Title: Transcriptomics Reveals Tumorigenesis and Pathogen Response in Scombrid Puffy Snout Syndrome

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Abstract:
Scombrids represent some of the most economically important fisheries globally. However, increased interest in creating aquaculture systems for these fish has increased the risk of disease emergence. One such disease, Puffy Snout Syndrome (PSS), causes collagenous tissue growths on the face, in numerous scombrid taxa. PSS has only been documented in captive-held fish populations and can lead to high mortality rates. Despite this, little is known about PSS's causative agent(s) and the immune response they elicit. Therefore, we leveraged metatranscriptomic data of symptomatic-captive, asymptomatic-captive, and healthy-wild Pacific Mackerel (*Scomber japonicus*) to evaluate the physiological characteristics of PSS infections and identify a mechanism of disease. PSS symptomatic-captive and asymptomatic-captive mackerel showed distinct gene expression patterns from their wild counterparts. Genes involved in tumorigenesis, immune response, and pathophysiology were over-expressed in captive-held fish. In particular, the *Wnt9* pathway was increased by more than 2 orders of magnitude in captive fish, potentially representing a diagnostic feature of this disease. Together, these host physiological data and our past visual identification of RNA virus-like particles in afflicted tissues suggest that viral-mediated oncogenesis may be driving PSS in captive mackerel.

Introduction:
The Scombridae fishes (tunas, bonitos, mackerel) comprise one of the largest global finfish economies, projected to generate ~$50-80 billion USD annually by 2027 [1]. Efforts have been undertaken in recent years to increase the efficiency of tuna ranching and farming, which involves keeping tunas captive for extended periods of time, potentially years, at high densities. Tunas and mackerels are robust species in aquaculture with negligible economic loss from diseases [2]–[4]. However, high fish-stock densities, low-quality food sources, and increasing temperatures in mariculture operations have contributed to large-scale disease outbreaks [4], [5]. Mass mortality outbreaks in tuna aquaculture are often associated with parasitic blood flukes (*Cordicola* spp.) ranging from 50-80% of an enclosure [6]. Incidences of mass mortality events from bacterial and viral diseases are less common than parasitic pathogens, but significant die-offs occur in scombrid aquaculture, mostly associated with juveniles. An outbreak of *Photobacterium damselae* contributed to over 2500 Atlantic bluefin tuna (*Thunnus thynnus*) fish deaths in a mariculture operation over a six-month period in 2004 [7]. This outbreak was directly correlated with increased water temperatures and compounded by the long-term feeding of low-quality baitfish [8]. Other bacterial

outbreaks reported in hatchery-reared tuna larvae include *Mycobacterium*, *Vibrio*, and *Tenacibaculum* sp. [10],[11]. Both nervous necrosis virus (NNV) and red seabream iridovirus (RSIV) have had high mortality rates in larval and juvenile Pacific Bluefin tunas (*Thunnus orientalis*)but have yet to be described in adults [12]–[14]. However, a study did describe that juvenile *T. orientalis* were highly susceptible to RSIV when experimentally challenged orally, with nearly 90% mortality in the infected group [15].

Puffy Snout Syndrome (PSS) is a disease of captive scombrids characterized by excessive collagen growths on the fish head. The first signs are a darkening and wrinkling of the skin, though this phenotype is more pronounced in tunas. As the disease progresses, a gelatinous substance overgrows on the dorsal area of the head. Work using Masson trichrome staining found that normal facial tissue was composed primarily of striated muscle fiber; however, in PSS-afflicted fish, this tissue broke down and was replaced by collagen [16]. Further, mucus-producing goblet cells which create a healthy slime coat on the fish, were noticeably decreased in PSS-affected tunas [16]. Severe degradation at the cellular level renders mitochondria one of the only recognizable structures [17]. A survey of 11 rearing facilities that held tunas, mackerels, or both found that of the total 22 rearing units in facilities, PSS developed in 68% of these units [16]. Fish with severe PSS cannot feed and, as a result, are either euthanized or starve to death. Further, PSS may be exclusively associated with fish held in captivity. None of the facilities surveyed reported seeing PSS in wild-caught fish, with PSS developing in the first 6 months of captivity [16].

Despite its unique disease characteristics, density-dependent prevalence, and wide host range, PSS is an understudied syndrome with mortality rates ranging from 20-100% of an enclosure [16], [18]–[20]. Only 9 published reports exist on the topic, and only two of those attempts to identify etiological agents [16], [17]. Recent work used electron microscopy techniques and identified viral-like particles (VLPs) within PSS-afflicted tissues of *S. japonicus* [17]. These VLPs shared morphology with small RNA viruses being amorphous in shape and ~100 nm in diameter. Along with VLPs, malformed mitochondria had a high abundance in PSS-afflicted tissues. The VLPs and disease prevalence in increased fish stock densities [16] strongly suggest that PSS onset is associated with a viral infectious agent.

As a next step to identify the underlying mechanisms of PSS, we leveraged high throughput sequencing methods to further characterize this disease at the physiological and genomic levels. Our aims were to 1) analyze comparative host gene expression dynamics in PSS of apparently healthy mackerel, asymptomatic-captive, and symptomatic-captive mackerel; 2) identify expression pathways indicative of specific diseases (i.e. viral, bacterial, cancerous). Here we present the results of differential gene expression analysis for *Scomber japonicus* afflicted with PSS and, for the first time, evidence of tumorigenesis in this species.

Methods and Materials:

Sample Collection:

Sample collection and euthanasia of captive-held and wild-caught mackerel were performed as described previously [17]. Briefly, captive *S. japonicus* were obtained from the Monterey Bay Aquarium (MBA) and euthanized with an overdose of MS-222 (i.e. tricaine mesylate). Wild-caught fish were captured from the coast of Monterey Bay, California, USA, using a hook and line and placed in a holding tank for 2-12 days before euthanasia via decapitation. All fish were placed in separate bags and placed on ice or at -20°C until facial necropsy. Epithelial and muscle tissue were taken from behind the nares and anterior to the eyes. A total of 3 symptomatic-captive, 3 asymptomatic-captive, and 3 healthy wild-caught mackerel were used for this analysis.

RNA Extraction, Sequencing, and Trimming:

At Oregon State University (OSU), total RNA was isolated from frozen *S. japonicus* tissue. All surfaces were sterilized with 95% ethanol. Frozen mackerel samples were removed from the -80℃ freezer and held in liquid nitrogen before RNA extraction to ensure RNA stability within tissues. Approximately 0.5 mg of tissue was removed with a sterile scalpel and forceps, homogenized in liquid nitrogen using an autoclaved mortar and pestle, and extracted using the E.Z.N.A DNA/RNA Isolation kit from Omega Bio-tek per manufacturer instructions [21]. RNA was eluted in 50 μl of nuclease-free water and stored at -80 ℃ until library preparation.

RNAseq library preparation and sequencing were performed at OSU's Center for Quantitative Life Sciences. Briefly, ribosomal RNA was removed from the total RNA extractions using an Illumina mouse/rat/human Ribo-Zero kit for host rRNA removal and then a second Ribo-Zero kit for bacterial rRNA removal. After rRNA removal, a stranded RNA-seq library was prepared using the Takara PrepX DNA library prep. Then samples were sequenced on one lane of a 2x150bp PE Illumina HiSeq 3000 run. Illumina HiSeq libraries ranged from 28,745,252 to 49,799,840 paired-end reads. The sequence data was quality-filtered by removing reads with a Phred score lower than 30. Adapters removal, poly(A) tail removal, and read normalized to 30x coverage were all done using the BBtools suite [22]. Library read counts after each quality control step can be found in Supplementary Table 1. The resulting 593,829,134 reads were merged with BBmerge [23].

Informatic pipeline:

Trinity v3.9.1 [24] was employed to build the transcriptome using default settings. Quality-controlled transcripts of individual libraries (i.e. samples) were mapped to the de novo assembled transcriptome using the Salmon Estimation Method packaged with Trinity (v.1.8.0 [25]). Compared to two additional reference-guided assemblies (*Scomber colias* genome and *Scomber scombrus* transcriptome), the de novo transcriptome recruited the most reads among libraries and was used in this study. All three assemblies' metrics and recruitment statistics can be found in Supplemental Material.

DESeq2 (v.3.16) [26] was employed with R statistical programming software (v.4.1.2) [27] to test for differential expression between treatment group pairs to determine whether each model coefficient differed significantly from zero. A two-way ANOVA was used for significance testing. The resulting *p*-values were adjusted for multiple comparisons using Tukey's honest significant difference test in PRISM by GraphPad (v.9.4.1) [28].

Results:

A total of 697 differentially expressed genes (DEGs) were identified with distinct differences between captive and wild-caught fish from the DeSeq2 package (Figure 1c). Genes with adjusted P-value less than 0.01 and an absolute value of log2 more than 1 were defined as differentially expressed. A two-way ANOVA to analyze the effect of captivity and disease status on the transcriptome of *Scomber japonicus* revealed a statistically significant difference between wild and captive status groups ($p < 0.0001$). This result was consistent for all pairwise comparisons between all DGEs in captive and wild groups (Tukey's multiple comparisons test $p < 0.0001$, n=697). No significant difference was seen between the asymptomatic and symptomatic-captive groups. Tumor suppression and oncogenesis genes were highly expressed in captive compared to wild-caught animals (Figure 2a). Immune-related genes and

pathophysiological markers were also significantly over-expressed (Figure 2b-c) (p-value < 0.01 n = 100). Wild-caught *S. japonicus* were assumed to be healthy in all cases as there were no visible signs of disease externally or internally.

Elevated Tumorigenesis Markers Are Characteristic of PSS

Wnt9 was identified as the most highly expressed DEG within captive-held *S. japonicus* tissue. *Wnt9* showed a two-fold difference in expression between captive and wild-caught animals (Figure 1d). This gene is the starting component of the canonical Wnt-signaling pathway responsible for cell proliferation and growth. Other genes identified as differentially expressed in this pathway were *LDLR*, *CCTNB1*, and *CNNA2* (Figure 2A).

Immune-Related Gene Expression Altered in Captive Fish

All immune genes identified as differentially expressed are innate immune system components with some of the most important in fish being *TRIM16* and *TLR8* (Figure 2b). Several of these genes trigger the inflammatory response such as: *HIF-PHI*, *il-11* and *HSP90A1*. All of the aforementioned genes had a 1-1.5 fold increase in expression in the captive-held mackerel. Only *USP25* appears significantly underexpressed in this system by a 1-1.5 fold. *USP25* has been implicated as an innate immune response to pathogens and some cancers.

Pathophysiology are overexpressed in Captive Fish

Genes related to tissue regeneration were overexpressed within captive-held mackerel (Figure 2C). The most abundant of these genes induces collagen production, which appears to be a hallmark of PSS. Genes associated with ossification (*NPPC*), glucose transport and tissue regeneration (*ADIPOQ*), fibrosis (*ENTPD2*), and vascular repair (*B2R*) were also overexpressed in captive-held mackerel. No genes associated with muscle regeneration were found to be differentially expressed.

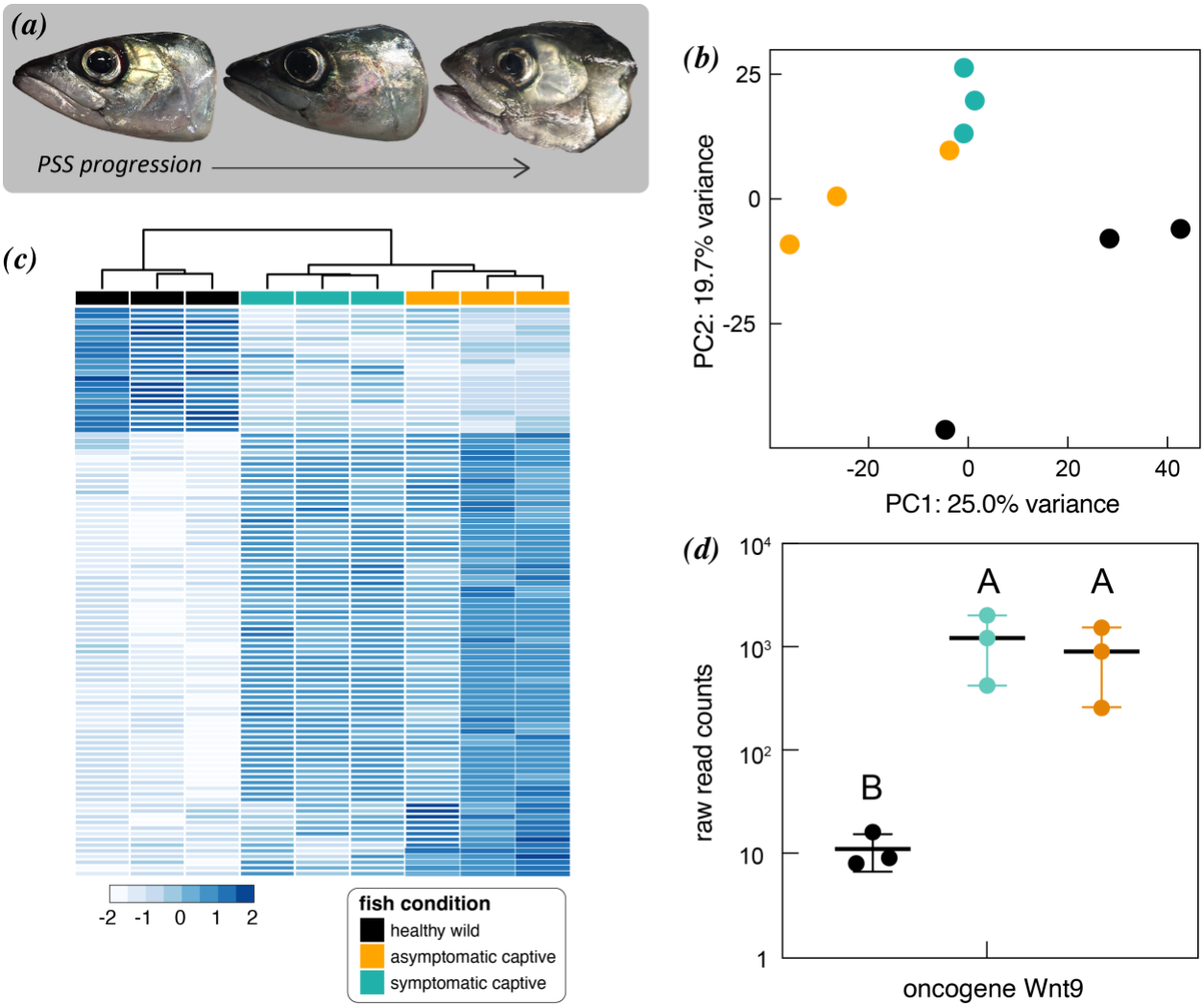

Figure 1: Transcriptomic data shows distinct differences between captive and wild mackerel A) Adapted from Miller et al., 2021[17]; From left to right images of healthy wild fish, asymptomatic-captive fish, and PSS symptomatic-captive fish. B) Principal component analysis plot of all transcripts showing the top 2 axes which explain the most variance. C) Top 100 differentially expressed genes in a heatmap showing clear differentiation by fish origin. D) Raw counts of the gene, *Wnt-9B* in each group. Black boxes and symbols represent healthy-wild mackerel, green represents symptomatic-captive mackerel, and yellow represents asymptomatic-captive mackerel.

Discussion

This work characterizes Puffy Snout Syndrome (PSS) at the transcriptomic level, showing facial tumorigenesis evidence through the pan-cancer marker overexpression within damaged tissues. However, PSS remains an enigmatic syndrome with multiple focal areas for future study. Transcriptomics offers insight into variations in animal physiology by highlighting differences in gene expression amongst healthy, asymptomatic, and symptomatic fish. Here, we found that gene expression in tumorigenesis, immune response, and pathophysiology were dramatically altered in captive fish compared to their wild counterparts. Many genes can be associated with PSS's gross pathology (Figure 2d).

PSS tissues are highly enriched in genes involved in tumorigenesis.

Transcriptomic data suggest that the primary cause of PSS is dysregulation of cell proliferation & differentiation. This could lead to the observed collagenous growths and progressive evolution through hyperplasia and dysplasia in the facial tissue, often characteristic of cancers. This is reflected in the largest detected shift in gene expression in *Wnt9* between wild and captive groups. *Wnt9* is a morphogen in adult animals' early development, cell differentiation/migration, and tissue homeostasis [29]. This gene was elevated ~2-fold in captive fish and may be diagnostic of PSS. The gene for beta-catenin, *CTNNB1*, the other major gene in the *Wnt9* pathway, was also overexpressed in captive fish. Genes in the canonical *WNT*/beta-catenin pathway (Figure 3) are often diagnostic for many human and non-human animal cancers [30]–[32].

Despite its role in tumor suppression, retinoblastoma protein (*Rb1)* was found to be significantly overexpressed in captive fish. *Rb1* regulates mitosis and gene expression by binding chromatin and limiting gene expression of proteins involved in moving cells from the G1 to the S stage [33]. However, *Rb1* phosphorylation by the cyclin-mediated pathway alters its ability to bind these genes [34]. *CCNA2*, which encodes cyclin-A2, which regulates this transition, is also highly expressed ~1.5 fold, suggesting that tumor suppression activity of Rb1 may be inhibited upstream by cyclin over-expression.

Pathogens may explain the differences in the immune gene expression.

We hypothesize that the immune-specific transcriptomic profile results from prolonged physiological stress in captivity or an underlying pathogenic infection such as a virus that captivity may exacerbate. Essential immune genes expressed in response to PSS are indicative of the viral pathogen response in fish and mammals. For example, *USP25* is a form of post-translational modification and has been shown to positively regulate RNA virus-induced signaling by the innate immune system, therefore playing a critical role in antiviral immunity [35]. Studies with *USP25* deficient mice showed they were more susceptible to infection to both herpes simplex virus 1 and influenza A than their wild-type counterparts [36]. *USP25* successfully activated the antiviral cascades of IFN3 and NF-κβ by stabilizing *Traf3* and *Traf6* from viral degradation [31].

TRIM16 is part of the fish-specific TRIM proteins and has been associated with viral infections in fish through negatively regulating interferon production [37]–[40]; higher expression of TRIM16 expression is associated with higher viral loads and poor survival outcomes of fish. Further, in fish, TLR8 primarily identifies single-stranded RNA molecules and is presumed to be a sensor for single-stranded RNA viral infection [41]. Further investigation is required to determine if USP*25, TRIM16, and TLR8* expression patterns are due to a stress response or virus.

Pathophysiology corresponds to the histological examination of tissues.

Often pathophysiological markers show an organism's response to tissue damage but do not necessarily provide information on the cause of that damage. Voorhees, 2015 reported an initial inflammatory response in PSS-afflicted tissue with extensive fibrogenesis followed by collagen's slow replacement of muscle tissue. The overexpressed pathophysiological genes in captive-held animals support this finding. A total of four different collagen types were reported to be over-expressed in captive-held animals. Likewise, no genes associated with muscle regeneration were found to be overexpressed in captive mackerel. Thus, the observed collagen gene expression profiles could be affiliated with the morphology of PSS increased tissue growth on the head and overall tissue inflammation.

We propose that PSS is a condition induced by an infectious oncogenic virus that induces tumor formation, physiological cancer induced by alternate factors and not previously described in wild fish, or transmissible cancer. Given that transmissible cancers are extremely rare in wildlife, with few exceptions, such as in marine molluscs, terrestrial canines, and rodents, we can mostly but not completely rule this out. Further, no transmissible cancer has been described in teleosts, so it remains an unlikely explanation. However, our previous microscopy work showed the presence of small viral particles and large numbers of deformed mitochondria in affiliated fish. Given these visual observations, the increased expression of tumorigenesis genes, and genes involved in the innate immune response, viral infection-inducing tumorigenesis seems most likely. Further characterization of the presence and abundance of viral-specific genes and genomes and additional viral-induced cytopathology will be required to confirm these hypotheses.

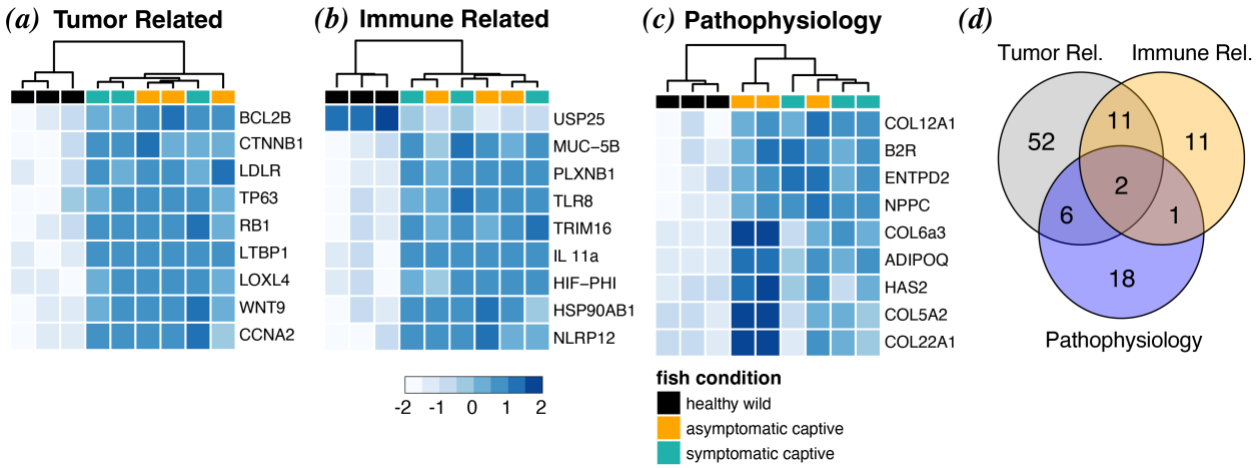

Figure 2: Selected DGEs: A) tumor-related genes; B) immune genes; C) and pathophysiological genes. D) Venn diagram showing the overlap of the top 100 genes within these three groups. Black boxes represent healthy-wild mackerel, yellow represent asymptomatic mackerel, green represents symptomatic mackerel.

Data Accessibility:

Raw reads and transcriptome were deposited to the figshare data repository under DOI: 10.6084/m9.figshare.22779725. Raw reads were also uploaded to NCBI under BioProject number PRJNA967399.

Authors' contributions:

All authors contributed to the writing in this manuscript. E.M performed all necropsies. S.L., M.D., and T.G. performed DNA/RNA extractions and library preparation. S.L and K.V created all figures. R.V.T. provided the grants for this work.

symptomatic-captive mackerel, and yellow represents asymptomatic mackerel.

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Citations:

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