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Electron microscopy reveals viral-like particles and mitochondrial degradation in scombrid puffy snout syndrome

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ABSTRACT: Aquaculture is an increasingly important food resource, but its sustainability is often limited by disease. In Scombridae fishes, puffy snout syndrome (PSS) is a debilitating condition where tumor-like collagenous growths form around the eyes, nares, and mandibles which impair vision and feeding and frequently lead to mortality. While PSS is considered an infectious or metabolic disease, no disease agents or promoters have been identified. Here, we used electron microscopy (EM) to describe the cellular pathology and search for etiological agents of PSS in Pacific mackerel *Scomber japonicus*, the first use of this approach for PSS. We examined aquaculture specimens across a range of apparent PSS severity, comparing the results to both wild and aquaculture asymptomatic mackerel. EM imagery consistently revealed viral-like particles in PSS samples, as well as the uniform absence of bacteria, protists, fungi, and other multicellular parasites. In addition to viral-like particles, symptomatic fish had a higher mean percentage of swollen and disintegrating mitochondria than both asymptomatic aquaculture and wild mackerel. This suggests that degraded mitochondria may be related to PSS and could be important to further understanding the origin, promoters, and prevention of PSS. This study serves as a first step in identifying the etiological agents of PSS.

KEY WORDS: Disease · Aquaculture · Scombridae · Mackerel · Electron microscopy · Viral infection · Mitochondria

1. INTRODUCTION

Aquaculture and aquaria are critical infrastructures in achieving food security and fisheries sustainability. Aquaculture contributes to over half of global seafood consumption and is an increasingly important component of many nations' GDPs (FAO 2018). Public aquaria, through their ability to keep live marine animals, are important outlets for research, education, and conservation. Both settings maintain animals at higher-than-natural densities, increasing the chance of disease outbreaks. As a result, diseases are frequently in greater prevalence or appear uniquely in fishes maintained in aquarium and aquaculture environments. Farmed salmonids contract infectious salmon anemia and infectious pancreatic necrosis virus (McAllister & Bebak 1997), cultured white sturgeon experience *Streptococcus iniae* outbreaks (Pierezan et al. 2020), and cultured channel catfish *Ictalurus punctatus* experience channel catfish virus

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(Ourth et al. 2017). An inability to manage infectious disease can threaten animal welfare and the health of wild populations should disease-carrying captive animals escape or be released into marine ecosystems (McAllister & Bebak 1997). In aquaculture settings, we have opportunities to learn about marine diseases and pathology.

Puffy snout syndrome (PSS) is exclusively associated with aquarium- and aquaculture-raised fish, has largely been documented in the Scombridae family (tunas and mackerels), and is a serious concern for industries that keep these fish captive long-term. This syndrome is characterized by excessive growth of connective tissue around the eyes, nares, and jaw, resulting in visual occlusion and gross morphological deformities (Voorhees 2015). Normal swimming behavior and feeding correspondingly become increasingly difficult for affected fish, resulting in slower growth, decreased meat quality, and eventual mortality. Global landings of scombrids are valued at $10–12 billion USD annually, with a point of sale value of over $42 billion (Galland et al. 2016). While it is below 1% of total global tuna production, 17–37% of highly valued bluefin tunas are farmed in growth pens for at least part of their lives before being brought to market (Metian et al. 2014, Benetti et al. 2015). This captivity can take the form of wild-caught fish kept in pens for short periods for pre-market conditioning, smaller wild-caught juveniles kept for a longer period until grown to market size, or closed-cycle breeding operations that culture tuna from broodstock (Sawada et al. 2005). With many wild tuna stocks currently being overfished (Metian et al. 2014) there is increasing interest in full reproductive-cycle production (Sawada et al. 2005). However, this shift in production requires long-term aquaculture, increasing the risk of PSS, and challenging industry sustainability at scale.

PSS was first described in yellowfin tuna *Thunnus albacares* at the Kewalo Basin Research Center in Honolulu, Hawaii, USA, in 1951 (Tester 1952). In addition to yellowfin tuna, PSS has been described in Pacific bluefin tuna *T. thynnus*, blackfin tuna *T. atlanticus*, skipjack tuna *Katsuwonus pelamis*, Pacific mackerel *Scomber japonicus*, and Atlantic mackerel *S. scombrus* (Tester 1952, Nakamura 1962, 1972, Dizon et al. 1978, Queenth et al. 1983, Benetti et al. 2009, Voorhees 2015). Anecdotally, the disease has also been reported in northern anchovy *Engraulis mordax*, Pacific sardines *Sardinops sagax*, California yellowtail *Seriola dorsalis*, and rainbow trout *Oncorhynchus mykiss*. Though studies have characterized the gross pathology of PSS, no causal agents have been identified (Nakamura 1972, Benetti et al. 2009, Voorhees 2015). Recent attention has screened for parasites, inoculated culture media for known disease-causing bacteria, and inoculated cell lines to test for a cytopathic effect (Voorhees 2015). The results showed no parasites, bacterial growth, or viral infections. However, the cell-lines inoculated with PSS were not tuna cell lines nor were they from a salt-water species, limiting inference about these negative results. Interestingly, the study found that prevalence of the syndrome increased with tank density, suggesting PSS has an infectious causal agent (Voorhees 2015).

In this study, we focused on identifying potential causal agents of the disease through electron microscopy (EM) imaging of symptomatic and asymptomatic Pacific mackerel from the Monterey Bay Aquarium (MBA) and wild Pacific mackerel caught in Monterey Bay (36.6305° N, 121.8907° W). Due to broad pathological similarities to fibropapillomatosis (Van Houtan et al. 2010), we hypothesized PSS has a viral etiology. Specifically, we identified key signs and symptoms of active viral infections including, but not limited to, the presence and morphology of viral-like particles (VLPs), mitochondrial degradation, and cell necrosis/apoptosis (Albrecht et al. 1996).

2. MATERIALS AND METHODS

2.1. Ethics statement

All live animal protocols described below were approved by the Animal Welfare Committee of the MBA. Animal housing facilities met the Association of Zoos and Aquariums accreditation standards.

2.2. Fish collection and care

We obtained aquaculture Pacific mackerel from an MBA exhibit during routine display deaccession (for collection and care; see the Supplement at www.int-res.com/articles/suppl/d147p025_supp.pdf). To serve as controls, we obtained 4 wild Pacific mackerel directly captured from Monterey Bay, CA, USA, using hook and line gears. These fish were collected during routine exhibit collections (California Department of Fish & Wildlife Scientific Collecting Permit SC-2026).

2.3. Sample collection and preparation

Aquaculture fish were euthanized via MS-222 and pithing followed by decapitation at the first vertebra. We immediately placed each fish in separate bags and sampled them. Wild control fish were euthanized upon capture and kept at 4°C for <24 h before being placed in separate bags and sampled.

We selected 3 asymptomatic and 6 symptomatic aquaculture fish. Asymptomatic Pacific mackerel were comparable to wild fish, only lacking the characteristic reflectivity of iridophores in recently caught fish. Symptomatic fish had darkened and raised tissue around the nares, eyes, operculum, and jaw. As the syndrome progressed, this connective tissue became thicker and more rugose. Eyes were sunken with laterally extended, thickened corneal epithelium.

We recorded curved total and fork lengths and mass and took dorsal, ventral, and lateral pictures. We made incisions anterior to the eyes and posterior to the nares to excise three <0.5 cm^3 samples $fish^{-1}$.

For EM analysis, we fixed samples in 2.5% glutaraldehyde and 1% paraformaldehyde in 0.1 M sodium cacodylate buffer and stored them at 4°C. We prepared samples following Deerinck et al. (2018), modified to use sodium cacodylate (see the Supplement). The tissue from one symptomatic fish did not correctly fix and was excluded from analyses.

2.4. Quantitative EM imagery analyses

A Helios 650 Ultra Resolution Dual Beam FEG Scanning Electron Microscope imaged the resin-embedded, stained samples at the Electron Microscopy Facility at Oregon State University. Multiple slides were imaged from each sample at 500 nm, 1, 2, 3, 4, 5, 10, 20, 30, 40, and 50 µm magnifications. Cellular features indicative of disease, such as VLPs, inclusions, or damaged mitochondria, were recorded. We counted healthy and unhealthy mitochondria. Other organelles were examined but did not exhibit the same degradation as the mitochondria. We reviewed images for each sample to provide a total count of healthy and unhealthy mitochondria and avoid pseudoreplication across magnifications.

A generalized linear mixed effects model (with a binomial distribution) investigated the effect of aquaculture and gross pathology on counts of mitochondria. For this analysis, we first calculated the percent of malformed mitochondria per fish. Individual fish identity was treated as a random effect. A post hoc Tukey test evaluated pair-wise differences. For all analyses, we used a significance level of $\alpha = 0.05$.

3. RESULTS

EM identified VLPs but no bacterial, fungal, or other pathogens in aquaculture Pacific mackerel externally expressing PSS as well as asymptomatic aquaculture fish (Fig. 1). We did not observe VLPs in the wild mackerel. We observed VLPs in 4 of 6 symptomatic aquaculture mackerel and 1 of 3 asymptomatic aquaculture mackerel. Of the 2 symptomatic aquaculture mackerel that did not have VLPs, one appeared to lack all cellular structures, likely due to poor fixation. The other had potential viral replication sites (Fig. 2B). VLPs were pleomorphic and spherical in shape, 63–125 nm in diameter. Some VLPs appeared hollow while others contained small amorphous subunits (see Fig. 1). Many VLPs had a concentric circle or inner membrane (see Fig. 1B), indicating they may generate envelopes using the host membrane (Fig. 1C). The VLPs were not morphologically identical to any viruses previously described in fish, though several virus families have similar pleomorphic spherical shapes in similar sizes. The highly degraded state of the cells challenged diagnostic identification.

VLPs were observed within the cell cytoplasm as well as within malformed mitochondria. EM images showed extensive cellular degradation with most intracellular structures unidentifiable, consistent with previous histological findings (Voorhees 2015). Degraded vesicles were filled with dense materials which possibly served as viral replication sites (Fig. 2). Asymptomatic and symptomatic aquaculture mackerel contained some mitochondria that were swollen relative to their standard diameter, with extensive degradation (see Fig. 2). The malformed mitochondria had lost distinct internal structures, with the cristae and inner membrane often appearing entirely degraded and/or absent (Fig. 2).

Swollen and degraded mitochondria were present in all treatments but their proportion varied by group. Symptomatic aquaculture fish had a higher percentage of malformed mitochondria than wild ($\beta = -4.9$, SE = 2.0, $p = 0.01$; Fig. 3). Asymptomatic aquaculture fish did not significantly differ from wild ($\beta = -4.2$, SE = 2.3, $p = 0.15$) or symptomatic aquaculture fish ($\beta = -0.7$, SE = 2.3, $p = 0.9$) (Fig. 3).

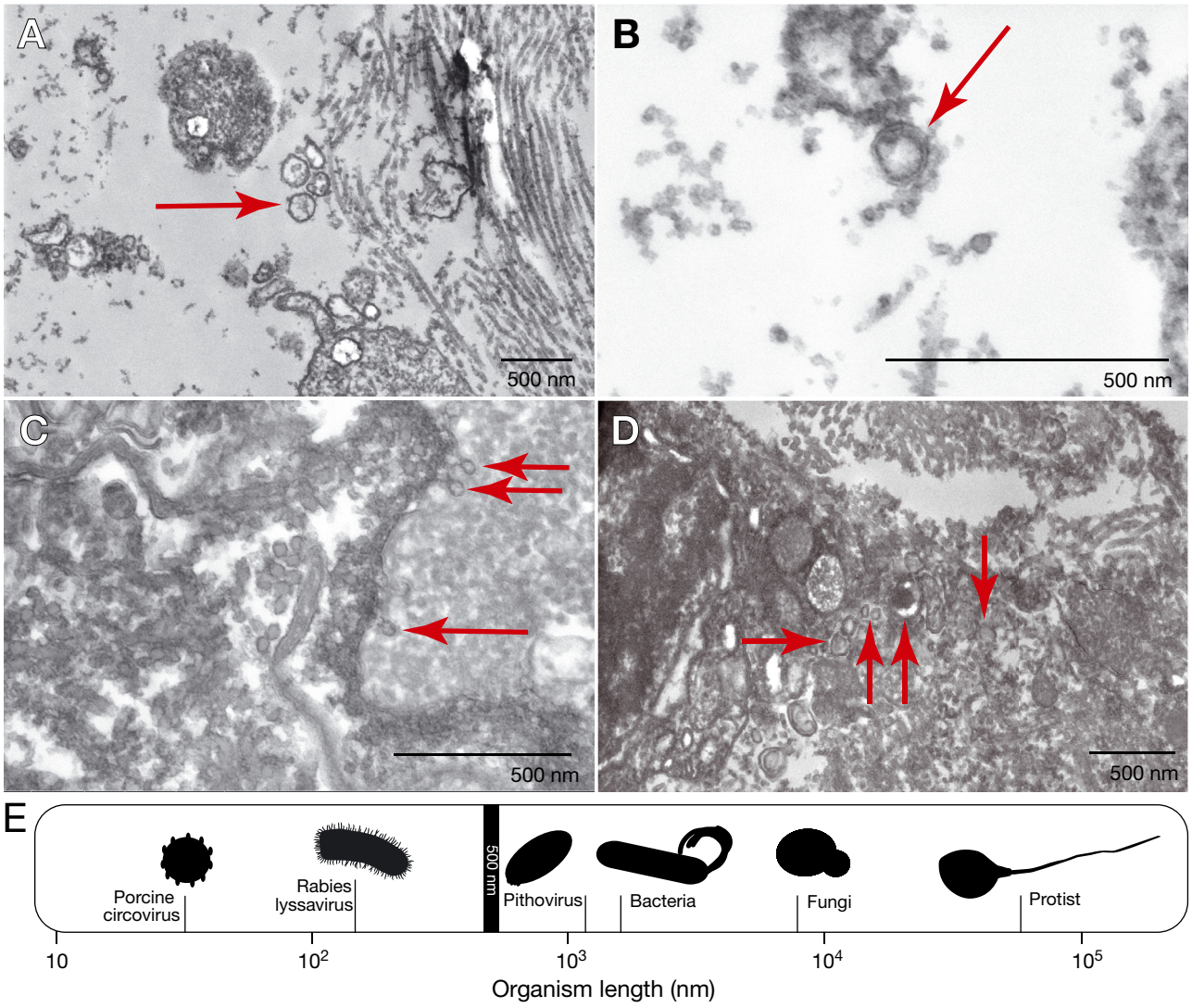

Fig. 1. Viral-like particles (VLPs) are common in facial tissues from Pacific mackerel afflicted with puffy snout syndrome (PSS). (A–D) Electron microscope images depict epithelium tissue collected anterior to the eyes, posterior to the nares. Red arrows: VLPs; in (C) they reveal VLPs in the process of budding. VLPs were more prevalent in symptomatic individuals with PSS. (E) A representative set of potential pathogens plotted along a logarithmic size scale. Images revealed zero examples of bacterial, fungal, or parasitic pathogens. VLPs fell within the size range of common viruses. Scale bars = 500 nm

4. DISCUSSION

PSS is a debilitating disease of scombrids that threatens animal welfare and sustainability in aquarium and aquaculture settings. Identifying the etiological agent and the suite of conditions that lead to increased syndrome presentation are the first necessary steps to identifying prospective therapeutics.

EM revealed VLPs across symptomatic and asymptomatic groups in aquaculture with a greater proportion of presence in symptomatic fish. Five of 6 symptomatic fish had VLPs or possible viral replication sites. The presence of VLPs in aquaculture fish, their absence in wild fish, and the increased occurrence in symptomatic relative to asymptomatic fish suggest a possible viral-mediated cause of PSS. Stocking at high densities allows for greater viral exposure and subsequent cellular degradation in aquaculture fish.

The observed VLP diameters overlap with the size range of many common viruses (~100 nm). The pleomorphic spherical-shaped VLPs in our study have a similar form to enveloped viruses that consist of a nucleic acid within a helical or polyhedral core enclosed by a lipoprotein envelope. In these viruses, the viral envelope is often derived from a host cell membrane via budding. Fig. 1C may document this

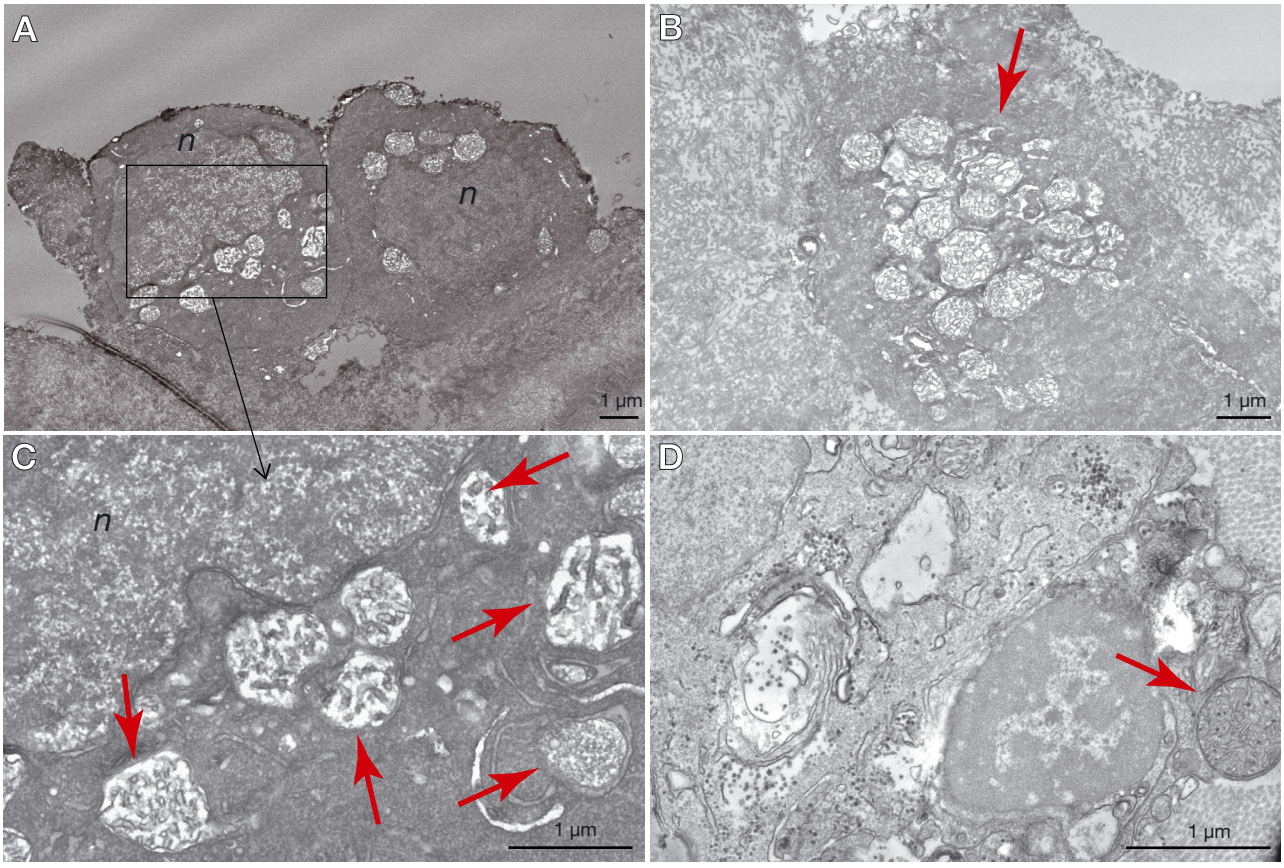

Fig. 2. Malformed mitochondria frequently appear in Pacific mackerel afflicted with puffy snout syndrome. (A–D) electron microscopy images of epithelium tissue collected between the nares and eyes; *n*: nuclei. (B) Possible cluster of viral replication vesicles (noted by red arrow). (C) Magnified inset image from within (A); red arrows identify malformed mitochondria that appear swollen and/or in a degenerative process. (D) A wild control sample containing a healthy mitochondrion (identified by a red arrow). Scale bars = 1 µm

budding process. The VLPs we observed likely have an RNA genome based on size. Double-stranded RNA (dsRNA) viruses are generally larger (>125 nm) and single-stranded DNA (ssDNA) viruses are generally smaller (<55 nm). Many RNA viruses replicate in the cytoplasm while many DNA viruses replicate in the nucleus (den Boon et al. 2010). EM images revealed sites of VLP presence and cellular degradation that indicate cytoplasmic replication.

We are possibly observing a novel virus in fish aquaculture. Described viruses common to aquaculture fish include aquabirnaviruses (non-enveloped icosahedrons, 60 nm, dsRNA) in salmon, betanodaviruses (spherical, non-enveloped, 25–30 nm, ssRNA) observed in 40+ species of marine and freshwater fish, infectious salmon anemia virus (*Orthomyxoviridae*; pleomorphic, enveloped, 90–130 nm, ssRNA), salmon alphavirus (*Togaviridae*; enveloped, spherical, 60 nm, ssRNA), infectious hematopoietic necrosis virus (*Rhabdoviridae*; bullet-shaped, 190 nm length, 65–75 nm width, ssRNA) in salmonids and sturgeon, and epizootic hematopoietic necrosis virus (*Iridoviridae*; icosahedral, outer limiting membrane from host, 175 nm, dsDNA) in perch and salmonids (Leong & Fryer 1993, Crane & Hyatt 2011, Yong et al. 2019). Of these common viruses, infectious salmon anemia virus has a similar shape and overlapping size range to VLPs in Pacific mackerel, but it has a replication site in the nucleus rather than cytoplasm and no gross morphological similarities. Molecular analyses are needed to confirm if PSS is caused by a virus novel to fish aquaculture.

While mitochondrial degradation is part of the normal process of cell turnover, it can also be emblematic of certain diseases (Anand & Tikoo 2013). Viruses may trigger mitochondrial death pathways and cellular apoptosis, or they may exploit mitochondrial functions for replication and translation (Anand & Tikoo 2013). Influenza A virus (*Orthomyxoviridae*) damages mitochondria and reduces mitophagy, re-

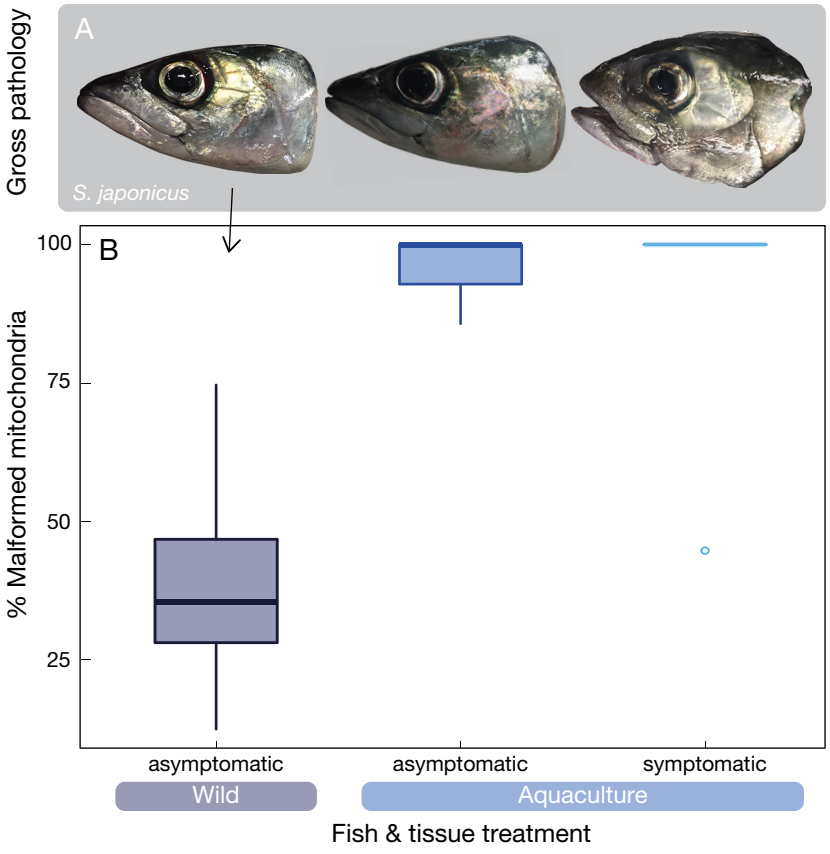

Fig. 3. Percentage of malformed mitochondria is highest in aquaculture fish with puffy snout syndrome (PSS) symptoms. (A) The progression of the gross pathology of PSS shown in asymptomatic wild (n = 4), asymptomatic aquaculture (n = 3), and symptomatic aquaculture (n = 6) Pacific mackerel. Lateral photographs of the head trace the posterior, trailing edge of the operculum. (B) Percent of malformed mitochondria observed in electron microscopy images increases in aquaculture settings and with the onset of PSS relative to asymptomatic wild fish. Nearly all (98%) mitochondria observed (n = 907) in facial epithelia cells from symptomatic fish were malformed. Box plots indicate median (bar), interquartile range (IQR; box), ±1.5× IQR (whiskers), and outlier (dot)

sulting in the accumulation of degraded mitochondria (Abdoli et al. 2018). Conversely, hepatitis C virus (*Flaviviridae*) induces mitophagy to promote viral replication (Abdoli et al. 2018). The VLPs and extensive mitochondrial damage we observed in aquaculture and symptomatic mackerel suggest PSS may also follow a mitochondria pathway. However, the presence of degraded cells in wild fish suggests any viral-mediated cause of PSS may be induced by additional stressors introduced by aquaculture. Whether the degraded mitochondria or wider cellular degradation observed are symptoms of viral infection, caused by viral replication within mitochondria, host response to shutting down viral activity, or related to the proliferation of tissue distinctive of PSS is unknown.

Even well-studied viral pathogens are difficult to identify and manage in the aquatic environment because the viromes of marine organisms are generally poorly understood. Additionally, treatments and vaccines are challenging to effectively develop and administer in aquatic settings (Leong & Fryer 1993). Yet environmental concerns related to aquaculture diseases are pressing. Infectious pancreatic necrosis virus occurs in wild fish populations but becomes an issue in high-density salmonid aquaculture and is documented in wild brook trout *Salvelinus fontinalis* downstream of salmonid farm effluent plumes (McAllister & Bebak 1997). Vaccine-induced immunity against some viruses has been demonstrated in farmed fish (Lauscher et al. 2011), and this field is advancing. Preventing viral outbreaks and mitigating infections are critical for sustainable commercial aquaculture. As aquaculture has expanded dramatically in the last decade, managing viral pathogens will remain a pressing sustainability and welfare concern.

5. CONCLUSIONS

PSS is likely caused by a naturally occurring virus that contributes to cellular damage when higher-density aquaculture introduces additional stressors. Like many syndromes, the causative pathways are complex and will require additional study following the characterization of PSS using EM. This study is the first in a series to identify the suspected virus and determine its effects on host cells. Metatranscriptomic analyses will follow, along with culturing techniques. Upon confirming viral identity, subsequent research is needed to develop automated methods to detect viral presence in enclosed aquatic environments. In addition to viral therapeutics, experimental trials can highlight preventative measures and best practices by manipulating stocking density, tank volume and shape, water quality parameters, and calories per biomass to mitigate its occurrence in aquaculture and aquaria settings.

Data availability. All data and code are available at https://osf.io/vaz9b/

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