nature portfolio

Peer Review File

Brown bullhead catfish melanoma represents a novel transmissible cancer.

Corresponding Author: Dr Julie Dragon

Any redactions in this file are there to maintain patient confidentiality, the confidentiality of unpublished data, or to remove third-party material.

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Referee #1

(Remarks to the Author)
We found the study by Dr Curd and co-authors (A novel fourth type of transmissible cancer; clonal malignant melanoma in the benthic fish species Ameiurus nebulosus), fascinating with results presenting of immediate interest to conservation and evolutionary biologists, disease epidemiologists and cancer researchers.

The article addresses a very important topic, the discovery of a fascinating infectious disease, a transmissible cancer, in a new vertebrate host, a freshwater benthic fish. Naturally occurring transmissible cancers (that have been proposed to be new parasitic life forms) have so far only been discovered in two vertebrate groups, in dogs and Tasmanian devils. Recently several transmissible cancers have been discovered in marine ecosystems, where their presence is becoming concerning as they can wipe out populations of ecosystem engineers, i.e. bivalves. Studies investigating these infectious diseases are therefore urgently needed. The study by Curd et al. highlights the importance of surveying other ecosystems, like freshwater bodies (that may or may not be exposed to pollution) to discover, mitigate and manage the emergence of these novel parasitic life forms.
While we found the article very interesting, we believe it would benefit from further polishing, clarifications and some additional analyses before the manuscript could be considered for publication.

We understand the samples were collected at different time points for different purposes, and we are aware of the difficulties in obtaining consistent wildlife samples. We also appreciate the longitudinal nature of the study, but we got lost in the sampling methods, sample compositions and such, the results and interpretations.
For example, we were excited to see Figure 2. “The average variant allele frequency (VAF) for mitochondrial SNV sites in heteroplasmic tissue samples”. Our immediate thought was that: could the Low frequency (LF) tumours represent a more older tumour lineage compared to the High Frequency (HF) tumours that appear to be more diverged? Figure 3. Phylogeny of phased mitochondrial genomes shows a similar pattern, where the high frequency tumours appear to be more diverged. Would this be an artefact of sampling from different time points or would this species be affected by multiple cancer lineages. If yes, have those lineages arisen independently (like in the Tasmanian devils)? Or have several tumours been sampled/fish and the low/high frequency tumours represent different stages of disease progression? For example, fish HC4T appears to have both LH and HF tumours, so probably the latter? Could different stages of cancer progression affect observed frequencies?

Under the supplementary materials the authors declare that:
“Histologically the LF samples did not show evidence of a tumour but rather proliferations of melanocytes in the dermis and epidermis, and the skin structure was intact with no sign of metastases and little to no invasion into underlying muscle, which is consistent with a low ratio of clonal tumour DNA sequencing reads to host DNA sequencing reads.”
Based on that statement, we would assume the low vs high frequency tumours represent the process of tumour progression in this fish, but we would be very keen to hear the opinion and a potential discussion by the authors. There is a min. 5 fold difference in VAF between LF and HF tumours, is this expected during melanoma development, or is it higher than in human cancers?

In Figure 3 and Supplementary Figure 2. It appears that tumours were detected in other populations (?) or at time points of sampling (years?) (again, hard to follow the sampling process) and we were curious about the opinion of the authors whether these tumour samples represent earlier/later/different lineages of the same transmissible cancer, or do they represent additional independently emerged cancers? We were hoping to see a phylogenetic tree just based on the tumour samples to understand their evolutionary history (are they independently emerged lineages, or one cancer lineages evolving over time?). For example, in Fig 3. samples SB14T-CT and SB14T-LCT and in Supp. Fig. 2. SB14-ST-TN seem to be highly diverged tumour lineages in a different population (or sampling point?), could this lineage be the ancestral cancer line that emerged first?
Would the authors conclude that the LF tumours are not transmissible cancers (but rather "classical" melanocytes) and the HF tumours are the transmissible cancer? Or would they believe the LF tumours present the first stage of transmissible cancer (right after transmission) and the HF tumours would be the late stage (cancer cells "ready to transmit")?

Including a molecular clock analysis of the tumour samples only (without the host tissue samples) would help deciphering the relationships between these fascinating cancers and add an exciting dimension and depth to the study. Since the authors sampled across an 8 year period (2015 to 2023), have access to epidemiological and historical data ("to infect 30% of fish in Lake Memphremagog by 2014, after not being present at noteworthy levels prior to 2012"), they could include those as priors in a Bayesian phylogenetic analysis (BEAST) to determine the evolutionary history, age, potential most recent common ancestor, the dynamics etc. of this novel transmissible cancer. That would also help to make the study a bit less descriptive and would allow a stimulating discussion on the evolution of transmissible cancers.

Would the authors have multiple tumour samples from the same fish? Or would the authors have been able to sample the same fish over time? According to the manuscript they were all sampled through electroshock, so we assume the fish have died and they were only sampled at one time point. We however couldn't see if multiple samples were taken from the same fish as the data presented (Supp table 3) has 'sample' ID only. Based on Supp table 3 it appears overall the authors sampled 85 fish, and they found 41 lesions, so if only one sample was taken/fish, that means almost 50% prevalence. Prevalence can be misleading when sample sizes are not representative of the populations surveyed. We noted the study cited (Blazer et al 2019) reported around 30% prevalence based on much higher sample sizes. Can the authors comment on how the prevalence estimates in the Blazer et al study differs from the ones presented in this study. Do these estimates reflect what could be expected from a clonal cancer with high transmissibility as postulated?

Detailed comments:
Line:
"To date no viruses or other microorganisms have been identified as related to the origination of the Lake Memphremagog melanomas (see Supplementary Section 7 and Supplementary Tables 8,9), …"
>After looking at section 7 and supp tables 8 and 9, the authors appeared to have found viruses in their samples. Could they please clarify if they can categorically demonstrate the lack of a viral agent in the sampled lake.

Line: "To date, transmissible cancers have only been observed in three types of animals: dogs, Tasmanian devils, and bivalve species4."
>Potentially add "naturally occurring transmissible cancers" as cancer transmissions have been observed in laboratory setting and under particular circumstances in humans.
Lines: "This supports clonal lineage of the tumor samples in Lake Memphremagog that predates 2015 and did not originate in Lake Memphremagog." and Figure 1. Brown bullhead sampling…
"do not know the extent to which this cancer lineage may already exist in other populations throughout North America."

>We understand this is the first study to describe the transmissible cancer, but we were wondering whether melanoma has been observed in other regions across the distribution range of the species. This is not a criticism to the sampling approach, but rather a reflection on the origin of the cancer and the susceptibility of the species.
Figure 2 (figures in general).
It might be due to converting image files to pdf, but some of the symbols appear to be not present on the figure. For example, there are filled circles with a dash line over them on Figure 2 in the legend, but they don't appear on the figure. Please check for consistency.

"We find tumors are enriched for arsenic (V. Blazer personal communication, 2025), but it is not clear if it plays a role in facilitating transmission of the tumor or is a result of the metabolism of the cancer cells."

>Reading the literature revealed that a drain beneath Coventry Landfill in Vermont has been discharging leachate containing arsenic and other chemicals into nearby wetlands that flow into Lake Memphremagog. Would the authors be able to link a pollution event prior to 2014 to the emergence/initiation of the melanomas? Have they observed any signs of cancer in other fish/species inhabiting Lake Memphremagog?

Line: "necessity of containment efforts."
>Can the authors expand and explain what containment efforts could be undertaken to prevent cancer spread? It seems to us that the cancer is spread throughout the lake, and we don't see much scope for containment.
It also appears that "it has been observed that older fish with extensive tumors in and around their mouths and gills persist seemingly with little impact from the tumors"
>This comment is not supported by any datapoint in the manuscript. Would the authors have a reference or pers.com to support this statement? Would the authors consider that the fish potentially have adapted to the disease (via tolerance or

resistance)? The tumours have only been observed since 2012 … that would mean very fast adaptation from the fish. What is the generation time of these bullfish? Or would the authors consider the cancer being highly transmissible but low pathogenic (but they also found metastasis to other organs in the fish)? Could the authors comment on the potential evolutionary path and trajectory of bullheads and their transmissible cancer might take?

Based on the comments and questions above, it's clear, we are very supportive of the publication of this manuscript, once our queries have been answered.

Beata Ujvari and Rodrigo M. Hamede

Referee #2

(Remarks to the Author)
The key finding here is that there is a new transmissible cancer, that is found in fish for the first time and also for the first time, is melanoma. This is highly original and exciting scientifically but could have major implications for the aquatic ecosystems. The writing is clear and very accessible to a wide audience.

Suggestions for improvements:

(1) The authors might want to frame their work within the context of other natural forms of pigmentary lesions in fish, such as Xmrk (Xiphophorus), melanoma in trout, salmon melanocytic lesions...This might help show that this is really distinctive from these natural pigment lesions.

(2) The quality of the Figure 1B should be improved (it is rather rudimentary).

(3) Is there evidence of the species origin of this melanoma? Can the genomic data be used to understand the species. The authors say that the cancer pre-dates 2015 and did not originate in Lake Memphremagog.

(4) Can the genomic data be used to understand if there is key driver mutation? This might also be useful for histopathology to see the "mosaic of clonal tumor and host cells" in the tumour tissue.

(5) I would suggest removing the "personal communications" bit in the discussion. If the data is too preliminary to publish, then best to not include.

Referee #3

(Remarks to the Author)
The manuscript tests the hypothesis that melanoma-type tumour lesions in a benthic fish species, Ameiurus nebulosus, are a transmissible cancer, in which the tumour cells are distinct from the host and can spread between individuals, akin to a parasite. The authors outline that these types of cancer are rare but have been described in two mammalian species and 10 species of marine bivalves. In this manuscript the researchers have used genomic tools to test the hypothesis of transmissibility by sequencing (using short read, long read and RNA) the mitochondrial and nuclear genomes of normal (no tumour lesions) brain tissue and skin tissue, as well as brain tissue and skin tissue from fish with black lesions. The samples were collected at three time points, 2015, 2022 and 2023.

Analysis of the mitochondrial genomes demonstrates heteroplasmy in all tumour lesions samples and a proportion of normal tissue. Phylogenetic analysis of mitochondrial SNVs shows that most of the tumour samples form a monophyletic clade, with the tumours more closely related to one another than to the normal fish samples, consistent with a transmissible cancer lineage. The tumours are separated into low frequency (LF) and high frequency (HF) variant samples, with HF samples more closely related to each other and exhibiting patterns of phylogeny consistent with a transmissible tumour. The LF samples are less clear cut, see further points below.

Analysis of the nuclear genomes supports the clustering of HF tumour samples in a monophyletic clade, distinct from normal fish samples. Further, analysis of shared variants in HF samples highlights the similarity of tumour samples (through shared variants) compared to normal samples. This data is contrasted to melanoma samples in humans, which demonstrate few shared variants between tumours from different individuals. Similarly, structural variants are more shared between the HF tumour samples than between normal tissues.

The evidence presented by the authors strongly suggests that this is indeed a transmissible tumour. This is challenging to prove conclusively, but the comparisons of SNVs and SVs, particularly among the HF tumour samples are compelling evidence. As such, it makes an original and impactful piece of work. However, I do have several concerns as to how the data is presented and the conclusions drawn in the paper, particularly in regards to the 'low frequency' (LF) tumours.
• The different sequencing methods utilised and the lack of consistency in methods across the samples causes issues in the analyses and conclusions that can be drawn. This is apparent in the division of samples into low frequency and high frequency tumours (Figure 2 and Figure 3), with the low frequency tumours clustering separately to the high frequency tumours, with SB14 tumour more closely related to normal tissues (Figure 3). The authors attribute this to host contamination

(ie fewer tumour cells) in the low frequency tumours, but this may also be an artefact of sequencing depth in these samples, or an independent cancer lineage. Can the authors normalise the data and SNV calling for read depth?
• I would also like to see a direct comparison of LF and HF tumours in terms of sequencing depth for the nuclear genome and shared SNVs, this is difficult to see from the supplementary material.
• In the supplementary material the authors note that 'histologically the LF samples did not show evidence of a tumor', but no histopathology is shown in the supplementary materials or main paper. Given the division into LF and HF tumours the pathology the authors refer to should be shown or histopathology should be included.
• The clustering of the low frequency tumours with the normal tissues (ie. Figure 3, Supp Figure 6) is attributed to chimeric tumour and host, but can the authors be sure of their interpretation? The data may indicate non-transmissible, independently arising tumours or an early lineage of the transmissible tumour. Again, histopathology and consistent methods of sequencing across samples would assist in making this point more robust.
• Given the reference genome generated and the extensive sequencing of non-tumour samples it should be possible to identify germline variants and tumour specific variants?
• The samples collected in 2015 were sequenced by RNA_seq. This data appears to be only used in Figure 2, but could provide insight into gene expression changes in the tumour? Is there a reason the data was not analysed further?
• The supplementary information also includes an analysis of the microbiome/mico-organisms, this is not included in the main paper, but a brief description of the findings would be valuable, given the possibility of tumour inducing viruses.

Referee #4

(Remarks to the Author)
The key results suggest the existence of a transmissible tumor in brown bullhead. However, some key steps, in my mind are missing to demonstrably demonstrate its existence. First, the authors rely heavily on mtDNA for their analysis, which could be simply different due origin from a different (migrant female). Overall, the results are not convincing. The authors could follow the processes used to demonstrate the existence of other transmissible cancers. First of all, there is no real histology to demonstrate the cancer or type. Secondly, Koch's postulates were not satisfied; the tumor could have been isolated in independent fish cell lines (which are easy to culture) and cultures could have been used to infect other fish. Therefore the data are lacking to convincingly demonstrate a transmissible tumor, and the blueprint is there to do so, which includes whole genome sequencing, demonstrating the tissue the tunmor is expressed in skin tissue using transcriptomics.

While well-written, the results of the paper do not convincingly demonstrate a transmissible tumor. As such, I cannot recommend the paper in its current form.

Referee #5

(Remarks to the Author)
I co-reviewed this manuscript with one of the reviewers who provided the listed reports.

Version 1:

Reviewer comments:

Referee #1

(Remarks to the Author)
The authors have satisfactorily addressed all previously raised concerns. Their methodology, sample collection procedures, and data analyses are now presented with greater clarity and coherence, making the study easier to follow. The manuscript is streamlined, well written, and demonstrates originality with significant implications for our understanding of transmissible cancer evolution and ecology. The conclusions are robust, supported by rigorous statistical analyses. We commend the authors on this excellent work and look forward to future studies that further explore this novel and compelling transmissible cancer cell line.
Beata Ujvari and Rodrigo Hamede

Referee #2

(Remarks to the Author)
I continue to find this a fascinating discovery that has significant implications for our understanding of cancer biology as well as ecology.

1. I disagree with the authors that their Figure 1F is clear. It requires reading their methods to understand their cohort of samples and apply it to this figure (what is normal tissue etc; samples numbers; controls). I think this figure is the opportunity to explain their cohort and study design, as well as the hypothesis. The authors even agree in response to Reviewer 1 that "We agree that the details of the study design were confusing." They also address comments about metastatic disease to the Reviewers, which are reasonable questions based on the biology of melanoma (the brain in the schematic looks like it is a metastatic site; which is a major problem in the human disease).

2. While the pathology of the lesions has been published previously, I think the paper could have improved impact with some basic H&E so we can see the pathololgy of the disease and see the cancer cells.

3. Line 431 discusses transmissionable cancers in over a dozen species, and yet in line 72 the authors say that the transmissionable cancers have "only been observed in three types of animals". Please clarify these statements.

4. Line 67 - I would recommend reconsidering how this sentence is cited. Based on the way it is written, I thought that the hypothesis was generated in the citation. Upon reading that (excellent) reference, I now understand it is meant as a reference about transmissible cancers generally.

Referee #3

(Remarks to the Author)
The revised manuscript is significantly improved from the original, in particular the evidence in support of these melanomas as transmissible tumours is strengthened. The re-sampling, additional samples from 2019 and associated sequencing has sufficiently resolved the ‘low frequency’ tumour samples showing that these were an artifact of the sampling and has confirmed the monophyletic nature of the tumour samples. The addition of the normal tissue samples from Maine and New Hampshire has also improved the analyses, suggesting an origin for the tumour beyond Lake Memphremagog. The authors have removed the long read sequence data from the analyses in Figure 2 and Supplementary Figure 3, confining these to short read sequence data, which improves consistency in the analysis. I am also satisfied with the description and analyses of viruses and microorganisms in the work.
A number of the suggestions (ie. identification of tumour specific variants and further use of RNA_seq data) have not been followed up by the authors, but I do accept their justification that the need for consistently higher sequencing depth across additional samples (ie more sampling) pushes these experiments outside the scope of the current work.
Two minor issues in the Introduction:
Line 83 – replace ‘an’ with ‘a’
Line 89 – I would begin this line with 'An indicator of a transmissible cancer…’ the way the sentence is currently phrased indicates that the relationship between cancer genomes and the host genome is the only parameter required to prove cancer transmissibility. The phrasing may also be confused with Hanahan’s papers on the Hallmarks of cancers.
Overall, the manuscript is now well written, and the data well supports its conclusion, that a transmissible tumour is circulating in Ameiurus nebulosus, the first contagious cancer to be identified in a fish.

Referee #4

(Remarks to the Author)
Significant improvement from original MS. Satisfactory responses to reviewer comments.

Referee #5

(Remarks to the Author)
I co-reviewed this manuscript with one of the reviewers who provided the listed reports.

We thank all reviewers for their thorough reading and insightful feedback on our manuscript. Below we provide responses in green text to respond to each reviewer concern. Most notably, in response to overlapping concerns by multiple reviewers we re-sequenced the low frequency samples and verified they were low purity samples rather than different lineages of tumor (see "The resolution of the low tumor purity samples" section below). All samples now definitively resolve as part of the same clonal cancer lineage and present a much clearer narrative without the need to present different sequencing types in the main body of the paper. We also add additional samples from 2019 that we sequenced since the initial submission of this manuscript, added an analysis of shared copy number variants that further supports a clonal origin, and added a results section to directly address the alternative hypothesis that a virus/microbe could be involved with the cancer. We were happy to see reviewers' enthusiasm suggesting additional analysis to identify such questions as cancer age, driver mutations, and differential expression, but explain our reasoning that these requests are out of scope of this paper. Together, these revisions address the reviewers' major concerns and reinforce the central conclusion of a transmissible clonal cancer lineage in brown bullhead.

Referees' comments:

The resolution of the low tumor purity samples:

The low frequency (LF) vs high frequency (HF) tumor samples were a major and valid cause for concern across reviewers. To resolve this issue, we went back to the frozen carcasses of fish identified as having low frequency tumors and collected new tissue samples from the center of tumors. The original tissues were collected from the margins of tumors. As an example, see the red circle on the picture of fish JR9 below (Figure 1). Notice that the majority of the skin removed (red circle) had little to no underlying melanistic tissue. It also resembles the samples collected from healthy skin (purple circles). In contrast, we resampled the center of the tumor (black tissue in the blue circle) and despite sampling the same depth into the tissue, we found no healthy tissue. When we sequenced the new tissue sample, the mitochondrial tumor tissue VAF increased from < 0.1 to > 0.7, indicating a much higher tumor purity comparable to the other samples. This re-sampling effort and resulting monophyletic phylogenies show that the low frequency samples were the result of methodological sampling artifact and do not represent a biologically meaningful difference between fish tumor genomes.

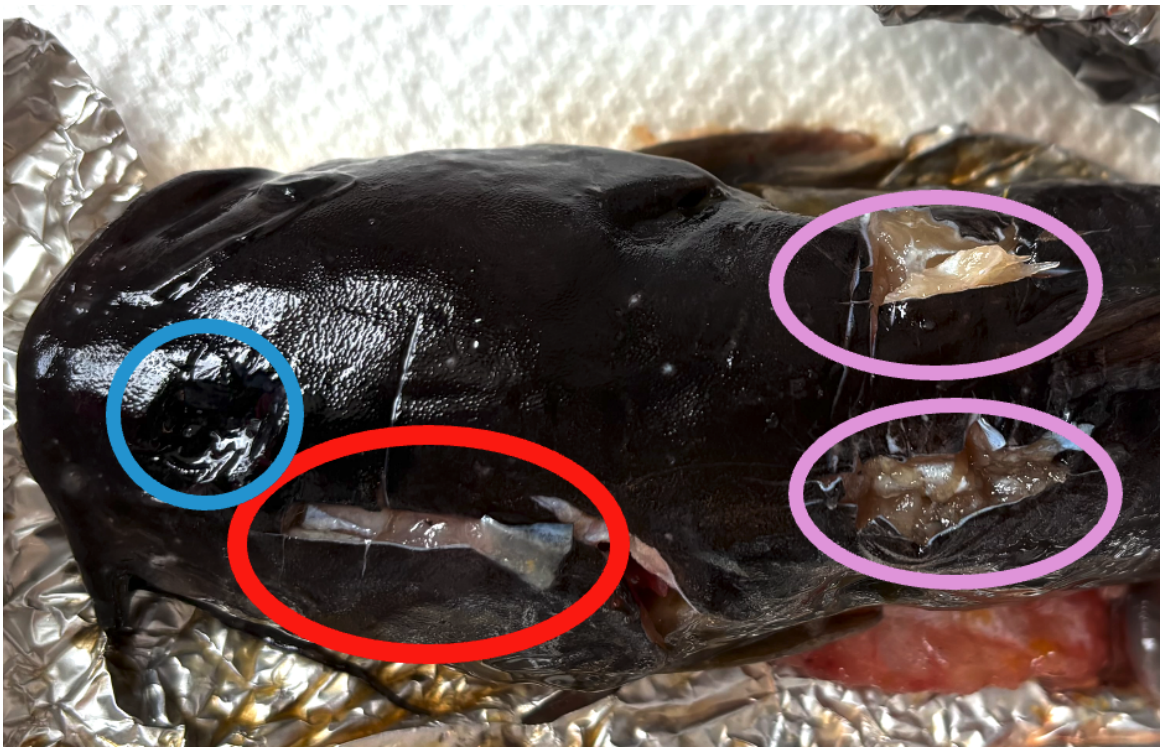

Figure 1. Image of the frozen carcass of fish JR9. The initial low frequency sample was collected at the left edge of the red circle. The purple circles indicate healthy skin samples. The blue circle indicates the re-sampled tumor tissue.

Referee #1 responses (lines 45-323)
Referee #2 responses (lines 324-377)
Referee #3 responses (lines 378-503)
Referee #4 responses (lines 504-555)

Referee #1 (Remarks to the Author):

We found the study by Dr Curd and co-authors (A novel fourth type of transmissible cancer; clonal malignant melanoma in the benthic fish species Ameiurus nebulosus), fascinating with results presenting of immediate interest to conservation and evolutionary biologists, disease epidemiologists and cancer researchers.

The article addresses a very important topic, the discovery of a fascinating infectious disease, a transmissible cancer, in a new vertebrate host, a freshwater benthic fish. Naturally occurring transmissible cancers (that have been proposed to be new parasitic life forms) have so far only been discovered in two vertebrate groups, in dogs and Tasmanian devils. Recently several transmissible cancers have been discovered in marine ecosystems, where their presence is becoming concerning as they can wipe out populations of ecosystem engineers, i.e. bivalves. Studies investigating these infectious

diseases are therefore urgently needed. The study by Curd et al. highlights the importance of surveying other ecosystems, like freshwater bodies (that may or may not be exposed to pollution) to discover, mitigate and manage the emergence of these novel parasitic life forms.

While we found the article very interesting, we believe it would benefit from further polishing, clarifications and some additional analyses before the manuscript could be considered for publication.

We would like to thank Beata Ujvari and Rodrigo M. Hamede for their careful consideration of and thoughtful comments regarding our paper submission. We too are fascinated by the discovery of this transmissible cancer and share your concerns about what it means for these fish. Please find our response to these comments below:

We understand the samples were collected at different time points for different purposes, and we are aware of the difficulties in obtaining consistent wildlife samples. We also appreciate the longitudinal nature of the study, but we got lost in the sampling methods, sample compositions and such, the results and interpretations.

We agree that the details of the study design were confusing. We complicated the design to explain the initial LF vs HF sample phenomenon. Since removing the LF vs HF sample issue (see the resolution of low tumor purity samples section above), we simplified our analyses by primarily presenting the short read DNA results in the main body of the manuscript. We address the analyses of other types of sequencing data in the supplementary materials. We did add an additional 9 melanistic fish (collected from Lake Memphremagog in 2019) to the short reads data analyses.

For example, we were excited to see Figure 2. “The average variant allele frequency (VAF) for mitochondrial SNV sites in heteroplasmic tissue samples”. Our immediate thought was that: could the Low frequency (LF) tumours represent a more older tumour lineage compared to the High Frequency (HF) tumours that appear to be more diverged? Figure 3. Phylogeny of phased mitochondrial genomes shows a similar pattern, where the high frequency tumours appear to be more diverged. Would this be an artefact of sampling from different time points or would this species be affected by multiple cancer lineages. If yes, have those lineages arisen independently (like in the Tasmanian devils)? Or have several tumours been sampled/fish and the low/high frequency tumours represent different stages of disease progression? For example, fish HC4T appears to have both LH and HF tumours, so probably the latter? Could different stages of cancer progression affect observed frequencies?

The revised analyses with the re-sequenced 2023, and the new 2019 samples are consistent with a single clonal origin transmissible tumor. We removed Figure 2 and

address variant allele frequency (VAF) for mitochondrial SNV and SNV sites in Supplementary Figure 2. The current phylogenetic trees for the mitochondria show that all tumor tissues have a similar mitochondrial genome, and the previously HF/LF differences noted were artifacts of low tumor purity rather than biological differences in the tumors. With the greater purity we could also confirm this clonal relationship with the nuclear genome, where all tumors formed a monophyletic clade.

Under the supplementary materials the authors declare that:
“Histologically the LF samples did not show evidence of a tumour but rather proliferations of melanocytes in the dermis and epidermis, and the skin structure was intact with no sign of metastases and little to no invasion into underlying muscle, which is consistent with a low ratio of clonal tumour DNA sequencing reads to host DNA sequencing reads.”
Based on that statement, we would assume the low vs high frequency tumours represent the process of tumour progression in this fish, but we would be very keen to hear the opinion and a potential discussion by the authors. There is a min. 5 fold difference in VAF between LF and HF tumours, is this expected during melanoma development, or is it higher than in human cancers?

The revised analysis suggests that the same tumor is responsible for the development and proliferation of the melanistic lesion, from small proliferations of melanocytes to raised black melanomas. The major consideration for current and future sampling is whether we can collect enough tumor cells in the tissue such that the ratio of tumor to host cells is high enough for genomic characterization of the tumor. We removed that particular passage from the Supplementary Materials, based on our success resampling and re-sequencing the former LF samples.

In Figure 3 and Supplementary Figure 2. It appears that tumours were detected in other populations (?) or at time points of sampling (years?) (again, hard to follow the sampling process) and we were curious about the opinion of the authors whether these tumour samples represent earlier/later/different lineages of the same transmissible cancer, or do they represent additional independently emerged cancers? We were hoping to see a phylogenetic tree just based on the tumour samples to understand their evolutionary history (are they independently emerged lineages, or one cancer lineages evolving over time?). For example, in Fig 3. samples SB14T-CT and SB14T-LCT and in Supp. Fig. 2. SB14-ST-TN seem to be highly diverged tumour lineages in a different population (or sampling point?), could this lineage be the ancestral cancer line that emerged first? Would the authors conclude that the LF tumours are not transmissible cancers (but rather “classical” melanocytes) and the HF tumours are the transmissible cancer? Or would they believe the LF tumours present the first stage of transmissible cancer (right

after transmission) and the HF tumours would be the late stage (cancer cells “ready to transmit”)?

Former Figure 3 and Supplementary Figure 2, are replaced with Figure 2 and Supplementary Figures 3 and 4. We dropped the long reads sequences (that we used to compensate for different tumor mitochondrial VAF in LF sequences) from these trees to minimize confusion and replaced the original LF data with the resampled and re-sequenced sample data. We also added the 2019 tumor normal pairs. Figure 2 now shows only Tumor and Normal mitochondria from tumor fish, and normal mitochondria from reference fish. All tumor samples presented in this manuscript were from Lake Memphremagog, albeit from different time points. We also added additional normal fish from a population in Maine and a population in New Hampshire. The resulting tree indicates that all tumor lineages are very similar indicating a clonal origin. We also added the mitochondrial variants found in RNASeq data to Supplementary Figures 3 to show that this clonal tumor was present in 2015, and the mitochondria are nested within the clonal cancer clade.

Including a molecular clock analysis of the tumour samples only (without the host tissue samples) would help deciphering the relationships between these fascinating cancers and add an exciting dimension and depth to the study. Since the authors sampled across an 8 year period (2015 to 2023), have access to epidemiological and historical data (“to infect 30% of fish in Lake Memphremagog by 2014, after not being present at noteworthy levels prior to 2012”), they could include those as priors in a Bayesian phylogenetic analysis (BEAST) to determine the evolutionary history, age, potential most recent common ancestor, the dynamics etc. of this novel transmissible cancer. That would also help to make the study a bit less descriptive and would allow a stimulating discussion on the evolution of transmissible cancers.

We would very much like to run a molecular clock analysis with the nuclear data collected from tumor fish, and plan to do this in the coming years. The current data set is lacking the sampling depth and the longitudinal coverage to easily address these questions. Unfortunately, no frozen carcasses or DNA sequences exist for the 2015 samples (which were only RNA sequenced), though we now have samples from 2019 and 2023, and expect to collect more over the coming years. We hope a few more time points will be sufficient to calibrate a molecular clock, alongside deeper sequencing for sensitive resolution of host versus tumor nuclear variants, though that will depend on the age of the cancer and the consistency of mutational processes, which are unknown. We are also sampling more geographic locations to find populations with and without tumors to address the other interesting evolutionary questions. Overall, this endeavor is likely to take multiple years and thus outside the scope of this initial paper.

Would the authors have multiple tumour samples from the same fish? Or would the authors have been able to sample the same fish over time? According to the manuscript they were all sampled through electroshock, so we assume the fish have died and they were only sampled at one time point. We however couldn't see if multiple samples were taken from the same fish as the data presented (Supp table 3) has 'sample' ID only. Based on Supp table 3 it appears overall the authors sampled 85 fish, and they found 41 lesions, so if only one sample was taken/fish, that means almost 50% prevalence. Prevalence can be misleading when sample sizes are not representative of the populations surveyed. We noted the study cited (Blazer et al 2019) reported around 30% prevalence based on much higher sample sizes. Can the authors comment on how the prevalence estimates in the Blazer et al study differs from the ones presented in this study. Do these estimates reflect what could be expected from a clonal cancer with high transmissibility as postulated?

We did not collect tissue for DNA sequencing for all of the healthy fish caught during the sampling, thus the incongruence in ratio of tumor prevalence in the supplementary data relative to the Blazer et al. 2019 paper. The Blazer et al. 2019 paper considers all of the fish caught during 2023 and has the correct ratio.

When these fish were collected in 2023 we had different hypotheses for the origination for the tumors and only sequenced the tumor tissues collected from the tumor fish samples included in this paper. We only sampled a single tumor per fish, and all fish were lethally sampled. We revised the text of the Supplementary Figure for clarification: Supplementary Table 2. Summary of the Lake Memphremagog DNA Samples sequenced for this study. This is a subset of the fish collected during the sampling events, where not all tumor fish were sampled, and only single tumors were sampled per fish.

We did not consider looking at two tumors per fish and think monitoring progression in individual fish over time would be helpful for understanding tumor evolution within and between fish. We are piloting non-lethal sampling methods for tumor tissue which would enable this type of study.

As to what frequency of transmissible cancer incidence is reasonable to expect in a population, it is difficult to say without knowing more about the biology of the tumor, mode of transmission, and lethality of the tumor, among other variables. Other transmissible cancers display wide ranges of incidences by locality. In soft-shell clams, some populations show up to 90% incidence while others have low levels (<5%) or a complete absence of transmissible tumors. Since the 30% incidence reported by Blazer et al. is within this wide range, it seems reasonable for a transmissible cancer.

Detailed comments:
Line: “To date no viruses or other microorganisms have been identified as related to the origination of the Lake Memphremagog melanomas (see Supplementary Section 7 and Supplementary Tables 8,9), …”
>After looking at section 7 and supp tables 8 and 9, the authors appeared to have found viruses in their samples. Could they please clarify if they can categorically demonstrate the lack of a viral agent in the sampled lake.

We added the following paragraph to **Supplementary Material**:

9.2 Viral Results:
Of the viruses found in our fish samples, the majority were viruses of prokaryotes. We identified three viral lineages that are known to infect animals, and two that generally infect Eukaryotes. Of the Viruses of Animals, a member of Amarillovirales was found in a single tumor tissue, a member of Blubervirales was found in a single normal tissue, and a member of Orthopolintovirales was found in a single normal tissue. Of the viruses of Eukaryotes, a member of the Picornavirales (originally classified from brown bullheads) was found in a single tumor and a single normal tissue. A member of the Ortervirales was found in four normal tissues.

We added the following to the **Materials and Methods** in the main manuscript:

Screening for Microorganisms in Tissue Samples:
We screened tissue DNA and RNA sequences for viral and other cellular organisms. Any sample read that did not map to the HL4 genome was processed used nf-core/mag (Ewels et al., 2020; Krakau et al., 2022) to generate assemblies (Li et al., 2016; Nurk et al., 2017) and run classification of mobile genetic elements (virus and plasmid) using geNomad on assembled contigs (Camargo et al., 2023) and classification of prokaryotic and eukaryotic organisms on sequencing reads with Kraken2 (Wood et al., 2019).

Kraken2 data were imported into R using phyloseq (McMurdie & Holmes, 2013). We explored differential abundance of taxa between tumor and normal samples for each dataset (RNA or DNA) using DESeq2 (Love et al., 2014). To remove false positives, we filtered out organisms with less than 50 counts across the data set, and that were present in fewer than a quarter of the sample subset prior to running the analysis.

We added the following to the **Results** in the main manuscript:

No viral or microbial agents associated with tumor tissues:
We screened the tumor and normal tissues for viral and cellular organisms (Supplementary Materials Section 9.) to identify potential agents associated with melanistic lesions. We found many viruses of prokaryotes but there were so few viruses of animals or eukaryotes found in our samples that we were unable to run a

formal statistical analysis (Supplementary Table 10) . We identified and ran a differential abundance analysis of cellular organisms found in tumor and normal tissue (Supplementary Table 11), and did not identify any microbial genera that were associated with tumor tissue. If there is a virus or microbial agent that facilitates tumorigenesis, it was not present in our tumor samples.

Line: “To date, transmissible cancers have only been observed in three types of animals: dogs, Tasmanian devils, and bivalve species4.”
>Potentially add “naturally occurring transmissible cancers” as cancer transmissions have been observed in laboratory setting and under particular circumstances in humans.

Added the following to the introduction: “To date, **naturally occurring** transmissible cancers have only been observed in three types of animals: dogs, Tasmanian devils, and bivalve species.”

Lines: “This supports clonal lineage of the tumor samples in Lake Memphremagog that predates 2015 and did not originate in Lake Memphremagog.” and Figure 1. Brown bullhead sampling… “do not know the extent to which this cancer lineage may already exist in other populations throughout North America.”

>We understand this is the first study to describe the transmissible cancer, but we were wondering whether melanoma has been observed in other regions across the distribution range of the species. This is not a criticism to the sampling approach, but rather a reflection on the origin of the cancer and the susceptibility of the species.

There are other populations in which similar melanistic lesions have been observed across the northeast. iNaturalist and the fisherperson social media app *Fishbrain* have quite a few pictures of likely tumor fish which phenotypically appear similar to the Memphremagog tumors. Additionally, the paper we cite from 1925 with similar tumors, the experimenters even transmit between fish themselves, makes it likely that this lineage is widespread or brown bullhead are very susceptible to incipient transmissible cancers. At this point it is not clear whether other observations fit one of these two possibilities, or represent phenotypically similar conventional melanomas, and will require widespread sampling and sequencing to address.

Figure 2 (figures in general).
It might be due to converting image files to pdf, but some of the symbols appear to be not present on the figure. For example, there are filled circles with a dash line over them on Figure 2 in the legend, but they don’t appear on the figure. Please check for consistency.

Thank you for pointing this out. We remade most of our figures and double checked them for consistency and completeness.

"We find tumors are enriched for arsenic (V. Blazer personal communication, 2025), but it is not clear if it plays a role in facilitating transmission of the tumor or is a result of the metabolism of the cancer cells."

>Reading the literature revealed that a drain beneath Coventry Landfill in Vermont has been discharging leachate containing arsenic and other chemicals into nearby wetlands that flow into Lake Memphremagog. Would the authors be able to link a pollution event prior to 2014 to the emergence/initiation of the melanomas? Have they observed any signs of cancer in other fish/species inhabiting Lake Memphremagog?

We cannot link the two events with the data available. The presence of other populations of brown bullhead with melanistic lesions in unrelated watersheds suggests that the arsenic is not causal, but we cannot rule out that it is influencing tumor growth and spread through this population. There are no other species in the lake that show a high prevalence of cancer.

We removed the mention of arsenic in the discussion because it is not published data and will not be publicly available for several months. In addition, the role of arsenic in the growth and spread of clonal tumorigenesis is potentially very interesting, but it is neutral relative to the clonal tumor hypothesis that we support throughout the paper.

Line: "necessity of containment efforts."
>Can the authors expand and explain what containment efforts could be undertaken to prevent cancer spread? It seems to us that the cancer is spread throughout the lake, and we don't see much scope for containment.
It also appears that "it has been observed that older fish with extensive tumors in and around their mouths and gills persist seemingly with little impact from the tumors"
>This comment is not supported by any datapoint in the manuscript. Would the authors have a reference or pers.com to support this statement? Would the authors consider that the fish potentially have adapted to the disease (via tolerance or resistance)? The tumours have only been observed since 2012 … that would mean very fast adaptation from the fish. What is the generation time of these bullfish? Or would the authors consider the cancer being highly transmissible but low pathogenic (but they also found metastasis to other organs in the fish)? Could the authors comment on the potential evolutionary path and trajectory of bullheads and their transmissible cancer might take?

We clarified the statement to "necessity of containment efforts to minimize spread beyond Lake Memphremagog."

We deleted the statement “it has been observed that older fish with extensive tumors in and around their mouths and gills persist seemingly with little impact from the tumors”

This was based on observations of the fish biologists during surveys. The fish with extensive tumors are not underweight or demonstrably in distress. However, we lack empirical data to support the statement, and we hope to learn more about the morbidity and mortality of fish with these tumors. In order to support the statement and answer many of your follow up questions we would need to study individual fish over many years to assess morbidity and mortality and run transmission studies between naive and infected individuals to address adaptation, transmissibility, and pathogenicity. We would like to learn the answers to these questions, but given our current data and analyses it would be hard to give definitive answers. Brown bullheads generally live to 7 years old but some are recorded to live into the middle teens.

Based on the comments and questions above, it’s clear, we are very supportive of the publication of this manuscript, once our queries have been answered. Beata Ujvari and Rodrigo M. Hamede

Referee #2 (Remarks to the Author):
The key finding here is that there is a new transmissible cancer, that is found in fish for the first time and also for the first time, is melanoma. This is highly original and exciting scientifically but could have major implications for the aquatic ecosystems. The writing is clear and very accessible to a wide audience.

We thank Referee #2 for their enthusiasm for our work and have incorporated their suggestions for improvement as indicated below.

Suggestions for improvements:
(1) The authors might want to frame their work within the context of other natural forms of pigmentary lesions in fish, such as Xmrk (Xiphophorus), melanoma in trout, salmon melanocytic lesions...This might help show that this is really distinctive from these natural pigment lesions.

We added the following line to our discussion to bring in the context of other fish melanoma/lesions "Other fish species have been reported with melanistic lesions, some with putative causes (e.g. Xiphophorus hybrids36) and others of unknown cause (e.g. coral trout37, smallmouth bass38), which may warrant testing for clonality."

(2) The quality of the Figure 1B should be improved (it is rather rudimentary).

Figure 1B is now Figure 1F. We agree that it is a simplistic illustration of our hypothesis, but think it effectively illustrates the difference between stochastic somatic mutations

resulting in tumorigenesis vs a clonal cancer hypothesis. We have spent much time presenting this data to broad (and sometimes skeptical) audiences. We found that having a simple cartoon representing the difference in hypotheses has been an effective tool in making the data more broadly comprehensible and contextualizing the striking clonality of the tumors in the real-data phylogenies.

(3) Is there evidence of the species origin of this melanoma? Can the genomic data be used to understand the species. The authors say that the cancer pre-dates 2015 and did not originate in Lake Memphremagog.

We used the black bullhead (*Ameiurus melas*) mitochondria to root our phylogenetic analysis of mitogenomes and found that the tumor lineage is a unique clade nested within the bullhead mitochondrial genome samples. Although we did not have nuclear genomic data from *Ameiurus melas*, we did add additional fish from populations in New Hampshire and Maine. These eastern population formed a monophyletic clade (mitochondrial, and nuclear trees) and tended to be the sister clade to our tumor clade. Together this makes it highly likely that our tumors originated in brown bullhead, and that they are more closely related to fish from eastern populations than to fish in Lake Memphremagog.

(4) Can the genomic data be used to understand if there is key driver mutation? This might also be useful for histopathology to see the "mosaic of clonal tumor and host cells" in the tumour tissue.

These are great suggestions. We agree that we have a treasure trove of genomic data to look at driver mutations, though a lot of sampling and sequencing resolution of tumors and healthy fish would be needed to separate out germline variants inherited from the founder fish and somatic mutations that happened early in the cancer's evolution. Conclusive driver mutations is still an area of investigation across other transmissible cancers, in part due to this complexity, so we do not believe this would be a quick and easy process. We also think it is a great idea to make histopathology probes, specifically for tumor SV regions, to explore the "mosaic of clonal tumor and host cells" in the tumor tissue, as would be the application of spatial transcriptomics. Though exciting, we believe these directions are out of scope to the goal of this paper to establish the clonality of the cancer.

(5) I would suggest removing the "personal communications" bit in the discussion. If the data is too preliminary to publish, then best to not include.

We agree, and since our collaborators will not be publishing this data for a few months, we deleted this reference to arsenic in tumors relative to healthy skin.

Referee #3 (Remarks to the Author):

The manuscript tests the hypothesis that melanoma-type tumour lesions in a benthic fish species, Ameiurus nebulosus, are a transmissible cancer, in which the tumour cells are distinct from the host and can spread between individuals, akin to a parasite. The authors outline that these types of cancer are rare but have been described in two mammalian species and 10 species of marine bivalves. In this manuscript the researchers have used genomic tools to test the hypothesis of transmissibility by sequencing (using short read, long read and RNA) the mitochondrial and nuclear genomes of normal (no tumour lesions) brain tissue and skin tissue, as well as brain tissue and skin tissue from fish with black lesions. The samples were collected at three time points, 2015, 2022 and 2023.

Analysis of the mitochondrial genomes demonstrates heteroplasmy in all tumour lesions samples and a proportion of normal tissue. Phylogenetic analysis of mitochondrial SNVs shows that most of the tumour samples form a monophyletic clade, with the tumours more closely related to one another than to the normal fish samples, consistent with a transmissible cancer lineage. The tumours are separated into low frequency (LF) and high frequency (HF) variant samples, with HF samples more closely related to each other and exhibiting patterns of phylogeny consistent with a transmissible tumour. The LF samples are less clear cut, see further points below.

Analysis of the nuclear genomes supports the clustering of HF tumour samples in a monophyletic clade, distinct from normal fish samples. Further, analysis of shared variants in HF samples highlights the similarity of tumour samples (through shared variants) compared to normal samples. This data is contrasted to melanoma samples in humans, which demonstrate few shared variants between tumours from different individuals. Similarly, structural variants are more shared between the HF tumour samples than between normal tissues.

The evidence presented by the authors strongly suggests that this is indeed a transmissible tumour. This is challenging to prove conclusively, but the comparisons of SNVs and SVs, particularly among the HF tumour samples are compelling evidence. As such, it makes an original and impactful piece of work.

We thank Referee #3 for an extremely thorough and thoughtful summary of our manuscript. Please find our responses to your concerns below.

However, I do have several concerns as to how the data is presented and the conclusions drawn in the paper, particularly in regards to the ‘low frequency’ (LF) tumours.

We resampled and re-sequenced the LF samples (See **the resolution of low tumor purity samples:** discussion above) and recovered the entire clonal cancer mitochondrial genomes in these fish. We are confident that the LF phenomenon was a methodological artifact and not biologically meaningful.

• The different sequencing methods utilised and the lack of consistency in methods across the samples causes issues in the analyses and conclusions that can be drawn. This is apparent in the division of samples into low frequency and high frequency tumours (Figure 2 and Figure 3), with the low frequency tumours clustering separately to the high frequency tumours, with SB14 tumour more closely related to normal tissues (Figure 3). The authors attribute this to host contamination (ie fewer tumour cells) in the low frequency tumours, but this may also be an artefact of sequencing depth in these samples, or an independent cancer lineage. Can the authors normalise the data and SNV calling for read depth?

We agree that the previous draft pulled from a variety of sequence types to compensate for the LF samples. We focus the current draft of the main manuscript on only short read DNA sequences, and only present data for long read DNA and RNA in the supplementary materials as supporting data. We also added 9 tumor-normal fish pairs collected from 2019 and found that they share the clonal cancer lineage.

We filtered but did not normalize the SNV data for read depth. The SNVs sites included in analyses had at least 10x coverage for 90% of the samples included in an analysis.

• I would also like to see a direct comparison of LF and HF tumours in terms of sequencing depth for the nuclear genome and shared SNVs, this is difficult to see from the supplementary material.

We added coverage information to Supplementary Table 4. Under the columns for *HL4 Genome Mapping.*

• In the supplementary material the authors note that ‘histologically the LF samples did not show evidence of a tumor’, but no histopathology is shown in the supplementary materials or main paper. Given the division into LF and HF tumours the pathology the authors refer to should be shown or histopathology should be included.

The revised analysis suggests that the same tumor is responsible for the development and proliferation of the melanistic lesion, from small proliferations of melanocytes to raised black melanomas. The major consideration for current and future sampling is whether we can collect enough tumor cells in the tissue such that the ratio of tumor to host cells is high enough for genomic characterization of the tumor. We removed the

particular passage from the Supplementary Materials, based on our success resampling and re-sequencing the former LF samples.

• The clustering of the low frequency tumours with the normal tissues (ie. Figure 3, Supp Figure 6) is attributed to chimeric tumour and host, but can the authors be sure of their interpretation? The data may indicate non-transmissible, independently arising tumours or an early lineage of the transmissible tumour. Again, histopathology and consistent methods of sequencing across samples would assist in making this point more robust.

We agree that if the resampling and resequencing had gone differently that we would need to show this, but resequencing clarifies that all tumors, including formerly LF samples, are the same clonal lineage.

• Given the reference genome generated and the extensive sequencing of non-tumour samples it should be possible to identify germline variants and tumour specific variants?

This would be possible to an extent, but two issues make this outside the scope of the current manuscript. First, we need higher sequencing depth for most samples to definitely resolve host versus tumor SNVs. Second, to resolve germline variants (inherited from the founder fish) from somatic mutations (occurring during the cancer's evolution), you need widespread sampling and sequencing of bullhead populations to characterize variants that exist in this population. Our current sampling was not set up to have such resolution, mainly focusing on Lake Memphremagog with a few other fish from other locations as reference fish. As is, our data set is best suited to test the clonal cancer hypothesis. Future sampling to locate populations closely related to the tumor origin will help resolve germline/somatic variants.

• The samples collected in 2015 were sequenced by RNA_seq. This data appears to be only used in Figure 2, but could provide insight into gene expression changes in the tumour? Is there a reason the data was not analysed further?

We agree that gene expression data are important for understanding what is happening in these tumor environments but believe this is out of scope for this paper. The major goal for using this archival RNAseq data in this paper was to call mitochondrial variants and test whether the lineage of clonal cancer has been in the lake since at least 2015, and that it is a single lineage that was present for that time frame.

• The supplementary information also includes an analysis of the microbiome/mico-organisms, this is not included in the main paper, but a brief description of the findings would be valuable, given the possibility of tumour inducing viruses.

We added the following to the **Materials and Methods** in the main manuscript:

Screening for Microorganisms in Tissue Samples:
We screened tissue DNA and RNA sequences for viral and other cellular organisms. Any sample read that did not map to the HL4 genome was processed used nf-core/mag (Ewels et al., 2020; Krakau et al., 2022) to generate assemblies (Li et al., 2016; Nurk et al., 2017) and run classification of mobile genetic elements (virus and plasmid) using geNomad on assembled contigs (Camargo et al., 2023) and classification of prokaryotic and eukaryotic organisms on sequencing reads with Kraken2 (Wood et al., 2019).

Kraken2 data were imported into R using phyloseq (McMurdie & Holmes, 2013). We explored differential abundance of taxa between tumor and normal samples for each dataset (RNA or DNA) using DESeq2 (Love et al., 2014). To remove false positives, we filtered out organisms with less than 50 counts across the data set, and that were present in fewer than a quarter of the sample subset prior to running the analysis.

We added the following to the **Results** in the main manuscript:

No viral or microbial agents associated with tumor tissues:
We screened the tumor and normal tissues for viral and cellular organisms (Supplementary Materials Section 9.) to identify potential agents associated with melanistic lesions. We found many viruses of prokaryotes but there were so few viruses of animals or eukaryotes found in our samples that we were unable to run a formal statistical analysis (Supplementary Table 10). We identified and ran a differential abundance analysis of cellular organisms found in tumor and normal tissue (Supplementary Table 11), and did not identify any microbial genera that were associated with tumor tissue. If there is a virus or microbial agent that facilitates tumorigenesis, it was not present in our tumor samples.

Referee #4 (Remarks to the Author):

We thank the referee for their consideration of the paper but disagree with their interpretation of what was presented in the original manuscript. We also disagree with their concept of the current gold standard for identifying clonal cancer. Please find responses to their remarks below.

The key results suggest the existence of a transmissible tumor in brown bullhead. However, some key steps, in my mind are missing to demonstrably demonstrate its existence. First, the authors rely heavily on mtDNA for their analysis, which could be simply different due origin from a different (migrant female).

We presented extensive mitochondrial and nuclear genome evidence in the original draft of this manuscript including phylogenetic and tumor specific analysis of SNV, and SVs (large inversions, duplications, insertions, deletions). We added an analysis of nuclear copy number variation to the current analysis.

We do not understand the statement “which could be simply different due origin from a different (migrant female)”. We know of no mechanism where the mitochondria from a migrant female would be present in the tumor tissue of over 28 melanistic fish (from 3 time points over 8 years) but not be present in the healthy tissue of those fish. If the normal tissue and the tumor tissue both contained only a single mitochondrial genome and that genome was present in all of the tissue in only the fish with tumors, we would agree with you. That, however, is not the case in this study.

Overall, the results are not convincing. The authors could follow the processes used to demonstrate the existence of other transmissible cancers. First of all, there is no real histology to demonstrate the cancer or type.

The histology for cancers for this population are published in Blazer et al., 2020., which is cited in the second line of the introduction:

Blazer et al. determined these raised black lesions to be malignant melanomas based on histopathological analysis and observed these lesions in 23%–37% of brown bullhead sampled between 2014 and 2017[1].

Secondly, Koch's postulates were not satisfied; the tumor could have been isolated in independent fish cell lines (which are easy to culture) and cultures could have been used to infect other fish.

Koch's postulate, while definitive, is not the gold standard for identifying transmissible tumors. The seminal publications presenting genomic evidence of transmissible cancers in Tasmanian devils (Pearse & Swift et al, 2006, Nature), dogs (Murgia et al., 2006, Cell), and bivalves (Metzger et al., 2015, Cell) did not perform transplantation experiments themselves, instead relying on overwhelming genetic evidence showing that tumors across multiple animals shared a common origin and were not related to their hosts. We are directly following this approach in our analyses.

Therefore the data are lacking to convincingly demonstrate a transmissible tumor, and the blueprint is there to do so, which includes whole genome sequencing, demonstrating the tissue the tumor is expressed in skin tissue using transcriptomics.

We used nuclear genome sequencing for both drafts of the manuscript (see Figure 3 and 4, Supplementary Figures 5-14, Supplementary Materials Section 6, the Methods section, and the Results sections: Nuclear single nucleotide variants also support a clonal tumor and Nuclear structural variants also support a clonal tumor). We disagree that transcriptomics are required to demonstrate a transmissible cancer, given that cancers of the same type often evolve convergently to express similar genes, but with differing mutations causing those mutations (which we capture in our DNA sequencing)

While well-written, the results of the paper do not convincingly demonstrate a transmissible tumor. As such, I cannot recommend the paper in its current form.

We hope that you will consider the evidence in the revised draft, and our responses to your concerns in evaluating the merits of our work.

Referee #5 (Remarks to the Author):
I co-reviewed this manuscript with one of the reviewers who provided the listed reports.

This email has been sent through the Springer Nature Manuscript Tracking System NY-610A-SN&MTS

We thank all reviewers for their thorough reading and insightful feedback on our manuscript. Below we provide responses in bold to respond to the remaining reviewer concerns.

Referees' comments:

Referee #1 (Remarks to the Author):
The authors have satisfactorily addressed all previously raised concerns. Their methodology, sample collection procedures, and data analyses are now presented with greater clarity and coherence, making the study easier to follow. The manuscript is streamlined, well written, and demonstrates originality with significant implications for our understanding of transmissible cancer evolution and ecology. The conclusions are robust, supported by rigorous statistical analyses. We commend the authors on this excellent work and look forward to future studies that further explore this novel and compelling transmissible cancer cell line.
Beata Ujvari and Rodrigo Hamede

Referee #2 (Remarks to the Author):
I continue to find this a fascinating discovery that has significant implications for our understanding of cancer biology as well as ecology.

1. I disagree with the authors that their Figure 1F is clear. It requires reading their methods to understand their cohort of samples and apply it to this figure (what is normal tissue etc; samples numbers; controls). I think this figure is the opportunity to explain their cohort and study design, as well as the hypothesis. The authors even agree in response to Reviewer 1 that "We agree that the details of the study design were confusing." They also address comments about metastatic disease to the Reviewers, which are reasonable questions based on the biology of melanoma (the brain in the schematic looks like it is a metastatic site; which is a major problem in the human disease).

> **We changed the figure so that the normal tissue sampled per fish was not specifically healthy brain tissue. Incidentally, we did not observe metastasis in the brain tissue for the fish for which we sequenced the melanistic lesions but appreciate the association with human disease and modified the figure to be more general.**
>
> **We agree that clarity regarding cohort structure is important. However, because Figure 1F is designed to illustrate the expected phylogenetic relationships under competing biological hypotheses, incorporating the full sampling design (including multiple years, tissue types, and fish conditions) would substantially complicate what is intended as a simplified conceptual diagram. In the previous revision, we substantially simplified and clarified the description of the cohort and study design in the main text and Methods. These changes were noted favorably by other reviewers and we therefore maintain the schematic nature of this panel while directing readers to those clarified sections.**

The legend now reads:

“F. Expected phylogenies from paired normal (N) and tumor (T) tissue genomes for fish with tumors that arise conventionally via somatic mutation or originating from the transmission of a clonal lineage of cancer.”

2. While the pathology of the lesions has been published previously, I think the paper could have improved impact with some basic H&E so we can see the pathology of the disease and see the cancer cells.

Histological images have been submitted for publication as part of a larger pathology study (Blazer et al, *In Submission*, Journal of Fish Diseases) and will not be shown here. We refer reviewers and readers to previous work by Blazer et al. 2020 for extensive discussion and representation of tumor histology.

While the histological images of the cancer cells in the melanomas are interesting, we think they are not specifically relevant to the primary inference presented in this manuscript, that the cells are clonal and transmissible. Multiple cell types are present in these tumors and identification of the transmissible cancer cells will be done using laser microdissection and subsequent single cell sequencing with freshly collected samples this spring.

3. Line 431 discusses transmissionable cancers in over a dozen species, and yet in line 72 the authors say that the transmissionable cancers have "only been observed in three types of animals". Please clarify these statements.

We clarified the text that was on line 72. The original read:

“To date, naturally occurring transmissible cancers have only been observed in three types of animals: dogs, Tasmanian devils, and bivalve species[4].”

The current text reads:

“To date, naturally occurring transmissible cancers have only been observed in three types of animals: dogs, Tasmanian devils, and several species of bivalve[4].”

4. Line 67 - I would recommend reconsidering how this sentence is cited. Based on the way it is written, I thought that the hypothesis was generated in the citation. Upon reading that (excellent) reference, I now understand it is meant as a reference about transmissible cancers generally.

We modified the way the sentence is cited. Initially it read:

“This led to the hypothesis that this cancer may be a transmissible cancer - a clonal cancer lineages that spreads through a population by engrafting on new organisms like a contagious parasite[4].”

The text currently reads:

"This led to the hypothesis that this cancer may be a transmissible cancer - <u>because this genomic observation is consistent with known</u> clonal cancer lineages that spread through a population by engrafting on new organisms like a contagious parasite[4]."

<u>Referee #3 (Remarks to the Author):</u>
The revised manuscript is significantly improved from the original, in particular the evidence in support of these melanomas as transmissible tumours is strengthened. The re-sampling, additional samples from 2019 and associated sequencing has sufficiently resolved the 'low frequency' tumour samples showing that these were an artifact of the sampling and has confirmed the monophyletic nature of the tumour samples. The addition of the normal tissue samples from Maine and New Hampshire has also improved the analyses, suggesting an origin for the tumour beyond Lake Memphremagog. The authors have removed the long read sequence data from the analyses in Figure 2 and Supplementary Figure 3, confining these to short read sequence data, which improves consistency in the analysis. I am also satisfied with the description and analyses of viruses and microorganisms in the work.
A number of the suggestions (ie. identification of tumour specific variants and further use of RNA_seq data) have not been followed up by the authors, but I do accept their justification that the need for consistently higher sequencing depth across additional samples (ie more sampling) pushes these experiments outside the scope of the current work.
Two minor issues in the Introduction:
Line 83 – replace 'an' with 'a'

We made the suggested replacement

Line 89 – I would begin this line with 'An indicator of a transmissible cancer…' the way the sentence is currently phrased indicates that the relationship between cancer genomes and the host genome is the only parameter required to prove cancer transmissibility. The phrasing may also be confused with Hanahan's papers on the Hallmarks of cancers.

We modified this sentence to start with "An indicator".

Overall, the manuscript is now well written, and the data well supports its conclusion, that a transmissible tumour is circulating in Ameiurus nebulosus, the first contagious cancer to be identified in a fish.

<u>Referee #4 (Remarks to the Author):</u>
Significant improvement from original MS. Satisfactory responses to reviewer comments.

Referee #5 (Remarks to the Author):

I co-reviewed this manuscript with one of the reviewers who provided the listed reports.