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TUNEL Apoptotic Cell Detection in Stony Coral Tissue Loss Disease (SCTLD): Evaluation of Potential and Improvements

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Abstract

Stony coral tissue loss disease (SCTLD) is a highly lethal coral disease that has caused a dramatic loss of coral tissue along the Florida Reef Tract and throughout the Wider Caribbean. This study seeks to understand whether programmed cell death (apoptosis) is involved in the pathology of the highly virulent SCTLD tissue loss lesion. Tissues from diseased colonies of *Pseudodiploria strigosa* collected in 2018 and 2020 were stained using the terminal deoxyribonucleotidyltransferase (TdT) mediated dUTP-biotin nick end labeling (TUNEL) assay to visualize areas of programmed cell death. The archived tissue samples collected in 2018 exhibited a significantly higher degree of positive staining for DNA fragmentation with high non-specific staining of cytoplasmic material, suggesting that the age of paraffin blocks (2+ years) and/or the length of fixation (100+ days) caused DNA damage that influences the samples' reactivity to TUNEL labeling. No step of the protocol tested here (epitope retrieval method, proteinase K concentration, proteinase K incubation time, TdT enzyme concentration) significantly altered the degree of positive staining, indicating that the influence of these upstream processing steps cannot be easily remedied through staining protocol manipulation. This study highlights the limitations of archived fixed coral tissues for immunohistochemical analysis.

Keywords: disease, cell death, apoptosis, stony coral tissue loss disease, histology

1. Introduction

Coral reefs are one of the most productive and ecologically valuable ecosystems on Earth (Odum & Odum 1955; Grigg et al. 1984). The structurally complex habitat and foundational nutrients provided by coral organisms support 25% of the world ocean's biodiversity while only covering 0.5% of ocean floor (Norse 1993; Reaka-Kudla et al. 1997). Despite their importance, coral reefs have been declining for several decades, and many are now at risk of extinction (Halpern et al. 2008; Carpenter et al. 2008, Jackson 2008). There are many factors that have contributed to this decline, both on a local scale, such as pollution and eutrophication of coastal waters, and global, such as ocean warming and acidification. Together these stressors have caused the loss of 60% of coral reefs worldwide (Jackson 2008).

In the last three decades, coral disease has emerged as another severe and highly unpredictable threat to coral reefs (Harvell et al. 2002; Maynard et al. 2015). Since the first coral disease was reported in the early 1970s, the number of new diseases, reef-wide prevalence, and frequency of large-scale epizootic events have risen drastically worldwide (Goreau et al. 1998; Richardson et al. 1998; Harvell et al. 1999; Green & Bruckner 2000; Porter et al. 2001; Wie 2004). There are now 40 described coral diseases that are reported to affect more than 200 species of scleractinian coral (Sutherland et al. 2004; Riegl et al. 2009, Bruckner 2009; Woodley et al. 2016). Infectious coral disease has been identified as one of the most severe threats facing coral reefs, alongside rising sea surface temperatures (National Marine Fisheries Service 2015). An increase in ocean temperatures can also exacerbate disease prevalence by influencing the fitness of coral hosts, the abundance and virulence of marine pathogens, and the potential interactions between them (Harvell et al. 2007; Maynard et al. 2015). The threat of coral disease is therefore expected to worsen in coming decades if climate change continues unabated (Field et al. 2014; Duarte et al. 2020).

Caribbean corals are particularly vulnerable to disease (Goreau et al. 1998; Green & Bruckner 2000; Wiel 2002; Wiel et al. 2006). Although they only account for 8% of global coral reefs, an estimated 66% of all coral disease reports by 2000 were from the Caribbean (Green & Bruckner 2000). The most common type of coral diseases present in this region are 'white syndromes,' which encompass a variety of coral diseases that are all characterized by the rapid

dissociation of coral tissue from skeleton (Wiel 2002; Raymundo et al. 2009) (Table 1). White syndromes can appear as highly variable lesions, affect a wide variety of coral host species, and seldom have a consistent known microbial pathogen present in symptomatic tissues (Richardson et al. 1998; Pantos et al. 2003; Pantos & Bythell 2006; Lesser et al. 2007). White syndromes therefore remain one of the most enigmatic coral diseases, despite their global prevalence and high lethality (Work & Rameyer 2005; Lesser et al. 2007; Raymundo et al. 2008).

White syndromes often occur as wide-scale multi-species disease outbreaks. In the Caribbean, and especially along Florida's coral reef (FCR, formerly known as the Florida Reef Tract, **FRT**), these recurring outbreaks are uniquely frequent and have contributed to substantial coral mortality in recent decades (Aronson and Precht 1997; Richardson et al. 1998a; Patterson et al. 2002; Williams and Miller 2005; Brandt et al. 2012). Repeated outbreaks of lethal white syndromes such as white plague (WP) (Bruckner & Bruckner 1997; Dustan 1997; Miller et al. 2003; Richardson et al. 2008; Ballantine et al. 2008; Hickerson et al. 2008; Rogers et al. 2008; Croquer & Weil 2009) and white-band disease (WBD) (Gladfelter 1982; Aronson & Precht 2001; Porter et al. 2001; Bruckner 2002; Gardner et al. 2003), coupled with other anthropogenic stressors, have contributed to the loss of 80% of Caribbean coral cover (Harvell et al. 1999; Gardner et al. 2003; Harvell et al. 2004; Eakin et al. 2008).

Today, Florida's coral reefs are experiencing a highly lethal tissue-loss disease outbreak that is unprecedented in its scale and anticipated damage (Precht et al. 2016; Walton et al. 2018). Reports of white-plague-like lesions on multiple coral species first emerged in September 2014 (Precht et al. 2016), and has since spread across 300 km of reef and resulted in a 30% loss of coral density and 60% loss of live-tissue cover in the northern portion of the FRT (Walton et al. 2018). The disease lesion presents as distinct and rapidly expanding focal or multi-focal areas of denuded skeleton, which can originate in the base, periphery, or center of the colony (SCTLD Case Definition 2018; Muller et al. 2020). Tissue loss continues across the surface of a colony at a rate of 5 to 40 cm^2 per day until the entire colony is devoid of tissue (Sharp & Maxwell 2018). This disease and its associated outbreak have been termed "stony coral tissue loss disease" (SCTLD) to reflect its characteristic lesion and indiscriminate ability to affect scleractinian hosts, as it has now been reported to affect as many as 22 species (SCTLD Case Definition 2018). At the time of writing, SCTLD remains active and spreading, and has been reported throughout the entirety of the Florida Keys and spreading throughout the Caribbean (SCTLD Case Definition

2018; Marks and Lang 2018; Dahlgren 2020). The total ecosystem impact is difficult to predict; however, data suggest that the effects of this expansive disease event will be transformative and complete recovery is uncertain (Walton et al. 2018).

Table 1: Coral tissue-loss diseases in Florida and the Caribbean, adapted from Woodley et al. 2016)

Disease	**Susceptible Hosts**	**Prevalence and Impact**	**Geographic Distribution**	**Rates of Tissue Loss**	**References**
White-band disease (WBD	*Acropora palmata, Acropoa cervicornis*	1-80% or more; up to 98% mortality over two decades	Florida and Caribbean	2 cm/day	Antonius 1981; Davis et al. 1986; Gladfelter et al. 1977
White plague Type I (WP-I)	13 species	1-4% average, up to 73% of individual species; maximum of 20-30% mortality	Florida	3.1 mm/day	Dustan 1977; Richardson 1995; Nugues 2002
White plague Type II (WP-II)	40 species	<1-58%; losses of up to 38% of corals from a single outbreak	Florida, Bermuda, Caribbean	2-10 cm/day	Richardson et al. 1998; Denner et al. 2003
White Plague Type III (WP-III)			Florida	2-5 cm/day	Richardson et al. 1998; Denner et al. 2003
White patch disease	*Acropora palmata*	<1-100%; caused 87% loss of *A. palmata* from 1996-2002	Florida, USVI, Puerto Rico, Mexico		Raymundo et al. 2008; Patterson et al. 2002; Sutherland and Ritchie 2004; Bruckner and Bruckner 1997a, b; Weil 2004; Rodriguez-Martinez et al. 2001
Stony coral tissue loss disease	22+ species		Florida, Bahamas, Jamaica, Mexico, St. Maarten, USVI, Dominican Republic, Turks and Caicos, Belize, St. Eustatius, Puerto Rico	5 to 40 cm^2/ day	Precht et al. 2016; Walton et al. 2019; Muller et al. 2019; Marks & Lang 2018; Sharp & Maxwell 2018; Dahlgren 2020;

The etiology of SCTLD is an area of active research. Studies have found the disease signs to be elicited via direct contact between individuals and between species and via a suspected waterborne agent (Muller et al. 2020; Aeby et al. 2019). This, coupled with successful treatment with antibiotics (Aeby et al. 2019; Neely et al. 2019) and a spatially-clustered distribution during its spread along Florida's coral reefs (Muller et al. 2020), suggests that a communicable agent is contributing to SCTLD, although whether it is a primary pathogen promoting disease onset or a secondary pathogen fueling lesion progression remains unclear. Early histological diagnosis of SCTLD have identified microlesions of lytic (apparent as the complete dissolution of tissues into a viscous, fluid-like mass, similar to liquefactive necrosis though without confirmation of hydrolytic enzymes) and coagulative (nuclear material is degraded but the tissue architecture is maintained) necrosis as the diagnostic criteria for this disease, which appear to be most heavily concentrated in deeper tissue layers (the basal body wall and oral gastrodermis) (Landsberg et al. 2020). In the oral gastrodermis, the *in hospite* photosynthetic zooxanthellae symbionts appear to be the first bodies affected by the disease, and can exhibit signs of degradation such as vacuolization, discoloration, swelling, pallor with nuclear loss ("ghosting"), and necrosis (Landsberg et al. 2020).

While the morphology of SCTLD tissues has demonstrated a heavy presence of necrotic microlesions, it is difficult to conclude whether necrosis is the sole mechanism of cell death responsible for lesions, or whether the necrotic morphology is more demonstrative of late-stage pathology. Programmed cell death (PCD), otherwise known as apoptosis, is another main type of cell death that is more difficult to identify by its morphology alone, and can be misidentified or underestimated in histological investigations of cell death (Jerome et al. 2000; Garrity 2003). While more difficult to visualize, PCD is a critical process in tissue homeostasis and disease, and its potential role in pathology can have important implications for describing the clinical process and ultimate etiological cause of disease.

Programmed cell death is a genetically regulated process of cell destruction that is systematically employed by an organism during development, to maintain homeostasis, and as an immune strategy (Kerr et al. 1972; Reef 1994; Ameisen 2002). In the latter, PCD is used as a means of quarantining an infection, by removing cells that have been infiltrated by a pathogen or are exhibiting another type of malfunction (King & Cidlowski 1998; James & Green 2004; Gao & Kwaik 2004; Zebell & Dong 2015). In this way PCD works to localize a threat and preserve

the health of the overall organism. However, when dysregulated, PCD can be the very cause of pathogenesis, either by inhibiting the death of damaged cells (Kerr et al. 1994; Worth et al. 2006; Elmore 2007) or by accelerating the death of large areas of tissue (Li et al. 1995; Ethell & Buhler 2003; Elmore 2007). In the "hypersensitivity response" of plants, detection of an extracellular microbial pathogen can induce a PCD cascade that causes rapid widespread tissue death (Levine et al. 1995; Liu et al. 2007; Zurbriggen et al. 2010). In a study characterizing cell death in the livers of rats, PCD has been shown to cause a rate of tissue recession as fast as 25% per day when expressed in less than 10% of the cell population (Bursch et al. 1990).

Although a less-studied component of coral disease than necrosis, PCD is an important mechanism in the coral immune system and has been shown to drive some tissue-loss coral lesions and stress responses. In an investigation of the histopathology of Pacific white syndrome affecting tabular acroporid corals (*Acropora cytherea*, *Acropora clathrata*, *Acropora hyacinthus*), which is similar to SCTLD in its characteristic lesion of rapid tissue loss, Ainsworth et al. (2007) found that PCD was the primary driving mechanism of cell death. This was observed without the presence of an intracellular pathogen, indicating that PCD in Pacific white syndrome is host-mediated and can be continuously expressed without chronic exposure to an infectious agent (Ainsworth et al. 2007a,b). Programmed cell death has also been identified as a pivotal mechanism in the bleaching process (expulsion of symbiotic algae during times of stress), and as such, can be triggered in the coral host by the abiotic stressors that contribute to the breakdown of the symbiotic relationship (temperature stress, irradiant stress, nutrient stress, etc.) (Dunn et al. 2004; Richier et al. 2006; Wies 2008; Tchernov et al. 2011).

Determining the role of PCD in SCTLD is critical for refining our diagnostic understanding of this disease and uncovering any potential parallels between this outbreak and similar tissue-loss diseases. Because of the comparable signs of this disease and that of Pacific white syndrome, and because of its reported involvement in other coral stress responses, it is possible that PCD is likewise playing an important role in SCTLD pathology. Because PCD is difficult to capture and adequately quantify in standard histological analyses, it is critical to target this process specifically to properly describe its role in disease.

In coral cells, the distinction between PCD and necrosis is most often made using transmission electron microscopy, which allows for visualization of ultrastructural changes when the resolution of light microscopy is inadequate. Apoptotic cells are characterized by distinct

changes such as chromatin condensation and margination, cell shrinkage, membrane blebbing, and division into apoptotic bodies (Kerr & Harmon 1991; Majo & Joris 1995; Darzynkiewicz et al. 1997), whereas necrotic cells are distinguished by cell swelling, rupture, and tissue damage and deterioration in surrounding areas (Wyllie et al. 1980; Kerr et al. 1994; Leist & Nicotera 1997). While this remains the gold standard for distinguishing between these two processes, there are limitations to basing estimates of cell death on morphology alone (Garrity 2003; Jerome et al. 2000). One such limitation is the overlap of necrotic and apoptotic morphologies under certain physiological conditions, which can obscure the ultimate mechanism of cell death. Stimuli that are specifically associated with the necrotic pathway can in some cases induce an apoptotic morphology (Laster et al. 1988; Portera-Cailliau et al. 1997; Raffray & Cohen 1997; Roy & Sapolsky 1999), and vice versa as an apoptotic pathway can induce a necrotic morphology (Nicoretta et al. 1998; Oppenheim et al. 2001). This later scenario most often occurs in cases where cellular ATP or caspases, which are integral to the apoptotic pathway and phenotype, become depleted (Nicoretta et al. 1998; Oppenheim et al. 2001). Cells exhibiting an apoptotic morphology can also be removed from mammalian tissues via phagocytosis within several hours of displaying detectable changes and leave no residua behind, which can make the actual prevalence of PCD in fixed tissues difficult to capture (Bursch et al. 1990).

To overcome these limitations and more accurately and efficiently locate PCD in tissues, assays have been developed that target the biochemical and genomic signatures of this process. The PCD pathway is associated with mitochondrial membrane permeabilization, activation of certain caspases, and DNA fragmentation (Thiry 1992; Migheli et al. 1995; Marino 2014). The principle of DNA fragmentation during apoptosis is the basis of the terminal deoxyribonucleotidyltransferase (TdT) mediated dUTP-biotin nick end labeling (TUNEL) assay, which enzymatically labels the 3'-OH termini of DNA fragments to be detected by immunoperoxidases. The TUNEL method is the most common technique for visualizing PCD *in situ* due to its ease and efficiency when compared to using visual analysis of morphology, and it has been successfully been applied to scleractinian tissues to study coral disease (Ainsworth et al. 2007a; Ainsworth et al. 2008; Yasuda 2012; Ainsworth et al. 2015) and stress (Pernice et al. 2011), as well as on a host of studies investigating cell death in the model cnidarian organism *Aiptasia* sp. (Dunn et al. 2002; Dunn et al. 2004; Richier et al. 2006). However, the TUNEL assay can also present challenges in reproducibility and accuracy if not properly optimized for

each specific application (Garrity 2003). Results of TUNEL method should therefore be interpreted with caution and should be validated with other histopathological indicators of disease and necrosis to disentangle how these two processes are contributing to a pathology.

The objectives of this study were to apply the TUNEL method to paraffin-embedded coral tissues to determine the role of PCD in SCTLD tissue loss. I compared the applicability of the TUNEL staining protocol for freshly collected and archived tissues (stored for 2+ years as paraffin-embedded block) to determine the potential of archived tissue collections for use in immunohistochemical analyses. Determining whether PCD is the primary mechanism underpinning the rapid tissue loss of this disease is a critical preliminary step in understanding the etiology of SCTLD and will refine our understanding of lethal tissue loss diseases.

2. Methods

2.1 Sample Collection

This study targeted the highly susceptible species *Pseudodiploria strigosa*, which has had regional population losses of up to 75% in southern Florida as a result of SCTLD and has been heavily impacted in other regions throughout the Caribbean (Precht et al. 2016; Lunz et al. 2017; Walton et al. 2018). Paraffin-embedded tissues of *P. strigosa* were acquired from the archived collection stored at the Florida Fish and Wildlife Research Institute (FWRI). These 2-cm tissue cores were extracted *in situ* via a hammer and corer from (1) D: diseased tissue from the active SCTLD margin (n=3), (2) U: apparently healthy unaffected tissue remaining on a diseased colony (n=3), and (3) H: healthy tissue from apparently healthy corals showing no signs of disease (n=3) in 2018. Tissue cores were immediately fixed in 1-part Z-Fix Concentrate (Anatech, Ltd.) diluted with 4-parts filtered seawater and were stored in a dark cooler for transport to the laboratory. Samples were stored in fixative for 103–256 days before being enrobed in agarose, decalcified in 10% EDTA, and embedded in paraffin blocks according to standard processing procedures for histology (Price & Peters 2018). Paraffin blocks were stored at FWRI facilities from April 2018–June 2020 (26 months).

In addition to these archived tissues, additional samples of corals with active SCTLD lesions were collected opportunistically from *P. strigosa* (n=3), *Dichocoenia stokesii* (n=1), and

Colpophyllia natans (n=1) from various locations in 2020 (Table 2). These corals were collected as whole colonies and transported to the laboratory via cooler for sample collection *ex situ*. Samples were then immediately fixed in 4% paraformaldehyde in phosphate buffered saline for 18 hours. After fixation, diseased tissues were trimmed and enrobed in agarose gel to preserve structure during further processing steps. All samples were decalcified in 10% EDTA pH 7, rinsed in water, and then embedded in paraffin blocks for sectioning onto glass slides following standard histology tissue processing procedures (Price & Peters 2018).

Table 2: Number of SCTLD cores sampled by species, site, year, and condition (diseased, unaffected, healthy)

Collection Date	Species	Condition			Collection Site
		Diseased (D)	Unaffected (U)	Healthy (H)	
2018	PSTR	N=3	N=3	N=3	Middle Keys
2020	PSTR	N=3			Lower Keys
	DSTO	N=1			Lower Keys
	CNAT	N=1			Lower Keys

Species acronyms: PSTR: Pseudodiploria strigosa; DSTO: Dichocoenia stokesii; CNAT: Colpophyllia natans.

2.2 In situ *detection of cell death activity*

Tissue sections (4-µm thickness) were prepared for *in situ* end labelling of fragmented DNA associated with PCD on charged glass slides (Apoptag *in situ* detection kit S7101 Millipore Sigma Inc. USA) (Ainsworth et al. 2007; Dunn et al. 2002). Tissue sections were first dewaxed in xylene and rehydrated in a series of alcohols before being treated with 20 µL/mL proteinase K in phosphate-buffered saline (PBS) for 15 minutes for epitope retrieval. Sections were washed in PBS and incubated with an equilibration buffer (Apoptag equilibration buffer S7106 Millipore Sigma Inc. USA) before incubation with dUTP-digoxigenin with terminal deoxynucleotidyl transferase (TdT enzyme) in a humidified chamber for 1 hour at 37°C. The reaction was stopped by placing the slides in stop wash buffer and tissue sections were then washed in PBS before incubation with an anti-digoxigenin antibody that is conjugated to a peroxidase reporter molecule in a humidified chamber for 30 minutes. This antibody conjugate binds to the fragmented DNA strands that were labelled during incubation with TdT enzyme and reacts with a peroxidase substrate to create the orange-brown staining indicative of apoptosis. After the incubation step with diaminobenzidine (DAB) peroxidase substrate, the slides were washed and counterstained with Mayer's hematoxylin to allow observation of intact nuclei alongside apoptotic nuclei. Slides

were then washed and cleared with n-butanol and xylene before applying the coverslip with Permount mounting medium.

2.3: Protocol optimization of TUNEL method for use on formalin-fixed paraffin-embedded (FFPE) coral tissues

Due to the sensitivity of the TUNEL assay to variations in processing, the protocol should be optimized for each particular use (Garrity 2003). In addition to staining slides according to the manufacturer's instructions, the following aspects of the protocol were tested on serial tissue sections taken from the same sample blocks containing diseased coral tissues (Table 3):

Table 3: Protocol modification trials for optimizing the TUNEL method for use on FFPE coral tissues

Protocol Step	Purpose	Treatments
Counterstain	Reduce background staining of coral mucus and tissues	A) 0.5% Methyl Green* B) Mayer's Hematoxylin
Addition of Blocking Buffer Step	Reduce background staining of coral mucus and tissues	A) Addition of 30-minute incubation step with Roche blocking reagent between exposure to TdT enzyme and antibody conjugate, B) Without exposure to blocking reagent*
Antigen retrieval method	Reduce potential for false positive staining by chemically induced DNA fragmentation	A) 20 µL/mL proteinase K 15 minutes* B) 10mM citrate buffer heated for three 3-minute cycles in microwave C) 0.5% TRITON X-100 for 10 minutes
Concentration of proteinase K	Reduce potential for false positive staining by chemically induced DNA fragmentation	A) 5 µL/mL, B) 10 µL/mL, C) 12 µL/mL, D) 15 µL/mL, E) 20 µL/mL*
Incubation time with proteinase K	Reduce potential for false positive staining by chemically induced DNA fragmentation	A) 5 minutes, B) 10 minutes, C) 15 minutes*

Pretreatment with EDTA	Chelate calcium-containing vesicles or residual calcium in tissues to reduce potential for false positive and background staining	Slides were boiled in 3% EDTA (pH 7.2) for 1 hour and incubated with the following concentrations of TdT Enzyme in Reaction Buffer (RB): A) 30% TdT B) 20% TdT C) 10% TdT D) 6% TdT
Pretreatment with proteinase K + incubation with 3% EDTA	To cleave calcium-containing vesicles or residual calcium in tissues to reduce potential for false positive and background staining while maintaining high resolution of nuclei	Slides were pretreated with 20 uL/ mL of proteinase K for 15 minutes and incubated in 3% EDTA (pH 7.2) for 1 hour, then incubated with the following concentrations of TdT enzyme: A) 40% TdT B) 11% TdT
Concentration of TdT Enzyme	Reduce potential for false positive staining by over-labelling DNA	A) 41% TdT B) 31% TdT C) 21% TdT D) 11% TdT

*Recommended by manufacturer

- **Alternative counterstains:** Alternative counterstains were explored to reduce the over-staining of coral mucus in the specimens, which obscures the nuclei that are the focus of this protocol. Sections were counterstained with either 0.5% aequeous methyl green in 0.1M sodium acetate buffer (free of crystal violet, recommended by manufacturer) or Mayer's hematoxylin for comparison.

- **Addition of a blocking step to reduce the amount of non-specific antibody binding**: After the incubation period with TdT enzyme and Reaction Buffer, a 30-minute incubation step with Roche blocking reagent was added before incubation with the anti-digoxigenenin conjugate.

- **Antigen-retrieval methods:** Formalin fixation causes protein cross-linking that masks target antigens. A pretreatment step is necessary for breaking these crosslinks and exposing the antigen that will be bound by the TdT enzyme and produce positive staining for apoptosis. The Z-fix (10% aqueous buffered zinc formalin) used in this study contains zinc ions that prevent to formation of excessive crosslinks, which can remove the need for this unmasking step. However, because the tissues were fixed for such an extended period, it is possible that crosslinks were created that do require removal. Instead of the proteinase-K digestive enzyme, which enzymatically breaks the protein cross links that are formed during fixation (called "proteolytic-induced epitope retrieval, or PIER), these links can also be broken by heat (called "heat-induced epitope retrieval, HIER), or through incubation with a detergent. These three antigen-retrieval methods were tested to ensure that the amount of positive staining was not related to the method, which would indicate that DNA fragmentation was being caused by the protocol and was not reflective of genuine cellular processes prior to fixation (Garrity 2003). Serial sections of the same tissue sample were treated with 20 µL/mL of proteinase-K in PBS for 15 minutes at room temperature (PIER method recommended by the manufacturer), treated with 10 mM citrate buffer and heated for three 3-min cycles in a microwave (HIER method) or incubated in 0.5% TRITON X-100 for 10 minutes at room temperature (detergent method).

- **Proteinase K concentration:** While an antigen-retrieval step is necessary for increasing the antibody-epitope binding that labels apoptotic cells, it has been reported that excessive incubation or excessive concentrations of proteinase-K can cleave DNA strands and cause false-positive staining for apoptosis (Garrity 2003). To ensure that this was not occurring, serial sections from the same tissue sample were exposed to the following proteinase-K concentrations: 5, 10, 12, 15, and 20 µL/mL (concentration recommended by manufacturer).

- **Proteinase K incubation time:** Numerous incubation times with proteinase-K were also tested to ensure that prolonged exposure to this digestive enzyme was not causing false positive staining by chemically cleaving DNA strands. The protocol was repeated on

serial sections of the same tissue sample with an incubation in 20 µL/mL for a duration of 5, 10, and 15 min (duration recommended by manufacturer).

- **Pretreatment with EDTA at varying concentrations of TdT:** There is some evidence that DAB can bind to calcium-containing vesicles within tissues (Morgan et al. 1994; Pileri et al. 1997). To chelate any residual calcium that may be contributing to non-specific staining, samples were pretreated by boiling samples in 3% EDTA for one hour. The EDTA pretreatment method is reported to cause high background staining, so it is recommended to reduce the concentration of TdT when applying this technique. To determine whether exposure to EDTA will sufficiently reduce non-specific staining with DAB, the following concentrations of TdT were tested in combination with this pretreatment method: 6%, 11%, 20%, and 30% TdT.

- **Proteinase K with EDTA pretreatment:** To determine whether incubation with 3% EDTA could be used to chelate tissue-bound calcium ions without being used as the primary epitope retrieval method, a combination of proteinase K (20 µg/mL, 15 minutes) and incubation in room temperature 3% EDTA for one hour was tested using two concentrations of TdT enzyme: 41% TdT (recommended by the manufacturer) and 11% TdT.

- **Concentration of terminal deoxynucleotidyl transferase (TdT):** High staining of DNA throughout the samples and high non-specific staining may be caused by too high of a concentration of TdT in the working strength solutions. To determine whether decreasing the concentration of TdT will increase the selectivity of DNA labelling, the following series of concentrations of TdT were tested: 41% (recommended by manufacturer), 30%, 20%, and 11% TdT. The volume of working strength solution was maintained by supplementing TdT volume with deionized water as recommended by manufacturer.

2.4: Analysis of cell death activity and staining quality

Photomicrographs of tissue sections were taken at 400x magnification at ten random locations along the surface body wall (SBW) and basal body wall (BBW), and at five random locations along mesenterial filaments and cnidoglandular bands for a total of thirty photographs per section. The high number of replicates within each sample was chosen to reduce the influence of biases towards areas of high- or low-positive staining with DAB and to capture a comprehensive representation of sample-wide patterns of cell death. To isolate the prevalence of PCD in different tissue layers, photomicrographs were pre-processed in Photoshop Software to separate tissue layers into individual files for further analysis using the Lasso tool. Photomicrographs were then analyzed using the Fiji2 plugin of ImageJ software to quantify the areas of DAB and hematoxylin (H)-stained nuclei (Figure 1). A color deconvolution filter was first applied to images using the H & DAB channels to separate the image into its blue and orange parts, thereby separating all tissues stained with DAB and those stained with hematoxylin into separate files for all further processing steps. To remove influence from background staining and to isolate the staining of nuclei only, the Photoshop Color Select tool was used to select the darkest hue of the photomicrograph and remove the inverse selection, leaving only the darkest-staining areas (nuclei) in each micrograph for further analysis. A Gaussian Blur (Sigma: 2.00) was then applied to the selection to reduce pixel noise. The image was then converted to an 8-bit grey-scale image and a threshold was applied to create a binary image with all objects (stained nuclei as isolated from prior steps) appearing as white against a black background. The extent of PCD and spatial patterns of cell death within tissues was determined by quantifying the area of white in each image. The labeling index was calculated by dividing the area of DAB-stained material by the total area of stained material for each micrograph. This was chosen as the metric for PCD prevalence rather than positive and negative cell counts due to the high overlap of nuclei in dense tissue layers (epidermis, cnidoglandular bands) and insufficient segmenting techniques using ImageJ Software. To determine how PCD is being expressed in the population of *in hospite* zooxanthellae, three micrographs of the gastrodermal layer were randomly selected for each of the 2020 tissue samples and cell populations were manually counted using ImageJ software.

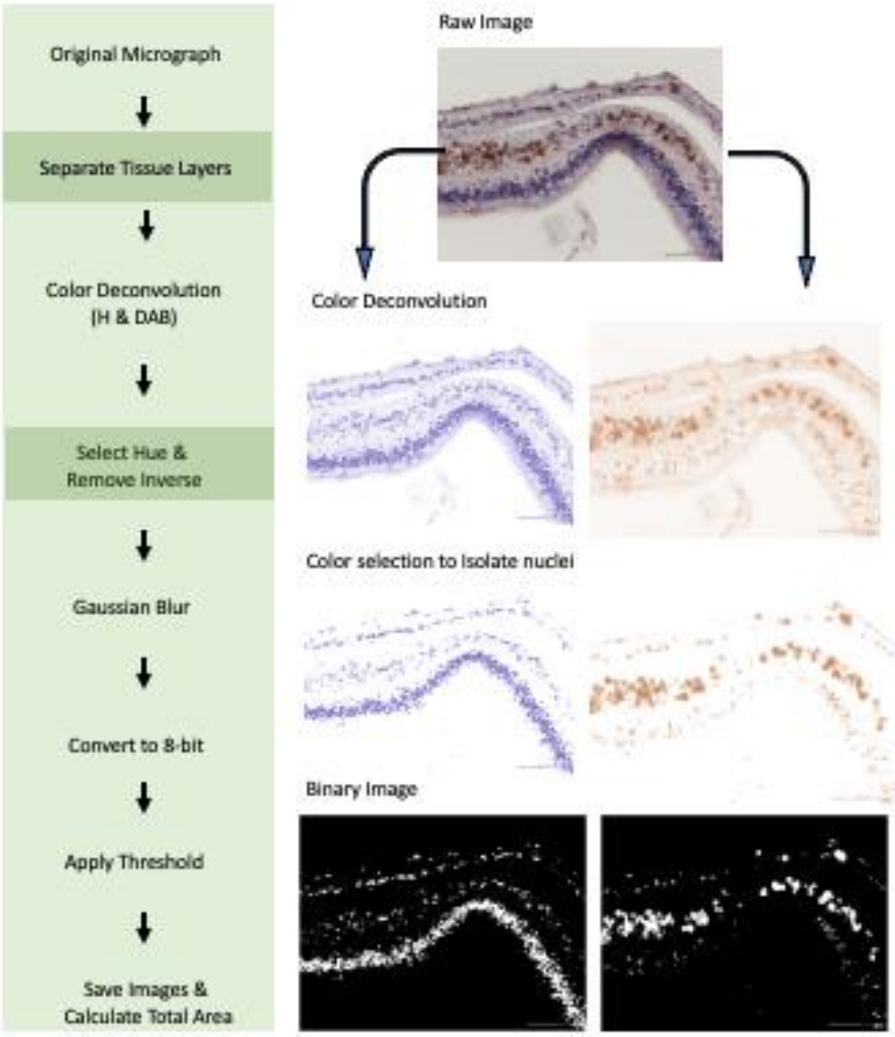

Figure 1: Image analysis pipeline to calculate total stained area using Photoshop and ImageJ (Fiji2) software. Image processing steps in dark green box were completed in Photoshop, steps in lighter green box were completed in ImageJ.

2.5 Statistical analysis

All statistical analyses were performed in R using the library for Mixed GAM Computational Vehicle with Automatic Smoothness Estimation (mcgv). Quasibinomial generalized additive models (GAMs) were used to determine the effect of treatments (proteinase K concentration, proteinase K incubation time, EDTA pretreatment, proteinase K and EDTA pretreatment, and TdT concentration), year (archived tissues collected in 2018, fresh tissues collected in 2020), and tissue condition (healthy, unaffected, or diseased conditions of archived

P. strigosa tissues) on labelling index (calculated as the proportion of DAB-stained area to total stained area). This model was selected as it produced a better fit to the data (smaller residuals) than a binomial distribution and a generalized linear model (GLM).

3. Results

3.1 Optimization and Recommendations for TUNEL assay on FFPE coral tissues

Every sample, including all species (*P. strigosa*, *D. stokesii,* and *C. natans*) (Figure 2) and all conditions (healthy, diseased, and unaffected archived samples; diseased freshly collected samples) (Figure 3; 4) exhibited a high degree of DAB staining indicating DNA fragmentation associated with apoptotic activity. The archived samples of *P. strigosa* obtained in 2018 exhibited a significantly greater degree of DAB staining than those collected in 2020 (p=3.84e-13, quasibinomial GAM) with an average labelling index of 73% with a high background signal, compared to an average labelling index of 49% in the freshly-collected samples (Figure 4). In addition to the higher labelling index in the archived tissues, there was no significant difference in degree of positive staining between any of the archived tissue conditions (healthy, diseased, unaffected; Figure 3) (p=0.205, quasibinomial GAM).

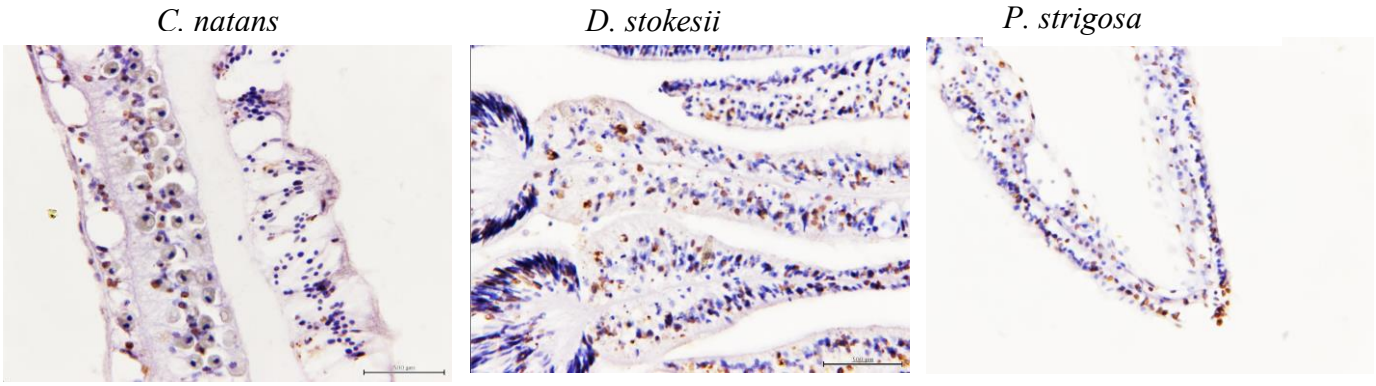

Figure 2: Example micrographs showing positive staining with DAB in tissues of (a) *C. natans*, (b) *D. stokesii*, and (c) *P. strigosa*.

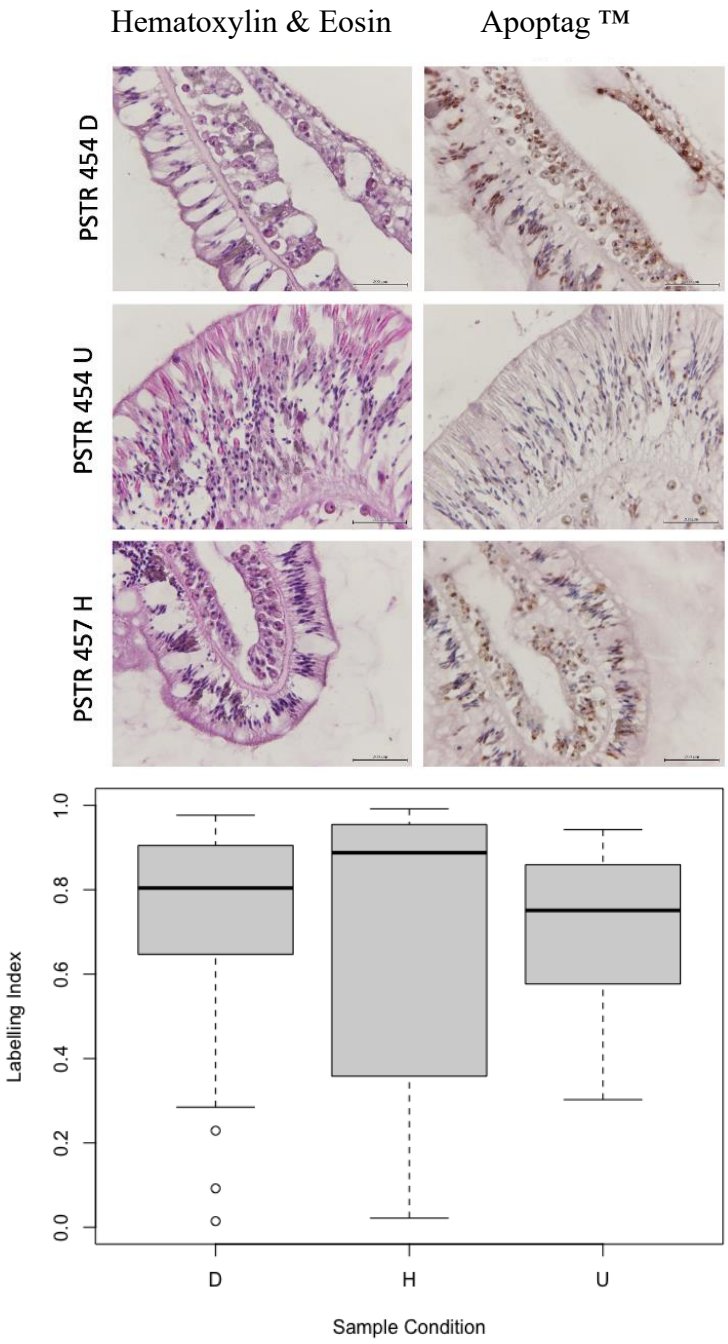

Figure 3: Comparison of tissue sections of healthy (H), diseased (D), and unaffected (U) tissues of *P. strigosa* stained with either hematoxylin and eosin or Apoptag Peroxidase In Situ Apoptosis Detection Kit. PSTR 454 D = Diseased, PSTR 454 U = Unaffected, PSTR 457 H = Apparently healthy. In micrographs stained with Apoptag, orange-brown staining indicates apoptotic nuclei, blue staining indicates intact nuclei. Apoptotic nuclei are present in all tissue types, diseased, unaffected, and apparently healthy. There was no significant difference in labelling index between tissue conditions (p=0.205, quasibinomial GAM).

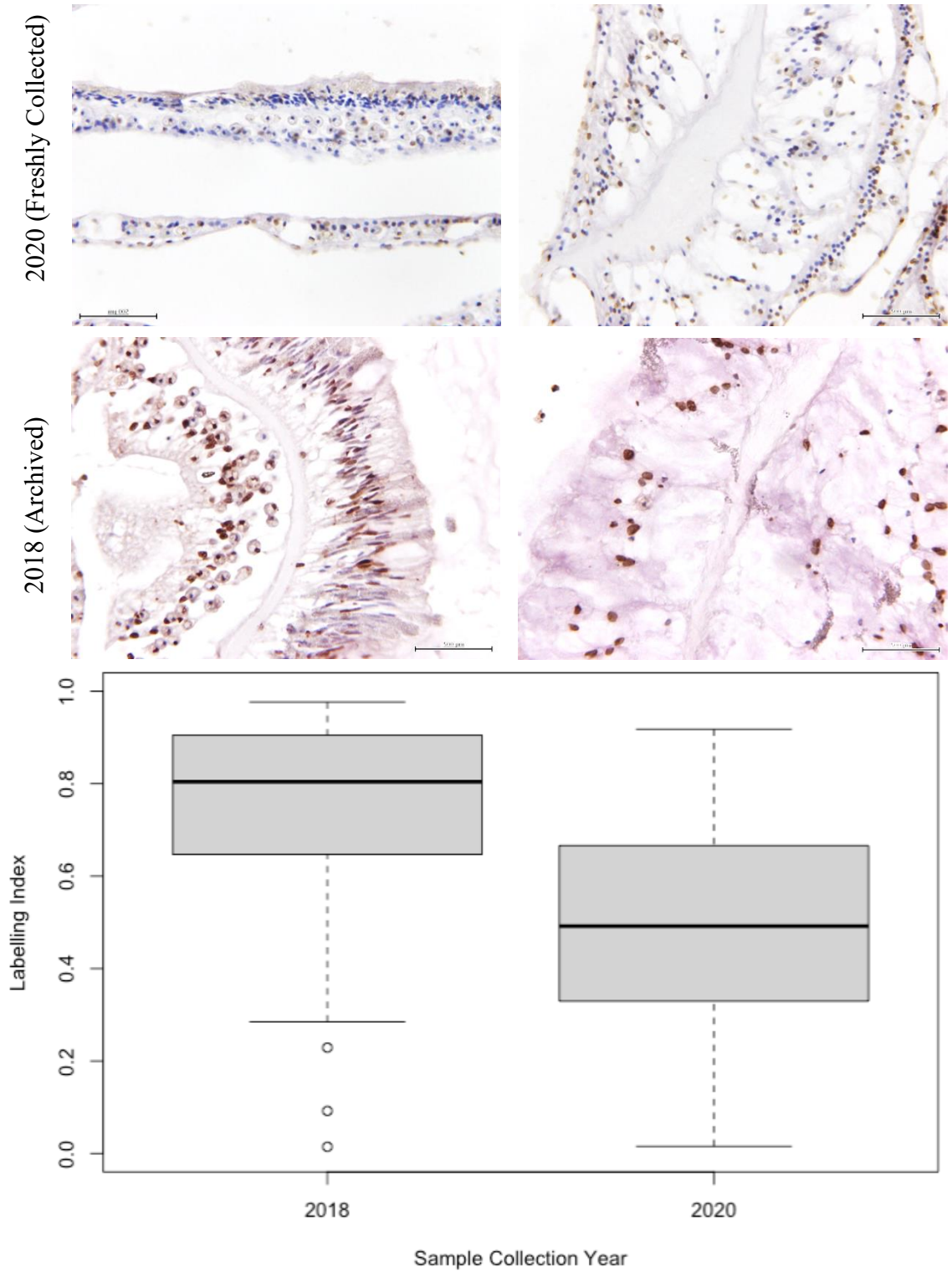

Figure 4: Example micrographs of diseased *P. strigosa* collected in 2020 (a, b) and 2018 (c, d). Samples collected in 2018 had a significantly higher area stained with DAB peroxidase than those collected in 2020, including a high degree of non-specific staining of cytoplasmic material ($p=3.84x10^{-13}$, quasibinomial GAM).

No part of the Apoptag Peroxidase *In Situ* Apoptosis Detection Kit protocol manipulated in this study significantly altered the degree of positive staining resulting from the TUNEL stain (Table 4). The proteolytic-induced epitope retrieval method with proteinase-K was the most effective method of exposing antigens, compared to the heat-induced epitope retrieval method (10 mM citrate buffer, three 3-min cycles in microwave) and detergent method (0.5% TRITON X-100 for 10 min at room temperature), which both damaged tissues and did not facilitate high resolution staining of nuclei (Figure 5). There was no significant difference in stain properties when varying the incubation time in or concentration of proteinase K ($p=0.628$, $p=0.9$, respectively) (Figure 6, 7). Therefore, using the original concentration and incubation time in the manufacturer's procedure is not suspected to have caused false-positive staining through chemically induced DNA fragmentation. Incubation with EDTA, either as the epitope retrieval method (boiled for one hour in place of proteinase K) or in combination with proteinase K (incubated for one hour at room temperature after incubation with proteinase K) was not effective at reducing the amount of positive staining, indicating that residual calcium in tissues is not contributing to non-specific staining seen here (Figure 8). Reducing the concentration of TdT enzyme in the working strength solution did not significantly reduce the amount of non-specific staining, either with proteinase K pretreatment ($p=0.23$, quasibinomial GAM; Figure 9), EDTA pretreatment ($p=0.427$, quasibinomial GAM), or with a combination of proteinase K and EDTA ($p=0.507$, quasibinomial GAM; Figure 10). While these modifications did not substantially alter the TUNEL reactivity, the following protocol alterations did improve the stain appearance and are recommended when applying the TUNEL assay to FFPE coral tissues. To reduce background staining of coral tissues and mucus, adding the additional blocking step between incubation with the TdT enzyme and the antibody conjugate is recommended. The progressive counterstain of Mayer's hematoxylin is more effective for staining only intact nuclei with minimal background staining, whereas the manufacturer-recommended counterstain of 0.5% aqueous methyl green was found to bind heavily to coral mucus and obscure cellular morphology from view.

Table 4: Results and observations from TUNEL optimization experiments.

Protocol Step	**Treatments**	**p-value**	**Suggestion**	**Observations**
Addition of Blocking Buffer Step	(A) Addition of incubation step with Roche blocking reagent between exposure to	N/A	A) Addition of Blocking Buffer	An additional blocking step reduced the background staining of DAB peroxidase substrate on cytoplasm on mucus, and is

	TdT enzyme and antibody conjugate, (B) Without exposure to blocking reagent*			recommended particularly for mucus-heavy species
Concentration of proteinase-K	(A) 5 µL/mL, (B) 10 µL/mL, (C) 12 µL/mL, (D) 15 µL/mL , (E) 20 µL/mL *	p=0.628	E) 20 µL/mL *	There was no significant difference in staining with an increasing gradient of concentration. 20 µL/mL was chosen to align with manufacturer's instructions, though lesser concentrations are acceptable to reduce chemical cost.
Incubation time with proteinase-K	(A) 5 minutes, (B) 10 minutes, (C) 15 minutes*	p=0.9	C) 15 minutes*	There was no significant difference in stain with an increasing incubation duration. 15 minutes was chosen to align with manufacturer's instructions, though shorter durations are acceptable to reduce overall protocol time.
Antigen retrieval method	(A) 20 µL/mL proteinase K 15 minutes* (B) 10mM citrate buffer heated for three 3-minute cycles in microwave (C) 0.5% TRITON X-100 for 10 minutes	N/A	(A) incubation with 20 µL/mL proteinase K for 15 minutes at room temperature	Exposure to the digestive enzyme proteinase-K resulted in the best resolution of both apoptotic and intact nuclei. The heated method with citrate buffer was damaging to tissue and did not allow for strong staining. The detergent method with 0.5% TRITON X-100 also did not produce intense or refined staining.
Counterstain	(A) 0.5% Methyl Green* (B) Mayer's Hematoxylin	N/A	(B) Mayer's Hematoxylin	0.5% Methyl green heavily stained coral mucus and obscured nuclei. Mayer's hematoxylin resulted in sharp staining of intact nuclei with very little-to-no background staining of coral tissues and mucus.
Pretreatment with EDTA	Slides were boiled in 3% EDTA (pH 7.2) for 1 hour and incubated with the following concentrations of TdT Enzyme in Reaction Buffer (RB): (A) 30% TdT (B) 20% TdT (C) 10% TdT (D) 6% TdT	p=0.427	Pretreatment with EDTA is not recommended	Tissues boiled with 3% EDTA as the epitope retrieval method incurred unpredictable damage from the boiling processes and displayed a high degree of non-specific staining of cytoplasmic material. There was no significant difference when stained with decreasing concentrations of TdT enzyme.
Pretreatment with proteinase K + incubation with 3% EDTA	Slides were pretreated with 20 µL / mL of proteinase K for 15 minutes and incubated	p=0.507	The addition of an EDTA incubation step	There was no significant difference in staining reactivity when a 1-hour incubation period with room temperature EDTA is

	in 3% EDTA (pH 7.2) for 1 hour, then incubated with the following concentrations of TdT enzyme: (A) 40% TdT (B) 11% TdT		is not recommended	added, so this additional step is not recommended.
Concentration of TdT Enzyme	(A) 41% TdT (B) 31% TdT (C) 21% TdT (D) 11% TdT	p=0.23	(A)41% TdT*	There was no significant difference in the degree of positive staining with varying concentrations of TdT enzyme. A concentration of 41% was chosen to align with manufacturer's recommendation.

* recommended by manufacturer
Steps with no p-value were analyzed using observations only.

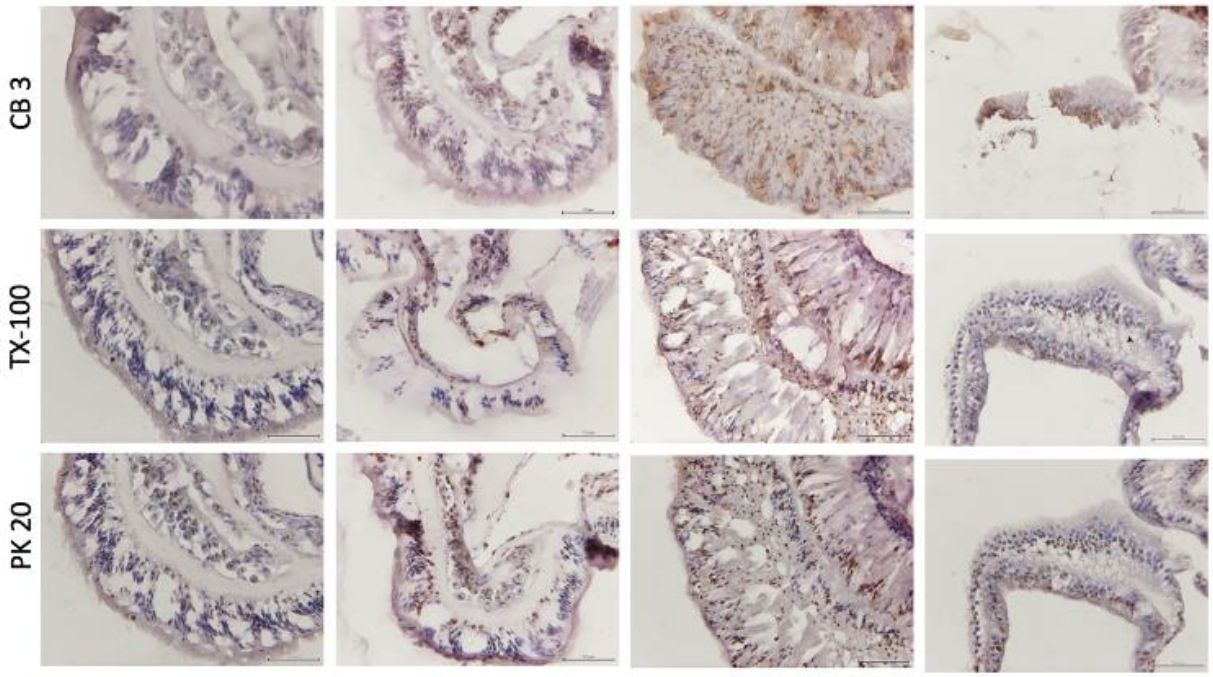

Figure 5: Comparison of staining quality using different epitope-retrieval methods in TUNEL method protocol on diseased *P. strigosa* tissues. CB 3 = 10 mM citrate buffer, heated for three 3-min cycles in a microwave; TX-100 = 0.5% TRITON X-100 for 10 min at room temperature; PK 20 = 20 µl/mL proteinase-K for 15 min at room temperature. Orange-brown staining indicates apoptotic nuclei, blue staining indicates intact nuclei. Specimens treated with proteinase-K (PK 20) showed best staining results with highly refined nuclei and strong specific staining. The heated treatment in citrate buffer (CB 3) appeared to damage tissues, the detergent treatment with Triton X-100 (TX-100) displayed lower resolution and intensity staining.

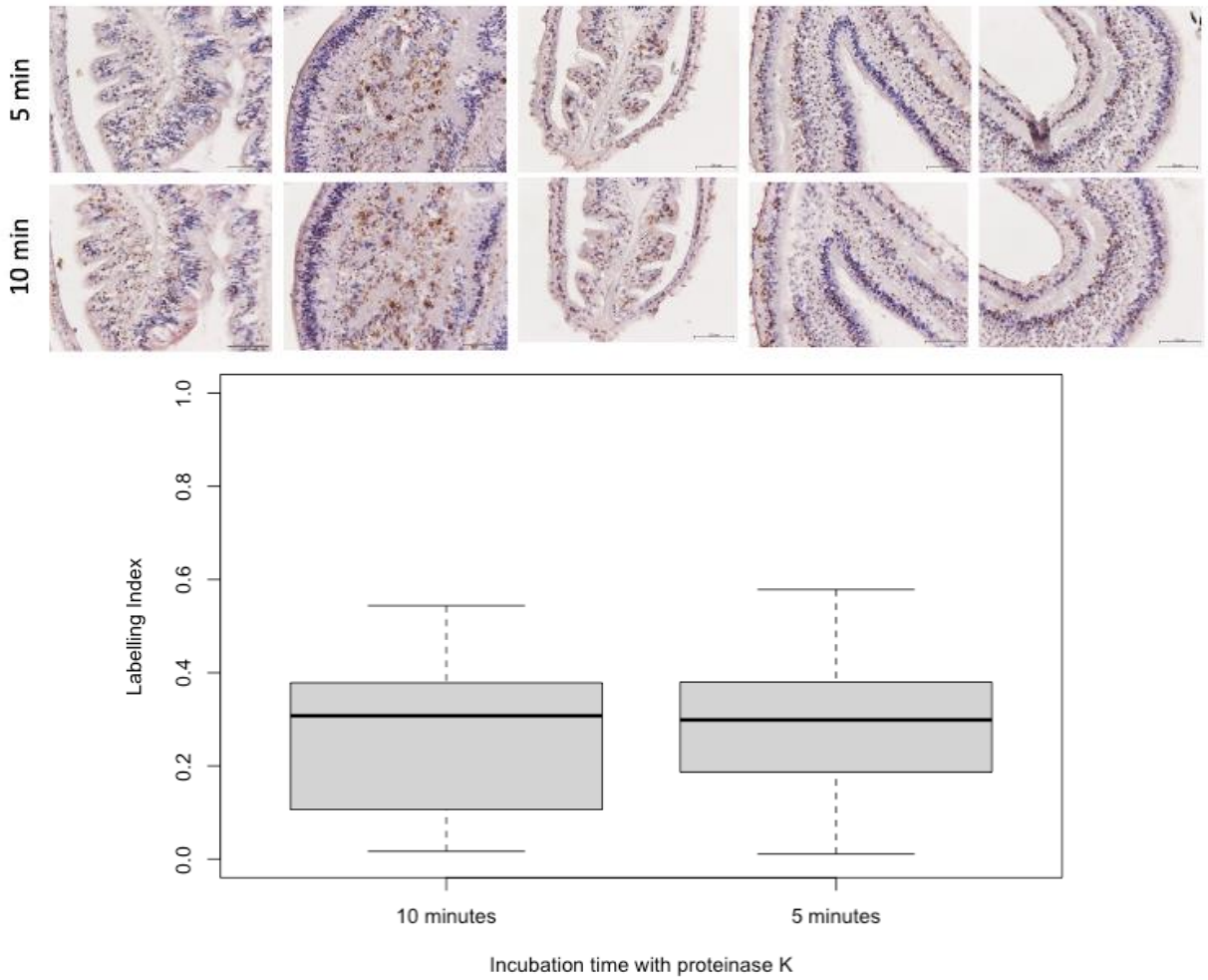

Figure 6: Comparison of TUNEL reactivity on diseased tissue of *D. stokesii* with 5- and 10-minute incubation times in proteinase-K. There was no significant difference between the degree of positive staining between treatments (p=0.9, quasibinomial GAM). Variations in staining between treatments is attributed to small differences between the sections as they were taken from the tissue block.

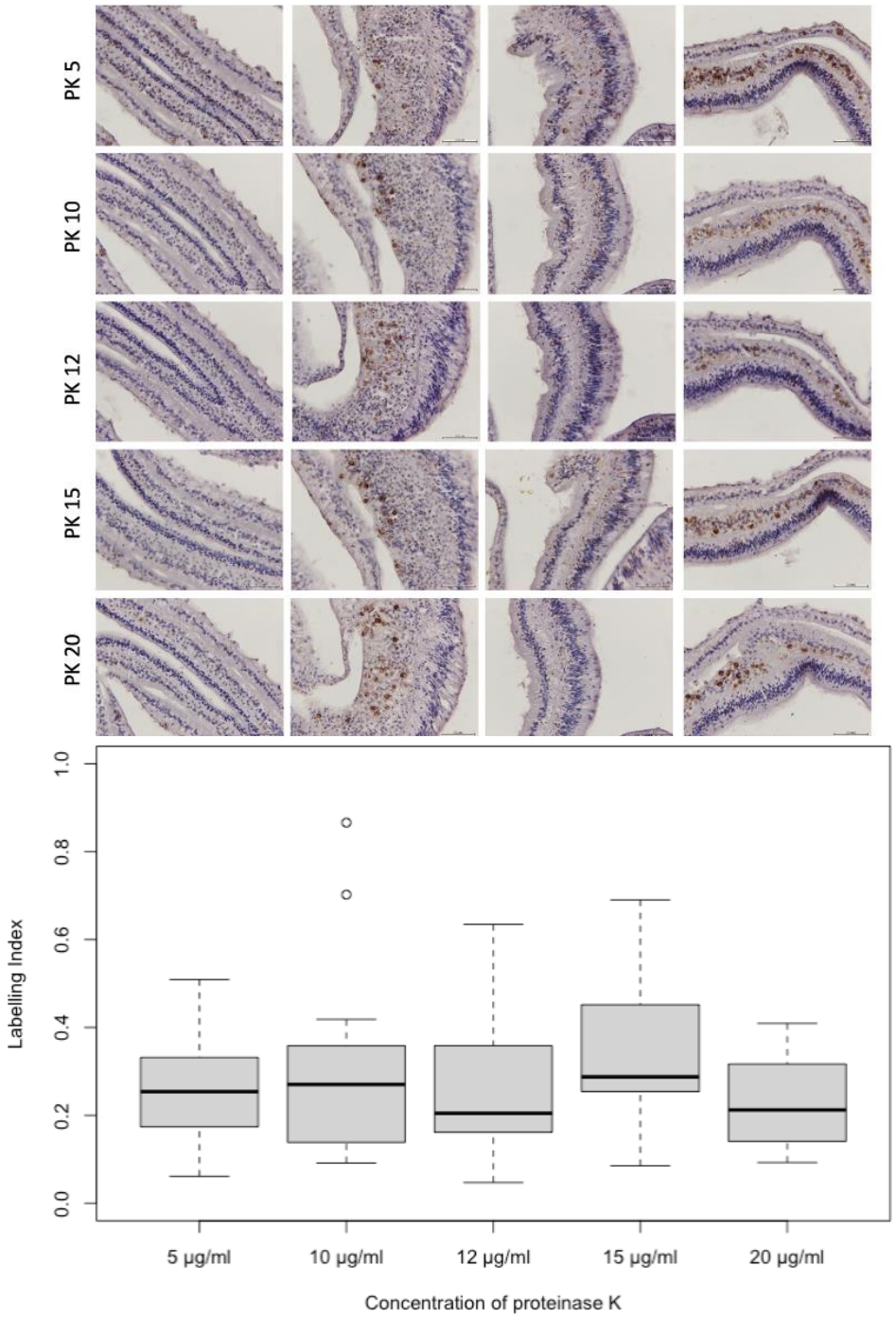

Figure 7: Comparison of TUNEL reactivity on diseased tissues of *D. stokesii* with varying concentrations of proteinase-K. PK 5 = 5 µL/mL. PK 10 = 10 µL/mL, PK 12 = 12 µL/mL , PK 15 = 15 µL/mL , PK 20 = 20 µL/mL . There was no significant difference in the degree of positive staining in any treatment (p=0.628, quasibinomial GAM). Variations in staining between treatments is attributed to small differences between the sections as they were taken from the tissue block.

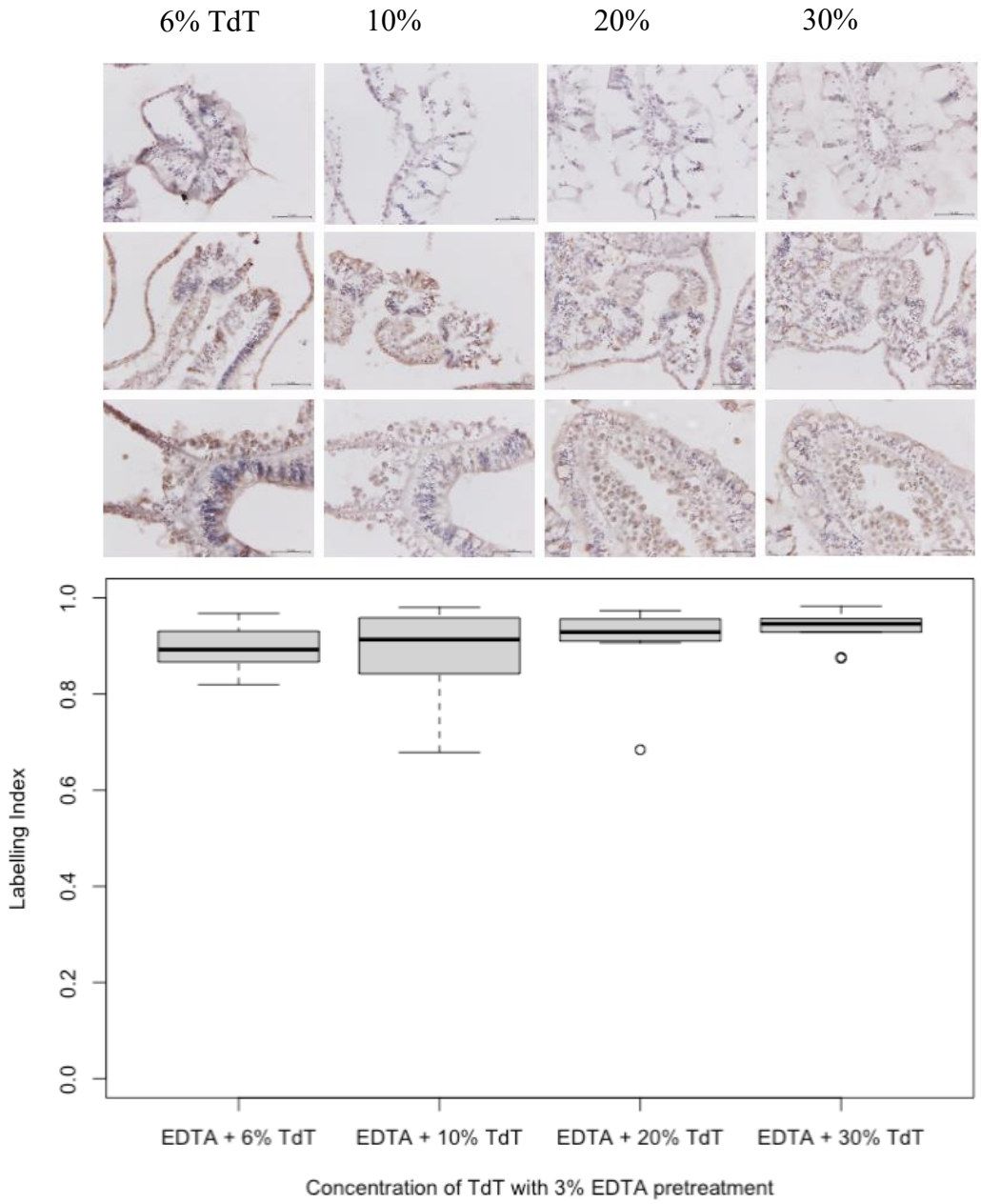

Figure 8: Comparison of diseased tissues of *P. strigosa* pretreated with boiling 3% EDTA (pH 7.2) for 1 hour and incubated with different concentrations of TdT enzyme to reduce background staining. No significant difference in degree of positive staining was detected between treatments (p= p=0.507, quasibinomial GAM). Tissues exhibited a high degree of background staining, low nuclear resolution, and apparent damage from boiling.

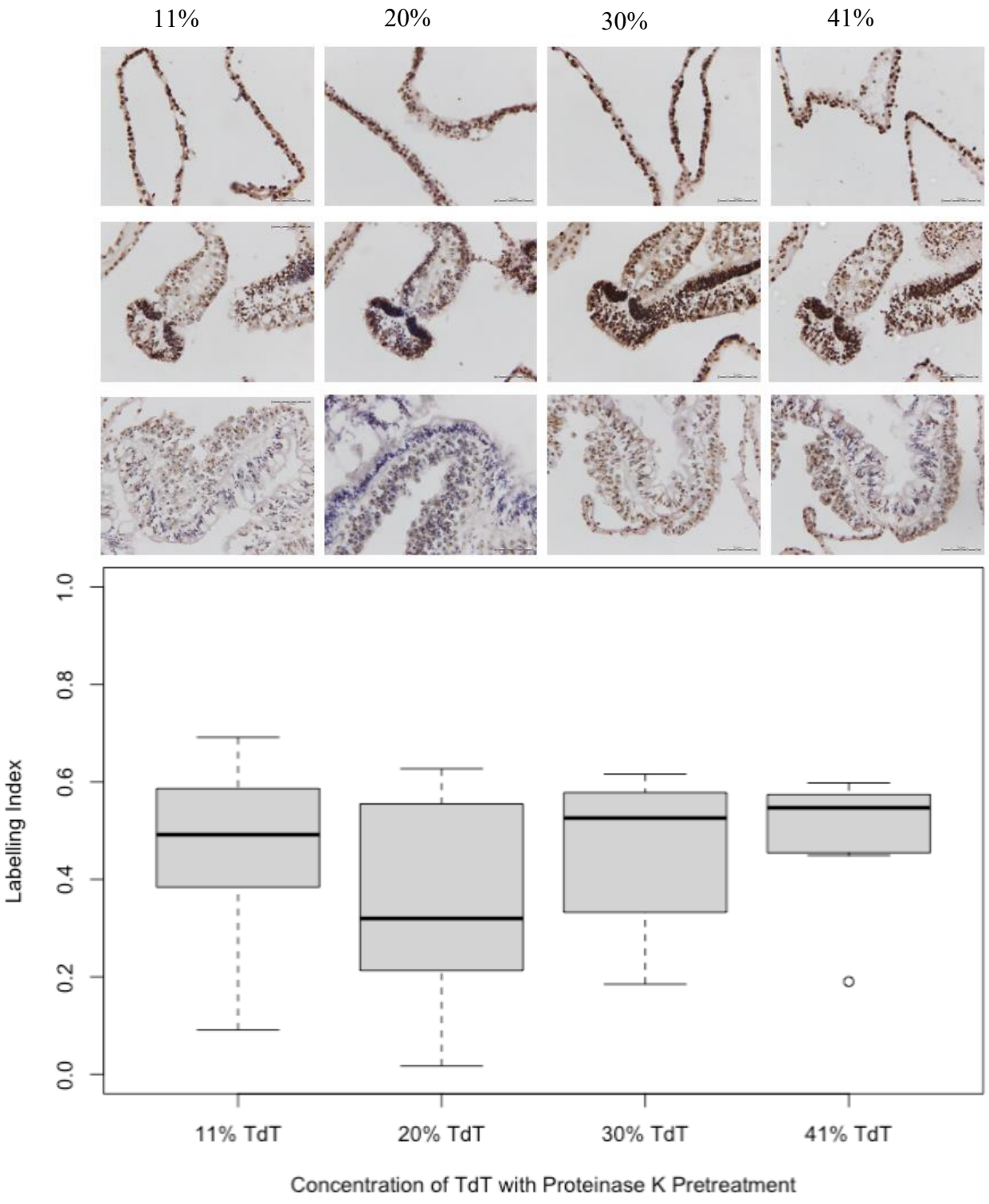

Figure 9: Comparison of tissues treated with varying concentrations of TdT enzyme. No significant difference in the degree of positive staining was detected between concentrations of 11%, 20%, 30%, or 41% TdT enzyme diluted in reaction buffer (p=0.23, quasibinomial GAM).

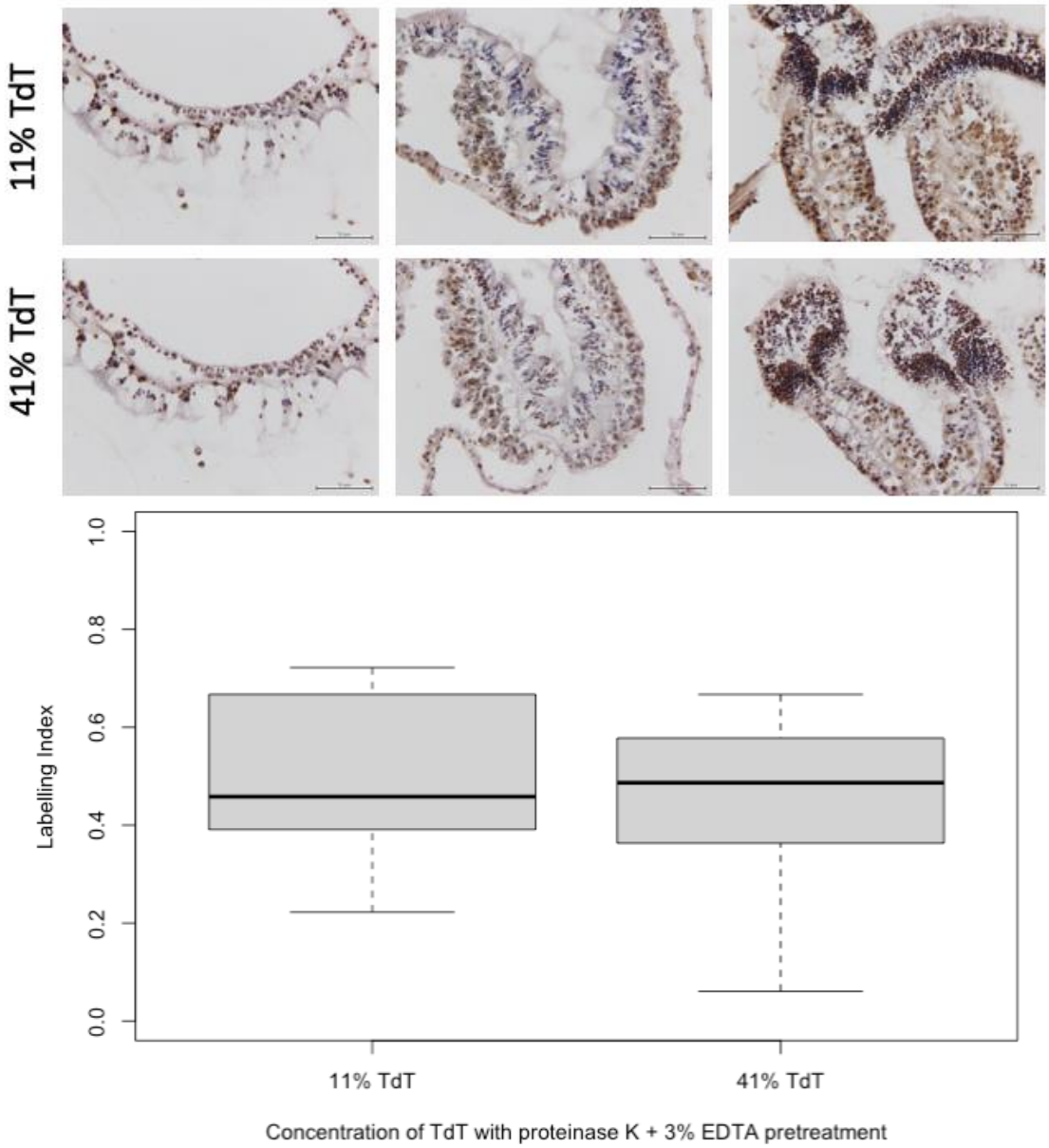

Figure 10: Comparison of tissues pretreated with 20 µL /mL of proteinase K for 15 minutes and incubated in 3% EDTA (pH 7.2) for 1 hour, then incubated with either 11% or 41% TdT enzyme. There was no significant difference between the degree of positive staining between concentration treatments (p=0.507, quasibinomial GAM).

3.2 Patterns of cell death in freshly-collected samples of SCTLD coral tissues

In the freshly collected *P. strigosa* samples, the epithelial cells of the basal body wall (calicodermis, aboral gastrodermis) exhibited a significantly higher apoptotic signal than all those of the surface body wall epidermis, oral gastrodermis, and mesenterial filaments ($p=3.56\times10^{-12}$, quasibinomial GAM) (Figure 11). Positive staining for DNA fragmentation was noted to be occurring in all areas with evident microlesions, such as areas of complete tissue rupture, cell swelling (particularly in the basal body wall), and in areas of necrosis of epithelial cells resulting in tissue layer dissociation (Figure 12). Positive staining for apoptosis was detected in coral cells of the gastrodermis and in the algal cells of *in hospite* zooxanthellae in all species tested (Figure 13). In the three freshly collected samples of *P. strigosa* available for analysis, there was no significant difference in the proportion of positively stained cells in these two cell populations ($p=0.423$, quasibinomial GAM; total cell count: 1,287), with the average proportion of positive-stained cells between 21–25% for both cell types. There was a substantial difference in the proportion of apoptosis observed in these cell populations between species, with diseased *D. stokesii* exhibiting the highest proportion of zooxanthellae cells positive for apoptosis (92–100%, total cell count: 703) and *C. natans* being the only species that exhibited less positive staining in zooxanthellae than coral cells (total cell count: 350)(Figure 13).

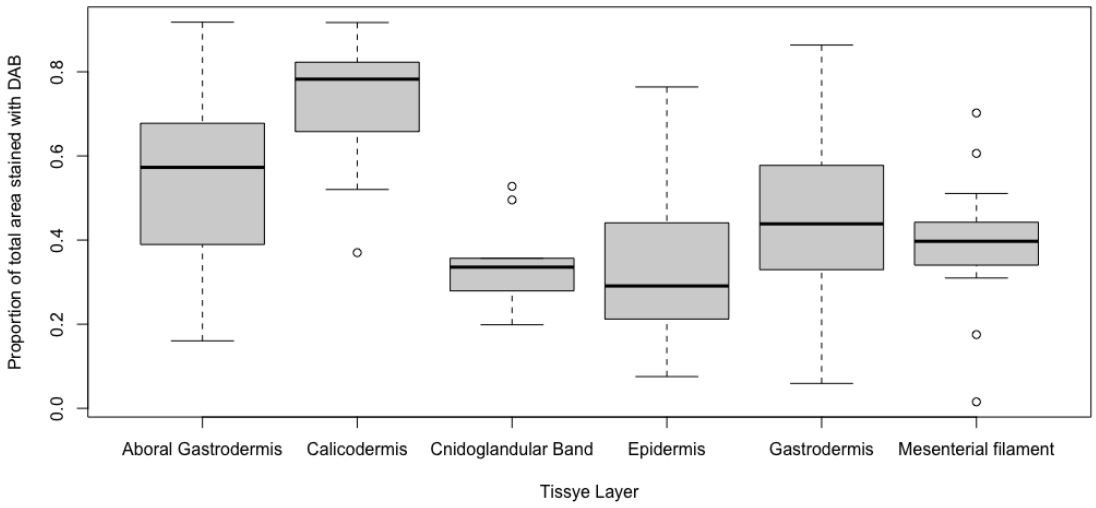

Figure 11: There was a significant difference in the degree of positive staining in different coral tissues of freshly collected diseased *P. strigosa*, with the highest signal in the calicodermis of the basal body wall ($p=3.56\times10^{-12}$, quasibinomial GAM).

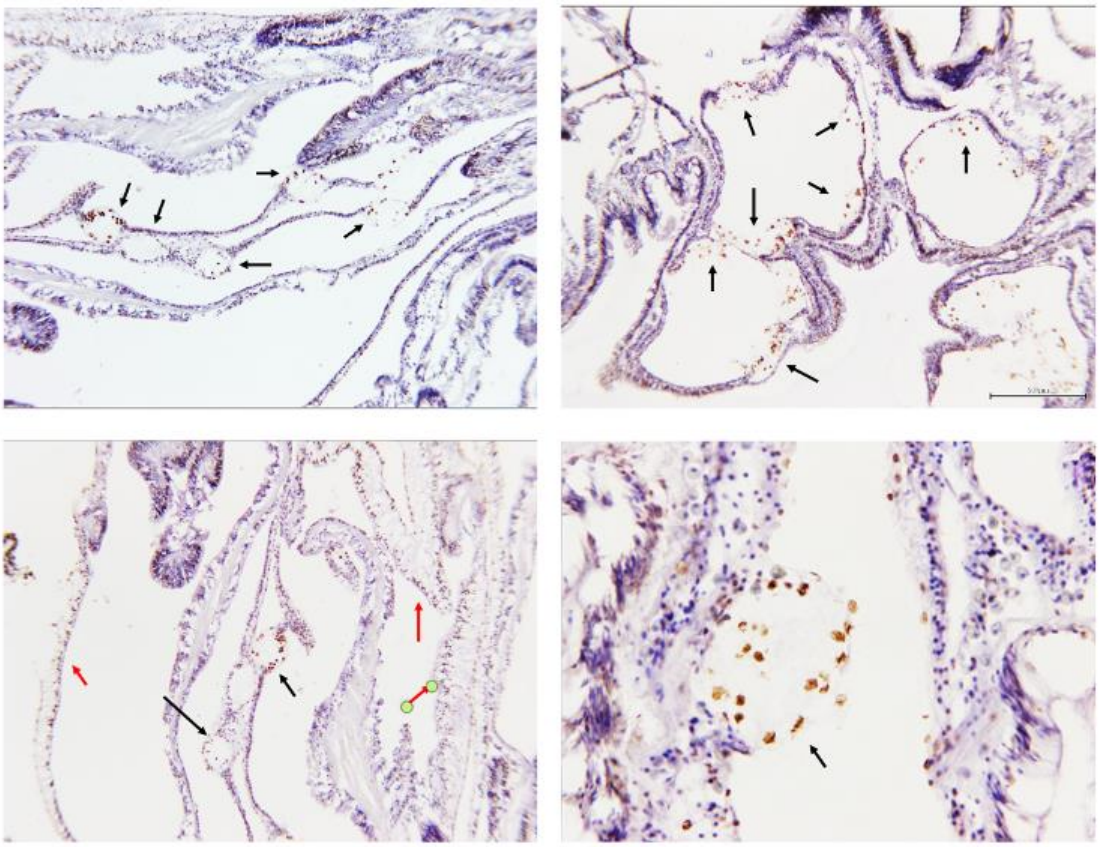

Figure 12: DNA fragmentation observed to be lining areas of tissue rupture and necrosis in the SCTLD lesion of *P. strigosa.* Orange-brown staining indicates apoptotic cells, blue staining indicates intact nuclei. Black arrows point to areas of tissue rupture and accompanying apoptosis, red arrows point to areas of apoptosis in a swollen basal body wall (BBW).

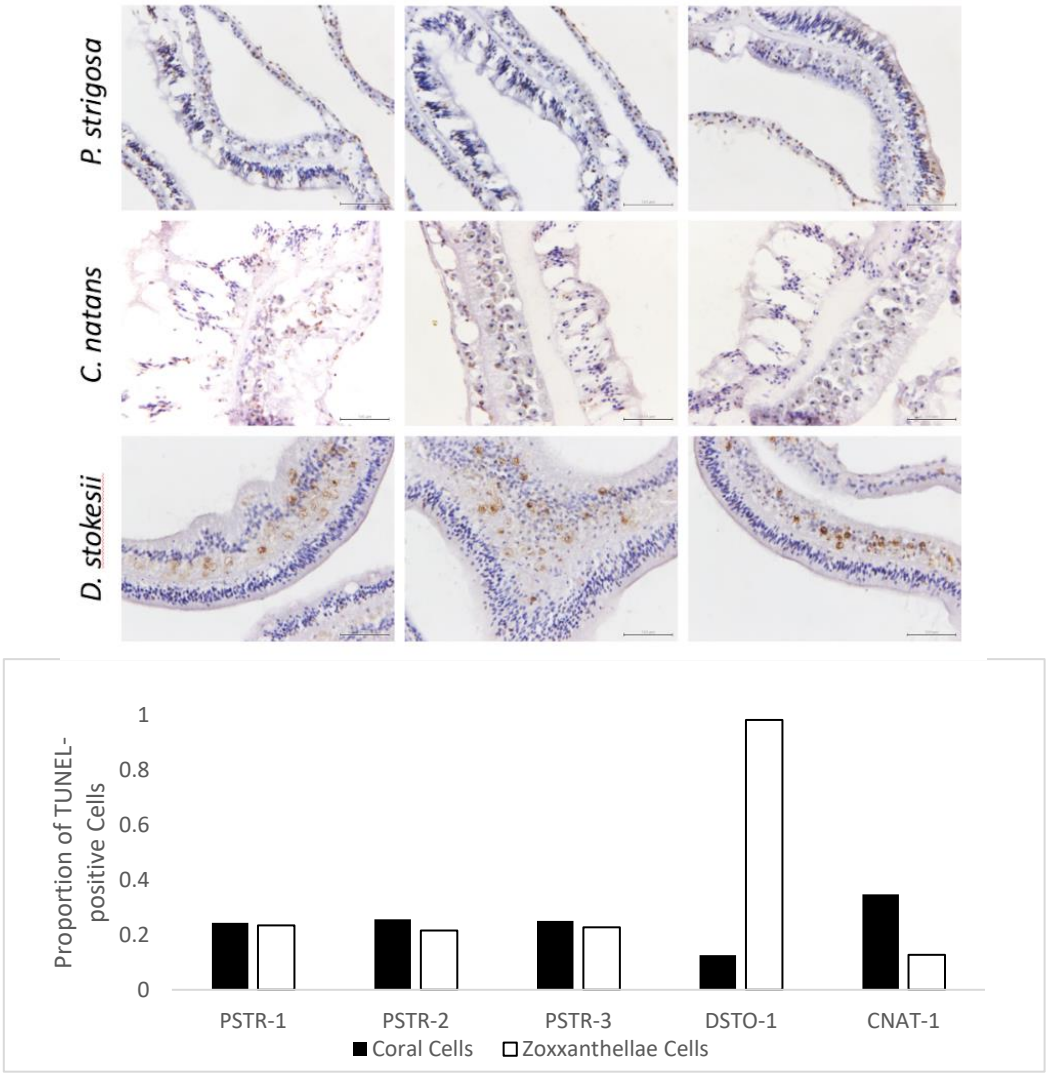

Figure 13: Comparison of TUNEL reactivity in the coral and Symbiodiniacea cell populations of the oral gastrodermis between species (*P. strigosa, C. natans, D. stokesii*). There was an apparent difference between the proportion of positive staining in these cell populations between samples, with the highest expression in zooxanthellae cells seen in the single sample of diseased *D. stokesii*.

4. Discussion

The TUNEL method is one of the most common techniques for visually assessing PCD in fixed tissues and has been successfully applied to scleractinian tissues in past studies investigating coral disease (Ainsworth et al. 2007a; Ainsworth et al. 2008; Yasuda 2012; Ainsworth et al. 2015) and thermal stress (Pernice et al. 2011), as well as studies investigating cell death in the model cnidarian organism *Aiptasia* sp. (Dunn et al. 2002; Dunn et al. 2004;

Richier et al. 2006). The TUNEL method overcomes many of the limitations of using morphological indicators to distinguish cell death mechanisms, and is thus a powerful tool for locating this process in pathological studies that might otherwise underestimate PCD in disease. Despite its wide applications, interpreting results from TUNEL-stained micrographs should be done with caution, as the technique has been shown to be highly sensitive to even small differences in processing steps (Garrity 2003), and can often cause false-positive staining (Stahelin et al. 1998; Pulkkanen et al. 2000) and labelling of DNA fragments in nuclei undergoing other cell death processes (Gold et al. 1994; Grasl-Kraupp et al. 1995; Dunn et al. 2002). The stain properties observed in this study and the significant difference between archived and fresh tissue samples validate these concerns and highlight the important influence that histological processing can have on tissue conditions and appearances. In particular, this study highlights the limitations of using fixed archived tissues for immunohistochemical analysis, which are at a greater risk of DNA degradation that may confound results. However, while this study was unable to fully optimize the TUNEL assay protocol for the full sample set of FFPE coral tissues used here, it does provide some potential insights into the possible involvement of PCD in coral tissue-loss diseases and in apparently healthy corals.

The staining properties observed in the archived tissue samples, which produced a high average DAB staining intensity of 73% and included a high degree of non-specific staining of cytoplasmic material, are believed to be the result of a faulty TUNEL stain reaction and may not be indicative of genuine cellular processes. Because a similar pattern of overstaining was consistent in all tissue conditions collected in 2018, including healthy tissue exhibiting no grossly detectable signs of disease, and because none of the protocol steps manipulated in this study had any significant influence on stain pattern, I concluded that the samples are likely incompatible with the TUNEL method. There are a few differences between the archived and freshly collected samples that may be the cause of their different reactivity to the TUNEL stain, including the duration of fixation, collection method, and the wait time between embedding and staining.

The extensive fixation time of the archived samples (103-256 days) may have contributed to DNA damage that influenced the reactivity to the TUNEL assay. Fixation is a critical step in histological processing and can profoundly influence tissue conditions (Garrity 2003). Most immunohistochemical procedures require that the tissue is fixed in a formalin-based solution,

such as paraformaldehyde, and require a precise incubation duration to prevent over- or under-fixation. Formalin-based fixatives preserve tissues by creating crosslinks between the amino acid groups in all proteins within the tissue. If a sample is over-fixed, which produces an excess of cross links that may be difficult or impossible to remove during the epitope retrieval process, it may result in an artificially lower signal of the target antibody (Davison et al. 1995). In some reports, over-fixation can also cause false-positive staining with the TUNEL method, as found by Tamura et al. (2000), which reported significantly higher positive staining in cardiac tissues fixed in formalin for 8 weeks compared to 3 days (Tamura et al., 2000). In studies that have applied this technique to fixed coral tissues, the fixation duration ranged from 8 hours (Ainsworth et al. 2007a, b, Pernice et al. 2001) to 12 hours, and the 2020 coral samples collected in this study were fixed for 18 hours. A number of studies investigating the molecular weight of DNA extracted from formalin-fixed tissues have found evidence of DNA degradation (Dubeau et al. 1986; Bramwell & Burns 1988; Hamazaki et al. 1993; Koshiba et al. 1993; Giorgi et al. 1994; Greer et al. 1994; Douglas & Rogers 1998), particularly at elevated temperatures (Haselkorn & Doty 1960; Fraenkel-Conrat 1954). An alternative to the neutral buffered formalin often used in IHC application is zinc-buffered formalin, which was used in the archived tissue collection analyzed in this study. The zinc ions present in these solutions prevent excessive cross linking and have been shown to be more effective at preserving DNA integrity than neutral buffered formalin (Wester et al. 2003). However, the extensive incubation time in this fixative and any temperature fluctuations that they might have incurred during transport and storage may have overcome these benefits and caused fixation-related DNA degradation. While archived tissues present a valuable resource for histological research, samples fixed in formalin and stored for extensive periods may have limited potential for immunohistochemical analyses. Future studies are needed to determine the maximum fixation time that coral tissues can endure before adverse molecular artifacts become apparent.

The archived samples used in this study experienced an extensive storage period as paraffin-embedded blocks, which may further increase the likelihood of DNA degradation over time and result in false-positive staining (Watanabe et al. 2017; Groelz et al. 2018). In prior studies that have used the TUNEL method on coral tissues and in the 2020 samples collected in this study, the collection, fixation, embedding, and full immunohistochemical processing have occurred in the minimum allowable time. A study by Watanabe et al. (2017) investigated DNA

degradation in FFPE tissues and found that there was significant age-related DNA damage in tissues stored for 0.5, 3, 9, and 12 years (Watanabe et al. 2017). In the archived tissue samples that were stored as paraffin blocks for more than 2 years, it is possible that over time the DNA began to degrade and produced artificial breaks that were labelled in the TUNEL method as apoptotic. However, some studies have found evidence that target antigens were still adequately detectable after storage in paraffin blocks for up to over 3 years (Beckstead 1994). Further studies are needed to determine how long-term storage in paraffin may change the morphological and genetic integrity of coral tissues.

A challenge to conducting standardized histological analysis on field-collected coral tissues lies in ensuring that collection techniques are consistent and do not create histological artifacts. On branching corals, such as those in the genus *Acropora*, collection can be completed by breaking or cutting the branch tips, which can be swift and minimizes mechanical damage to tissues away from the cut margin. On boulder and brain corals, such as the *P. strigosa* that was the focus of this study, collection can be more difficult and may present multiple opportunities for collection-related stress. Tissue cores are most often extracted using either a hammer and sharpened corer or using a hole drill-bit on a pneumatic drill, which can damage the tissues of the sample and of the surrounding area by heavy vibration and mechanical lacerations. Vibrational stress may be a particular problem for the gastrodermis and its resident algal symbionts, as applying repeated shocks to coral tissues is a common method for intentionally dislodging symbiotic endodermal cells from intact coral tissues (Nielson et al. 2018). In addition to the physical collection from the colony, field collections can also incur a variable amount of time between extraction and fixation, which can provide additional opportunities for stress exposure that might influence sensitive cellular mechanisms, such as apoptosis or mucus secretion. It is possible that even acute exposure to sampling stress might prompt an upregulation of the PCD pathway, which has been shown to be activated immediately (<2 minutes) after exposure to thermal stress in *Aiptasia* sp. (Dunn et al. 2004). Particularly because there was no significant difference between the degree of positive staining between the diseased and healthy samples, and because preliminary results from concurrent studies on the general histopathology of these samples reported substantial microlesions in all sample conditions (Landsberg et al. 2020), it is possible that the collection technique produced stress-induced lesions that are difficult to disentangle from SCTLD. Future studies should optimize coral tissue collections to minimize the

risk of such collection-related artifacts, particularly in boulder corals and for the purpose of sensitive immunohistochemical analyses.

The freshly collected tissues of *P. strigosa*, *D. stokesii*, and *C. natans* exhibited a more comparable degree of positive staining for PCD (average proportion of positively stained area 49%) to that seen in past studies that have successfully applied the TUNEL assay in coral disease and quantified PCD using cell counts (24–58%, Ainsworth et al. 2007). While this gives us a degree of confidence of the results of TUNEL labeling as applied here, these results are only suggestive—future studies are needed to apply this technique to a more complete sample set of diseased and apparently healthy coral tissues to fully determine the role of PCD in SCTLD lesions, as the opportunistically acquired samples tested here did not include asymptomatic controls. It is also important to note that the TUNEL assay has been reported to incorrectly label fragmented DNA in heavily necrotic areas as apoptotic (Grasl-Kraupp et al. 1995; Cummins et al. 1997; Jerome et al. 2000), so it is important to confirm the positive staining in these tissues with morphological data via transmission electron microscopy. Nevertheless, the results of this preliminary work can provide some potential insights into the role of PCD in SCTLD and provide some foundational information for future studies.

In the freshly collected samples of *P. strigosa*, *D. stokesii*, and *C. natans*, PCD was apparent in all areas with an evident histopathological lesions, such as areas of necrosis, tissue layer rupture, or epithelial cell dissociation, and was also present in many areas with no apparent lesion. Because of the overlap between apoptotic and necrotic phenotypes under certain cellular conditions (Nicoretta et al. 1998; Oppenheim et al. 2001), and because necrotic cells can also contain fragmented DNA that can be incorrectly labelled as apoptotic (Gold et al. 1994; Grasl-Kraupp et al. 1995; Cummins et al. 1997; Jerome et al. 2000; Dunn et al. 2002), it is difficult to discern from these tissues which process is driving lesion development or whether they co-occur independently of one another. In all of these samples, PCD was detected in all epithelia (epidermis, gastrodermis, calicodermis, and mesenteries) but was highest in the calicodermis of the basal body wall. This is consistent with the reported histopathology of SCTLD, which notes that this epithelium has a high abundance of necrotic microlesions (Landsberg et al. 2020; SCTLD Case Definition 2018). It is possible that the calicodermis is an area particularly prone to energy depletion, which may cause an initially apoptotic phenotype to devolve into a necrotic one as ATP and caspases become depleted (Nicoretta et al. 1998; Oppenheim et al. 2001). The

specialized cells in the calicodermis facilitate the chemical reactions that create the coral's aragonite skeleton, provide the first line of defense against endolithic invasions (Renegar et al. 2008), and have been shown to be heavily involved in wound-repair processes (Work & Aeby 2010). There is also evidence that energy depletion plays an important role in the pathology of white syndrome lesions, as seen as diminishing lipid reserves in diseased colonies vs. apparently healthy colonies of acroporids (Smith et al. 2020). Past studies on the histopathology of *Acropora cervicornis* with WBD, another lethal white syndrome, have likewise shown pathogenesis to be originating in the basal body wall (Hightshoe et al. unpublished; Miller et al. 2014). It is not currently known whether endolithic organisms are contributing to SCTLD, or whether the high energy input in this epithelium makes it more vulnerable to disease during times of stress. The many functions of this tissue layer perhaps suggest that it is dynamic and pluripotent, but rates of routine cell proliferation and death are not known. Like epithelial cells in mammals, the cells of the calicodermis may routinely express high rates of cell turnover (Green 2010), which may contribute to the high positive signal observed here. Further analysis is needed to determine the normal range of apoptosis expression in optimally functioning coral epithelia to better understand how expression changes under stressed and diseased conditions.

Apoptosis was detected in both the coral host cells and in the zooxanthellae of all samples tested. Vacuolization and various forms of degradation of zooxanthellae within the surface body wall gastrodermis have been identified as a diagnostic signature of SCTLD, although the precise role of the symbionts in the disease remains unclear (SCTLD Case Defnition 2018; Landsberg et al. 2020). It is possible that the algae are vulnerable to changing conditions within the coral's tissues as it becomes affected by SCTLD and their *in hospite* environment becomes toxic. Alternatively, it is also possible that the symbionts themselves are the target of a pathogen, which may be either biotic or abiotic in nature, and that this pathology indirectly causes the lethal tissue-loss lesion in the coral host. It is not possible to discern from the patterns of cell death in these tissues which of these scenarios appears to be more likely, although it is possible for PCD to be activated in either situation. PCD has been shown to be an important process in the bleaching response in scleratinian corals and in the sea anemone *Aiptasia* sp. (Dunn et al. 2002; Richier et al. 2006; Wies 2008; Tchernov et al. 2011), and it has been shown to be expressed in both the algal symbiont and in the coral gastrodermal (endoderm) cell (Dunn et al. 2004). Programmed cell death can also be activated in response to an

intracellular pathogen (King & Cidlowski 1998; James & Green 2004; Gao & Kwaik 2004; Zebell & Don 2015), and Symbiodiniaceae have been shown to be vulnerable to pathogens within the coral host, which is associated with coral diseases such as yellow-band disease (Cervino et al. 2004), *Porites* white patch syndrome (Lawrence et al. 2015), and some instances of coral bleaching (Correa et al. 2016).

One possible way for an apoptotic cascade in the population of algal symbionts to initiate a similar response in surrounding coral cells is through the release of reactive oxygen species. Reactive oxygen species (ROS) are a necessary signal in both the intrinsic (prompted by intracellular stress) and extrinsic (prompted by extracellular signals) PCD pathways (Liu et al. 2007; Redza-Dutordoir & Averill-Bates 2016). The excessive production of ROS is cited as the primary promoting signal of upregulated PCD in diseases of plants and higher organisms (Levine et al. 2005; Zurbriggen et al. 2010); and it is well documented that Symbiodiniaceae and other marine algae have been shown to produce excessive ROS in response to stress (Franklin et al. 2004; Ross et al. 2005; Lesser & Farell 2004; Lesser 2006; Richier et al. 2006; Saragosti et al. 2010; Tchernov et al. 2011). Indeed, this mechanism has been implicated as a defining factor in the selective survival of corals to bleaching (Dunn et al. 2004; Richier et al. 2006; Weis 2008; Tchernov et al. 2011). More work is needed to determine the roles of PCD in both the symbiont and gastrodermal (endoderm) cells of diseased coral tissues.

While PCD was evident in the Symbiodinaceae populations in all species, the dramatic difference in PCD expression between *P. strigosa*, *D. stokesii*, and *C. natans* may suggest interesting differences in PCD expression among species. In *P. strigosa*, there was no significant difference in the frequency of PCD between the coral host cells and the Symbiodinaceae. In *D. stokesii*, the zooxanthellae cells in the gastrodermis of the surface body wall were almost exclusively stained positively for PCD, with 92–100% of algal cells within the gastrodermis expressing PCD compared to only 12% of the coral host cells in the same region. Alternatively, *C. natans* was the only one to express more PCD in the coral host cells than the algal symbionts (34% and 12%, respectively). While these results are only suggestive, this may indicate that differences in coral or symbiont species may differentially express PCD in SCTLD. A study by Feuss et al. (2017) found that corals of different phylogenetic lineages utilized the PCD pathway as an immune strategy differently, and that the differences aligned with the species' susceptibility to disease (Fuess et al. 2017). Alternatively, it is possible that the species of

Symbiodinaceae themselves are differentially susceptible to a PCD cascade. Future studies are needed to confirm these patterns of cell death in more replicates of each species of both coral and symbiont.

This study was unable to definitively determine the role of PCD versus necrosis in stony coral tissue loss disease. The non-specific staining properties of the TUNEL assay applied to archived paraffin embedded coral tissues highlights the limits of archived collections for this technique and suggests that fixation and storage durations can impact DNA integrity in coral tissues. However, applying the TUNEL assay to freshly collected samples of corals with SCTLD did provide some potential insights into the involvement of PCD in this disease, although further work is needed for fully describing the mechanisms of cell death driving SCTLD lesions. Doing so will uncover important information regarding the coral's immune response to this lethal disease, which will inform future studies investigating its ultimate etiological cause and mitigation strategies.

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