Gill lesion assessment in greenlip abalone (*Haliotis laevigata*) held at elevated water temperature.

Histopathology of *Haliotis laevigata* gills

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Abstract

Water temperatures which exceed thermal optimal ranges (~19-22°C for greenlip abalone (*Haliotis laevigata*), depending on stock genetics) can be associated with abalone mortalities. We assessed histopathological changes in *H. laevigata* gills held in control (22°C) or elevated (25°C) water temperature conditions for 47 days by developing a new scoring protocol which incorporates histopathological descriptions and relative score summary. Lesions were allocated to one of three reaction patterns: (1) epithelial, (2) circulatory or (3) inflammatory and scored based on their prevalence in gill leaflets. Indices for each reaction pattern were calculated and combined to provide an overall gill index. *Haliotis laevigata* held in 25°C water temperature had significantly more epithelial lifting and hemolymph channel enlargement and significantly higher gill and circulatory reaction pattern indices than *H. laevigata* held in 22°C water temperature. Only live or moribund *H. laevigata* were assessed; therefore, lesions are not necessarily reflective of abalone mortality. One *H. laevigata* had proliferation of un-identified cells in the v-shaped skeletal rod of a gill leaflet. The un-identified cells contained enlarged nuclei, a greater nucleus-cytoplasm ratio and in some cases, mitotic figures. This cell population could represent a region of haematopoiesis in response to hemocyte loss or migration to a lesion. Without thorough diagnostic testing, the origin of these larger cells cannot be confirmed. The new scoring protocol developed will allow the standard quantification of gill lesions for *H. laevigata*, specifically for heat-related conditions, and could further be adapted for other *Haliotis* spp..

Keywords: hemocytes, abalone, gill histopathology, summer mortality, scoring protocol.

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1. INTRODUCTION

Greenlip abalone (*Haliotis laevigata*) are economically important shellfish cultured in South Australia. In 2017/2018, the South Australian abalone aquaculture industry produced 399 tonnes of abalone valued at $14.2 million AUD and contributed ~$42.3 million AUD to the state economy (BDO EconSearch 2019). *Haliotis laevigata* are commonly cultured in land-based systems which are subject to large fluctuations in water temperature (10°C to 25°C) (Stone et al. 2013, Stone et al. 2016). When water temperatures increase beyond those optimum for *H. laevigata* growth (~19-22°C, depending on stock genetics), farms can experience abalone mortality events for weeks afterwards referred to by farmers and in literature as summer mortality. Research in stock genetics has allowed for better understanding of *H. laevigata* susceptibility to summer mortality; Shiel et al. (2020) identified 487 *H. laevigata* genes which express differently for abalone tolerant to thermal stress than abalone susceptible to thermal stress. During a 75 day laboratory induced heat wave experiment involving water temperatures of 18°C, 20°C and 21°C, 54 genes were significantly differentially expressed (Shiel et al. 2020). Data from Shiel et al. (2020) supports potential of selective breeding and genetic improvement programs to decrease the stock mortalities (of up to 50%) (Vandepeer 2006, Stone et al. 2013) and economic losses which can be associated with summer mortality. Research and implementation of genetic improvement programs for *H. laevigata* remains in its infancy and studies that offer practical solutions to summer mortality now are crucial (Shiel et al. 2018).

Water temperature-related mortality events in molluscs are common and represent an interaction between infectious and non-infectious disease (Cheng et al. 2004a, Travers et al. 2008a). Temperature-related mortality events have been described for various cultured *Haliotis* spp., including summer immune depression in the European green ormer (*Haliotis tuberculata*) (Travers et al. 2008a) and bacterial disease outbreaks in the culture of small Taiwan abalone (*Haliotis*

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diversicolor supertexta) (Cheng et al. 2004a) and *H. laevigata, H ruba* and their hybrids in Tasmania (Handlinger et al., 2005). Withering syndrome is a disease affecting *Haliotis* spp. caused by *Xenohaliotis californiensis* whereby bacterial cells infect and damage abalone epithelial cells in the gastrointestinal tractinfects which can lead to starvation and death in abalone (Braid et al. 2005, Friedman et al. 1997). While a direct relationship between withering syndrome in *Haliotis* spp. and water temperature has not been identified, elevated water temperatures likely play a role in exacerbating mortalities (Braid et al. 2005, Friedman et al. 1997).

Haliotis spp. are ectothermic, meaning their metabolism is directly affected by environmental water temperature (Abele & Puntarulo 2004, Lushchak 2011, Vosloo et al. 2013). Increased water temperature can increase the metabolic activity, oxygen and energy demand of *Haliotis* spp. (Vandepeer 2006, Day et al. 2010, Lushchak 2011). Elevated metabolic activity increases the production of reactive oxygen species (ROS) as a by-product of cellular respiration (Vosloo & Vosloo 2010, Lushchak 2011, Hooper et al. 2014b). Harmful ROS are primarily stabilised by endogenous antioxidant enzymes, which are produced in response to oxidative stress (De Zoysa et al. 2008, 2009, Luschack 2011, Lange et al. 2014). When endogenous antioxidants in *Haliotis* spp. are depleted, the antioxidant/ROS balance becomes de-stabilised, resulting in excess ROS (Lushchak 2011). Excess ROS can damage abalone deoxyribonucleic acid (DNA), proteins and lipids, specifically the oxidation of polyunsaturated fatty acids (Abele & Puntarulo 2004, Lushchak 2011). The cellular damage induced by ROS can manifest as changes to *Haliotis* spp. cell structure, necrosis and tissue damage (Lushchak 2011) which have been observed in previous histopathological studies of thermal stress in *Haliotis* spp. (Day et al. 2010, Hooper et al. 2014b).

Infection with *Vibrio* spp. bacteria (vibriosis) is often associated with *Haliotis* spp. summer mortality (Nicolas et al. 2002, Pichon et al. 2013, Cardinaud et al. 2015, Handlinger et al. 2015). *Vibrio* spp. optimially grow in 30°C water temperature (Travers et al. 2008a). High bacterial

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abundance of *Vibrio* spp. and depression of *Haliotis* spp. immune systems in warmer water temperature (Nicolas et al. 2002, Cheng et al. 2004a, Travers et al. 2009, Hooper et al. 2014b) is the likely cause of more *Vibrio* spp. infections in abalone during summer. *Haliotis* spp. possess an innate immune system containing both cellular and humoral components (De Vico & Carella 2012). Travers et al. (2009) demonstrated increasing water temperature from 17°C to 18°C significantly increased *Vibrio harveyi* associated mortalities in two year old *H. tuberculata* from 0 to ~80% respectively. Blacklip abalone (*Haliotis rubra*) held at elevated water temperatures (21°C or 24°C) for seven days had significantly decreased antibacterial activity (Dang et al. 2012). Reduced antibacterial activity at higher water temperatures may signify a stress induced exhaustion of this aspect of the *Haliotis* spp. immune system (Dang et al. 2012).Vibriosis outbreaks may therefore be a consequence of bacteria exploiting the compromised health of *Haliotis* spp. at elevated water temperatures (Hooper et al. 2007, Lange et al. 2014).

Haliotis spp. gills are the primary organ impacted by infections from different pathogens and gill damage can provide an entry portal for bacteria (Pichon et al. 2013, Hooper et al. 2014b). Mucus which bathes gill epithelium may provide a rich growth medium for bacteria (Sharon & Rosenberg 2008). Gills share direct contact with the surrounding environment and consequently, are vulnerable to environmental change (Pichon et al. 2013, Yifan et al. 2015). Gills can therefore provide a visual indicator of oxidative stress and/or inflammation occurring within *Haliotis* spp. (Day et al. 2010, Harris et al. 1998, Hooper et al. 2014b). Elevated water temperature may therefore, be favourable for bacterial infiltration into *Haliotis* spp. gills. Infiltration of *Haliotis* spp. gills by bacteria may also be encouraged by structural damage resulting from by oxidative stress (Pichon et al. 2013, Hooper et al. 2014b). Oxidative gill stress, induced by elevated water temperatures can be visualised by histopathology and has been described for different marine fish species (Madeira et al. 2014, Yifan et al. 2015). Japanese flounder (*Paralichthys olivaceus*) gills illustrated serious damage at an elevated water temperature of 32°C and were more sensitive to thermal stress than the other

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assessed tissue sections (Yifan et al. 2015). When water temperatures exceeded 28°C, gills of gilthead seabream (*Sparus aurata*) (Madeira et al. 2014) contained structural damage including epithelial lifting, oedema and convoluted lamellae (Madeira et al. 2014). Examining abalone gills is therefore a strategy that offers high potential to reveal the processes occurring near their upper thermal limit.

Histopathology has been used to describe how environmental stressors such as hypoxia and hyperoxia (Harris et al. 1998), anaesthesia and movement (Hooper et al. 2014a), water pollutants (Harris et al. 1998) and pH changes (Harris et al. 1999a) may affect the health of *Haliotis* spp.. No studies involve histopathological assessment of *H. laevigata* gills maintained at elevated water temperatures long-term are currently available. Hooper et al. (2014b) and Day et al. (2010) assessed the histopathological response of hybrid tiger abalone (*H. laevigata* x *H. rubra)* gills with short term exposure (seven days) to an elevated water temperature of 26°C. Results from short-term heat exposure studies do not accurately represent physiological responses of *Haliotis* spp. to chronic thermal stress. Long-term heat exposure studies involving histopathology provide a better understanding of *H. laevigata* responses to conditions experienced on farm during a summer mortality event and allow biomarkers of thermal stress to be identified. Identificaton of biomarkers for thermal stress in *Haliotis* spp. is increasingly important as sea water temperatures are expected to rise with global climate change (Shiel et al. 2017). Histopathology is a practical diagnostic tool that generally relies on qualitative descriptions (Costa 2018). Semi-quantitative histopathological scoring protocols have been developed for freshwater (Bernet et al. 1999) and marine fish (Mitchell et al. 2011). To our knowledge, there is no standardised, quantitative scoring protocol for *H. laevigata* gill histopathology. Using quantitative histopathological analyses may help define significant relationships between *H. laevigata* lesions and elevated water temperatures. Our study used a semi-quantitative analysis to determine histopathological changes in *H. laevigata* gills in response to long-term elevated water temperatures. In addition, we proposed a scoring protocol

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142 adapted from Bernet et al. (1999), which can be used to assess the histopathological response of *H.*
143 *laevigata* to thermal stress. This protocol provides a flexible template which can be further adapted
144 for histopathological scoring of the gills belonging to any *Haliotis* spp. amid environmental
145 stressors beyond elevated water temperatures.
146
147 **2. MATERIALS AND METHODS**
148
149 **2.1. Experimental animals**
150
151 Experimental *H. laevigata* were used from temperature trials described by Buss et al. (2017).
152 *Haliotis laevigata* were purchased from Pure Australian Abalone (Boston Point, Port Lincoln,
153 South Australia) and held in flow-through systems at the South Australian Research and
154 Development Institute Aquatic Sciences Centre (West Beach, Adelaide, South Australia). Ten
155 individuals were randomly distributed into three replicate tanks (n = 60) which were then subject to
156 either a control (22°C) or elevated water temperature (25°C) treatment. One week after stocking,
157 water temperature for all treatment tanks was increased from the intial temperature of 15°C by
158 ~0.6°C every second day until 22.0°C (30 days). Water temperature for the elevated water
159 temperature treatment group was then further increased by ~1.0°C every second day until 25.0°C
160 (35 days). Water temperature for the control and elevated water temperature treatment groups were
161 maintained for 17 and 12 additional days, respectively, resulting in a total trial period of 47 days.
162 *Haliotis laevigata* were fed a commercial diet from Eyre Peninsula Aquafeeds (Lonsdale, South
163 Australia) to excess (0.6% body weight day^{-1}). Any mortalities were removed and replaced with
164 unmarked similar-sized *H. laevigata* to maintain stocking density within the trial of Buss et al.
165 (2017) and were not sampled for this experiment. After 47 days, two *H. laevigata* from each tank (n
166 =12) were euthanised and a portion of the left gill was placed into separate histology cassettes in
167 seawater-buffered formalin for >24 h and stored in 70% ethanol solution at room temperature.

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Fixed tissue samples were dehydrated through an ethanol series to histolene, embedded in paraffin wax and sectioned at 5 µm using a Leica RM 2235 rotary microtome (Leica Microsystems GmbH, Wetzlar, Germany). Sections were then floated onto Objekttrager microscope slides (90-degree ground edges, twin frosted, ProSciTech Pty Ltd, Kirwan, QLD, Australia.) and dried before staining.

2.2. Histopathology

Deparaffinised *H. laevigata* gill tissue sections were stained using haematoxylin and eosin and viewed using light microscopy. Two histopathological *H. laevigata* sections were excluded from analysis due to poor staining resulting in a total sample size of five slides each for the control and elevated water temperature treatment groups. All ten slideswere scanned using a Hamamatsu NanoZoomer S360 Digital slide scanner 2.0HT and viewed using NDP.view2 software (NanoZoomer Digital Pathology, Hamamatsu Photonics K.K., Iwata City, Japan). *Haliotis laevigata* gill leaflets were assessed from the side of the central gill axis (rachis) containing the greatest number of intact leaflets (Fig. 1). Starting at the base of the central gill axis, every second leaflet was assessed (n = 15 gill leaflets). Any gill leaflet <900 µm in length or lacking well defined v-shaped skeletal rods was excluded from examination. Leaflets containing significant artefacts were ignored where possible. Descriptions of *H. laevigata* gill lesions used in histopathological scoring are in Table 1.

2.2.1 Hemocyte descriptions.

Haliotis laevigata hemocytes were viewed histopathologically using the NDP.view2 software and classified by diameter, nucleus-cytoplasm ratio and nucleus size (Table 4). Hemocyte types were

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given putative identifications based on previous descriptions of *Haliotis* sp. cell types (Table 4) (Elston 1983, Ottaviani 2011, De Vico and Carella 2012, Pila et al. 2016).

2.2.2 Scoring

Histopathological lesions for *H. laevigata* were classified using an adapted version of the protocol proposed by Bernet et al. (1999). To do this, we modified the protocol of Bernet et a. (1999) by assigning reaction patterns, lesions and pathological omportance factors which were specific to the morpholpogy and histopathology of *H. laevigata*. Each *H. laevigata* histopathological gill lesion was categorised into one of three different reaction patterns: (1) epithelial, (2) circulatory or (3) inflammatory. Epithelial reaction patterns were lesions leading to an alteration in *H. laevigata* epithelium structure surrounding the hemolymph channel. The circulatory reaction pattern included pathological lesions resulting from changes to *H. laevigata* tissue or hemolymph (Table 1) (Bernet et al. 1999). Inflammation reaction patterns included sites of hemocyte infiltration. Specific histopathological gill lesions for each reaction pattern are described in Table 2.

Haliotis laevigata histopathological gill lesions were assigned pathological importance factors between 1-3 (Bernet et al. 1999) (Table 1). Pathological importance factors were based on the degree of negative impact to the organ and apparent capacity for the lesion to be resolved (Bernet et al. 1999) (Table 1).

1 = minor pathological importance: limited impact on organ function and lesion expected to be resolved with improvements to water quality.

2 = moderate pathological importance: moderate impact on organ function and in most cases, can expected to be resolved if water quality is improved.

3 = substantial pathological importance: total loss of organ function and lesion apparently unable to be resolved.

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Haliotis laevigata gill leaflets were allocated scores of 1 for lesion presence or 0 for lesion absence. The term, lesion prevalence, was used to define lesion occurance within each *H. laevigata* gill assessed (Bush et al. 1997). Lesion prevalence was then calculated as the percentage of leaflets containing that lesion (Bernet et al. 1999) (*equation 1*):

$$Leaflet\ lesionlprevelance = (number\ of\ leaflets\ with\ lesion/15) \times 100 \quad \text{[equation 1]}$$

Each *H. laevigata* gill leaflet was scored between 0-5, based on lesion prevalence. Lesions were described as minor, moderate or extensive (Table 3). An alteration index (I_a) for each lesion was then calculated (Bernet et al. 1999) (*equation 2*):

$$I_a = a_x \times w_x \quad \text{[equation 2]}$$

where a_x represents the lesion score and w_x represents the corresponding importance factor. A reaction index (I_r) for each reaction pattern was then calculated as the sum of the alteration indices for that pattern (Bernet et al. 1999) (*equation 3*):

$$I_r = \sum(a_x \times w_x) \quad \text{[equation 3]}$$

A gill index (I_g) for the gill was calculated as the sum of each reaction index (Bernet et al. 1999) (*equation 4*):

$$I_g = \sum(I_r)_x \quad \text{[equation 4]}$$

2.3 Statistical analyses

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Data for leaflet lesion prevelances along with alteration, reaction and gill indices are presented as mean ± standard deviation. *Haliotis laevigata* gill index, lesion prevalences and alteration indices were tested for normality using the Shapiro-Wilk test (n = 10). Equality of variance based on the means was tested using Levene's test of homogeneity. Total gill and epithelial reaction pattern indices were log transformed while inflammation and circulatory reaction pattern indices were square root transformed. Lesion prevalence (where relevant) and alteration indices for epithelial lifting and necrosis were square root transformed. Transformations were performed to allow indices to meet the assumptions for an independent sample t-test. An independent sample t-test was used to compare means between water temperature groups at a significance level of < 0.05. A non-parametric Mann Whitney U test was used to compare the medians for alteration indices belonging to the epithelial reaction pattern (hypertrophy, focal hyperplasia, goblet cell hyperplasia and atrophy), circulatory reaction pattern (hemolymph channel enlargement, vascular occlusion and vascular congestion) and inflammation reaction pattern (hemocyte infiltration). All statistical analyses were conducted using IBM SPSS version 26 for Apple Mac (IBM SPSS Inc., Chicago IL).

3. RESULTS

3.1 Survivial

The survival rate for abalone held at 25°C was below 80% while no mortalities occurred in the 22°C group.

3.2 Histopathological descriptions

3.2.1 Lesion descriptions – Epithelial reaction pattern

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The most extensive epithelial reaction patterns (score 5, Table 3, Table 5) across both temperatures included epithelial hypertrophy and focal hyperplasia (Fig. 2). Epithelial lifting at v-shaped skeletal rods (Fig. 2) occurred extensively (score 5) in *H. laevigata* held at 25°C but was only minor (score 2) in *H. laevigata* held at 22°C (Table 3, Table 5). Moderate epithelial atrophy and minor goblet cell hyperplasia (Fig. 2) were observed in both water temperature treatments (scores 3-4, Table 3, Table 5). Moderate epithelial necrosis (Fig. 2) was present in both water temperature treatments (score 2-3, Table 3, Table 5). Minor lamellar fusion (Fig. 2) was observed at 25°C (score 1, Table 3, Table 5), however, did not occur in *H. laevigata* held at 22°C (score 0, Table 3, Table 5).

3.2.2 Lesion descriptions – Circulatory reaction pattern

Lesions belonging to the circulatory disturbance reaction pattern (Table 1) ranged from minor to moderate (scores 1-4, Table 3, Table 5). Hemolymph channel enlargement (Fig. 2) was moderate (score 4) in the 25°C water temperature treatment while *H. laevigata* held at 22°C only had minor levels of this lesion (score 1, Table 3, Table 5). Haemorrhage and rupture of the hemolymph channel and vascular occlusion (Fig. 2) in *H. laevigata* were minor (scores 1-2 respectively, Table 3, Table 5) at both 22°C and 25°C. Vascular congestion (Fig. 2) did not occur in *H.laevigata* held at 22°C and was only minor for *H. laevigata* held at 25°C (score 1, Table 3, Table 5).

3.2.3 Lesion descriptions – Inflammation reaction pattern

Hemocyte infiltration (Fig. 2), in the inflammation reaction pattern (Table 1), was minor (score 1) in the 22°C temperature group and moderate (score 4) in abalone held at 25°C (Table 3, Table 5).

3.2.4 Hemocyte descriptions

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297 Three different immune-responsive cells were observed in *H. laevigata* gills: two circulating
298 hemocytes (small hyalinocytes, larger hyalinocytes), and/or granulocytes (non-circulating, fixed
299 granular cells) (Table 5). Small hyalinocytes appeared undifferentiated and blast-like with a smaller
300 diameter, when compared to larger hyalinocytes and a large nucleus-cytoplasm ratio (Table 5).
301 Small hyalinocytes were most prevalent in hemolymph channels, within *H. laevigata* gills. Larger
302 hyalinocytes had a smaller nucleus-cytoplasm ratio when compared to small hyalinocytes and
303 granulocytes. Granulocytes were the least observed cell type and were distinguished by a large
304 diameter, low nucleus-cytoplasm ratio, the presence of granules and in some cases, pseudopods
305 (Table 5).
306
307 One *H. laevigata* gill from the 22°C water temperature group contained larger hemocytes than any
308 other *H. laevigata* gill assessed. Large hemocytes were only present near a substantial area of
309 epithelial loss at the tip of a v-shaped skeletal rod (Fig. 3). Cells contained enlarged nuclei, a high
310 nucleus-to-cytoplasm ratio (Table 5), were often pleomorphic, with mitotic figures and in some
311 cases, two dividing nuclei (Table 5).
312
313 **3.3 Histopathological scoring**
314
315 Epithelial reaction pattern indices were 28.00 ± 8.60 and 41.00 ± 12.94 for *H. laevigata* exposed to
316 22°C and 25°C respectively (Fig. 4, Table 5). Differences between epithelial reaction pattern
317 indices at 22°C and 25°C were not significant ($p = 0.087$, Table 5). Water temperature had no effect
318 on the prevelance of focal hyperplasia ($p = 0.690$), goblet cell hyperplasia ($p = 0.080$), lamellar
319 fusion ($p = 0.310$), atrophy ($p = 0.715$) or necrosis ($p = 0.548$) in *H. laevigata* (Table 5). At 22°C,
320 epithelial hypertrophy occurred in 97.33 ± 5.96% of assessed leaflets, which was similar to *H.*
321 *laevigata* held at 25°C (80.00 ± 18.86%) ($p = 0.095$, Table 5). The prevalence of epithelial lifting
322 was significantly greater in *H. laevigata* held at 25°C (93.33 ± 11.55%) than for *H. laevigata* held at

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22°C (34.67 ± 28.83%) (p = 0.008 , Table 5).

The circulatory reaction pattern index was greater for *H. laevigata* held at 25°C (13.60 ± 8.99) than for *H. laevigata* at 22°C (2.8 ± 2.2) (p = 0.039, Fig 4, Table 5). Water temperature had no effect on the occurrence of hemolymph channel rupture (p = 0.069), vascular occlusion (p = 0.151) or vascular congestion (p = 0.310) in *H. laevigata* (p > 0.05, Table 5). Hemolymph channel enlargement was significantly more common in *H. laevigata* exposed to 25ºC (74.67 ± 40.66%) than *H. laevigata* exposed to 22ºC (8.00 ± 8.69) (p = 0.032, Table 5).

Inflammatory reaction pattern indices were 0.60 ± 0.50 and 4.20 ± 2.68 for *H. laevigata* exposed to 22°C and 25°C respectively (Fig. 4, Table 5). Differences between inflammatory reaction pattern indices at 22°C and 25°C were not significant (p = 0.095, Table 5). Water temperature had no significant effect on the occurrence of hemocyte infiltration in *H. laevigata* (p > 0.05, Table 5).

The overall gill index for *H. laevigata* held at 22°C was 31.40 ± 9.80, which was significantly lower than for *H. laevigata* held at 25°C (58.80 ± 20.78) (p = 0.026, Table 5).

4. DISCUSSION

Quantitative assessment of histopathological slides can be complicated when little reference information is available. Prior studies used different semi-quantitative approaches to score histopathological lesions in *H. laevigata* (Harris et al. 1999a, Hooper et al. 2014a, b). Hooper et al (2014a) used semi-quantitative scales for assessing hemocyte prevalence and intensity of eosinophilia in the papilla of the left kidney following anaesthesia and movement of *H. laevigata* x *H. rubra*. A similar semi-quantitative scoring protocol was used to quantify the intensity of eosinophilia in gills and oedema and hemocyte infiltration in the digestive gland of

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H. laevigata x *H. rubra* after acute thermal stress (Hooper et al. 2014b). Harris et al. (1998a) used a scale system (1-3) to categorise lesions in the gill and left kidney of *H. laevigata* exposed to high ammonia, nitrate and low dissolved oxygen rates. Bernet et al. (1999) developed a protocol to assess histopathological responses of freshwater fish to waterborne pollutants. Semi-quantitative scores are created by consolidating large amounts of observational data into numerical semi-quantative scores. Loss of data and information during this process can therefore lead to misinterpretation of histopathological lesions and the associated relationship between lesion presence with a cause (Wolf, 2018). To address this limitation, our semi-quantitaive scoring system provides individual histopathological descriptions and summary values which are specific to morphology and histopathology observations of *H. laevigata*. Together, the use of histopathological descriptors and summary values offers a stronger approach than the use of either alone. Our adapted protocol can be used as a standardised method for scoring histopathological changes in *H. laevigata* gills amid environmental stress to complement histopathological descriptions. Our histopathological scoring protocol also provides a template which can be further adapted to different species using additional or alternative histopathological changes.

We are the first to assess histopathological responses of *H. laevigata* to long-term elevated water temperatures. We selected *H. laevigata* which survived the temperature trial; relative scores and lesion prevalences determined in this study therefore denote lesion characteristics associated with either *H. laevigata* survival or progression towards mortality. Gill index describes the number of gill lesions, with a higher index indicating a higher number of lesions. *Haliotis laevigata* held in warmer water had higher gill indices than *H. laevigata* held in cooler water, indicating chronic, long-term temperature stress results in increased lesion prevalence. Gill indices, using our scoring protocol may therefore be a reliable quantitivate method to identify summer mortality in *H. laevigata*.

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375 Greater epithelial lifting occurred in *H. laevigata* held at elevated water temperatures when
376 compared to the control group (Table 4). In some cases, the subsequent epithelial loss following
377 epithelial lifting led to complete fusion of the epithelium lining adjacent leaflets (Fig. 2, Table 4).
378 Hooper et al. (2014b) found greater epithelial loss in the gills of acute heat stressed
379 *H. laevigata* x *H. rubra* compared to controls. The only published description of persistent
380 epithelial lifting in *H. laevigata* without overt epithelial loss was reported in *H. laevigata* exposed
381 to sub-lethal concentrations of nitrite (Harris et al. 1998). Epithelial lifting has been described in
382 other molluscs and is a major histopathological symptom of oyster oedema disease, an
383 economically troubling disease affecting the pearl oyster (*Pinctada maxima*) (Jones et al. 2010,
384 Goncalves et al. 2017). The aetiology of oyster oedema disease remains unknown, however, it is
385 typically accompanied by tissue oedema and lifting of intestinal and stomach epithelium (Jones et
386 al. 2010, Goncalves et al. 2017).
387
388 Epithelial lifting has been observed in the gills of various fish species exposed to elevated water
389 temperatures (Madeira et al. 2014, Yifan et al. 2015), waterborne pollutants (Mallat 1985, Zhihao et
390 al. 2012, Hassaninezhad et al. 2014, Lujic et al. 2015) and both stressors simultaneously (Salazar-
391 Lugo et al. 2011). Mallat (1985) reviewed fish gill histopathological alterations and suggested that
392 epithelial lifting was the most commonly reported lesion and may result from an infiltration of fluid
393 into the interlamellar space. Epithelial lifting is common in many types of epithelial cell
394 degeneration and may also serve as an indicator of early sloughing or loss of epithelium. The
395 deleterious effects of waterborne pollutants, specifically transition metals, may be exacerbated by
396 elevated water temperatures (Sokolova 2004, Cherkasov et al. 2007). Epithelial lifting in *H.*
397 *laevigata* gills during periods of elevated temperature may therefore be a consequence of increased
398 sensitivity to pollutants at elevated water temperature rather than thermal stress alone. This
399 suggestion cannot be confirmed as the concentration of waterborne pollutants were not measured by

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400 Buss et al. (2017), however, an association of this lesion with elevated water temperature allows
401 epithelial lifting to be used as a histopathological biomarker of thermal stress.
402
403 Hemolymph channel enlargement was more prevalent in *H. laevigata* held in warmer water
404 temperature (Table 4) and potentially occurred to accommodate for a greater quantity of hemocytes
405 and hemolymph volume similar to vessel dilation in vertebrates held in warm conditions.
406 Hemolymph channel enlargement may be a progressive alteration which, possibly due to vascular
407 wall damage, can gradually result in a focal distention of the hemolymph channel. Consequently,
408 hemolymph channel enlargement may act as a precursor for vascular occlusion which may be
409 associated with a reduction in haemolymph channel function following severe thermal stress. Of the
410 three reaction patterns analysed, only the circulatory reaction pattern index increased with elevated
411 water temperature suggesting hemolymph channel structure is more affected by thermal stress than
412 epithelial or inflammatory reaction patterns.
413
414 Hemocyte infiltration in *H. laevigata* gills was observed but did not differ between water
415 temperature treatments, which may reflect the ability of this lesion to be resolved if water
416 conditions improve. Day et al. (2010) described hemocyte infiltration in the gills of *H. laevigata* x
417 *H. rubra* held at 26°C for seven days however, these results were not quantified. Following this,
418 Hooper et al. (2014b) quantitatively demonstrated greater infiltration in the digestive gland of
419 hybrid *H. laevigata* x *H. rubra* held at 26°C for seven days. No hemocyte infiltration in the gills of
420 *H. laevigata* x *H. rubra* was reported by Hooper et al. (2014b).
421
422 Substantial epithelial necrosis in the gills of hybrid *H. laevigata* x *H. rubra* held at an elevated
423 water temperature of 26°C has been described (Day et al. 2010, Hooper et al. 2014b). Similarly,
424 severe epithelial necrosis was observed in *P. olivaceus* gills with acute exposure (24 h) to an
425 elevated water temperature of 32°C (Yifan et al. 2015). Necrosis in the tissue of aquatic organisms,

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426 such as finfish and molluscs, can indicate oxidative stress (Lushchak 2011). An accumulation of
427 ROS can result in damage to DNA and proteins as well as the oxidation of polyunsaturated fatty
428 acids, which can all manifest as cell necrosis (Abele & Puntarulo 2004, Lushchak 2011). We
429 showed elevated water temperature can increase necrosis in *H. laevigata* gills, however, the
430 difference was not statistically significant to *H. laevigata* gills held in cooler water (Table 4). Only
431 *H. laevigata* which survived the water temperature trial were assessed and tissue necrosis may have
432 been more apparent in dead *H. laevigata*. In live *H. laevigata*, however, necrosis was not a reliable
433 indicator of summer mortality.
434
435 In addition to circulating hemocytes, fixed granular cells were seen at low prevalence within *H.*
436 *laevigata* gill tissue, fitting prior descriptions of molluscan granulocytes (De Vico & Carella 2012).
437 There are inconsistent reports of granulocyte cells in *Haliotis* spp.. Granulocytes have been
438 described in the hemolymph of *H. diversicolor* (Hong et al. 2019) and Donkey's ear abalone
439 (*Haliotis asinina*) (Sahaphong et al. 2001), but were absent in the disk abalone (*Haliotis discus*
440 *discus)* (Donaghy et al. 2010). Travers et al. (2008b) failed to distinguish granulocytes in *H.*
441 *tuberculata* using either flow cytometry, phase contrast microscopy or transmission electron
442 microscopy, despite being visible histopathologically. Pichon et al. (2013) later identified
443 granulocytes in primary gill cultures of *H. tuberculata* using phase contrast microscopy. An
444 alternative suggestion is that gill granulocytes may resemble the hemocyanin synthesising
445 rhogocytes previously described in the mantle tissue of *H. laevigata* (Sairi et al. 2015). Sairi et al.
446 (2015) described rhogocytes as having an irregular and ovoid shape ranging in size from 10 to
447 30µm. Descriptions of rhogocytes in molluscs vary and therefore, describing the non-circulating,
448 fixed granular cells observed in this study as rhogocytes is putative.
449
450 One *H. laevigata* held at 22°C had a proliferation of larger, multinucleate hemocytes which were
451 often pleomorphic and illustrated a greater nucleus-to-cytoplasm ratio (Table 5, Fig. 33). These

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452 novel cells are undescribed in the literature. A known consequence of stress in *Haliotis* spp. is a
453 decrease in the quantity of hemocytes (hemocytopenia) within hemolymph fluid (Cheng et al.
454 2004b, Day et al. 2010). A proposed explanation for hemocytopenia following environmental stress
455 is the migration and infiltration of hemocytes to areas damaged by physical affliction or oxidative
456 stress (Malham et al. 2003, Day et al. 2010), which may deplete hemocyte reserves. The area
457 responsible for hemocyte production (haematopoiesis) in *Haliotis* spp. is yet to be identified (Day et
458 al. 2010) and very little is understood about haematopoiesis in invertebrates (Pila et al. 2016).
459 Considering the close proximity of the cells in this study to a large infiltration of cells in a v-shaped
460 skeletal rod (Fig. 3), it is possible that they represent a region of haematopoiesis in response to
461 hemocyte loss or migration to a lesion. An alternative suggestion is that these cells may be
462 neoplastic. There are two important neoplastic lesions described for bivalve molluscs: disseminated
463 neoplasia (DN) and gonadal neoplasia (Aguilera 2017, Barber 2004, Metzger et al. 2016).
464 Disseminated neoplasia is defined by frequent mitosis of anaplastic cells with enlarged nuclei and
465 considerably greater nucleus-to-cytoplasm ratios. Disseminated neoplasia has commonly been
466 identified in the circulatory systems and hemolymph channels of bivalve molluscs (Aguilera 2017,
467 Barber 2004, Carballal et al. 2013, Ciocan & Sunila 2005, Le Grand et al. 2010, Metzger et al.
468 2016). However, neoplasia has not been previously described in *Haliotis* spp.. The only tumours
469 described in gastropoda are benign lesions in land snails (Tascedda & Ottaviani 2014). In bivalves,
470 DN can be transmitted from diseased to healthy individuals (Barber 2004, Carballal et al. 2013,
471 Ciocan & Sunila 2005) and infectious behaviour may be heightened by environmental stress
472 (Barber 2004), although no direct association with thermal stress has been identified (Carballal et
473 al. 2013). Common diagnoses of DN use histopathology of the diseased region and/or direct
474 examination of the hemolymph, referred to as hemocytology (Barber 2004). These novel cells were
475 a secondary finding and histopathology was the only diagnostic tool available at the time of
476 discovery. Observed cells, however, did not exceed the size of normal replicating cells, were
477 restricted to one region and were absent from adjacent folia which are characteristics not typical of

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DN. Ideally, other diagnostic tools such as hemocytology would be used to provide a confirmatory identification of this cell population.

This study relied on gill histopathology slides from *H. laevigata* used in previous water temperature trials and was limited by a small sample size. Limited samples within each water treatment group should be considered when using these results to draw conclusions about the histopathological response of larger *H. laevigata* cohorts to elevated water temperature. Furthermore, the potential for some lesions to be artefactial or a result of handling *H. laevigata* gills during sample preparation needs to be acknowledged. Future studies regarding thermal stress in *H. laevigata* should consider a concurrent analysis of histopathology with biomarkers for oxidative stress, allowing more accurate conclusionsconclusions to be made about the origin of lesions. Gene expression paired with quantitative histopathology (such as our scoring protocol) may also help increase understanding of the genes associated with resistance to lesions induced by thermal stress. This is increasingly important as the potential for selective breeding and genetic improvement of *H. laevigata* as means of combating summer mortality is recognised. Histopathological assessment in all areas of *Haliotis* spp. research will be greatly enhanced through the use of the standardised semi-quantitative scoring protocol proposed in this study.

5. CONCLUSION

We adapted and created a standardised method to semi-quantitatively score the histopathological response of *H. laevigata* to environmental stress. This protocol was successfully used to assess the histopathological response of *H. laevigata* gills to long-term elevated water temperatures. Overall gill index was higher for *H. laevigata* held at 25°C than at 22°C suggesting *H. laevigata* gills experience pathological changes with long-term exposure to elevated water temperatures. These pathological changes may represent defence mechanisms associated with survival or characteristics

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504 which are progressive towards mortality. *Haliotis laevigata* held at 25°C had a significantly higher
505 circulatory reaction pattern index than *H. laevigata* at 22°C therefore, hemolymph channels may be
506 more vulnerable to thermal stress than reaction patterns associated with epithelium or inflammation.
507 Increased prevalence of epithelial lifting at 25°C may allow this lesion to be used as a
508 histopathological biomarker for thermal stress in *H. laevigata*. An area of apparent hemocyte
509 proliferation was identified in the tissue surrounding the v-shaped skeletal rod of one leaflet. These
510 cells were characterised by the presence of enlarged nuclei, a greater nucleus-to-cytoplasm ratio and
511 in some cases, mitotic figures, which may represent a region of hematopoiesis in response to
512 excessive loss of hemocytes or migration to a lesion. Given that no specific site has been found for
513 hematopoiesis in *H. laevigata*, the presence of proliferating cells near a lesion, with evidence of
514 continued cells loss is of interest and warrants future research.
515
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527

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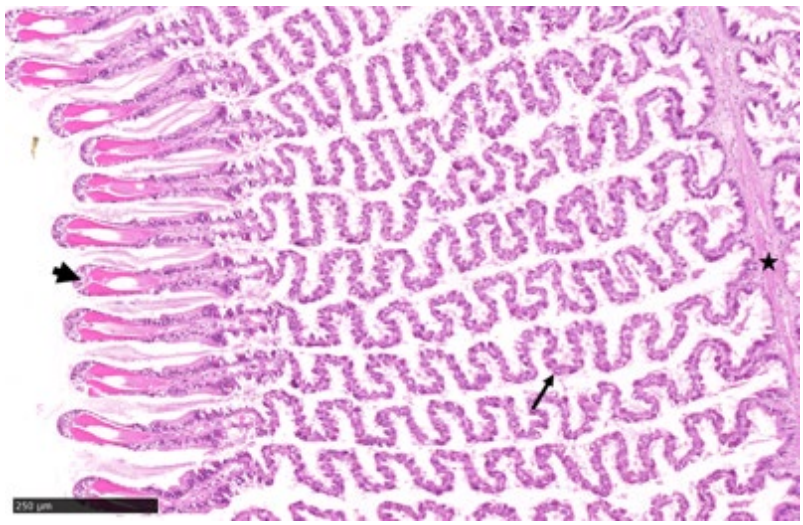

724

725 Fig. 1. Example of a region used to score greenlip abalone (*Haliotis laevigata*) gills. This region has
726 relatively normal histopathology and lacks gross lesions, with alignment including the primary
727 rachis (star), the convoluted area of thin epithelium (arrow) and terminating in v-shaped skeletal
728 rods (arrowhead). Bar = 250µm

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729 Table 1. Semi-quantitative scoring protocol: Reaction pattern, lesion and assigned importance factor
730 (ω) for histopathological assessment of greenlip abalone (*Haliotis laevigata*) gills.

Reaction Pattern	Lesion	Importance Factor (w)*
Epithelial	Epithelial hypertrophy	1
	Focal epithelial hyperplasia	1
	Goblet cell hyperplasia	1
	Lamellar fusion	2
	Epithelial atrophy	2
	Epithelial lifting at v-shaped skeletal rods	2
	Epithelial necrosis	3
Circulatory	Hemolymph channel enlargement	1
	Vascular congestion	2
	Vascular occlusion	2
	Hemorrhage and rupture of hemolymph channel	3
Inflammation	Hemocyte infiltration	1

731 *Importance factors were assigned based on lesion impact on organ function and reversibility: 1 =
732 minor pathological importance and can be reversed with improvements to water quality, 2 =
733 moderate pathological importance and in most cases, can be reversed if water quality is improved, 3
734 = significant pathological importance, the lesion is irreversible and will lead to either partial or total
735 loss of organ function (Bernet et al. 1999).

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736 Table 2. Reaction pattern, lesions, and the description used for histopathological assessment of
737 greenlip abalone (*Haliotis laevigata*) gills.

Reaction Pattern	Lesion	Description
Epithelial	Epithelial hypertrophy	Increase in size and/or intracellular contents of epithelial cells.
	Focal epithelial Hyperplasia	Increase in the number of epithelial cells at a particular focus point without a change in cell volume or intracellular components.
	Goblet cell hyperplasia	Sporadic increase in the number of clear goblet cells without a change in cell volume or intracellular components.
	Lamellar fusion	Integration of adjacent gill leaflets.
	Epithelial atrophy	Reduction in the size and/or intracellular contents of epithelial cells.
	Epithelial lifting at v-shaped skeletal rods	Visible negative space between epithelial cells and the hemolymph channel of a v-shaped skeletal rod.
	Epithelial necrosis	Visual deterioration of epithelial cells often accompanied by hemocyte infiltration.
Circulatory	Hemolymph channel enlargement	Increase in the width of hemolymph channel.
	Hemorrhage and rupture of hemolymph channel	Obvious rupture of vessels walls, accompanied by the release of excessive hemolymph fluid and hemocytes into the interlamellar space.
	Vascular occlusion	Clotting and organisation of hemocytes within a ballooned hemolymph channel.
	Vascular congestion	Visual clogging and interruption of hemolymph flow often accompanied by hemolymph channel enlargement.
Inflammation	Hemocyte infiltration	Visible migration of hemocytes to lesion and/or significant increase in the number of hemocytes within hemolymph channel.

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739 Table 3. Lesion score for each prevalence range and its relevant description for histopathological
740 assessment of greenlip abalone (*Haliotis laevigata*) gills.
741

Score (a)	Leaflet prevalence range (%)	Description
0	0	No lesions
1	1-19	Minor lesions
2	20-39	
3	40-59	Moderate lesions
4	60-79	
5	80-100	Extensive lesions

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743 Table 4. Cell types, light microscopy images and descriptions from the hemolymph of greenlip

744 abalone, *Haliotis laevigata*. Arrows point to described features, scale = 50µm.

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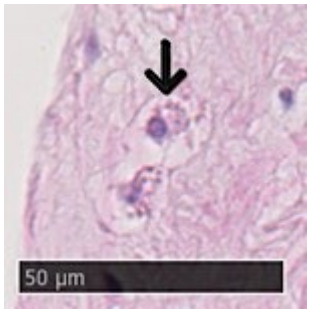
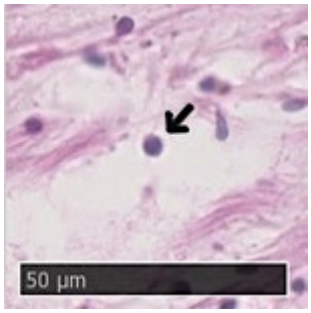
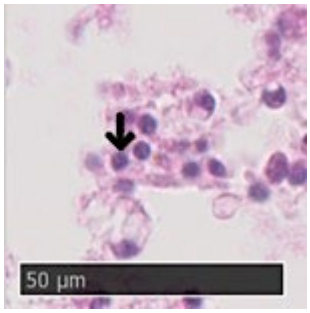
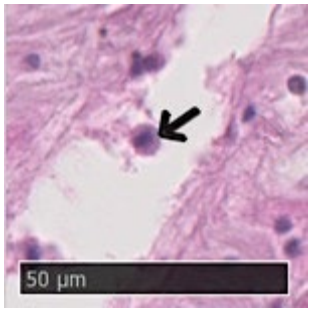
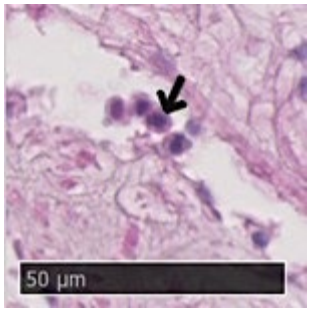
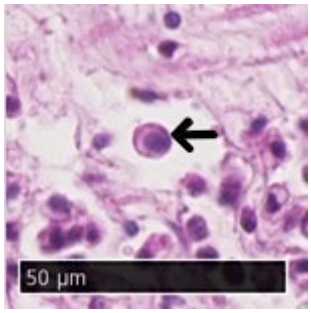
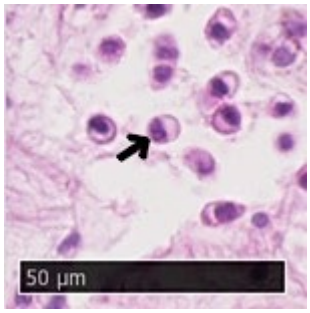
Hemocyte type	Image	Description
Fixed granulocyte	 	Low nucleus-to-cytoplasm ratio. Large diameter (10-15µm) Granular cytoplasm. Irregular cell shape. Can project pseudopodia.
Small hyalinocyte (blast-like cells)	 	High nucleus-cytoplasm ratio. Small diameter (2-4µm). Agranular cytoplasm. Round cell shape.
Medium hyalinocyte	 	Low nucleus-cytoplasm ratio. Moderate diameter (4-6µm). Agranular cytoplasm. Round cell shape.
Large hyalinocyte cell population	 	Enlarged diameter (5-9µm) and nuclei (3-6µm). High nucleus-cytoplasm ratio. Round cell shape. Some containing mitotic figures.

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746 Table 5. Alteration indices (AI) and lesion prevalence (LP) for gills of greenlip abalone (*Haliotis*
747 *laevigata*) held at either a control (22°C) or elevated temperature (25°C) for 47 days. AI and LP
748 expressed as mean ± standard deviation.

Reaction Pattern	Lesion	AI		LP (%)	
		22°C	25°C	22°C	25°C
Epithelial	Epithelial hypertrophy	5.80 ± 0.45	4.80 ± 0.84	97.33 ± 5.96	80.00± 18.86
	Focal epithelial hyperplasia	4.80 ± 1.64	5.60 ± 0.55	82.67 ± 28.13	94.67 ± 7.30
	Goblet cell hyperplasia	1.20 ± 1.30	1.40 ± 2.07	16.00 ± 19.20	22.67 ± 39.89
	Lamellar fusion	0.00 ± 0.00	0.80 ± 1.30	0.00 ± 0.00	12.00 ± 20.22
	Epithelial atrophy	3.80 ± 1.79	4.20 ± 1.48	60.00 ± 36.82	68.00 ± 29.59
	Epithelial lifting at v-shaped skeletal rods	2.20 ± 1.64^{A}	5.40 ± 0.89^{B}	34.67 ± 28.83^{a}	93.33 ± 11.55^{b}
	Epithelial necrosis	1.40 ± 1.14	2.80 ± 2.59	22.66 ± 20.87	42.67 ± 44.37
Circulatory	Hemolymph channel enlargement	0.80 ± 0.84^{A}	4.60 ± 2.19^{B}	8.00 ± 8.69^{a}	74.67 ± 40.66^{b}
	Hemorrhage and rupture of hemolymph channel	0.40 ± 0.55	1.80 ± 1.30	4.00 ± 5.96	24.00 ± 19.22
	Vascular occlusion	0.40 ± 0.55	1.20 ± 0.84	2.67 ± 3.65	10.67 ± 8.94
	Vascular congestion	0.00 ± 0.00	0.80 ± 1.30	0.00 ± 0.00	9.33 ± 17.38
Inflammation	Hemocyte infiltration	0.60 ± 0.55	4.20 ± 2.68	5.33 ± 5.58	70.67 ± 44.37
		31.40 ± 9.80^{A}		58.80 ± 20.78^{B}	

749 Within rows, different letters in the superscript indicate a statistically significant difference between
750 temperatures ($p < 0.05$).

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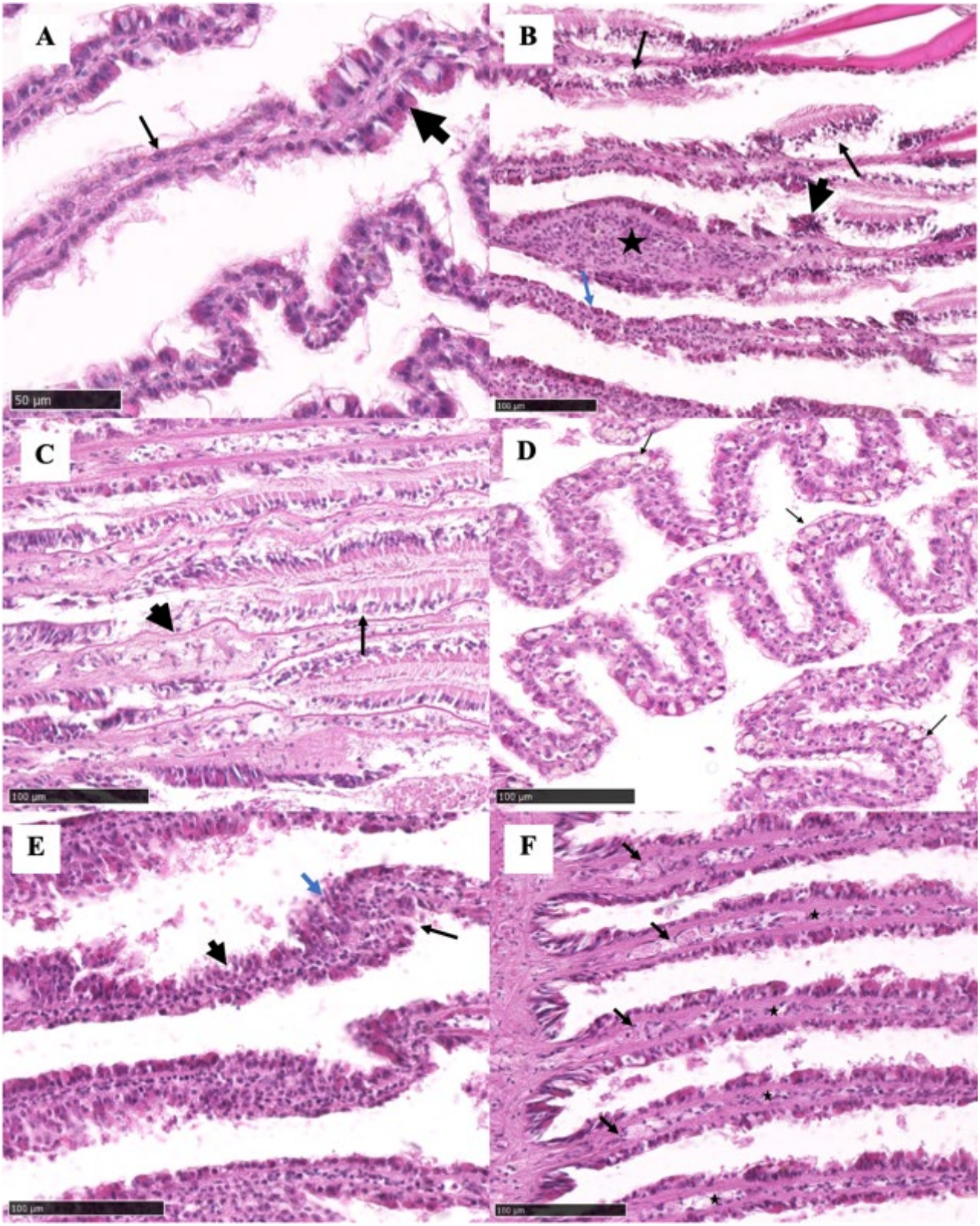

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755 Fig. 2. Left gill of greenlip abalone (*Haliotis laevigata*) displaying multiple lesions. (A) Normal
756 focus of elongated epithelium proximal to supporting cartilage, with goblet cells containing visible
757 mucus (black arrowhead), plus other paler cells with unstained mucus, plus mild epithelial swelling
758 without lifting from the basement membrane in a section of thin convoluted epithelium (black

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759 arrow). Bar = 50µm. (B) vascular occlusion accompanied by hemocyte infiltration (black star),
760 focal disruption and possible hyperplasia of epithelial cells (black arrowhead), epithelial lifting
761 from the basement membrane (black arrow) and disruption of adjacent thin epithelium (blue arrow).
762 Bar = 100µm. (C) epithelial lifting (black arrow) with adjacent epithelial loss is severe (epithelial
763 necrosis, black arrow). Remnant epithelial tags suggest this has led to fusion of adjacent gill
764 epithelium. Bar = 100µm. (D) goblet cell hyperplasia throughout the length of the thin convoluted
765 epithelium (black arrow), most with pale or unstained mucus. Bar = 100µm. (E) Epithelial
766 hypertrophy and fragmentation (mild focal necrosis), (black arrowhead). and epithelial folding
767 (black arrow), opposite a deep fissure in a region of epithelial hyperplasia (blue arrow). Bar =
768 100µm. (F) Vascular congestion (black arrow) accompanied by hemolymph channel enlargement
769 (black star). Bar = 100µm.
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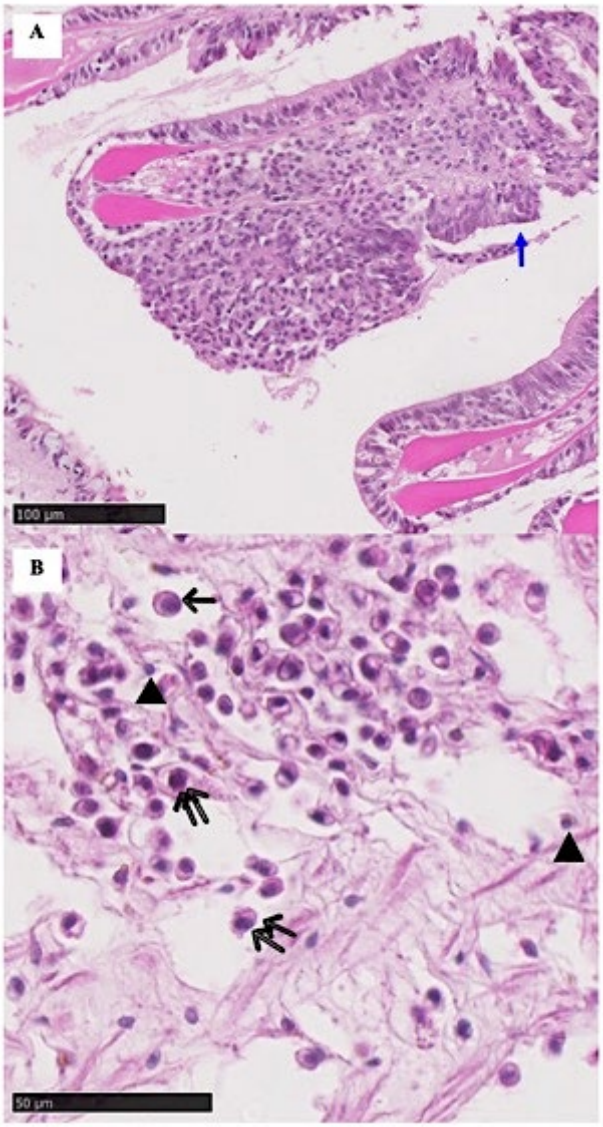

774
775 Fig. 3. (A) Infiltration of hemocytes extending from the v-shaped skeletal rod to a large area of
776 surface epithelial loss in the gill of greenlip abalone (*Haliotis laevigata)*. Note the extended margin
777 suggesting continued cell loss from this area (blue arrow). Bar = 100µm. (B) Enlargement showing
778 normal (arrowhead) and considerably larger hemocytes (arrow) some of which are illustrating
779 obvious mitotic figures (double arrow), in the left gill of *H. laevigata*. Bar = 50µm.

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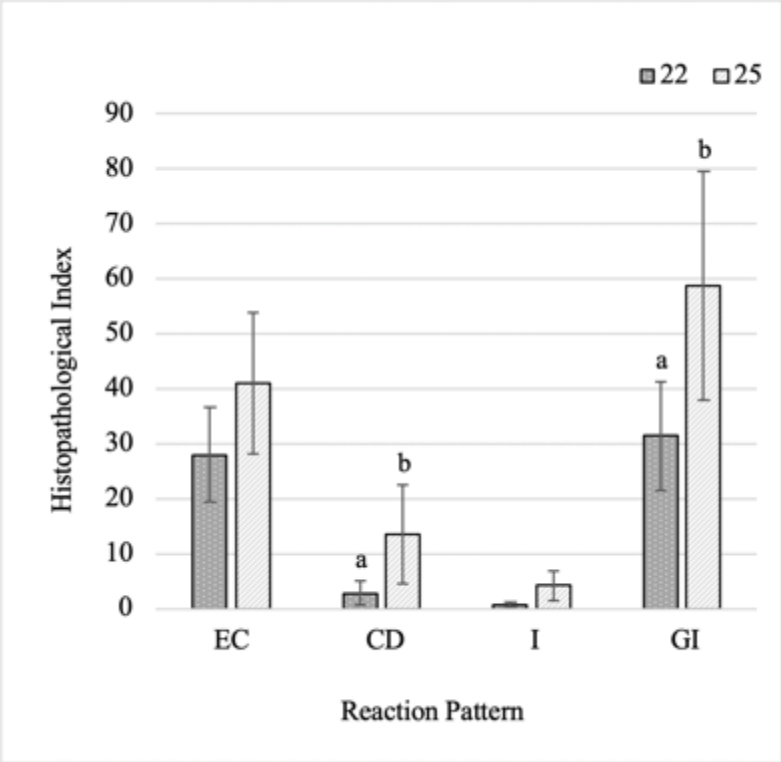

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781
782 Fig. 4. Reaction and gill indices (mean ± SD) for greenlip abalone (*Haliotis laevigata*) held at either
783 22°C or 25°C. Indices represented are epithelial (EC), circulatory (CD), inflammatory reaction
784 patterns (I) and overall gill index (GI). Letters indicate significance ($p < 0.05$).

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