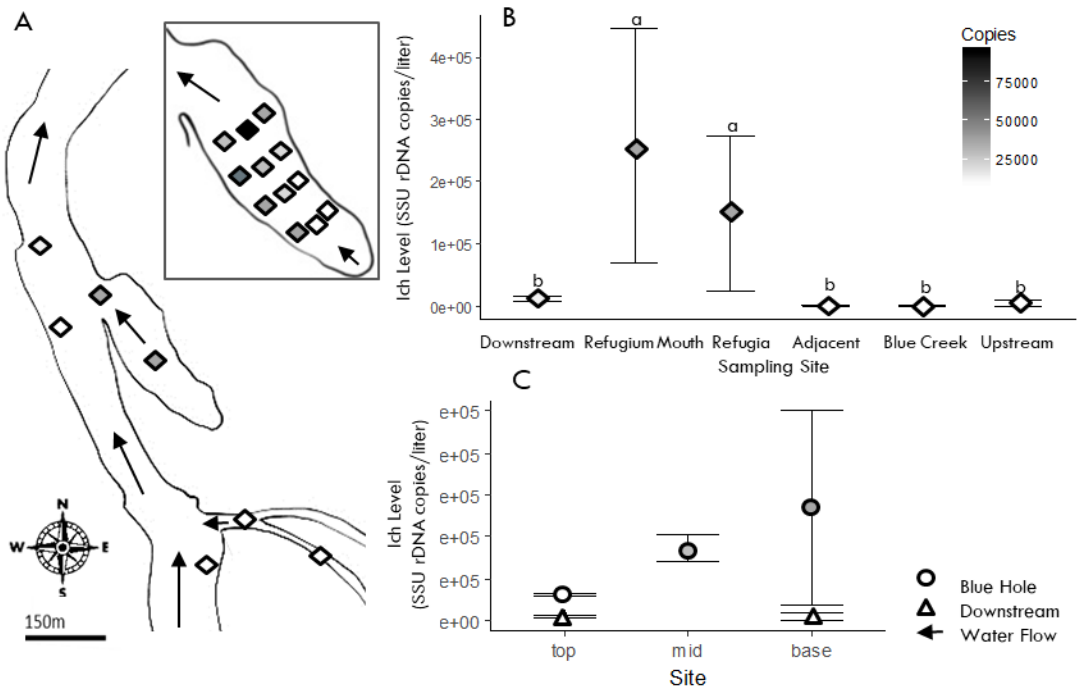

Figure 3.2 August 25, 2016 distribution of Ich in and around the Blue Hole cool water refugium. Points represent means and error bars represent standard deviation between biological replicates (*N*=3, at minimum). a) Ich levels in surface waters of sampling locations. Individual samples were collected in a grid pattern across Blue Hole, b) Ich levels within the refugium were significantly higher than levels in the unconnected river mainstem and Blue Creek (p<0.05). c) Vertical stratification of Ich at Blue Hole (circles) and downriver (triangles). Samples in the refugium were collected at depths of 0 m, 3 m and 6 m, and samples collected downriver were collected at 0 m and 3 m.

DISCUSSION

Application of the qPCR assay to environmental samples collected in and around the Blue Hole refugium confirmed the presence of the parasite in the refugium waters, and indicate that salmon congregating in the refugium acquire new infections at that location. Water sampling detected levels of Ich in the surface waters of the refugium that were significantly elevated compared with the Klamath River mainstem and Blue Creek surface waters. Water sampling may ultimately supplement or replace lethal sampling as a regular monitoring method for Ich in the Klamath River. In consideration to this application, we investigated the spatial distribution of the parasite within the refugium, and compared the degree and variation between triplicate water samples collected in the mainstem. We detected horizontal and vertical stratification of the waterborne parasite within the refugium, and a high amount of variation between samples collected at the refugia mouth. These factors indicate that selection of sampling method and location is critical, and will need to be considered as part of any monitoring effort.

Detections of elevated levels of Ich in the refugium were consistent with current knowledge of Ich infection dynamics. Ich has a direct life cycle in which the parasite matures on the fish, and then exits and attaches to the substrate where it replicates (Mathews 2005). The parasite replicates to produce infective stages (theronts), which enter the water column and can infect fish hosts. In favorable environmental conditions, locating and successfully infecting a host is one of the primary limits on Ich population levels (McCallum 1982), and host disease is dependent on the density of feeding parasites (Xu et al. 2011). Therefore, the majority of Ich associated mortality has been observed in dense captive fish populations such as catfish (Dickerson 2006; Xu et al. 2011), or trout (Picón-Camacho et al. 2012). These outbreaks typically occur at the upper end of Ich's thermal tolerances (Karvonen et al. 2010), as higher temperatures facilitate rapid proliferation (Aihua and Buchmann, 2001; Mathews 2005)

While it is unusual for these conditions to be met in wild systems, conditions in the Klamath river during fall-run Chinook salmon migrations can generate Ich outbreaks (Belchik et al. 2004). While Ich is continuously present in the Klamath mainstem, likely sustained by reservoir hosts such as speckled dace (*Rhinichthys osculus*) (Foott et al. 2016), the severity of Ich infections on migrating salmon varies from year to year. This variation appears to be driven by fish density, which is determined by migrating salmon return numbers, and salmon behaviors such as the timing and rate of river entry and upriver migration (Belchik et al. 2004). When upriver migration is delayed by elevated mainstem temperatures, salmon congregate in cool water refugia such as the Blue Hole refugium. The refugium is hydrologically isolated from the mainstem, therefore, parasites shed by these fish are not flushed out of the area of host concentration. This has the potential to increase the risk of density-dependent disease such as Ich, as the parasite's direct life cycle enables it to proliferate rapidly when environmental conditions are favorable, and hosts are plentiful (Dickerson 2006).

Our water sampling results indicate that Ich levels in the refugium were elevated in comparison to mainstem sites. This finding supports the interpretation that congregating salmon in the Blue Hole refugium amplify Ich levels, and that these salmon may be at an elevated risk for developing serious Ich infections. This scenario is consistent with the YTFP monitoring results from 2014, in which severe Ich infections were detected on salmon congregating in the refugium prior to severe levels being detected on fish in the mainstem (Belchik 2015). This suggests that the congregation of salmon in the Blue Hole, and other similar refugia, could be a risk factor for more serious river-wide outbreaks of Ich, such as those that occurred in the Klamath River in 2002 and 2014 (YTFP Technical Memorandum 2016).

There are several important limitations to consider when interpreting this data. While infective units are the basis for interpreting water samples, the assay also detects other life stages (e.g. tomont stages shed from fish) and non-viable stages (old, dead or fragmented parasite material). The infective theront likely comprises the bulk of water sample detections, as it is the most numerous stage in the life

cycle (Dickerson 2006), and remains waterborne for several days, whereas tomonts typically settle onto the substrate within an hour (under laboratory conditions) (Dickerson 2006; Lynn 2008). However, the inclusion of a dividing tomont or fragments of non-viable parasites would indicate a higher level of infective stages than was present in the sample, and so could skew sample interpretation. Additionally, more information is needed to conclude that Ich levels in the refugium were amplified by congregating salmon. Using these methods, we cannot distinguish between infections that occurred within or outside of the refugium. It is possible that highly infected salmon enter the refugium and shed the parasite, but were not re-infected in the refugium. Future research could address this by analyzing salmon infection levels and waterborne Ich levels in the refugium before, during and after salmon arrival. Similarly, although it is logical that salmon in the refugia may be exposed to more parasites than in the mainstem, and are therefore at an elevated risk for developing severe infections, we cannot confirm this without describing parasite dose in the refugia compared to mainstem. Dose is a factor of parasite concentration and host contact, therefore a comprehensive description of salmon movement, water velocity and circulation patterns in the mainstem and refugium is needed. Sentinel fish exposures in the refugium and mainstem could help address these questions.

To inform the application of this method as a supplement or replacement to lethal sampling, we investigated that spatial stratification of Ich levels in water samples. The ideal water sample collection site must reliably reflect infection levels on salmon, generate consistent Ich levels between replicates, and remain relevant from year to year. River dynamics such as water flow and topography have the potential to physically influence Ich distribution, while environmental factors such as temperature or light could influence parasite behavior (Wahli et al. 1991). Thus, the variability in parasite densities detected within the refugium could skew water sample interpretations. The stratification observed within the refugium was consistent with the flow of water, with higher levels of the parasite present in the surface waters towards the mouth of the refugium. The highest detections were at the mouth of the refugium,

in the mainstem. This site would be the most sensitive water sample collection site, however the samples at this point were highly variable. This is likely due to uneven mixing between the mainstem and refugium water. Therefore, a useful detection site may be just below the refugia mouth, where Ich levels remain elevated while being better mixed. This site would still be relevant in years that the salmon congregate in the mainstem refugia formed by Blue Creek, and should be unaffected by interannual changes to the configuration of the refugium. Additionally, variation between samples could be reduced by changes to the sample collection technique. Instead of grab samples, a larger sample of water could be collected by an automatic sampler, such as an ISCO, over a 24 hr period, pooled, and then triplicate samples collected from that pool.

In conclusion, although cool water refugia can facilitate disease recovery (Mathes et al. 2009), and can act as disease refugia (Chiaramonte et al., 2016), this is likely not the case when migrating Chinook salmon congregate in the Blue Hole refugium. Elevated levels of waterborne Ich were detected in the refugium, indicating that refugia use may amplify parasite numbers, resulting in severe Ich infections on migrating pre-spawning adult salmon. The primary management action to prevent Ich outbreaks in the lower Klamath River is the release of flows from the Trinity River (Lewiston Dam). These flows facilitate salmon migration, therefore reducing the density of congregating fish. This research emphasizes that waterborne Ich levels, fish concentration and refugia use should be included along with measures of salmon parasite load in discussions of implementing this management option. These findings contribute to our understanding of Ich infection dynamics in the lower Klamath basin, and illustrate the complexity of host, parasite and environment interactions in disease dynamics.

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CHAPTER 4: GENOTYPIC VARIATION OF *ICHTHYOPHTHIRIUS MULTIFILIIS* SSU RIBOSOMAL DNA AND MITOCHONDRIAL *COX1*

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ABSTRACT

Ichthyophthirius multifiliis is a ciliate parasite of freshwater fishes. Multiple isolates have been differentiated based on phenotypic differences, including relationships between Ich isolates and the environment (e.g. thermal and salinity tolerances), and interactions with the host immune system (e.g. confirmation of i-antigen on the parasite's surface). Description of the genetic diversity within the species has only recently begun, despite the potential for this information to improve our capacity to recognize, and thereby manage, a greater diversity of Ich. This project builds on the MacColl et al. 2015 description of the diversity of *cox1* gene sequences in relation to factors such as year collected, geographic origin and host species. We had two goals: determine if genetic variation exists in the *cox1* gene of Ich isolates collected from the Klamath River, and assess the specificity and sensitivity of previously developed primers that target SSU. To this end, we assessed genetic differences among Ich isolates collected from wild fishes in the Klamath River, CA, and pet shops in Oregon, USA. We determined that, while *cox1* genetic variation was low, there was a unique SNP present in all isolates collected from the Klamath River. Variations within the *cox1* gene did not give adequate resolution to describe genetic variation within Klamath river Ich isolates. Analysis of the SSU sequences revealed that previously developed primers were not adequately specific for many Ich isolates, and informed the development of novel qPCR primers targeting SSU.

INTRODUCTION

The ciliate protozoan *Ichthyophthirius multifiliis* (Ich) is an economically and ecologically significant parasite of freshwater fishes. Ich has been implicated in numerous die-offs in aquaculture, ornamental and free-ranging fish (Traxler et al. 1998; Belchik et al. 2004; Matthews 2005). The global distribution of Ich, coupled with its diverse physiology and environmental tolerances, have suggested that multiple strains of the parasite exist (Nigrelli et al. 1976). Isolates have been defined based on

biological variations, such as thermal and salinity tolerances (Aihua and Buchmann 2001), and differences in surface immobilization antigens (i-antigens) (Clark et al. 1995). I-antigen confirmation is the basis for classification of isolates into serotypes, the traditional method for describing Ich variation in the context of vaccine development (Dickerson et al. 1993). While biologically informative, these methods do not allow for comparisons of evolutionary relationships or exploration of functional genetic differences.

Ich has contributed to mortality of wild freshwater fishes in the Pacific Northwest of North America, including Pacific salmon (Traxler et al. 1998) (Belchik et al. 2004). Description of the genetic diversity of the Ich isolates could aid in salmonid conservation efforts and fisheries management, whereby identification and tracking of strain variations would describe connections between outbreaks, informing the implementation of biological controls and other outbreak prevention measures. Pathogen strain can impact the severity of host pathology that results from infection (Atkinson and Bartholomew 2010). Therefore, differences in Ich strains may correlate with progression and severity of disease outbreaks, and necessitate strain-specific control protocols. An understanding of the diversity of Ich strains would also inform the laboratory research on this pathogen, much of which uses uncharacterized isolates from aquaculture, to “wild” strains of Ich (e.g. Ewing et al. 1986, Dickerson et al. 1993, MacColl et al. 2015).

Gene-based definition of strains or parasite diversity should consider the evolution rate of the genes targeted, and their biological (i.e. functional) relevance. Protein coding genes can directly impact disease-relevant processes, such as parasite metabolism and host immune avoidance (Dzikowski et al. 2006), and are influenced by changes in environmental parameters such as temperature and host availability (Wolinska and King 2009; Tack et al. 2012). However, loci such as the nuclear small subunit ribosomal DNA (SSU) and the mitochondrial *cytochrome oxidase subunit 1* (*cox1)* have more constant

rates of evolution , and so are typically have used for intra- and inter-species comparisons (Hajibabaei et al. 2007; Zhao et al. 2013).

Previous studies describing the genetic diversity of Ich have identified at least two mitochondrial haplotypes (MacColl et al. 2015). Accordingly, we compared variations between both the *cox1* and SSU gene loci of Ich isolates collected from the Klamath River, CA, pet shops in Oregon, USA, and available GenBank data from isolates collected globally. Our analysis addressed two specific questions: 1) does genetic variation exist in Klamath River Ich isolates at either or both the *cox1* and SSU loci? and 2) can existing rDNA primers (Jousson et al. 2005; Miller et al. 2016, Howell et al. IP) amplify all Ich sequence types/isolates available? These questions were designed to inform the application of a molecular detection assay (qPCR) for the parasite (Chapter 2), and to investigate Klamath River Ich dynamics (Chapter 3).

METHODS

Collation of available Ich sequence data. The NCBI nucleotide database ("GenBank") was queried for sequence entries with title keyword "*Ichthyophthirius*" with "mitochondrial" or "cytochrome" or "cox", and "*Ichthyophthirius*" with "18S". Sequence hits were filtered to remove short entries (<~800bp) or genome reads (>~3000bp). The remaining rDNA and *cox1* sequences were downloaded in FASTA format and aligned in BioEdit (Hall 1999). We identified SSU sequences from 9 Ich isolates, and *cox1* sequences from 16 Ich isolates. To these reference alignments we added primer sequences from Jousson et al. (2005), and MacColl et al. (2015) for *cox1* and SSU, respectively (Figure 4.1). Accession numbers are given in Table 4.1.

Ich collection and DNA extraction. We obtained fish infected with Ich from 3 pet shops in Oregon, in an "as available" manner. Fish species included: goldfish (*Carassius auratus*), tiger barb

(*Puntigrus tetrazona*), and black tetra (*Gymnocorymbus ternetzi*). Ich isolates from "wild" environments were obtained from Chinook salmon *(Oncorhynchus tshawytscha)*, and speckled dace (*Rhinichthys osculus*) in the Klamath River, CA, and 'sentinel' rainbow trout (*Oncorhynchus mykiss*) from the Sacramento River, CA (Table 4.1). Fish were euthanized with an overdose of tricane methansulfonate (MS-222, Argent Laboratories, Redmond, Washington), and individual trophonts were removed using a dissecting scope and pipette. Parasites were placed individually into 2 mL collection tubes, and stored at -20°C. DNA was extracted and purified according to the manufacturer's protocol with a DNeasy Blood and Tissue Kit (Qiagen). Purified DNA was eluted into a final volume of 50 µl buffer AE.

PCR amplification and sequencing. Portions of the *cox1* and SSU gene loci of Ich isolates were amplified via nested PCR. The *cox1* gene was amplified with forward primer COXf1 (5' -TAT CAG GTG CTG CAT TAG CTA CT- 3') (MacColl et al. 2015), and reverse primer COXr1 (5' -TAA ACC TAA AGT AGA TGA AGT GTG AAG- 3') (MacColl et al. 2015). The SSU was amplified using over-lapping sequences generated by forward primers ERIB1 (5' -ACC TGG TTG ATC CTG CCA G- 3') (Barta et al. 1997), or 415F (5' – GCA GCA GGG ACG TAA ATT ACC- 3') (current study), with reverse primers IMRr1 (5' -ACC CAG CTA AAT AGG CAG AAG TTC AA- 3') (Jousson et al. 2005) or ALL1r (5' -GTG GTG CAT GGC CGT TCT TAG TT- 3') (Hallett et al. 2002). The 5' amplicon was ~650 bp, and the 3' amplicon was ~850 bp. PCR was performed in 20 µl reactions, comprising: 1X Titanium buffer (Takara), 0.5 µl REDiload dye, 0.5 µl BSA, 0.4 µl dNTPs, 1 Unit Titanium Taq (Takara), 0.5 µl of each primer at a 1:100 dilution from stock concentrations, 10.2 µl water and 2 ul sample DNA. Amplification conditions for SSU were: 95°C for 2 min, followed by 34 cycles of 94°C for 30 s, 62°C for 30 s, and 72°C for 1 min, to finish with 72°C for 10 min. Amplification conditions for *cox1* were: 95°C for 2 min, followed by 34 cycles of 94°C for 30 s, 60°C for 30 s, and 78°C for 2 min, to finish with 68°C for 7 min. A second-round PCR was used for the *cox1* gene only, using a 1:100 dilution of the first-round product, and the same primers, PCR reagents and cycling conditions. PCR products were stored at -4°C. Each PCR included positive and negative controls. PCR products were

visualized with gel electrophoresis, and those that generated a strong band were prepared for sequencing using the QiaQuick DNA clean up kit (Qiagen). Purified DNA was assessed with a Nanodrop ND-1000 Spectrophotometer (NanoDrop Technologies, Inc.) to determine yield and purity, and then submitted for Sanger sequencing at OSU's Center for Genome Research and Biocomputing. SSU amplicons were sequenced with the forward primers ERIB1 or 415f, and reverse primer ALL1r. *cox1* amplicons were sequenced with the forward primer COX1f.

Isolate comparisons. cox1 and SSU sequence alignments were constructed with BioEdit (Hall, 1999), including our newly sequenced isolates, and Ich sequences from GenBank. Available relevant information (year, location, host collected from) on each isolate was compiled into Table 1. SNPs were identified by eye, and phylogenetic relationships were visualized using the online program Phylogeny.fr (default "1-click" mode) (Dereeper et al. 2008).

Table 4.1 information on isolates analyzed at the cox1 and SSU loci. Information on isolates analyzed for *cox1* was adapted from Table 1 of the MacColl et al. 2015 publication. Information on isolates analyzed for SSU was adapted from Table 1 of the Zailya et al., 2017 publication. Isolates collected for this research and sequenced at the SSU loci were either sequenced for the smaller fragment (5' end), larger fragment (3' end), or both.

cox1

Accession Number	Sample	Year	Location	Fish Host (common name)	Environment Type
NA	FW#16.3	2016	Sacramento river basin, CA	Sentinel rainbow Trout	River
NA	Ich c	2014	Klamath River, CA	Chinook Salmon	River
NA	Ich d	2014	Klamath River, CA	Chinook Salmon	River
NA	Ich 71	2016	Klamath River, CA	Chinook Salmon	River
NA	Ich 75	2016	Klamath River, CA	Chinook Salmon	River
NA	Ich 78	2016	Klamath River, CA	Chinook Salmon	River
NA	Ich 31	2015	Corvallis, OR	Goldfish	Pet shop
NA	Ich 32	2015	Corvallis, OR	Goldfish	Pet shop
NA	Ich 33	2015	Corvallis, OR	Goldfish	Pet shop
NA	Ich 129	2016	Corvallis, OR	Barb, skin scrape	Pet shop
NA	Ich 130	2016	Corvallis, OR	Barb, skin scrape	Pet shop
NA	Ich 131	2016	Corvallis, OR	Barb, skin scrape	Pet shop
NA	Ich 134	2016	Corvallis, OR	Barb, gill	Pet shop
NA	Ich 135	2016	Corvallis, OR	Barb, gill	Pet shop
NA	Ich 136	2016	Corvallis, OR	Barb, gill	Pet shop
KT783594.1	BR1	2014	Paulo Lopes, Brazil	Silver catfish	Unknown
KJ690549.1	NY3	2004	Ithica, NY	Oscar	Pet shop
KT783591.1	ARK12	2014	Hotsprings, AR	White bass	hatchery
KJ690550.1	NY4	2004	Ithica, NY	Oscar	Pet shop
KT783595.1	NY6	2004	Ithica, NY	Freshwater shark	Pet shop
KJ690551.1	NY7	2010	New Hartford, NY	Oscar	supermarket
KJ690548.1	G15	2011	Athens,GA	Red parrot fish	supermarket
GU439200.1	G3		GA	Unknown	Unknown
GU439201.1	G2		GA	Unknown	Unknown
GU439199.1	G4		GA	Unknown	Unknown
KJ690547.1	G5		GA	Unknown	Unknown
GU439203.1	G6		GA	Unknown	Unknown
GU439204.1	G7		GA	Unknown	Unknown
KT783596.1	TW1	2015	Chyatyi, Taiwan	Rosy Bitterling	Unknown
KT783597.1	TW5	2015	Chyatyi, Taiwan	Rainbow fish	Unknown
KT783598.1	TW7	2015	Chyatyi, Taiwan	Kuhli loach	Unknown

SSU rDNA

Accession Number	Sample	Year	Location	Fish Host (common name)	Environment Type	Fragment
NA	Ich e	2014	Klamath River, CA	Chinook Salmon	River	both
NA	Ich a	2015	Klamath River, CA	Dace	River	small
NA	Ich c	2014	Klamath River, CA	Chinook Salmon	River	large
NA	Ich d	2014	Klamath River, CA	Chinook Salmon	River	large
NA	Ich 71	2016	Klamath River, CA	Chinook Salmon	River	large
NA	Ich 73	2016	Klamath River, CA	Chinook Salmon	River	both
NA	Ich 77	2016	Klamath River, CA	Chinook Salmon	River	both
NA	Ich 17	2015	Eugene, OR	Barb	Pet shop	both
NA	Ich 45	2015	Corvallis, OR	Black Tetra	Pet shop	both
KJ690565.1	G15	2011	Athens, GA	Red parrot fish	supermarket	total
KJ690566.1	NY3	2004	Ithica, NY	Oscar	Pet shop	total
KJ690567.1	NY4	2004	Ithica, NY	Freshwater shark	Pet shop	total
KJ690568.1	NY7	2010	New Hartford, NY	Oscar	supermarket	total
KJ690569.1	Ark5	2005	Central Arkansas	Channel catfish	unknown	total
KJ690570.1	Ark7	2008	Stoneville, MS	Channel catfish	unknown	total
KJ690571.1	Ark10	2011	Stuttgart, AR	Blue catfish	unknown	total
KJ690572.1	Ark9	2008	Lonoke, AR	Golden shiner	unknown	total
U17354.1	Unknown	Unknown	Unknown	Unknown	Unknown	total

RESULTS

We generated *cox1* sequences from 6 Ich isolates collected from wild environments and 9 Ich isolates collected from pet shops in Oregon (Table 1). These sequences were ~900bp long. When we compared the *cox1* sequence from these isolates with the 16 sequences from GenBank, there were two haplotypes identified, with a maximum difference of 2% between isolates (Figure 4.1). One group consisted of three GenBank isolates: KT783598.1 (2015, Taiwan, kuhli loach), KT783594.1 (2014, Paulo Lopes, Brazil; silver catfish) and KJ690549.1 (2004, New York, USA; Oscar), and the second group comprised the remaining sequences, including the 5 isolates collected from Klamath River Chinook salmon (2014 and 2016) (Figure 4.1 A). The isolates collected from Klamath Chinook included a unique single nucleotide polymorphism (SNP) not present in other isolates (Figure 4.1 B). The *cox1* sequences from Klamath River isolates were very similar, with a maximum difference of 0.1%.

We generated 14 SSU segments either ~650 bp, ~850 bp, or ~1,000 bp. These fragments were comprised of 1 or both overlapping fragments. The low diversity within the Ich SSU prevented phylogenetic analysis. The most distinct isolate was the oldest Ich representative in GenBank, U17354.1 (Wright and Lynn 1995). This sequence included 2 unique SNPs in the ~650bp segment, and 6 SNPs in the ~850 bp region. The second most divergent isolate was Ichc (2014, Klamath River, USA, Chinook salmon), with 3 SNPs in the ~850 bp segment. We observed in our SSU alignment that 2 of the SNPs were present in the primer regions of the Jousson et al. 2005 assay and 1 was present in a primer region of the Miller et al. 2016 assay; we designed our assay to avoid these variable regions (See Chapter 2).

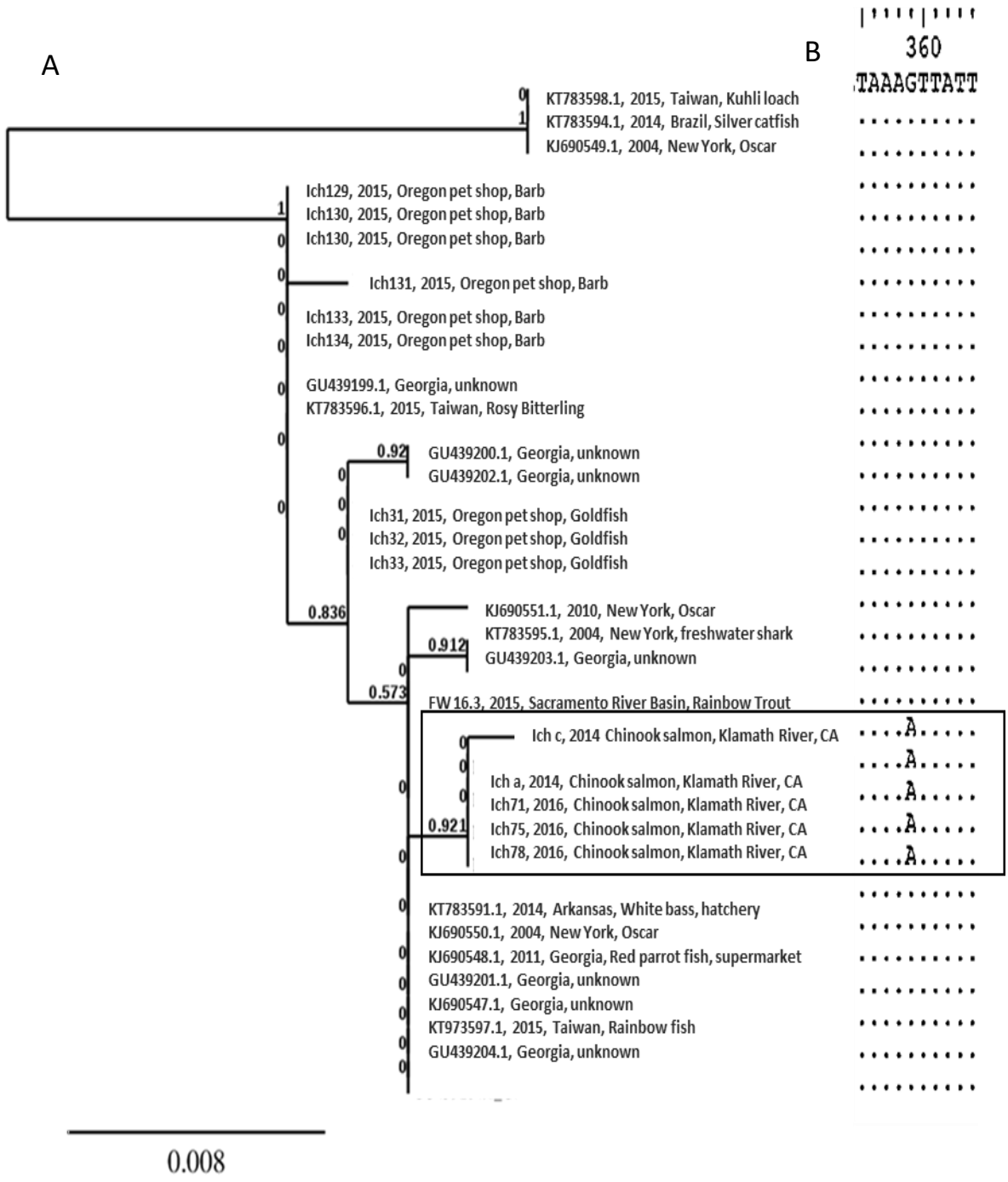

Figure 4.1 phylogenetic relationship between cox1 sequences from Ich isolates. A) *cox1* phylogenetic tree based on 900 bp. Branch length is proportional to the number of SNPs per site, tree constructed by Phylogeny.fr (Dereeper et al. 2008). B) Section of the sequence showing the unique SNP associated with Klamath River Ich isolates.

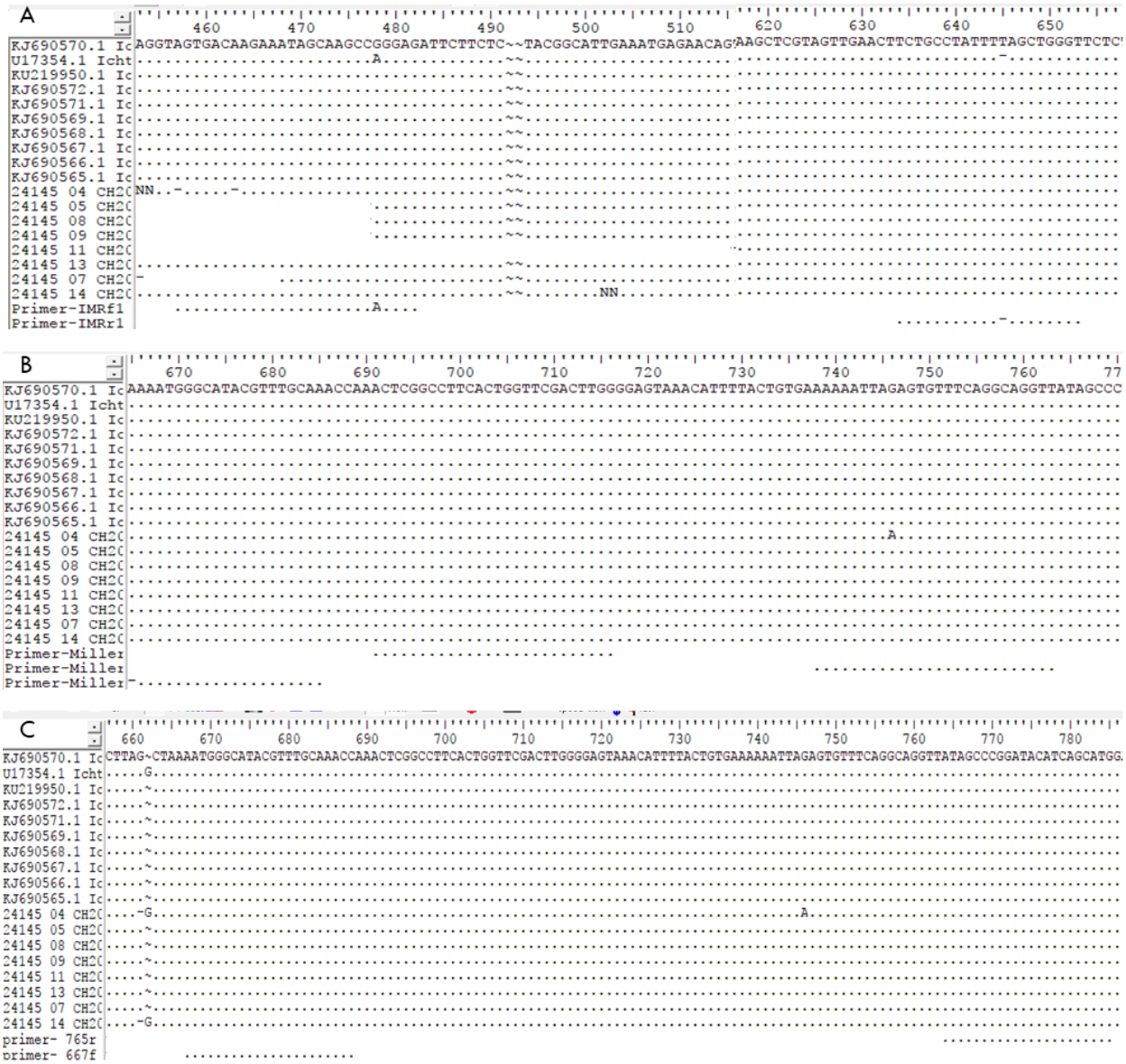

Figure 4.2 SSU sequences and primers for Ich isolates: downloaded from GenBank (first 10 sequences), sequenced in this study (next 8 sequences). A) Jousson et al. (2005) primers included a SNP in the forward primer and an insertion/deletion (indel) in the reverse. B) Miller et al. (2017) primers included one SNP in the reverse primer region. C) Primers from our assay were in conserved regions.

DISCUSSION

Analysis of the *cox1* and SSU loci of Ich isolates from GenBank and our newly sequenced isolates demonstrated that, while the amount of genetic variation is small in these regions, there is some resolution of within-species differences among Ich isolates. The *cox1* gene contained more variation

than the SSU, and provided our first insight into unique genetic variation (a single SNP) present in all Klamath River Ich isolates (Figure 4.1). The *cox1* gene sequences did not resolve genotypic differences among Klamath River isolates. Although we suspect that there is variation elsewhere in the genomes of Klamath River isolates, the *cox1* appeared highly conserved, and we observed <2% variation among all isolates, which was consistent with previous studies on Ich and other ciliates (Wright and Lynn 1995; Kher et al. 2011; MacColl et al. 2015). The SSU appeared conserved among all isolates, and was informative for the assessment of primer specificity.

The unique *cox1* SNP observed among Ich Isolates collected from the Klamath River (from 2 Chinook salmon in 2014 and 2016) provided a counter-example to previous research that did not find a relationship between geographic origin or fish host species and mitochondrial diversity (MacColl et al. 2015). This research did not include enough fish host diversity to distinguish between these two factors (each collection location was only represented by one host species), however, the presence of a relationship appears likely. Of the 15 isolates we collected and sequenced, the *cox1* sequences were conserved and distinct for each collection location/host species. Due to the low host specificity of Ich isolates, the collection location is likely more of a driver of this diversity. This could be confirmed by sequencing multiple fish hosts from the same area. The difference in our findings from previous research likely reflects methodological differences in sample collection and categorization. In the MacColl et al. (2015) study, the origin of an isolate was defined solely on the geographic location from which it was collected. However, isolates collected from the same geographic location were collected from multiple captive populations of fish. This could mask a relationship between geographic origin and mitochondrial diversity, because this nominal “geographic” location may not describe the Ich populations in any meaningful way. For example, tropical aquarium fishes and fishes being sold live at supermarkets may be transported globally and in the company of many other fishes. The confinement of multiple fishes from multiple locations in high concentrations (such as those in pet shops) allow ample

opportunities for pathogens such as Ich to infect new hosts. If the intention of looking at Ich origin is to identify discrete populations, then it makes sense to include other factors such as environment type (i.e. river or fish hatchery) when describing collection of Ich isolates.

Our SSU alignment included sequences from GenBank and novel data. We included published primers in this alignment, and observed that these were not optimally located. Although there were few SNPs observed in this gene segment, SNPs in some isolates were in the primer regions. The Jousson et al. 2005 primers included 2 SNPs, and the Miller et al. 2016 reverse primer included one SNP. These SNPs in the primer regions were located on isolates that were not yet sequenced at the time of primer development.

In conclusion, the *cox1* sequences from Klamath River Ich demonstrated that geographical origin may be a relevant factor for resolving Ich genotypes. The *cox1* did not resolve any genetic differences between Klamath isolates collected in 2014 vs 2016. Our analysis of the SSU demonstrated that previously published primers that targeting this locus were not sensitive to the full diversity of Ich isolates now known, and so we developed alternate primers for a novel Ich assay (Chapter 2), to incorporate the full diversity of available Ich isolates. Although we did not detect genetic variation between the isolates from the river at either the SSU or *cox1* loci, we continue to hypothesize that variations in fish host response in the different years, indicates that parasite genetic variation exists elsewhere in the genome. We sequenced only a few isolates from the river, and all were collected from Chinook salmon. We suspect that intra-basin differences occur in the Ich population, as the river is a complex environment, with numerous selection pressures such as spatial and temporal variations in temperature and host availability, which could drive parasite evolution. Although previous work did not find a correlation between host species and Ich isolate (MacColl et al. 2015), much of what is known about Ich infection dynamics is from captive fishes. Further research to investigate genotypic diversity of Ich should involve isolates from other fish species and from microhabitats within the Klamath River

basin, and could explore variation at other gene loci, for example the more variable Internal Transcribed Spacer region 1.

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CONCLUSIONS

This research indicates that qPCR analysis of environmental water samples could be a valuable monitoring tool for waterborne Ich the Klamath River. An effective monitoring protocol for Ich in the Klamath River requires 2 primary components:

1. A robust qPCR assay with adequate specificity and sensitivity to detect the full diversity of Ich, while excluding closely-related organisms present in environmental samples.
2. A water sampling protocol capable of detecting disease-relevant waterborne Ich levels.

This thesis describes the development of the first component (Chapters 2, 4), and begins to investigate the second (Chapters 2, 3).

I developed a qPCR assay targeting the SSU of Ich. The assay is sensitive to Ich isolates collected from Klamath River Chinook salmon, as well as to the full diversity of Ich sequences available on GenBank. The assay can detect SSU levels equivalent to a single waterborne parasite processed from 1-liter Klamath River water samples. The assay was designed to span a region of genetic variation between Ich and closely related organisms, thus permitting the identification of non-target amplification through sequencing analysis. Melt curve and sequencing analysis of qPCR amplicons indicated good specificity of the assay.

Initial application of the qPCR assay to water samples generated promising results, and will inform the development of a water sampling protocol for Ich. Waterborne Ich parasite stages appeared to distribute both horizontally and vertically based on infected host distribution and water flow (Chapter 3). This information will help identify relevant locations to establish index sites for monitoring Ich infection dynamics. Water samples captured enough resolution between Ich levels to describe variations in time (inter- and intra-annual variation in mainstem Ich levels at Tully Creek, Chapter 2), and space

(horizontal and vertical stratification of waterborne Ich in and around the Blue Hole refugium, a location with a known Ich outbreak, Chapter 3).

Future work will define disease-relevant waterborne Ich levels in the context of environmental and host parameters in the Klamath River. Several disease-relevant parameters have been identified for Ich infections in general, and in the Klamath River specifically. These include environmental parameters such as water temperature and flow, and host-related parameters such as behavior (e.g. congregating in thermal refugia), density, and physiological condition. Establishing these relationships will inform the application of management actions to control Ich infections in the Klamath River.

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APPENDIX

APPENDIX: PERSISTANCE OF THERONT DNA IN WATER SAMPLES

INTRODUCTION

The development of a water sampling based monitoring program for the parasite *Ichthyophthirius multifiliis* (Ich) requires a clear understanding of the relationship between Ich levels detected in water and disease risk to fish. Disease risk will be interpreted in the context of environmental and host conditions, and must address the assumption that detections in water samples consistently correspond to viable parasite numbers. This may not be the case, as theronts that are unable to locate and infect a fish host will perish after approximately 4 days (Dickerson 2006), and qPCR assays do not discriminate between live and dead cells (Sheridan et al. 1998). The interpretation of Cq values in reference to numbers of viable theronts could overestimate the exposure dose to fish and misinform management actions if this is not considered. Therefore, establishing the duration of non-viable theront detections in river water will refine water sample interpretation. Potential factors influencing the duration of detection include; the speed at which bacteria and other organisms digest the theront, the temperature of the water, and the amount of mixing in the water. This study will establish the duration of detection for filtered theronts in river water held at 20°C.

METHODS

Individual theronts were grown from trophonts collected from infected goldfish at the J. L. Fryer Aquatic Animal Health Laboratory in Corvallis OR. 12 theronts were visualized and collected using a dissecting scope and 10µl pipette. Once captured, theronts were placed into individual 2ml collection tubes and killed by exposure to 65°C for 5 minutes. After the heat exposure, 100 µl of river water from the Willamette River was added to each collection tube, and samples were incubated in a water bath at 20°C. At day 0, 7, 14 and 21, the contents of 3 collection tubes from each temperature treatment were

transferred into individual 1-liter bottles of UV sterilized river water, and then filtered through a 5µm nitrocellulose filter. Samples were stored at -20°C until future processing.

After the final collection point, the filtered samples were processed, and the DNA extracted and purified according to the protocols outlined in Hallett et al. (2012). The samples were assayed using a previously published assay targeting Ich SSU rDNA (Jousson et al. 2005), and the Cq values were compared across timepoints. Reactions consisted of: 5 µl of ABI master mix, 0.8 µl of each primer at a 1:100 dilution from stock concentrations, 0.25 µl of BSA, 0.3 µl of SYTO9 at a 1:100 dilution in TAE, 0.85 µl of water, and 2 µl of sample DNA. Samples were diluted 1:5 in AE to reduce potential environmental inhibition. The cycling protocol was as follows: 2 min at 50°C, 10 min at 95°C, followed by 40 cycles of 15 s at 95°C and 1 min at 60°C. A melt curve analysis was performed to verify the PCR products. Final products were brought from 64 °C to 88°C in increments of 0.3°C in 15s steps.

RESULTS

Ich DNA was detected at all timepoints, at lower Cq values (and therefore higher DNA levels) than expected (Figure A.1). Detection levels from day 0 had a significantly lower Cq value than those collected at later timepoints (t-test, $p<.05$).

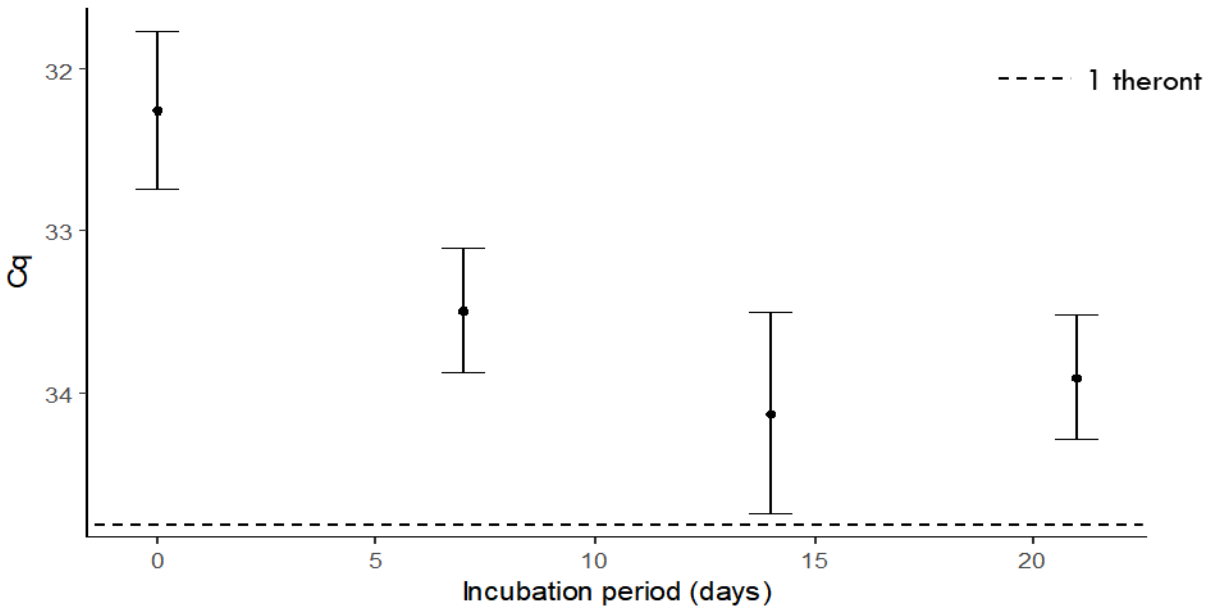

Figure A.1 Detection level (Cq) of SSU Ich DNA from a single theront at day 0,7,14 and 21. Circles represent average Cq, and error bars reflect standard deviation between sample replicates. n=3. Y-axis is in reverse order to reflect the inverse relationship between Cq value and DNA quantity. The average Cq value for a single theront assayed using this protocol and the Jousson 2005 assay is ~35. All samples had Cq values lower than the expected value.

DISCUSSION

SSU rDNA was detected in water samples 21 days after the parasite was killed. This result countered our expectation that microbial activity in river water would break down the nucleic acids over this period and temperature range (Josephson et al. 1993). It appears likely that there was a source of contamination in the samples, because all samples had more DNA than the average value for a single theront (Cq 35). The negative control for the qPCR assay was unaffected, therefore any contamination occurred prior to the qPCR assay.

The experiment should be repeated with several alterations to investigate this unexpected result. A negative control consisting of a 2ml tube of river water without a theront added should be included for each time point to identify sources of contamination during collection. Additionally, A

negative control consisting of a 1 liter water sample of UV sterilized river water should be included for each filtering time point to account for any wild Ich DNA that persisted through UV treatment. A final negative control should be included during DNA extraction and purification.

There was a significant drop in DNA after week 1. Assuming that background levels of Ich contamination were constant, this result indicates that SSU rDNA is no longer detected beyond 1 week post mortality. The interpretation of qPCR detection levels from environmental samples will need to take this into account. Future laboratory experiments should establish the relationship between temperature and non-viable Ich DNA detection across time, as well as investigate other factors influencing the spatial and temporal distribution of live and dead theronts prior, during and after an outbreak. Possible influencing factors include; theront hydrodynamics, water flow, Ich population levels and growth rate, and host availability.

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