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RESEARCH

Investigation of Molecular Pathogen Screening Assays for Use in Delta Smelt

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ABSTRACT

Pathogen surveillance must be part of any population supplementation or reintroduction program for the conservation of threatened and endangered species. The unintended transmission of pathogens can have devastating effects on these already at-risk populations or the natural ecosystem at large. In the San Francisco Estuary (estuary), abundance of the endemic Delta Smelt (*Hypomesus transpacificus*) has declined to the point where regulatory managers are preparing to augment the wild population using fish propagated in a hatchery to prevent species extinction. Although disease is not an overt cause of population decline, comprehensive pathogen presence and prevalence data are lacking. Here, we performed a pilot study that applied molecular assays originally developed in salmonids to assess the presence of a wide variety of pathogens in the gill tissue of cultured and wild Delta Smelt—as well as cultured fish—deployed in enclosures in the estuary. We found the assays to be highly sensitive, and observed positive detections of a single pathogen, *Ichthyophthirius multifiliis*, in 13% of cultured Delta Smelt. We also detected ten other pathogens at very low levels in cultured, enclosure-deployed, and wild Delta Smelt that likely represent the ambient pathogen composition in the estuary (as opposed to actual infection). Our results corroborate previous work that cultured Delta Smelt do not appear to present a high risk for pathogen transmission during population supplementation or reintroduction. However, the molecular pathogen screening assays tested here have great utility as an early warning system indicator of when further diagnostic testing might be necessary to limit the extent and frequency of disease outbreaks; their utility will be further increased once they are customized for Delta Smelt.

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KEY WORDS

Delta Smelt, pathogen, San Francisco Estuary, supplementation, *Ichthyophthirius multifiliis*

INTRODUCTION

Pathogen screening is an integral component of any population supplementation or reintroduction program (Viggers et al. 1993; Leighton 2002; Kock et al. 2010). Before release into the wild, source individuals must be tested to confirm that they do not carry infectious pathogens which may be transmitted into naïve recipient populations or to other susceptible sympatric taxa. Conversely, released individuals may also be vulnerable to pathogens in the wild environment; native populations and putative release sites should also be screened to prevent population supplementation or reintroduction failure due to disease. Pathogen screening paired with the collection of baseline health status data from source and recipient populations can help minimize risk and contribute to risk assessments of these high-profile conservation and management actions (Leighton 2002; Mathews et al. 2006; Muths and McCallum 2016).

Disease-causing agents may be bacteria, viruses, fungi, or parasites; and the nature and type of test required for their detection is species- and ecosystem-dependent. Common screening methods for these pathogens include microbial culture, microscopy, histology and immunohistochemistry, serology, and molecular methods such as Polymerase Chain Reaction (PCR) (Adams and Thompson 2011). Despite the breadth of methods, there are many inherent challenges associated with pathogen surveillance in wild populations: the species of interest may be rare and difficult to locate; the habitat may be vast or inaccessible to humans; invasive or lethal sample collection (e.g., liver, lung, or heart tissue) of threatened and endangered species may not be permissible under state and federal Endangered Species Acts; and collection of samples in the field setting may introduce contamination or cause degradation. Additionally, pathogen screening tests may not capture novel or emerging pathogens. For these reasons, instances of disease in wild populations are often under-reported or may rely on anecdotal evidence (Naish et al. 2007; Kock et al. 2010), but with supplementation and reintroduction the potential for pathogen transmission to wild populations should not be underestimated.

In fish, individuals used for population supplementation and reintroduction are often propagated in a hatchery. History has repeatedly shown that a lack of pathogen knowledge and inadequate pathogen surveillance can have dire consequences when cultured fish are released into the wild. The release of hatchery trout infected with the myxosporean parasite *Myxobolus cerebralis*, which causes whirling disease in salmonids, is believed to have contributed to wild trout population declines throughout the United States (Hedrick et al. 1998; Bartholomew and Reno 2002). Several mass mortalities of wild Pilchard (*Sardinops sagax*) in Australia were preliminarily traced to herpesvirus-infected Pilchard feedlots introduced into the marine environment as part of Tuna (*Thunnus maccoyii*) aquaculture (Gaughan 2002). Threatened and endangered wild fish populations are at even greater risk of adverse outcomes with unintentional pathogen transfer from cultured fish because of small population size, potentially low genetic diversity, and concomitant reduced ability to respond to stochastic pressures (Lively et al. 1990; Frankham 2003). Conservation aquaculture programs therefore routinely monitor for pathogens perform pathogen surveillance, and the results of these tests direct the treatment, release, and adaptive management actions for hatchery fish, such as in Silvery Minnow (Woodland 2009) and numerous salmonid species (Brenkman et al. 2008; Barry et al. 2014; Hardiman et al. 2017).

In the San Francisco Estuary (hereafter "estuary"), the endemic osmerid, Delta Smelt (*Hypomesus transpacificus*) is a state (CDFW 2021) and federal (USFWS 1993) Endangered Species Act listed species that needs more monitoring for pathogens. Delta Smelt were once ubiquitous in the estuary, but anthropogenic ecosystem change has caused a precipitous decline in the wild population (Moyle et al. 2016, 2018). Many are concerned that wild Delta Smelt are now so rare that the species may soon become extinct (Baumsteiger and Moyle 2017; Hobbs et al. 2017). To prevent extinction, regulatory agencies are

planning experimental release and subsequent population supplementation of Delta Smelt using hatchery fish reared at the University of California Davis Fish Conservation and Culture Laboratory (FCCL; Byron, CA) (Lessard et al. 2018; USFWS 2019, 2020; CDFW 2020); to date, only very small numbers of cultured Delta Smelt have been released in the estuary for special studies (Castillo et al. 2012) and not for the purpose of reinforcing the wild population.

Overt signs of widespread disease have not been observed in wild Delta Smelt, and disease is therefore not known to be a driving factor of population decline (USFWS 1993); however, comprehensive baseline disease status data is lacking (Teh 2007; Miller et al. 2012; Teh et al. 2020). Cultured Delta Smelt reared at the FCCL are routinely screened for pathogens by a US Fish and Wildlife Service pathologist at the California–Nevada Fish Health Center (Anderson, CA) before fish are transferred to the back-up facility at the Livingston Stone National Fish Hatchery (Shasta Lake, CA). Current regulations only require screening for six pathogens according to the standard US Fish and Wildlife Service protocol for salmonids: *Aeromonas salmonicida* (bacterium), *Yersinia ruckeri* (bacterium), infectious hematopoietic necrosis virus, infectious pancreatic necrosis virus, *Oncorhynchus masou* virus, and viral hemorrhagic septicemia virus; a screen for *Mycobacterium* spp. (bacterium) is also performed occasionally, upon request. Previous studies have documented sporadic infections of *Mycobacterium* (Antonio et al. 2000) and *Ichthyophthirius multifiliis* (*Ich*; ectoparasite) (Frank et al. 2017) in Delta Smelt propagated at the FCCL; *Mycobacterium* and helminth parasites were also detected in wild Delta Smelt (Foott and Bigelow 2010; Baxa et al. 2015). Chronic infection with *Mycobacterium* is typically well-tolerated in Delta Smelt but can cause reduced swimming performance (Swanson et al. 2002), whereas infection with *Ich* if left untreated may result in gill and skin defects, behavioral changes, or mortality (Frank et al. 2017). In preparation for population supplementation and reintroduction, it will be necessary, in following best scientific practices, to expand the scope of pathogen testing and to understand the potential for pathogen transmission between the wild and hatchery environments.

Here, we conducted a pilot study using high-throughput microfluidic quantitative PCR (qPCR) with assays originally developed for salmonid pathogen testing (Miller et al. 2016; Teffer et al. 2017) to assess differences in pathogen presence between Delta Smelt reared at the FCCL and in the estuary. In the absence of a Delta Smelt-specific pathogen panel, this pathogen panel is an appropriate proxy because salmonids, specifically Rainbow Trout (*O. mykiss*) and Chinook Salmon (*O. tshawytscha*), are found in the estuary, and some pathogens may infect both salmonid and smelt species; it is not possible to know definitively whether the pathogens on the panel can infect and cause disease in Delta Smelt without extensive pathogenicity studies. The assays are specific to 47 pathogens (Table A1), including *Ich*, significantly increasing the range of pathogen testing that has been performed in on Delta Smelt in the past. Four of the six pathogens tested for by the California–Nevada Fish Health Center (*A. salmonicida*, *Y. ruckeri*, infectious hematopoietic necrosis virus, and *O. masou* virus) are represented on the Miller et al. (2016) panel; an assay for *Mycobacterium* spp. was not developed and validated as part of this original panel, but given its relevance to cultured Delta Smelt, should be in the future. Molecular assays are well-suited for pathogen screening in rare, listed species because they are highly sensitive, require minimal starting material (e.g., such as what could be obtained with a mucus swab for some pathogens), can be fully customized to accommodate novel or emerging pathogens, and can be used to detect pathogens in the environment (e.g., environmental DNA [eDNA] in water and soil samples). Despite their benefits, molecular methods are most appropriately and powerfully used as a complement to standard disease diagnostic tests rather than as a replacement. DNA-based molecular assays, such as in Miller et al. (2016), can only confirm pathogen presence and relative quantities. A positive detection does not necessarily mean that a pathogen is infective or that there is an active

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infection. Instead, molecular pathogen screening assays can be performed routinely and serve as an early warning system that signals when other, "gold standard" diagnostic tests should be conducted to confirm a disease outbreak. Also, as with any molecular test, assays in the Miller et al. (2016) panel can only be used to the detect specific pathogens for which they were designed, which may limit the scope of information gleaned and conclusions drawn from any analyses.

In this exploratory study, our specific objectives were to identify pathogens in (1) cultured Delta Smelt reared at the FCCL, (2) cultured Delta Smelt that originated from the FCCL deployed in enclosures in the Estuary, and (3) the wild Delta Smelt population using the molecular assays developed by Miller et al. (2016) and to (4) evaluate the utility of these assays for pathogen screening in future population supplementation and reintroduction.

MATERIALS AND METHODS

In July 2018, we collected gill tissue (lethally) from 60 cultured pre-spawning adult Delta Smelt produced and held at the FCCL from across multiple tanks that had been stored at –20 °C. Hatchery staff observed signs of disease in some of these fish at the time of collection, but they did not communicate this information to us before our laboratory analyses. In January 2019, Delta Smelt (243 dph) propagated at the FCCL from the same cohort (but reared in an unconnected culture system) were deployed in enclosures for 4 weeks in the natural estuary environment at Rio Vista, CA for a separate feeding and survival study (see Baerwald et al., forthcoming). The authors chose the Rio Vista site and deployment time of year because typical environmental conditions are favorable for the survival of adult Delta Smelt, and the Enhanced Delta Smelt Monitoring program is still able to occasionally capture wild fish in this area (USFWS 2021). Baerwald et al. measured several environmental parameters during the deployment period, including temperature (range: 8.40 to 11.80 °C), turbidity (range: 13.90 to 99.63 FNU), and dissolved oxygen (range: 8.45 to 10.90 mg L^{-1}) which were in range of what is expected for preferred Delta Smelt habitat (Sommer and Mejia 2013). Upon completion of the study, we opportunistically took gill tissue (lethally) from 60 of these fish. We also obtained gill tissue from 60 archived wild individuals collected between June 2011 and December 2016 that had been stored at –80 °C, which were provided by the California Department of Fish and Wildlife (Table 1); we selected samples that represented a wide geographic range throughout the estuary. The sample number of 60 is derived from a 5% assumed pathogen prevalence level (APPL) in a population with greater than 100,000 individuals (USFWS and AFS–FHS 2014) and is what is used for diagnostic disease testing at the California–Nevada Fish Health Center. We selected gill tissue for this analysis because, as a filtering organ, the gill is a first contact site and thus has a high chance of detection for many pathogens of interest, and because it was the only tissue available to us in all cultured, enclosure-deployed, and archived wild Delta Smelt samples.

We extracted DNA from tissue samples with DNeasy Blood and Tissue kits (Qiagen, Venlo, Netherlands), using a QiaCube robotic liquid handler (Qiagen) following the manufacturer's protocols. For pathogen screening of all samples, we conducted high-throughput qPCR on a BioMark HD system (96.96 dynamic array; Fluidigm Corp., South San Francisco, CA)

Table 1 Location, number, and date of collection of archived wild Delta Smelt samples. *n* = number of samples.

Location	*n*	Year	Month
Sherman Lake/ Sacramento River	2	2011	June
	10	2012	October
Decker Island/ Sacramento River	9	2016	December
Lindsey Slough/ Cache Slough	3	2011	June
	9	2012	March
	7	2012	May
Prospect Island/ Deep Water Ship Channel	4	2012	May
	16	2014	June

according to the methods described by Teffer et al. (2017). In brief, we used a 96.96 Dynamic Array IFC for Gene Expression (Fluidigm) with primer sets for 47 pathogens (Table A1) developed by Miller et al. (2016); we ran each sample in duplicate. We included positive controls consisting of pooled gBlock synthetic DNA of all pathogens in the panel, and negative controls consisting of suspension buffer both in duplicate alongside our samples for each assay during pre-amplification and qPCR cycling. For each positive control, to estimate relative assay efficiency and approximate pathogen concentration per sample, we prepared 5-fold serial dilutions with concentrations that ranged from 2.0x104 to 32 DNA molecules/µl. To ensure qPCR suitability for these assays, we calculated individual assay qPCR efficiencies separately for each assay on each plate. We analyzed qPCR results with Fluidigm Real-Time PCR Analysis Software v 4.5.2.

In qPCR, a fluorescent reporter dye is released when a target sequence (here, specific to a particular pathogen) is identified by a complementary oligonucleotide primer and amplified via DNA polymerase. If the target sequence is present, the resulting fluorescent signal will increase and accumulate with each PCR amplification cycle. The PCR cycle number at which the fluorescent signal surpasses the background fluorescence of the negative control is known as the cycle threshold (Ct). The amount of the target sequence in the sample is therefore inversely proportional to the Ct value. For all samples, we averaged the Ct values of the duplicates; we discarded data from samples in which only one of the two duplicates amplified. The limit of detection (LOD) for each assay was the Ct of the lowest dilution of the positive control, or 32 DNA molecules μl^{-1} (see "Results"). The sensitivity of the BioMark instrument is as low as one DNA molecule, which corresponds to a Ct value of 27 to 28 (Fluidigm Corp). We therefore categorized a sample as "negative" (meaning the pathogen was not detected) if the Ct was (1) greater than that of the lowest concentration of the positive control, (2) greater than 27, or (3) if there was no detectable fluorescence. For three assays, we obtained Ct values greater than 27 at the lowest dilution of 32 DNA molecules μl^{-1}, meaning beyond the sensitivity of the BioMark instrument. However, we consistently obtained a Ct value less than 27 for each assay's positive control at a concentration of 160 DNA molecules/µl and all other concentrations. We subsequently considered a detection to be "marginal" if the Ct value was greater than that of the 160 DNA molecules μl^{-1} concentration of the positive control, but less than that of the 32 DNA molecules μl^{-1} concentration of the positive control or 27. We conservatively labeled samples with Ct values less than that of the 160 DNA molecules μl^{-1} positive control concentrations as "positive" detections.

RESULTS

Each pathogen assay amplified the positive control samples across all dilutions, including the lowest concentration tested (32 DNA molecules/µl). All qPCR efficiencies ranged between 80 and 120%, except for the *Moritella viscosa* assay from plate 1, which had a qPCR efficiency of 74.4% (Figure A1). Most assays (57 of 94, or 61%) had an efficiency between 90% and 110%, or the desirable target efficiency level for quantitative assays (Bustin et al. 2009). Because we used these assays to detect pathogen presence alone and only quantified relative levels of pathogen concentrations based on serial dilutions, efficiencies outside of the ideal range (i.e., between 80% to 90% and 110% to 120%) are acceptable, and likely caused by primer competition and low binding affinity as a result of the complex (i.e., 47 pathogens) multiplex reaction in the Fluidigm pre-amplification reaction step.

There were robust positive detections of *Ich* in 13% of Delta Smelt reared and held at the FCCL (Figure 1). There were also marginal detections of 10 pathogens, including *Ich*, among the three fish groupings, namely: *Ceratonova shasta*, *Flavobacterium psychrophilum*, *Kudoa thyrsites*, *M. viscosa*, *O. masou* virus, P*arvicapsula minibicornis*, *Piscichlamydia salmonis*, infectious salmon anemia virus, and viral encephalopathy and retinopathy (Figure 1, Table A2); the assay and detection

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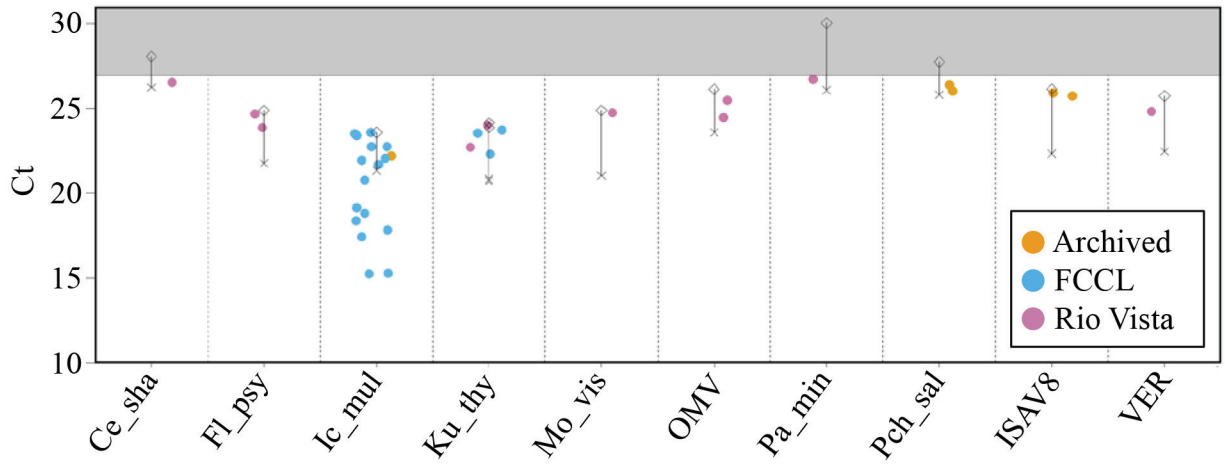

Figure 1 Average Ct values obtained from Delta Smelt gill tissue using Miller et al. (2016) qPCR assays corresponding to positive and marginal pathogen detections. The *gray shading* denotes Ct values above 27, or the sensitivity of the BioMark instrument. The average Ct value of positive control duplicate samples at concentrations of 32 and 160 DNA molecules μl^{-1} are shown as *diamonds* and *cross-marks*, respectively. Positive detections are samples with Ct values less than that of the positive control at a concentration of 160 DNA molecules/µl. Marginal detections are samples with Ct values greater than that of the positive control at a concentration of 160 DNA molecules μl^{-1} but less than that of the positive control at a concentration of 32 DNA molecules μl^{-1} or 27. Marginal detections are considered unreliable but presented for transparency on system limitations. *Archived* = amples from archived wild-caught fish provided by CDFW (n = 60); *FCCL* = samples from fish produced and held at the FCCL (n = 60); *Rio Vista* = samples from fish propagated at the FCCL and deployed in enclosures at Rio Vista (n = 60). Ce_shasta = Ceratonova shasta; Fl_psy = *Flavobacterium psychrophilum*; Ic_mul = *Ichthyophthirius multifiliis*; Ku_thy = *Kudoa thyrsites*; Mo_vis = *Moritella viscosa*; OMV = salmonid herpesvirus/*Oncorhynchus masou* herpes virus; Pa_min = *Parvicapsula minibicornis*; Pch_sal = *Piscichlamydia salmonis*; ISAV8 = infectious salmon anemia virus; VER = viral encephalopathy and retinopathy virus.

system used is highly sensitive, and the Ct values of these samples were near the LOD for the assays and the sensitivity of the BioMark instrument, and therefore should be interpreted with caution.

DISCUSSION

When releasing captively bred individuals into natural ecosystems, the potential for disease transmission must not be overlooked. Here, we report the results of the first effort to conduct pathogen screening of cultured and wild Delta Smelt using high-throughput qPCR and assays developed for salmonid pathogen screening by Miller at al. (2016). Based upon our control data, we found the assays to be highly sensitive, with detectable amplification at the lowest DNA concentration tested, consistent with Miller et al. (2016). Our most notable finding was the detection of *Ich* in 13% of Delta Smelt tested, which originated from and were held at the FCCL. As a ciliate protozoan, *Ich* infiltrates epithelial tissue and rapidly causes the formation of trophonts (visible as white spot disease) that can be particularly damaging to gill tissue and prevent proper osmoregulation and gas exchange (Ewing et al. 1994; Tumbol et al. 2001). *Ich* is common in hatcheries, including the FCCL, and if treated early should not cause lasting damage (2021 email communication between T-C Hung and DG, unreferenced, see "Notes"). It is not surprising that we found *Ich* in FCCL fish in this study, given that this pathogen is ubiquitous in the estuary (Lehman et al. 2020), appears following thermal stress (Frank et al. 2017), and hatchery staff noted disease in some tanks at the time of fish collection. Our positive results are therefore congruent with this data and suggest that the assays of Miller et al. (2016) can be used to accurately detect the presence of pathogens in Delta Smelt; further histological examination of skin and gill tissue would be required to definitively diagnose disease in these specimens. Another important outcome of this study was

the absence of positive pathogen detections in fish deployed in enclosures at Rio Vista or in archived wild fish. This finding corroborates previous, albeit limited, work on the presence and prevalence of pathogens in Delta Smelt Delta Smelt pathogen presence and prevalence, and points to a low pathogen burden in wild fish and the estuary, at least for pathogens that affect salmonids; to expand upon this conclusion, the screening of other tissues that are specific targets of certain pathogens would be useful.

Although close to the LOD of our assays or the sensitivity of the qPCR instrument, we obtained marginal detections of 10 pathogens (Table A2). The greatest number of marginal detections were for *Ich* and *K. thyrsites*. We posit that marginal detections of *Ich* in FCCL fish could represent one of the many stages of the *Ich* life cycle present in the FCCL environment, or potentially low-level infection. The large, single-celled parasitic trophonts that are visible on infected fish eventually fall off the host, at which point they are called tomonts. When a tomont settles on a substrate, it undergoes reproduction (binary fission) and becomes a tomocyst containing as many as 1,000 tomites (McCartney et al. 1985; Wei et al. 2013). The tomites then break through the tomocyst as free-swimming, infective theronts (Dickerson and Clark 1998). Given the extreme sensitivity of our pathogen assays and *Ich* outbreak at the time of fish collection when fish were collected from the FCCL, it is not unreasonable to suppose that marginal detections of *Ich* could be of individual tomonts, or theronts, or multicellular tomocysts that have been filtered by gill tissue or that are merely present in the water and environment. Marginal detections of *Ich* in our study could also denote early infection with just a few established trophonts. A similar justification could be made for the marginal detection of *Ich* in an archived wild Delta Smelt, especially because *Ich* is pervasive in the estuary (Lehman et al. 2020). Detection of *K. thyrsites* was puzzling and unexpected because these myxosporean parasites are typically found in marine host species, although they can infect anadromous fishes such as salmonids that move through estuaries (Whipps and Kent 2006; Eiras et al. 2014). It is possible that the highly sensitive molecular screening approach is detecting DNA contamination sourced from other fish species (Burger and Adlard 2011) because this microparasite has not been reported in the Delta; further investigation is warranted.

The remaining eight pathogens (Table A2) with marginal detections were in enclosure-deployed or archived wild Delta Smelt only. Given the high Ct values, incidence in only one or two individuals, and specificity of the pathogens to salmonids, it is likely that these marginal detections reflect the ambient pathogen community of the estuary rather than infection in Delta Smelt. However, subclinical, or asymptomatic infection in which an individual is infected but does not show overt signs of disease cannot be ruled out without conventional diagnostic testing. *F. psychrophilum* is endemic to California, including the estuary, and causes bacterial coldwater disease in salmonids, namely Rainbow Trout, as well as in other non-salmonid fishes (reviewed in Starliper 2011; Sebastião et al. 2020). Similarly, *C. shasta* and *P. minibicornis* are myxosporean parasites of the digestive tract and kidneys, respectively, that are known to be present and infect salmonids throughout the estuary and its tributaries year-round (Lehman et al. 2020). The other five pathogens with marginal detections in enclosure-deployed or archived Delta Smelt are found globally but have not been associated with large-scale infection or mortalities in California, and so are unlikely to represent true infection. Yet, it is important to note that not all pathogens are equal and affect hosts in the same way, and some could be highly virulent even at very low concentrations.

This pilot study showed that the assays developed by Miller et al. (2016) could successfully detect pathogens in Delta Smelt gill tissue, but further validation and customization will be necessary for the assays to be informative about the presence and load of pathogens in Delta Smelt. An immediate next step would be to sequence the PCR amplicons of positive or marginal detections and confirm that the resultant sequence matches that of the expected pathogen and not a closely

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related pathogen or an analogous region of the Delta Smelt genome; this step should be repeated for every pathogen at first detection. Another important experiment to ensure applicability for use in an endangered species would be to evaluate and confirm the success of these assays in detecting pathogens on non-invasive samples such as a mucus swab (applicable for select pathogens). It would also be prudent to expand the temporal range of archived wild Delta Smelt samples to span at least a 5- to 10-year period, as well as numbers of samples per year, to gain a more comprehensive understanding of the scope of the presence of pathogens and potential differences among water year types (e.g., wet versus dry) or other environmental conditions in the estuary. Similarly, collecting samples from Delta Smelt across seasons could inform seasonal trends in the presence of pathogens in wild or hatchery fish. Using these same assays, distinct changes in pathogen profiles have been observed in Chinook Salmon in the Fraser River among seasons (Tucker et al. 2018). Increasing the spatial range of wild Delta Smelt samples collected to be from throughout the estuary could improve our understanding of whether some pathogens are localized or widespread. Pathogens that affect Delta Smelt which the Miller et al. (2016) assays do not cover could be identified through an RNA-seq, metagenomics, or multi-locus sequence typing approach, using multiple tissues from wild, cultured, and enclosure-deployed fish (Urwin and Maiden 2003; Houldcroft et al. 2017; Fu et al. 2019). Since the BioMark HD system is fully customizable, once Delta Smelt-specific pathogens are identified, developed, and validated, they can be screened alongside those from Miller et al. (2016). The addition of an assay for *Mycobacterium* spp. would be particularly beneficial because this pathogen has been reported in wild and hatchery Delta Smelt previously (Antonio et al. 2000; Swanson et al. 2002; Foott and Bigelow 2010). Finally, although qPCR can be used to assess relative pathogen load per fish (only presence was considered here), it does not necessarily predict pathogenicity. Gene expression assessments in conjunction with histopathology can confirm disease progression, which could be correlated with pathogen load (Connon et al. 2012; Teffer et al. 2017).

Once pathogen screening assays have been honed for Delta Smelt, they can be applied in the health management of cultured Delta Smelt. Knowing that disease outbreaks are not out of the ordinary in hatcheries and that we detected *Ich* in Delta Smelt at the FCCL, pathogen prevention measures are equally if not more important than pathogen monitoring, and the two can be coupled to help ensure the healthiest possible cultured stock for population supplementation or reintroduction. The first line of defense against pathogen contamination at the FCCL is sand filtration and ultraviolet (UV) irradiation of water pumped in from the Clifton Court Forebay reservoir. In sand filtration, water percolates through a column of sand particles that is enriched with microorganisms. Mechanical and biological processes in the column, such as biofilm formation, result in adherence to and removal of bacteria, viruses, and parasites (Maurya et al. 2020). UV irradiation causes the formation of thymine and uracil dimers and prevents replication of DNA and RNA, respectively, in all waterborne pathogens but to varying degrees (Harris et al. 1987; reviewed in Hijnen et al. 2006). Next, the recirculating systems at the FCCL are bleached before fish are brought in, and maintenance equipment is disinfected daily with high concentrations of brine and iodine. Additionally, wild broodstock are kept in a separate holding area at the FCCL (never integrated with the refuge population), treated with antibiotics for 3 consecutive days (4 h per day) after capture (2022 email communication between T-C Hung and DG, unreferenced, see "Notes"); FCCL staff are trained to recognize the onset of potential infections and disease. Despite these rigorous pathogen prevention procedures, some potential routes for pathogen introduction cannot be controlled. The FCCL is a large-scale hatchery and, as such, requires more water than can be sourced from a well, which would pose a much lower risk of harboring pathogens (Yanong and Erlacher–Reid 2012). Sand filtration and UV irradiation are not infallible, and some pathogens are resistant to these methods. Even

with regular maintenance and exchange, UV bulbs become less potent and reliable with age. Lastly, pathogen dispersal is always possible by bringing in wild broodstock and live feed or excretion by birds (e.g., Arsan and Bartholomew 2009). Periodic screening of water before and after sand filtration and UV irradiation, scrapings of water samples from across tanks, or mucus swabs of wild broodstock for some pathogens using the molecular assays described above could help anticipate and limit the spread of any disease outbreaks.

Pathogen screening using the Miller et al. (2016) assays, especially with species-specific modifications, would be invaluable in guiding cultured Delta Smelt population supplementation and reintroduction efforts. A recent health evaluation performed by fish pathologists at the California–Nevada Fish Health Center indicated that cultured Delta Smelt were at a "low health risk" based upon the findings of previous disease studies mentioned earlier (Teh 2007; Foott and Bigelow 2010; Baxa et al. 2015) and the absence of viral disease in all fish tested from the FCCL since 2005 (2022 email communication between S. Foott and DG, unreferenced, see "Notes"). However, movement or release associated with population supplementation and reintroduction could be restricted by an unexplained large die-off or if a significant pathogen is detected in the cultured population (2022 email communication between S. Foott and DG, unreferenced, see "Notes"). A pathogen is considered significant if it is virulent and untreatable, novel to the estuary, or capable of causing high mortality (2022 email communication between S. Foott and DG, unreferenced, see "Notes"). To defend against possible interruption or termination of cultured Delta Smelt release for population supplementation or reintroduction, molecular pathogens could be screened for regularly (e.g., monthly or quarterly) and before release, and serve as an early warning system for some of these significant pathogens. As stated earlier, the assays used for qPCR on the dynamic array can be customized and made specific to pathogens that are of greatest concern to fish pathologists. Any positive detections could point to active infection and be a trigger for fish pathologists to move forward with gold-standard diagnostic tests. Depending on the pathogen and whether fish pathologists believe it is significant to the natural population, a marginal detection may not necessitate additional diagnostic testing but, instead, increased watchfulness and possibly repeated molecular screening. By screening for molecular pathogens alongside standard health monitoring, the risk of unintended introduction of pathogens into the estuary is further reduced. Moreover, the collection and screening of eDNA or eRNA in water and soil samples from putative release sites before release occurs, and regularly throughout the year, could tell managers when conditions for population supplementation and reintroduction are not optimal because of a high risk for significant pathogen exposure and disease. More generally, pathogen screening using molecular assays could be conducted on any field-collected mortalities, and as part of laboratory-controlled experimental studies, to gain a better understanding of why wild fish die, as well as the presence and prevalence of pathogens in the estuary.

CONCLUSIONS

The study presented here and previous work indicate that the pathogen burden in cultured and wild Delta Smelt is low. However, to minimize the risk of unintended pathogen transmission, vigilance in pathogen monitoring of cultured and wild fish and environmental samples should continue in preparation for the release of cultured Delta Smelt into the estuary. Our principal finding was the detection of *Ich* in 13% of cultured Delta Smelt tested. If *Ich* infection were confirmed by diagnostic tests, these fish could be deprioritized for potential future release until after treatment. Given that *Ich* is known to be present in the estuary, the potential release of fish infected with *Ich* does not pose a substantial risk to the ecosystem, but could affect the success of population supplementation and reintroduction efforts because *Ich* typically elicits a stress response in the fish host (Frank et al. 2017). We also obtained marginal detections of several other pathogens in archived wild and enclosure-

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deployed cultured Delta Smelt (e.g., *O. masou* virus) which suggest that the ambient pathogen community in the estuary may not yet be fully understood; further investigation of assay and host specificity is warranted to clarify whether these pathogens are indeed present, and whether they can infect are infective to Delta Smelt. The assays used herein were developed for salmonid species but could be customized for Delta Smelt through conducting RNA-seq, metagenomics, or multi-locus sequence typing approaches using multiple tissue types from cultured, wild, and enclosure-deployed fish, along with assessments of confirmed infection infectivity confirmation assessments within laboratory-controlled studies. A valuable future application could be pairing of primers in these assays with the CRISPR-Cas13a platform Specific High-sensitivity Enzymatic Reporter unLOCKing (SHERLOCK) (Baerwald et al. 2020) to develop a rapid, non-lethal (e.g., a mucus swab) screening approach for certain pathogens in Delta Smelt. Molecular pathogen screening assays will have a critical function in future Delta Smelt population supplementation and reintroduction.

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NOTES

Hung T-C. Email dated May 18, 2021, to Daphne Gille regarding Ich occurrence and treatment at the University of California Davis Fish Conservation and Culture Laboratory.

Hung T-C. Email dated February 6, 2022, to Daphne Gille regarding University of California Davis Fish Conservation and Culture Laboratory quarantine protocols for wild Delta Smelt broodstock.

Foott S. Email dated February 7, 2022, to Daphne Gille regarding pathogen screening at the University of California Davis Fish Conservation and Culture Laboratory and population supplementation.