Evidence of Melanoma in Wild Marine Fish Populations

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Abstract

The increase in reports of novel diseases in a wide range of ecosystems, both terrestrial and marine, has been linked to many factors including exposure to novel pathogens and changes in the global climate. Prevalence of skin cancer in particular has been found to be increasing in humans, but has not been reported in wild fish before. Here we report extensive melanosis and melanoma (skin cancer) in wild populations of an iconic, commercially-important marine fish, the coral trout Plectropomus leopardus. The syndrome reported here has strong similarities to previous studies associated with UV induced melanomas in the well-established laboratory fish model Xiphophorus. Relatively high prevalence rates of this syndrome (15%) were recorded at two offshore sites in the Great Barrier Reef Marine Park (GBRMP). In the absence of microbial pathogens and given the strong similarities to the UV-induced melanomas, we conclude that the likely cause was environmental exposure to UV radiation. Further studies are needed to establish the large scale distribution of the syndrome and confirm that the lesions reported here are the same as the melanomas in Xiphophorus, by assessing mutation of the EGFR gene, Xmrk. Furthermore, research on the potential links of this syndrome to increases in UV radiation from stratospheric ozone depletion needs to be completed.

Introduction

Prevalence and occurrence of novel diseases are reported to be increasing in many organisms worldwide. Understanding the etiology of these diseases, the host organisms they affect and potential causes and consequences are a vital first step in the development of control and management strategies. Many diseases are caused by microbial pathogens, and fish diseases in particular have been shown to be caused by a diversity of such pathogens including bacteria, parasitic copepods, viruses and fungi [1,2,3]. Historically, diseases in fish have been recorded more commonly in species of commercial value, usually farmed fish. This may be due to the higher than normal stocking densities which in turn can lead to higher levels of infections and/or the ease of sampling large numbers and continuous monitoring capabilities. Furthermore, there is also significant economic benefit to identifying pathogens of these commercially reared fish with the aim of ultimately curing them. In aquaculture systems, diseases cause a significant economic loss, with bacteria, viruses and fungi being the dominant pathogens involved [1,3]. In contrast, diseases of wild fish have received considerably less attention and their economic impact on commercial and recreational fisheries is unknown. In addition to microbial diseases common in fish, other diseases such as carcinomas have been extensively studied in the laboratory using fish model systems, including the Xiphophorus (swordtail) [4,5] and, more recently, the Danio (zebrafish) models [5,6]. To date, however, there are no reports of cancers occurring in wild fish populations. This study aimed to describe a previously unknown disease lesion, which was observed affecting large numbers of a commercially important reef fish, the coral trout Plectropomus leopardus.

Methods

Sampling

Individual coral trout, Plectropomus leopardus, were line caught with barbless 8/0 hooks using pilchard bait, following methods employed by commercial fishers. Four fishing trips were completed between Aug 2010 and Feb 2012 off the east coast of Australia, at Heron Island (23.4°S, 151.9°E) and One Tree Island (23.5°S, 152.0°E). In total 136 fish were sampled and photographed, 20 of which showed signs of skin abnormalities. From healthy individuals and those with the syndrome, two sets of samples were taken; one for microbial analysis and the other for histological examination. Additional affected individuals were observed during snorkel and dive activities, but only those individuals captured via fishing were included in this analysis.

To test for differences in bacterial, fungal and ciliate molecular diversity between healthy and lesion samples, we analyzed tissue sections collected from individuals captured in August 2011. Three replicate tissue sections (~10×3 w×3 d cm), separated by ~5 cm were cut using a sterile scalpel blade from n = 5 non-diseased (ND) fish, n = 5 diseased (D) fish (Fig. 1a) at the lesion interface and n = 5 apparently healthy (AH) tissues adjacent to the lesion on a
disease fish. Samples were placed directly into 100% EtOH and stored at −20°C until extraction and further analysis. A further set of samples, aimed at sampling the surface associated microbes, utilised sterile swabs. The surface of the fish scales were swabbed and these were placed directly into sterile micro-centrifuge tubes with 100% EtOH, stored at −20°C until extraction and further analysis. Further samples, aimed at sampling the surface associated microbes used sterile swabs. These were placed in sterile micro-centrifuge tubes and stored in 100% EtOH at −20°C until extraction. Samples for histology were collected as for microbial analysis (see above), with the same sample number of samples however, they were preserved in 5% paraformaldehyde made up with Phosphate Buffer Saline (PBS). Samples were fixed for 24 hr, dehydrated in a dilution series of EtOH from 50 to 100% and stored at 4°C until embedding and sectioning. DNA was extracted using the QIAGEN DNeasy Blood and tissue extraction kit.

Fungal PCR amplification and denaturing gradient gel electrophoresis (DGGE) of tissue samples/swabs

For DGGE analysis a portion of the fungal ITS rRNA gene was amplified using universal fungal primers; a nested PCR approach was utilised to yield the most complete diversity [7]. 1st round; fungal primers ITS1F (5’-CTGTCGATTTAGAGGAAGTAA-3’) and ITS4 (5’-TCTTCCTGATATTGATATGC-3’) were used. Samples were stored at 4°C until extraction and further analysis. A further set of samples, aimed at sampling the surface associated microbes used sterile swabs. These were placed in sterile micro-centrifuge tubes and stored in 100% EtOH at −20°C until extraction. Samples for histology were collected as for microbial analysis (see above), with the same sample number of samples however, they were preserved in 5% paraformaldehyde made up with Phosphate Buffer Saline (PBS). Samples were fixed for 24 hr, dehydrated in a dilution series of EtOH from 50 to 100% and stored at 4°C until embedding and sectioning. DNA was extracted using the QIAGEN DNeasy Blood and tissue extraction kit.

Bacterial PCR amplification and denaturing gradient gel electrophoresis (DGGE) of tissue samples/swabs

Extraction was the same as above. For DGGE analysis a portion of the bacterial 16S rRNA gene was amplified using universal eubacterial primers [12]; (357F-GC) (5’-CCTACGGAGGCAGCAG-3’) and (518R) (5’-CGCCCGCGCGCGCGCGGCGG-GGCAGGACGGGAGG-ATTACCGCGGC-GTCTG-G3’) PCR reaction mixtures and program were as described by [11]. PCR products were resolved on 10% (w/v) polyacrylamide gels that contained a 30–60% formamide (denaturant) gradient for 13 h at 60°C and a constant voltage of 50 V. Gels were stained with SYBER gold as described by [11]. Bands of interest (those which explained the greatest differences/similarities between samples) were excised from DGGE gels, left overnight in Sigma molecular grade water, vacuum centrifuged, re-amplified with the specific primers, labelled using Big Dye (Applied biosystems) transformation sequence kit and sent to Genevision (Newcastle University, UK) for sequencing. Fungal operational taxonomic units (OTUs) were defined from DGGE band-matching analysis using BioNumerics 3.5 (Applied Maths BVBA).

Ciliate PCR amplification and denaturing gradient gel electrophoresis (DGGE) of tissue samples/swabs

Ciliates 18S rRNA genes were amplified with an un-nested PCR approach. PCR mixture was as above with the forward primer CilF (5’-TGGTAGTGTATTGGACWACCA-3’) and a constant voltage of 50 V. PCR products were resolved on 10% (w/v) polyacrylamide gels that contained a 30–60% formamide (denaturant) gradient for 13 h at 60°C and a constant voltage of 50 V. Gels were stained as above and bands of interest were excised from DGGE gels, labelled and sent to Genevision (Newcastle University, UK) for sequencing. Bacterial OTUs were defined from DGGE band-matching analysis using BioNumerics 3.5 (Applied Maths BVBA).

Statistic analysis of microbial communities

In order to assess variation in microbial assemblages (bacterial, fungal and ciliate), matrices consisting of OTU’s and samples were generated using both presence/absence and band intensities data, using marker lanes for between-gel comparisons. Changes in microbial assemblages were evaluated with a one-way analysis of similarity (Primer, ANOSIM) and multi dimensional scaling (MDS), based on Bray-Curtis similarities, which was performed on all samples sets, healthy, apparently healthy and diseased.

Histology

Samples were collected as for microbial analysis; however tissue samples were preserved with 5% paraformaldehyde for 24 hrs.

Figure 1. Lesions were present on approximately 15% of the sampled population of Plectropomus leopardus; a) affected individual showing <10% coverage of body surface; b) P. leopardus with almost complete coverage >90%; c) healthy tissue under light microscope and d) the lesion. Scale bars = 20 μm.
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then stored in 100% EtOH until resin embedding in LR white\(^9\). For each tissue type, the location of bacteria was recorded using fluorescent in situ hybridisation (FISH) and the general fluorescent stain, 4'-diamidino-2-phenylindole (DAPI). For FISH, samples were stained and sectioned following the protocols in\(^{14}\), with the addition of an equimolar mix (EUBMIX). Oligonucleotide probes were purchased from Interactiva (http://www.interactiva.de) with an amonolink C6/MMT at the 3' end. Four probes were used: the 'universal' eubacterial probes EUB338\(^{,9}\), EUB338-II (5'-GCC GGC ACC CGT AGG TGT-3'), EUB338-III (5'-GCT GCC ACC CGT AGG TGT-3') and the 'non-sense probe' NONEUB (5'-ACT CTC AGG GGA GCC AGC-3'), which has the complementary sequence to EUB338, used to determine non-specific binding of EUB338. The three eubacterial probes were used in an equimolar mix (EUBMIX) and the NONEUB probe was used singly. DAPI staining followed the protocol by\(^{11}\), whereby each section was stained with 100 µl of 4% PBS buffered paraformaldehyde solution containing 4'-diamidino-2-phenylindole (final concentration 5 µg ml\(^{-1}\)) for 10 minutes, rinsed with filtered 1× PBS pH 7.4. All sections were viewed under epifluorescence microscopy with an FTTC-specific filter block (Nikon UK Ltd, Surrey, UK) and images recorded using an integrating camera (Model JVC KY-SSSB: Foster Findlay and Associates, Newcastle upon Tyne, UK). Samples of pure cultured \(E. coli\) were run alongside each section and for each staining protocol as a positive stain.

Further histological samples were stained with the melanin specific stain, Fontana-Mason, melanin granules reduce silver nitrate to metallic silver, which results in a histochemical reaction that participates black material wherever melanin is located\(^{15}\).

Samples for Transmission Electron Microscopy (TEM) were dehydrated using EtOH and PBS; 25% EtOH, 50% EtOH, 75% EtOH (30 mins each), then a further (2×1 hr) in 100% EtOH, with final dehydration using carbon dioxide in a Baltec Critical Point Dryer. Specimens were then mounted on an aluminium stub with Achesons Silver Dag (dried overnight) and coated with gold (standard 15 nm) using a Polaron SEM Coating Unit. Specimens were examined using a Stereoscan 240 Scanning Electron Microscope, and digital images collected by Orion6.60.6 software.

Samples for Transmission Electron Microscopy (TEM) were dehydrated using 25% acetone, 50% acetone, 75% acetone, (30 min each) and 100% acetone (2×1 hr). Then impregnated with 25% LR White resin in acetone, 50% resin/acetone, 75% resin/acetone (1 hr each), then 100% resin for minimum of 3 changes over 24 h, with final embedding in 100% resin at 60°C for 24 hrs. Survey sections of 1 µ were cut and stained with 1% Toluidine Blue in 1% Borax. Ultrathin sections (30 nm approx) were then cut using a diamond knife on a RMC MT-XL ultramicrotome. These were then stretched with chloroform to eliminate compression and mounted on Ploifoil filmed copper grids. Staining was with 2% aqueous Uranyl Acetate and Lead Citrate (Leica). The grids were then examined using a Philips CM 100 Compustage (FEI) Transmission Electron Microscope and digital images were collected using an AMT CCD camera (Deben) at the Electron Microscopy Research Services Laboratory, Newcastle University.

**Results and Discussion**

Approximately 15% of samples from a population of *Plectropomus leopardus* line caught at two locations in the southern Great Barrier Reef Marine Park - Heron Island and One Tree Island - showed evidence of a dark growth lesion (Fig. 1a,b), similar in appearance to those reported from laboratory induced melanomas seen in the fish *Xiphophorus*\(^{16}\). Prevalence of skin lesions was not significantly different (Chi Square = 0.063, df = 1, p = 0.803) between reef platforms with 14.1% of individuals at Heron Island and 15.7% of individuals at One Tree Island affected. In this study, the fish displaying these skin lesions struck fishing hooks as strongly as healthy individuals, appeared to have good muscle tone and were assessed by external examination as healthy aside from the skin discoloration. Coverage of the lesion on individual fish varied from <10% of body surface (Fig. 1a) to almost complete coverage (Fig. 1b). Although the size range of individuals sampled was limited (344–639 mm fork length), there was no relationship between percent cover and fish size \((r^2=0.02)\). Small individuals (468 mm) could show up to 98% lesion cover and larger individuals (639 mm) showed as little as 30% cover, indicating that prevalence can occur at varying sizes and ages. Lesions affected the surface of the fish caught, with a change from the characteristic blue-spotted patterning (Fig. 1c) in healthy individuals to raised lesions which were darker black/brown in coloration (Fig. 1d). Location of the lesions on the body varied between individuals.

**Associated microbial (rRNA gene) communities**

Analysis of microbial communities associated with healthy (non-diseased) and diseased fish, which would highlight potential pathogenic agents (those present in lesions and absent in healthy samples\(^{17,18}\)), was conducted using culture-independent (rRNA gene) molecular screening techniques. Swabs of the mucus and tissue samples from healthy fish, apparently healthy tissues on affected fish, and the lesion itself were sampled. Microbial (rRNA gene) diversity assessed using bacterial-, fungal- and ciliate-specific PCR primers showed no significant difference \((p>0.45)\) between the sample types. No known microbial pathogen sequences were found in lesion samples that were absent or in lower numbers within healthy and/or apparently healthy samples (Fig. 2a). The technique utilised in this study has routinely been used successfully in other studies to highlight potential microbial pathogens\(^{19,20,21}\). Furthermore, no significant differences \((p = 0.12)\) was found between tissue sections and non-invasive surface mucus swabs, suggesting that the microbial communities detected were mainly present on the surface of the fish and not in the dermis or muscle tissues, where the lesion recorded. Histological sections visualised with either Fluorescence In Situ Hybridisation (FISH) using eubacterial probes\(^{14}\) or the general nucleic acid stain DAPI (Fig. 2b,c), showed no microbial populations within the dermis, which supports the conclusion that the microbes detected using culture-independent screening were associated with the surface mucus layer of the fish. No evidence of these or other microbes such as virus like particles (VLPs) were detected using either Scanning Electron Microscopy (SEM) for surface microbes (Fig. 3a,b), or Transmission Electron Microscopy (TEM) (Fig. 3c, f, g, h) for those within the tissues. Processing for SEM and TEM would have removed the surface mucus layer; again supporting the conclusion that few, if any, microbes were present within the dermis at the sites of pathogenesis.

**Histopathological analyses**

Melanin-containing cells (melanosomes) were found to be in higher density, more widespread and with a deeper distribution within the lesions than compared to healthy tissue sections (Fig. 1c, d and Fig. 3c, d). In normal fish skin these cells are restricted to the immediate subepithelial dermis and are responsible for the pigment patterns in the integument\(^{22}\). Melanosomes are normally found to be well organised and clustered in tight groups throughout the dermis beneath the epidermal basement mem-
The samples were examined using Denaturing Gradient Gel Electrophoresis (DGGE) to determine the bacterial community composition in mucus samples from healthy and diseased fish. The DGGE fingerprints showed distinct patterns for each tissue type, enabling the identification of specific bacterial species associated with melanoma development in Xiphophorus model. These findings suggest that bacterial factors may play a role in the development and progression of melanoma in wild fish populations.

The histological examination of the lesions revealed a variety of cell types, including melanocytes, epithelioid-like cells, and macromelanophores. Melanin-specific staining was used to highlight the different stages of melanoma development, from stage I (cellular melanosis) to stage III (intradermal melanoma). The lesions were characterised by the presence of disorganised pleomorphic cells containing melanin granules, which were more heavily pigmented in advanced stages of melanoma development compared to stage I lesions.

In conclusion, the study provides new insights into the role of bacterial factors in the development of melanoma in wild fish populations, and highlights the need for further research to understand the potential environmental and genetic factors that contribute to this disease.

**Figure 3. Microscopic images of Plectropomus leopardus tissues; a) Scanning Electron Micrograph (SEM) of the healthy tissue; b) SEM of the lesion. MGC = mucus goblet cells, M = mucus. c) Light microscope image of a healthy scale and d) light microscope image of a diseased scale, showing disorganisation of natural melanin patterns seen in (c). Scales bars = 10 μm.**

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exposed to UV radiation over a wide depth range. Individuals in
this study were all captured in less than 20 m depth, well within
the UV-B exposure range of 30 m [27]. Interestingly, juvenile
hammerhead sharks have been shown to have the ability to 'sun
tan' [30], whereby integumental pigments such as melanin
increased in direct response to increases in solar radiation. The
juvenile shark's skin responded similarly to that observed in
humans and other vertebrates in response to direct sunlight,
turning from brown to black. Although a similar melanin response
was seen in this study (i.e. increased melanin concentration), the
sharks in this previous study showed no visible lesions or growths
and were therefore not shown to contract melanomas or dermal
carcinomas.

With regard to the *Xiphophorus* induced melanoma model, it had
long been assumed that only hybrid crosses of *Xiphophorus* (those
bred in captivity), could be induced to contract melanomas or
experience extensive melanosis. The wild (parental) types of these
species in comparison, were non-susceptible to neoplasia, even
after exposure to high doses of physical and chemical carcinogens
[4,31]. However, in addition to this study illustrating melanosis/
melanoma induction in wild type *Plectropomus*, one further study on
*Xiphophorus* also showed non-hybrid melanoma formation in a wild
captured fish, however this was accredited to a build up of androgen
metabolites within the holding tank [22]. Hybrid strains of
*Xiphophorus* have been noted to have differing susceptibility to
carcinogens suggesting a genetic basis for susceptibility to
melanoma formation [32]. Furthermore, it has been shown that
melanoma in *Xiphophorus* is caused by a mutated EGFR gene,
*Xmrk*, with constitutive expression of growth factors. When *Xmrk*
is transplanted into another fish *Oryzias latipes*, they subsequently
contract melanomas themselves [33,34]. Therefore, this suggests
an underlying genetic predisposition to the disease that is
expressed with the loss of tumour suppressor genes caused by
the onset of hybridisation. The occurrence of melanoma in a wild
population, particularly, at the levels observed in this study is
unusual. The relatively high (15%) prevalence of this syndrome
within the sampled *P. leopardus* population may be indicative of a
similar genetic defect as that experienced during hybridisation in
the laboratory, or alternatively it may be due to potential
inbreeding in this portion of the *P. leopardus* population resulting
in recessive susceptibility genes becoming homozygous. In
the latter instance, inbreeding may be potentially proliferated in the
local area due to recruitment of genetically related individuals to
the same reef system [35]. However, hybridisation has frequently
been shown to occur in wild populations of many fish species
[36,37], including populations of *P. leopardus* which have been
shown to hybridise with other *Plectropomus* species, such as the Bar-
cheeked coral trout, *P. maculatus* [38]. Frisch and van Herwerden
(2006) concluded that despite behavioural barriers to reproduction
(such as assortative mating), there was considerable opportunity
for hybridisation between different species of coral trout. Indeed,
the same macroscopic signs of this disease have been noticed on *P.
maculatus* and one further species, the blue spotted coral trout, *P.
laevis*, suggesting hybridisation between these species may be the
most likely cause of predisposition of *Plectropomus* to melanomas.
Current information suggests this syndrome is present throughout
the Great Barrier Reef (MRH unpublished data), but prevalence
appears to be highest in the southern Great Barrier Reef. This
high prevalence recorded in this study further supports the
presence of a genetic component to this syndrome, yet detailed,
broad sampling is required to confirm the extent of prevalence in
other Great Barrier Reef regions.

Coral trout, *P. leopardus*, is an iconic and highly valued species
and the Great Barrier Reef is one of the world’s most pristine and
carefully managed reef habitats. Successful management of these
resources is a crucial and challenging task [39]. The implications
of extensive melanosis/melanoma in wild coral trout will depend
on the prevalence of the syndrome outside the study region, the
causal factors and the proportion which develop into fatal
melanomas. However, this syndrome will no doubt have
implications for the management of fish populations and the
GBR marine park. Beyond health implications for individual fish,
this syndrome may have implications for the population as a whole
and the commercial and recreational fisheries that exploit this
species. In *Xiphophorus*, fish with tumours usually survive around 6
months, compared to an average of 4 years in healthy fish, but any
change in their environment, such as a drop in temperature can
rapidly lead to death [5]. It is unclear whether future changes in
the ocean environment or climate will similarly exacerbate the

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Figure 4. Histological section of LR white resin embedded samples of healthy and diseased *Plectropomus leopardus*: a) Healthy section stained with toluidine blue; b) lesion stained with toluidine blue; c) healthy section stained with melanin specific stain Masson-Fontana; d) lesion stained with Masson-Fontana; e) Transmission Electron Micrograph (TEM) of healthy section; f) TEM of lesion; g) higher magnification of TEM in (e); h) higher magnification of TEM in (f). Scale bars for (a–f) = 10 μm; scale bars for (g) and (h) = 2 μm. E = epithelium, M = melanosome, N = cell nucleus, CBM = caliginous basal membrane. Double headed arrows shows thickening of the integument, characteristic of laboratory induced-melanomas in the *Xiphophorus* model.
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Figure 5. Transmission Electron Micrographs of different samples of *P. leopardus* exhibiting: (a) healthy tissue showing the two cell types (A & B) associated in the dermis along the collagenous basal membrane (CBM). Cell A shows localisation of melanosomes and Cell B shows absence of melanosomes in the same area. (b) Lesion showing disorganisation of pleomorphic cells (A & B) with an increase in number and spread of melanosomes. This lesion is an example of a *P. leopardus* suffering from stage II melanoma, where the melanosomes are restricted to the dermal. Scale bars = 10 μm. doi:10.1371/journal.pone.0041989.g005

effect of melanomas in wild *P. leopardus* populations, but clearly further research is urgently needed to understand the distribution, prevalence, ecological and fisheries significance of this syndrome. In particular, further studies should focus on UV exposure as a risk factor and confirm whether there is a genetic effect to susceptibility of the syndrome. Utilising molecular markers used to study melanosomas in humans and laboratory fish models e.g. those that target the B-Raf protein [40], the EGFR gene, *Xmrk*, or other mitochondrial DNA status markers [41] would highlight this genetic aspect.

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Author Contributions

Conceived and designed the experiments: MS MH LC. Performed the experiments: MS MH LC MB. Analyzed the data: MS. Contributed reagents/materials/analysis tools: MS JB MH. Wrote the paper: MS JB MH NK.

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