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Morphological and Optical Modification of Melanosomes in Fish Integuments upon Oxidation

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Abstract: Reactive oxygen species (ROS) such as superoxide radicals O_2^-, hydroxyl radicals OH^-, and hydrogen peroxide H_2O_2 may have detrimental effects on marine organisms, including their integuments and visual appearances. Although some studies have described the impact of ROS on marine ecosystems and species ecology, the influence on the optical response of the integuments of marine species and on their visual appearances remains unknown. In this article, we used histology and optical characterisation to show, for the first time, that skin melanophores (melanin-containing chromophores) of the coral reef fish, *Stegastes apicalis*, change their shapes and fluorescent proprieties upon oxidation with H_2O_2 radicals. Our observations also suggest that pheomelanosomes may occur in fish integuments, where, previously, it was thought that fish melanosomes only contain eumelanin. This investigation relied on light and electron microscopy and steady-state fluorimetry, as well as time-resolved streak imaging systems. We suggest that the changes in the morphological and spectral characteristics of melanophores can be used as a marker of physiological stress induced by environmental factors such as ROS. Moreover, *S. apicalis* may be used as a potential model for studying the interaction between the surrounding environment and natural organisms in biologically diverse ecosystems, such as the Great Barrier Reef in Australia.

Keywords: water contamination; marine pollution; clean water; water quality; ecosystem protection; coastal biodiversity; marine biodiversity; reactive oxygen species; coral reef fish; gregory; *Stegastes apicalis*; melanophores; melanosomes

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1. Introduction

Colors in nature play an essential role in various biological phenomena such as visual communication, camouflage, and aposematism [1–3]. Their origin may be either pigmentary or structural. In fishes, colors originate from different cells in the skin, chromatophores (i.e., containing pigments), leucophores (i.e., comprising light scatterers), and iridophores (containing photonic structures), and from the structure of the scales covering a fish's body [1,3–7]. In addition to pigments, fish integuments can also emit light at different wavelengths by fluorescence decay [8–10]. Fishes use colors and patterns, amongst others, for social communication, mimicry, or camouflage and background adaptation [11–14].

Reactive oxygen species (ROS) such as superoxide radicals (O_2^-), hydroxyl radicals (OH^-), and non-radical hydrogen peroxide (H_2O_2) significantly impact sea organisms [15–18], including their integuments. ROS can be produced in seawater via various

abiotic and biotic processes, leading to the ionisation of organic or inorganic compounds, including the metabolisms of phytoplankton [19]; photolysis of water molecules; or radiolysis arising, e.g., from cosmic rays or ^{40}K decay [15,16]. In addition, climate changes and anthropogenic marine pollution appear to increase the concentration of ROS in seawater, especially in surface layers [18,20,21].

Despite the observed effect of ROS on marine organisms [15–18], how ROS affect the visual appearance and the optical proprieties of animal skin had not yet been described. In this study, we investigated the influence of ROS on the skin melanophores occurring in a marine fish, namely *Stegastes apicalis* (De Vis, 1885), through histological and optical characterization. This damselfish species of the family Pomacentridae, commonly known as the Australian gregory or the yellowtip gregory, lives on the Great Barrier Reef off the Australian East coast, in depths of 1–5 m. *S. apicalis* has a dark brown body coloration, with yellow, orange, or red margins on the dorsal fin and the tip of the upper lobe of the caudal fin (Figure 1a) [22]. Melanophores are cells that appear black or dark brown, as they contain vesicles of melanin called melanosomes within the cytoplasm [23,24]. This research allowed us to get insights into the naturally occurring interactions between the surrounding environment and the visual appearances of marine animals, by tracking the physical and optical properties of the fish melanophores upon oxidation with H_2O_2 [25], akin to oxidation with ROS within the marine environment. The goal of our study was to highlight the potential effects of oxidation on the visual appearance and optical properties of the integuments of a marine species. Since this study is a new topic of research, we aim at setting a baseline upon which future research using more naturalistic scenarios can build. In addition, microscope-based observations of the integuments unveiled the likely presence of pheomelanosomes, contradicting the common assumption that fish melanophores purely consist of eumelansosomes [23]. Finally, the changes that we found in this study can serve as an indicator of environmentally induced physiological stress. Hence, *S. apicalis* emerges as a potential model to study the effects of ROS and similar stressors on the reef.

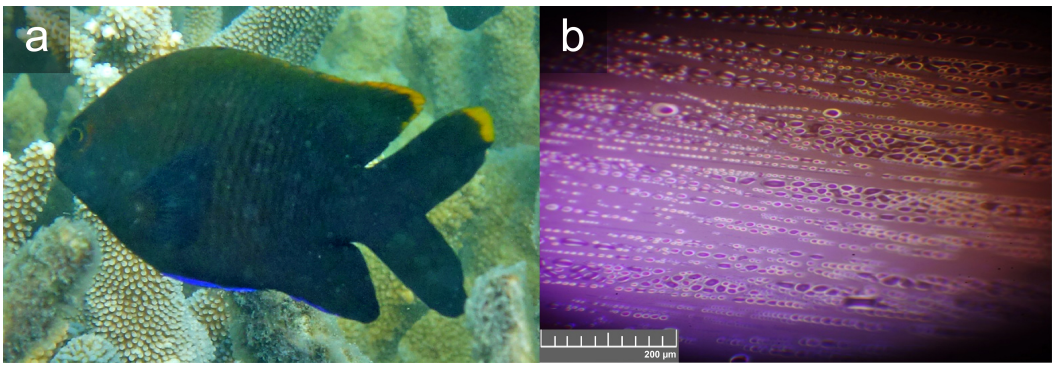

Figure 1. (**a**) *Stegastes apicalis* displays a brown color in daylight with yellow, orange, or red margins on its dorsal fin and the tip of the upper lobe of its caudal fin. (**b**) The brown color is due to melanophores occurring in the integuments of the fish; (**a**) was reproduced from Dr Anne Hoggett, https://commons.wikimedia.org/wiki/File:StegastApicalAnneHoggett.jpg, accessed on 26 June 2023.

2. Materials and Methods

2.1. Sample Preparation and Treatment

Specimens ($n = 4$) of *S. apicalis* were collected (GBRMPA permit number G17/381601; fishery permit number 180731) off the coast of Lizard Island, Queensland, Australia (S 14°41′16.5″, E 145°27′09.6″), at depth of 1–4 m in August 2018. All four dorsal fins were fixed overnight in a 0.01 M phosphate buffer solution (PBS) containing 4% paraformaldehyde (PFA) and 2.5% glutaraldehyde (GA) before being transferred to a PBS solution with a pH ranging from 7.2 to 7.4. Such fixation protocols are widespread in the scientific literature for histological observations of melanosomes in biological organisms, including fish [26–30]. Ideally, our study should have been carried out with fresh or living tissues. However, in this pioneering study, practical feasibility left us with no choice but to fix the

samples. Small areas of the dorsal fins were sampled using a scalpel and tweezers that are typically used for atomic force and electron microscopy following a standard procedure for the integuments of other biological organisms [31,32]. The tools were cleaned with 99.9999% isopropyl alcohol (IPA) prior to dissection. To assess the effect of the oxidative agent on the fluorescence emission from the integuments, some samples were incubated for 5 h in a 10% H_2O_2 weak-acid solution (pH between 4.5 and 5.0).

2.2. Light Microscopy

The instrument is based on a modified JENAVAL microscopic frame (manufactured by Carl Zeiss, Munich, Germany). The sample was placed on the existing mechanical stage, which was powered by a stepper motor, moving the sample vertically, with a 0.3-µm resolution. The removable prism deflected the beam and enabled the capture of bright field images on a Canon EOS 50D (Tokyo, Japan) digital camera. The images were recorded in reflection mode with a $40 \times \backslash 0.65$ objective.

2.3. Scanning Electron Microscopy

The histological observations were performed using a Tescan MIRA3 (Brno, Czech Republic) high-resolution field emission scanning electron microscope (FEG-SEM) with an accelerating voltage lower than 29 kV. Prior to any observation, the surface of samples was coated with a 2–3-nm-thin gold layer using a Quorum Technologies SC7620 Mini (Lewes, United Kingdom) sputter coater to prevent charging effects.

2.4. Time-Resolved and Steady-State Fluorimetry

The fluorimetry analysis was conducted using a pulsed light beam produced by a tunable Nd:YAG laser system (duration of 5 ns and repetition rate of 10 Hz) and by an FLSP920 UV–Vis–NIR spectrometer device (manufactured by Edinburgh Instruments, Livingston, UK). In the latter case, time-resolved measurements were recorded using a 60-W xenon lamp operating at a frequency of 100 Hz, and a Hamamatsu R928P (Hamamatsu City, Japan) photomultiplier tube was used to detect the emission signal in the visible region. In the former case, an optical parametric oscillator (OPO) was pumped by the fourth harmonic of the laser at 266 nm. The output of the OPO could be continuously tuned over a spectral range from 320 nm to 475 nm, and served as excitation beam. A time-resolved streak imaging system acquired the fluorescence steady-state emission spectra from the samples as a function of time. Full widths at half maximum (FWHM) were estimated using Origin 8.5.0 software, assuming a Gaussian function. The time-resolved streak imaging system used a Princeton Instruments SpectraPro 2300i (Trenton, NJ, USA) spectrograph, a Hamamatsu C4334-01 streak camera system, and the High-Performance Digital Temporal Analyzer Streak Camera Control software. The fundamental advantage of the streak camera is its two-dimensional nature that enables the acquisition of the temporal evolution of laser-induced phenomena. The camera was equipped with an image intensifier so that single photons could be detected, allowing the measurement of very-low-fluorescence intensities from the analyzed sample. The excitation and detection optical axes were aligned using a beam splitter to make it possible to adjust the angle of the excitation beam with respect to the sample surface, allowing a high sensitivity of detection using the single-mode fibers. The streak images were acquired in photon-counting mode with 1300 expositions. The time-resolved emission dynamics were observed at the maximum of the emission peak and fitted to single exponential functions, enabling the assessment of the related decay times using Origin software. Such fits by single exponential functions indicate that the excited molecules do not give rise to any non-radiative decay [33].

3. Results and Discussions

The integuments of the brown parts of the *S. apicalis* dorsal fin exhibit dark spots when observed by optical microscopy (Figure 1b). These spots are due to melanophores that contain melanin vesicles, called melanosomes, that abundantly cover the surface of the scales (Figure 2a) [34–38]. In nature, there are two types of melanosomes: eumelanosomes and pheomelanosomes. The former are rod-shaped and contain eumelanin, giving rise to black, dark brown, gray, and blonde appearances. The latter are spherical and contain pheomelanin, leading to red and brown colorations. In the case of the *S. apicalis* dorsal fin, the observed organelles were spherical with a diameter of 450–500 nm, likely corresponding to pheomelanosomes (Figure 2a). The consensus in the scientific community is that fish melanosomes only contain eumelanin [39]. However, it has previously been suggested that phenomelanin occurs in the integuments of a strain of the common carp (*Cyprinus carpio*) [40]. It is likely that pheomelanin in fishes is more common than assumed. Future studies accounting for phylogeny and species richness should be conducted to investigate its prevalence in fishes.

In addition to light absorption, melanin, as a polymer, and regardless of the type, is also believed to strengthen biological materials, thanks to covalent cross-linking processes [41–48]. For instance, melanin in birds has been shown to contribute to the mechanical properties of feathers [42,45–47].

Melanins usually do not fluoresce in their natural state under excitation by visible or UV light [49,50]. However, under excitation by UV light with a wavelength within the range of 340–380 nm, the *S. apicalis* integuments surprisingly emitted light (Figure 3a,c). The main advantage of using the streak camera as a detection system in fluorescence experiments lies in the fact that it allows the simultaneous recording of the time-resolved fluorescence spectra responses (Figure 4). The emission peaks of the *S. apicalis* integuments were found to be located in the range of 474–478 nm with a FWHM ranging from 86 nm to 101 nm. This light emission corresponds to fluorescence, with a decay time of about 2.2–2.7 ns (Table 1). We believe that this is mostly due to the autofluorescence of molecules such as collagen, flavins, and reduced nicotinamide adenine dinucleotide (NADH) [51,52]. In particular, the NADH fluorescence spectrum matches our measurements [52].

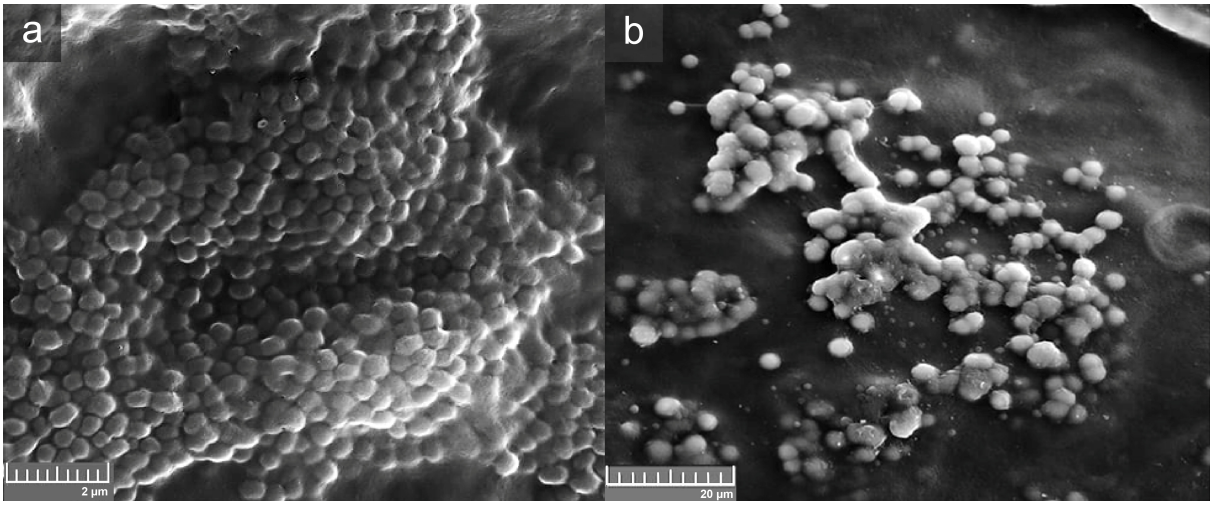

Figure 2. Representative micrographs of *Stegastes apicalis* dorsal fin skin sections. (**a**) Melanin granules, called melanosomes, are found in the integuments of the *S. apicalis* dorsal fin, as observed here by scanning electron microscopy, prior to oxidation by H_2O_2. (**b**) Upon oxidation by H_2O_2, these melanosomes agglomerate. Please note that (**a**,**b**) correspond to different magnifications.

Upon oxidation by H_2O_2, both the structure and the optical properties of the melanosomes were modified. Histologically, they agglomerated into more substantial and connected structures (Figure 2b). In addition, the fluorescence emission spectra of the fish integuments were modified in terms of shape, peak position, and FWHM (Figure 3b). When excited with UV light (from 340 to 380 nm), the emission peaks were longer shifted in the range of 523–531 nm, with a larger FWHM of 122–130 nm. The decay time (Figure 3d and Table 1) was also significantly shorter (from 1.66 to 2.02 ns), as also seen on the streak camera (Figure 4). The different values of peak location, FWHM, and decay time qualitatively indicate that, when treated with H_2O_2, very different states were excited, resulting in significantly different relaxation kinetics. Most likely, the difference in the fluorescent signals was caused by different chemical entities.

On one hand, the oxidation of melanin is known to give rise to fluorescence [50,53,54]. The spectra measured in this study are very close to the spectra reported for oxidized melanin [50,54]. Organic fluorophores and pigments like melanin are usually cyclic or polycyclic compounds with OH and COOH functional groups [33,54]. These groups are possible sites for reduction–oxidation reactions [25,54,55] caused by reactive peroxide and oxygen anions arising from H_2O_2 decomposition. Oxidation by H_2O_2 affects the electronic configuration of the molecules, leading to fluorescence emission. On the other hand, the oxidation process quenches the autofluorescence from some of the fluorescent materials of the fish integuments. ROS, therefore, have a direct influence on the integumental structure and the chromophores of *S. apicalis*. The fluorescence emission measured in this study are likely to be negligible for the signaling and behavior of *S. apicalis*. This is because the quantum yields of natural fluorophores are usually low [56–60], UV light propagates only over short distances in seawater [61], and the proportion of UV light in sunlight and moonlight is limited (i.e., there are ca. 6% of UV and 50% of visible light in the sunlight at sea level) [62]. However, oxidation affects the fluorescence emission of the fish integuments and most likely their color, which could affect the ecology and behavior of fishes, including in intra- and interspecific communication, temperature control, reproduction, and survival. Moreover, older fishes may be more impacted by such ROS oxidation, due to the longer exposure time. This might explain the degradation of their visual appearance and the loss of color during aging. Further physiological and behavioral investigations are needed to test these hypotheses with living animals in their natural marine environment. If the fluorescence emission may only be a likely by-product of another biological function, it could be exploited to detect, identify, and quantify the effects of ROS on fish integuments.

Table 1. Upon oxidation, the fluorescence response of the *Stegastes apicalis* dorsal fin integuments changed in terms of shape, peak wavelength λ_{max}, decay time τ, and FWHM.

	Wavelength (nm)			
	340	**360**	**370**	**380**
$\lambda_{max,\ no\ oxidation}$ (nm)	474	476	474	478
$\lambda_{max,\ oxidation}$ (nm)	523	524	528	531
$\tau_{no\ oxidation}$ (ns)	2.70	2.25	2.69	2.61
$\tau_{oxidation}$ (ns)	1.66	1.75	1.92	2.02
$FWHM_{no\ oxidation}$ (nm)	93	94	86	101
$FWHM_{oxidation}$ (nm)	122	130	126	126

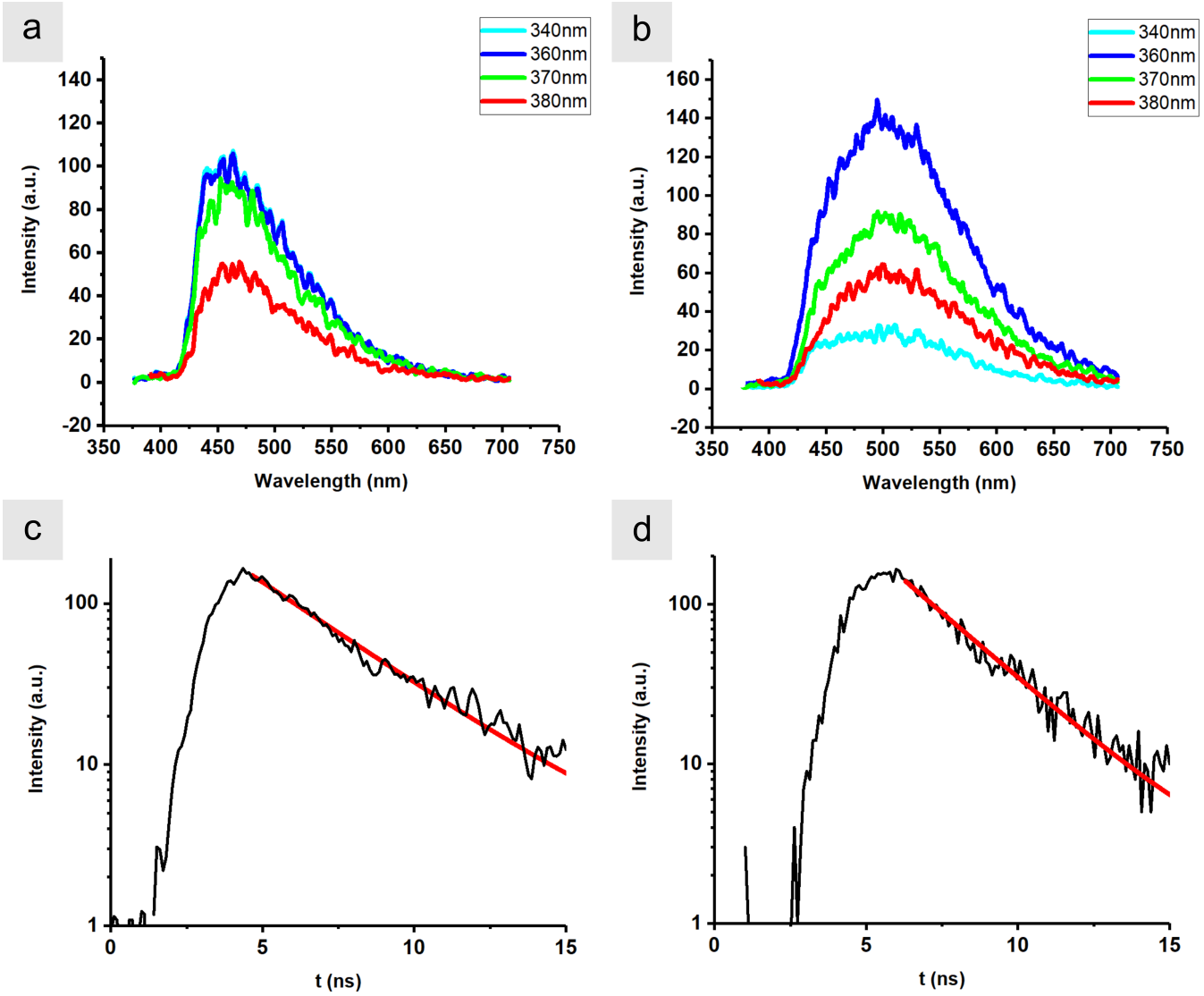

Figure 3. Upon excitation by UV light (the wavelength of which ranged from 340 nm to 380 nm), the integuments of the *Stegastes apicalis* dorsal fin emitted light by fluorescence. (**a**) The emission peak was located around 475 nm, with a FWHM of ca. 90-95 nm. (**b**) Upon oxidation by H_2O_2, the emission peak changed shape and shifted to ca. 530 nm, with a FWHM larger than 120 nm. The time-resolved measurements of the emitted intensities with an excitation wavelength of 360 nm were fitted with single exponential functions (the quality of the fits is given by coefficients of determination $R^2 = 0.980$ (**c**) and $R^2 = 0.983$ (**d**), respectively), allowing the assessment of the decay times of the integuments (**c**) before and (**d**) after oxidation.

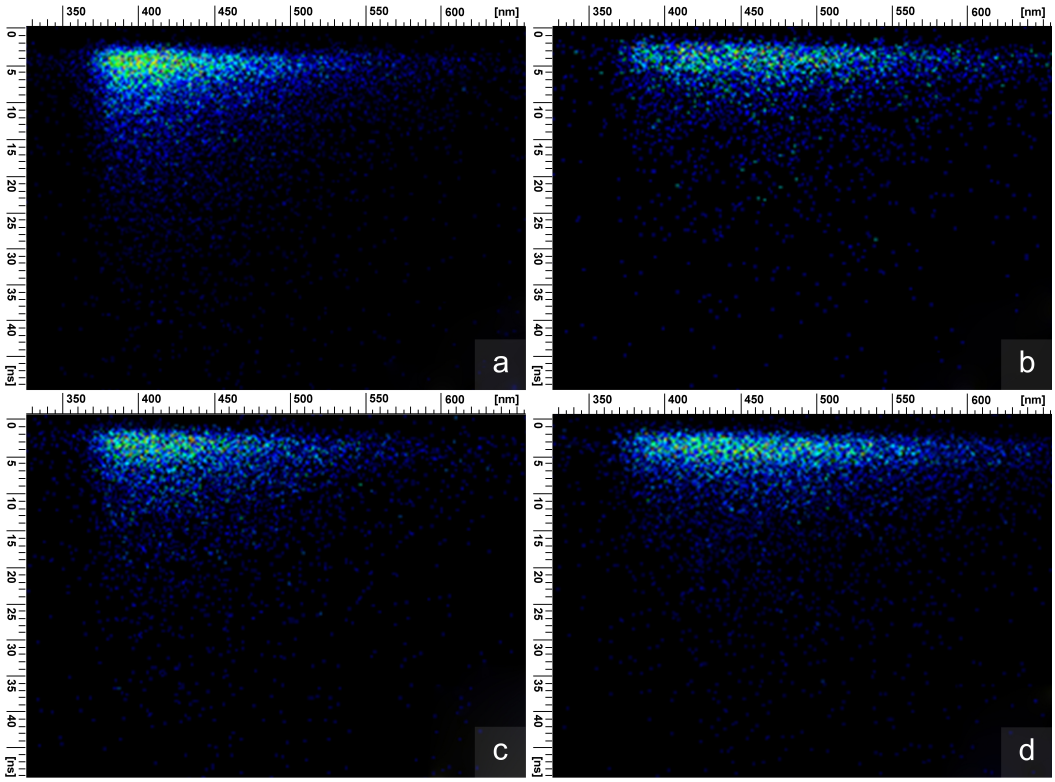

Figure 4. Upon oxidation with H_2O_2, the time-resolved fluorescence response of the *Stegastes apicalis* dorsal fin integuments considerably changes. Fluorescence emission was recorded with a streak camera with various excitation wavelengths, before (**a**,**c**) and after (**b**,**d**) oxidation. Horizontal profiles represent emission spectra and vertical profiles represent time-resolved measurements at selected excitation and emission wavelengths. (**a**) $\lambda_{ex} = 340$ nm before oxidation, (**b**) $\lambda_{ex} = 340$ nm after oxidation, (**c**) $\lambda_{ex} = 360$ nm before oxidation, (**d**) $\lambda_{ex} = 360$ nm after oxidation.

4. Conclusions

This study contributes to the growing body of scientific literature describing the effects of oxidation on biological organisms under different environmental stressors. We first observed the presence of spherical melanosomes in the integuments of *S. apicalis*, which most likely correspond to pheomelanosomes. As it appears that pheomelanin in fishes could be more common than assumed, we call for future investigations accounting for phylogeny and species richness to establish its prevalence in fishes. In addition, and most importantly, we provide some of the first data on how ROS might impact the visual appearance of biological organisms in the marine environment. Following the oxidation of the integuments, the observed pheomelanosomes agglomerated and the fluorescence response was considerably modified, most likely due to the quenching of the integuments' autofluorescence and the activation of fluorescence in the oxidized melanin. Hence, these changes can be tracked as markers for physiological stress caused by the surrounding environment, such as ROS. *S. apicalis* is here identified as a potential model for highlighting the consequences of such stress. The baseline data on how ROS affect the color of fishes that we have provided here create the possibility for more detailed investigations into the effects of ROS under natural conditions (e.g., clean versus polluted seawater). They also provide avenues to study, in detail, the ROS-induced agglomeration of melanosomes and the effects that changes in pH (e.g., due to ocean acidification) might have on these processes, as well as understanding the contributions from biotic and abiotic processes, including metabolic paths in marine environments.

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Institutional Review Board Statement: The animal study protocol was approved by the Animal Ethics Committees of the Queensland Brain Institute of The University of Queensland (QBI/304/16-2018) and of the University of Belgrade (2019). The samples were collected in the framework of the Great Barrier Reef Marine Park Authority (GBRMPA) permit number G17/38160.1 and Fisheries Queensland permit number 180731. This research complies with European Community (EC) health rules as regards animal by-products and derived products not intended for human consumption (Regulation (EC) n°1069/2009—University of Namur, n°BE 705/0089).

Data Availability Statement: The data that support the findings of this study are available from the authors, upon reasonable request.

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Conflicts of Interest: The authors declare no conflict of interest.

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