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Elevated seawater temperatures have a limited impact on the coral immune response following physical damage

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Abstract

Recurrent disturbances on coral reefs that cause injuries, like predation and storm damage, and elevated seawater temperatures reduce coral fitness and immunocompetence. An effective immune response is essential to prevent disease and enhance colony survival. To evaluate how elevated seawater temperatures affect the coral immune response following injury, fragments of *Acropora aspera* were exposed to ambient (27-29°C) or elevated (32-33.5°C) seawater temperatures for 8 days and subsequently experimentally injured. Expression patterns for 15 immune genes 24 hours post-injury revealed that most genes involved in the Toll-like receptor (TLR) pathway were unaffected by elevated seawater temperatures. Exceptions to this pattern were *cFos* and *cJun*, which were upregulated and likely played a role in repair processes, and *TRAF-6* and *NFκB*, which were downregulated suggesting reduced immune function. Components of the complement system were upregulated (*millectin*, *C3*) or downregulated (*Bf*, *Tx60*, *apextrin*) in corals at high temperatures. However, corals that also sustained injury, showed normal *Tx60* and *apextrin* expression, suggesting roles in the wounding response. Overall, basal expression levels of immune genes are sufficient to mount a response to injury in the short term, and the immune response of *A. aspera* following injury is not significantly affected by minor elevations in seawater temperatures.

Introduction

Physical injury presents a major challenge to an organism's immune system, providing a point-of-entry for pathogens and requiring energy for tissue repair. On coral reefs, as many as 21-56% of coral colonies are commonly damaged (Lindsay, 2010). Predictions of increasing severity and/or frequency of impacts that cause loss of tissue integrity, including physical damage following severe tropical storms and dredging associated with escalating coastal developments, as well as more localised

impacts (e.g. destructive fishing techniques, anchoring, reef-based tourism activities and entangled fishing line), highlight the importance of understanding the capacity of the coral's immune system to respond to injury. In addition, demands on a coral's energy budget associated with climate-change related stressors, particularly ocean warming and acidification, are likely to increase in coming decades. Given the important role of the immune system in wound healing, understanding how the coral's immune response to injury is likely to be affected by additional stressors is becoming increasingly important.

Maintaining tissue integrity is of vital importance for the maintenance of fitness in any animal, including corals. To re-establish tissue integrity following injury, corals have a wound healing process similar to those in higher organisms (Palmer et al., 2011). In this process, the immune system aids in wound sealing, elimination of microbes and tissue regeneration by activating transglutaminase and phenoloxidase and recruiting immune cells to the lesion site (Palmer et al., 2011; Palmer et al., 2012). In addition, molecular studies have revealed that corals possess components of a range of innate immune mechanisms that are involved in eliminating potential harmful microbes and maintaining healthy microbial communities in other invertebrates (Fraune & Bosch, 2007; Franzenburg et al., 2012; Franzenburg et al., 2013).. Various genes encoding the microbe-associated molecular patterns (MAMP)-detecting Toll-like receptors (TLR) and nucleotide-binding oligomerisation domain (NOD)-like receptors (NLR) have been reported in corals, as well as components of their respective downstream signalling pathways, such as the JNK, MAPK p38, ERK-2 and NFκB pathways (Miller et al., 2007; Shinzato et al., 2011; Hamada et al., 2013) (Fig. 1-I). Signalling via these pathways, results in the transcription of genes involved in the regulation of an immune response, as well as cell proliferation (Fig. 1-II). The existence of genes involved in these pathways in corals suggests that they may play a significant role in their immune responses following wounding. Nevertheless, few studies have investigated the functions of various homologues and their roles in

innate immunity, defence and disease in corals, and therefore little is known about the molecular mechanisms that regulate the injury-induced immune response.

Discovery of various components of the complement system in corals, including complement C3, Factor B (Bf), lectins and mannose binding lectin (MBL)-associated serine protease (MASP) (Miller et al., 2007, Kvennefors et al., 2010, Shinzato et al., 2011), suggests that corals also possess a functional complement system (Fig. 1-III). Binding of an MBL to the microbial cell wall, results in the activation of MASPs, which subsequently cleave complement C3 and Bf (Cerenius et al., 2010) (Fig. 1-III). Together, C3 and Bf form a C3-convertase that hydrolyses C3 into C3a and C3b. Whereas C3a functions as a chemotactic cytokine, C3b is deposited on the microbial surface (Fig. 1-III), promoting phagocytosis of the microbes by C3a-recruited immune cells (Cerenius et al., 2010) (Fig. 1-IV). Moreover, it initiates the formation of a microbicidal membrane attack complex (MAC) (Cerenius et al., 2010) (Fig. 1-V), for which several candidate MAC/PF (membrane attack complex / perforin) domain-containing genes have been identified in *Acropora millepora* (Miller et al., 2007). Studies of two complement system proteins in corals have shown that the MBL millectin agglutinates bacteria and is upregulated upon challenge with immunostimulants (Kvennefors et al., 2008). In contrast, complement C3 was upregulated following injury (Kvennefors et al., 2010). The presence of multiple components of the TLR pathway and complement system in corals suggests that they possess complex wound healing and immune systems, similar to those seen in higher organisms, although few studies have focused on functional responses of these pathways.

Various environmental and biological factors are known to significantly affect the process of wound healing in corals, including elevated nutrient levels (Renegar et al., 2008; Denis et al., 2013), sedimentation (Mascarelli & Bunkley-Williams, 1999; Cróquer et al., 2002), light intensity (Titlyanov & Titlyanova, 2009), cyanobacteria (Titlyanov et al., 2005) and algae (Mascarelli & Bunkley-Williams, 1999; Titlyanov et

al., 2005), and the climate change-related variables pCO_2 and temperature. Increases in both pCO_2 levels and seawater temperatures have been shown to reduce wound healing rates (Hess et al., 2004; Henry & Hart, 2005; Renegar et al., 2008; Edmunds & Lenihan, 2010; Lenihan & Edmunds, 2010; Denis et al., 2013). For example, corals that partially bleached following exposure to high temperatures healed at significantly lower rates than controls, and corals that fully bleached were unable to regenerate following injury (Meesters et al., 1997; Mascarelli & Bunkley-Williams, 1999; Fine et al. 2002). In contrast, regeneration capacity may also increase due to higher metabolic rates associated with a Q_{10} effect (Kramarsky-Winter & Loya, 2000). Increasing seawater temperatures predicted for the coming decades could have dire consequences for corals living near their thermal thresholds (Berkelmans & Willis, 1999), with large scale losses of coral reefs predicted (Hoegh-Guldberg et al., 2007). In addition, as seawater temperatures continue to rise, elevated seawater temperatures have also been linked to the recent rise in coral disease (Willis et al., 2004; Sokolow, 2009; Ruiz-Moreno et al., 2012). Shifts in coral-associated bacterial communities (Ritchie, 2006; Bourne et al., 2008; Mouchka et al., 2010; Littman et al., 2011; Witt et al., 2011), increases in coral pathogen virulence and a change in coral immune function (Palmer et al., 2011; Vidal-Dupiol et al., 2011; Mydlarz et al., 2008) have all been demonstrated to occur following exposure to elevated seawater temperatures. However, the combined effects of heat stress and injury on the various biochemical components and pathways involved in the coral's immune response are still largely unknown.

In this study, we used a manipulative aquarium-based experiment to assess the impacts of elevated seawater temperatures on the coral immune response exhibited in the first 24 hours following injury. In particular, we analysed the expression of several genes involved in the TLR pathway and complement system, to determine how the short-term wounding–induced immune response of corals is compromised by heat stress.

Material & Methods

Experimental design and sampling – Branches (approximately 10 cm in length, n=56) of the scleractinian coral *Acropora aspera* were collected from 3 large patches of *A. aspera* (approximately 100 m^2) across a 50 m length of the reef flat of Heron Island, Great Barrier Reef, Australia (23°26′31.20″S, 151°54′50.40″E) and transported in seawater to holding tanks at the Heron Island Research Station. Branches were held upright in 4 racks (n=14 fragments per rack) and acclimated at ambient reef flat temperatures (27.1°C ± 1.3°C (mean ± SE)) for a period of 7 days in flow-through seawater. Racks were randomly assigned to 50 litre aquaria (n = 4) (1 rack per aquarium) supplied with seawater at ambient temperatures. Daytime seawater temperature in two aquaria was gradually increased by 1°C per day over 4 days using thermostat-controlled heaters until the desired sub-lethal heat stress temperature of 32°C was reached. To simulate day-night temperature fluctuations on the reef flat, heaters were turned on at 07:00 and off at 19:00 hours each day. Therefore, temperatures in the elevated treatment tanks fluctuated between 27°C and 33.5°C (mid-day maximum because of solar heating) each day once the target temperature was achieved. This temperature regimen has previously been described to induce heat stress responses in *Acropora aspera* (Leggat et al., 2011; Ainsworth et al., 2011). Similarly, because of natural fluctuations in daily ambient temperatures of seawater supplied from the reef, corals in the control temperature treatment were exposed to temperature fluctuations of 26°C – 28.5°C (the maximum daily water temperature on the reef flat at time of collection). After 8 days in each temperature treatment, half of the coral fragments in each tank were injured (n=7 uninjured; n=7 injured per aquaria) by removing tissue using a high-pressure airgun at 15.00 hours. Lesions comprised a strip of exposed skeleton approximately 1 cm wide along a third of the length of each branch (~4 cm) (Fig. 2). Whole branches were collected 24 hours post-injury and snap-frozen in liquid nitrogen.

166

167 *Messenger RNA isolation*

168 Frozen samples of each experimental branch of *A. aspera* were crushed in a
169 stainless steel mortar and pestle, pre-chilled with liquid nitrogen, using a hydraulic
170 press. Messenger RNA (mRNA) was isolated from the crushed coral using the
171 Dynabeads mRNA DIRECT kit (Invitrogen Dynal AS, Oslo, Norway) according to a
172 modified protocol based on the manufacturer's recommendations. In summary,
173 approximately 100 mg of crushed coral was added to 400 µl lysis buffer, incubated
174 on a vortex at low speed for 7 min and centrifuged for 2 min at 12000 *g*. The
175 supernatant was added to oligo(dT)-Dynabeads (pre-washed in lysis buffer) and
176 incubated on the vortex at medium speed for 8 min to allow mRNA annealing. Tubes
177 were placed on a DynaMag-2 magnetic particle concentrator for 5 min and
178 supernatant was removed. Using the DynaMag-2, oligo(dT) Dynabead/mRNA
179 complexes were washed twice with 300 µl of Buffer A and subsequently twice with
180 400 µl of Buffer B. Complexes were resuspended in 27 µl ice-cold 10 mM Tris-HCl,
181 incubated at 80°C for 2 min and rapidly cooled down on ice. Oligo(dT)-Dynabeads
182 were concentrated on the DynaMag-2 and mRNA-containing supernatant was
183 collected and stored at -80°C until use.

184

185 *Quantification of gene expression*

186 Expression levels of 20 immune system-related genes (Siboni et al., 2012) and 4
187 reference genes (Csaszar et al., 2009; Seneca et al., 2010) (Table 1) were analysed
188 using the GenomeLab GeXP Start Kit and the CEQ-8800 Genetic Analysis System
189 (Beckman-Coulter, Brea, CA, United States of America) following the protocols
190 described in Siboni *et al*. (Siboni et al., 2012) with minor modifications. In summary,
191 cDNA was generated from 9 ng of mRNA. Reverse primer concentrations were
192 optimised for the multiplex to ensure signals in the electropherogram were within the
193 CEQ-8800 detection range: 500 nM for *TRAM*, *TIR-1*, *MEKK-1*, *TRAF6*, *Tx60*, *ERK2*,

Bf, *MAPK p38*, *Millectin*, *ctg_1913*, *Apextrin*, *cJun*, *HL-1*, *HL-2*, *HL-3*, *CTL-1* and *CTL-2*; 250 nM for *GAPDH* and *RPL9*; 62.5 nM for *NFκB* and *C3/A2M-2*; 12.5 nM for *cFos* and *ATF4/5*; 3.75 nM for *RPS7*. Forward primer concentrations were 200 nM. All details for genes of interest and primer sequences can be found in Table 1. Prior to loading on the CEQ-8800 Genetic Analysis System, PCR products were pre-diluted 1:20. Data were filtered and analysed using the GeXP Fragment Analysis module and the eXpress Profiler software (Beckman-Coulter, Brea, CA, United States of America) (Souter et al., 2011). Gene expression levels were normalised to the internal control (Kan^R) and to the geometric mean of the expression levels of three reference genes (*RPL9*, *RPS7* and *ctg_1913*) selected using geNorm (Vandesompele et al., 2002). Data were obtained for three technical replicates per sample.

Statistical Analysis

For each gene, expression data were analysed for differences in expression using a two-factor analysis of variance (ANOVA), where health status (healthy versus injured) and temperature (ambient versus elevated) were both treated as fixed factors. Differences between treatments were analysed using Tukey's HSD post-hoc tests. Differences were considered significant when $p<0.05$ (ANOVA) or when the value 0 did not fall within the 95% confidence interval (Tukey's HSD). All analyses were performed in the statistical software package S-PLUS 8.0.

Results

Genes involved in the Toll-like receptor pathway were largely unaffected by injury or heat stress in the first 24 hours, with no significant differences in expression patterns found between health states or temperature treatments for *TIR-1*, *MAPK p38*, *MEKK-1* and *ERK-2* (Fig. 3A, C-E). In contrast, injured corals had significantly lower *TRAF-6* expression compared to uninjured corals at ambient temperatures, although

expression levels did not differ between health states under mild heat stress (Fig. 3B). The transcriptomic response of both *ATF4/5* to injury was dependent on seawater temperature, being downregulated at ambient temperatures, but upregulated in the elevated temperature treatment as evidenced by a significant interaction effect between temperature and injury (Fig. 3B, F). The transcription factors *cFos* and *cJun* were significantly upregulated in response to injury, by up to 1.6-fold for *cJun* (Fig. 3G, H). Moreover, exposure to high temperatures caused further increases in expression of *cJun* in injured corals in comparison to controls (Fig. 3H). *NFκB* was downregulated 24 hours post-injury, but this downregulation was only significant when corals were exposed to high seawater temperatures (Fig. 3I).

Expression levels of genes involved in the complement system were affected by both elevated seawater temperatures and injury. Corals downregulated Factor B (*Bf*) by 1.2-fold, as well as the MAC/PF domain-containing genes *apextrin* and *Tx60* by 2.2 and 2.1-fold, respectively, when exposed to elevated temperatures (Fig. 4C, E-F). Interestingly, no difference in *apextrin* and *Tx60* expression was observed between corals injured under heat stress compared to injury at ambient temperatures (Fig. 4E-F). The lectins *millectin* and *HL-2* were both significantly upregulated under heat stress, but expression was not altered following injury (Fig. 4A, B). Although, complement C3 was upregulated in response to heat stress for both healthy and injured corals (Fig. 4D), expression levels were significantly lower in injured corals relative to those that were uninjured. Expression of *HL-1*, *HL-3*, *CTL-1*, *CTL-2* and *TRAM* was undetectable in all coral samples, potentially due to low expression levels. All results from statistical analyses can be found in Table 2.

Discussion

Studies of the wounding response in invertebrates have addressed the cellular and molecular processes in tissue regeneration, but the effects of confounding

environmental stressors on the immune response are largely unknown. Here, we demonstrate that components of the Toll-like receptor pathway and complement system involved in the wounding response are robust to mild increases in seawater temperatures in *A. aspera* 24 hours post-injury.

Toll-like receptor pathway

In the current study, we found that exposure of corals to mild heat stress for 8 days did not significantly affect the regulation of the TLR pathway, except for an increase in *cJun* expression. Injury, on the other hand, resulted in upregulation of both *cJun* and *cFos*, whereas *TRAF-6* was downregulated. Interestingly, the combination of injury and heat stress increased *cJun* expression in corals further, suggesting that it is involved in both the thermal stress response and wound repair. The transcription factors *cJun* and *cFos* together form the AP-1 transcription factor complex (Hess et al., 2004). AP-1 is involved not only in the TLR pathway, but also plays a role in the regulation of cell proliferation, differentiation and apoptosis. These processes are essential for sealing lesions and development of tissue layers and polyp structures, and are regulated via different signal transduction pathways (Hess et al., 2004). Since no other genes in the TLR pathway were upregulated following injury, our interpretation is that *cJun* and *cFos* are primarily involved in lesion regeneration rather than an immune response in the initial stages following injury.

Although no major changes in the expression of genes involved in the TLR pathway were detected under mild heat stress, injured heat-stressed corals did have reduced levels of NFκB. Because the TLR signalling pathway regulates the expression of antimicrobial peptides (AMP) primarily via NFκB, these corals may have reduced AMP production and may thus be more susceptible to disease. Although no AMP has been identified in *Acropora* yet, AMP-encoding genes are present in scleractinian corals and other cnidarians (Fraune & Bosch, 2007; Fraune et al., 2011; Vidal-Dupiol et al., 2011; Burge et al., 2013; Vidal-Dupiol et al., 2014).

Studies on the (septic) wounding response in a range of invertebrates (Fehlbaum et al., 1994; Rodriguez de la Vega et al., 2004; Pujol et al., 2008; Xu et al., 2010) have also shown upregulation of AMPs. The limited transcriptomic response of genes involved in the TLR pathway under heat stress observed in this study could indicate that these corals were capable of maintaining stable bacterial communities under these conditions. While no evidence of active regulation of bacterial community composition by corals has been presented to date, AMPs have been shown to be crucial in maintaining healthy tissue-associated bacterial communities in the cnidarian *Hydra* (Fraune & Bosch, 2007; Franzenburg et al., 2012; Franzenburg et al., 2013).

In summary, we were unable to establish a link between the TLR pathway and the response to injury in *A. aspera*. As our study is preliminary, consisting of a single post-injury time point, potential earlier or later changes in gene expression levels may have been overlooked. Based on this study, the TLR pathway in *A. aspera* was uncompromised, suggesting that the coral has good potential to respond to injury under mild heat stress. However, understanding the impact of heat stress on the full dynamics of the wounding-induced immune response in corals requires study over longer time-frames and with greater sampling resolution.

Complement system

The expression of genes involved in the complement system was affected by both elevated seawater temperatures and injury. Whereas heat stress caused downregulation of the MAC/PF domain-containing *Tx60 and apextrin*; at ambient temperatures, expression of *Tx60* and *apextrin* was similar between healthy and injured corals. This suggests that not only are *Tx60* and *apextrin* involved in the wounding response, but also that heat stressed corals are capable of inducing an immune response similar to those at ambient temperatures. Our results are in line with a previous study on the bumble bee *Bombus terrestris*, where upregulation of

genes encoding MAC proteins were observed following injury (Erler et al., 2011). The function of these genes in the recovery process in corals remains to be determined; however the presence of a MAC/PF domain suggests a role in the anti-microbial response and could indicate infection of the lesion by microbes. The involvement of *Tx60* and *apextrin* in the wounding response, but the absence of upregulation in expression at ambient temperatures in response to injury, indicates that the initial gene expression levels under normal conditions may be sufficient to prevent infection. This suggests that normal expression levels for other genes may also be sufficient, and that potentially, the immune protein expression at 24 hours post-injury is sufficient for an appropriate response. Alternatively, protein expression may be primarily regulated through increased mRNA stability (Wu & Brewer, 2012) rather than an increased transcriptomic response. This would enable the coral to direct its limited resources towards wound sealing and regeneration in the initial stages of the wounding response, although further studies are required to address this question.

The upregulation in expression of complement C3 and millectin observed in response to 8 days of exposure to elevated seawater temperatures may indicate that these heat-stressed corals were directing resources to preserve the coral-*Symbiodinium* endosymbiosis. This is in accordance with previous studies that have implicated these proteins in the maintenance of the coral-*Symbiodinium* endosymbiosis in *Acropora millepora* (Kvennefors et al., 2008; Kvennefors et al., 2010). The downregulation of *Bf* under heat stress suggests that this gene, which is a key player in the alternative complement pathway, may not be involved in the activation of C3 for the maintenance of symbiosis. This confirms a role for the lectin-complement pathway, with millectin as the MBL. In addition, our results for *millectin* are consistent with reports that injury does not change expression of this gene (Kvennefors et al., 2010). However, we show reduced C3 expression 24 hours post-injury, which is in contrast to results from a recent study showing upregulation of C3 within 12 hours after corals were injured (Kvennefors et al., 2010). Although this may

appear inconsistent, very little is known about expression dynamics of immune genes in corals, and the 12-hour time difference between sampling in the two studies could explain differences in results. Investigations into expression levels over time will be required to gain more insight into these dynamics.

Conclusion

In this study, we demonstrate that the immune response of *A. aspera* exhibited 24 hours post-injury, is not significantly affected by mild temperature stress. While heat stress did influence the coral's immune effector capacity, responses to injury under heat stress were as strong as those of non-heat stressed corals. Gene expression levels of both the TLR pathway components and other immune effectors were largely unaffected at 24 hours post-injury, while genes potentially involved in wound repair and tissue regeneration were upregulated. In addition, upregulation of components of the lectin-complement system, which is involved in the coral-*Symbiodinium* symbiosis, under heat stress suggests that corals were attempting to maintain the endosymbiosis. Normal expression levels of immune genes in the initial response following injury may be sufficient to ward off invading microbes, enabling the coral to invest more heavily in tissue repair. This study provides evidence that some corals are capable of withstanding the adverse effects of physical damage under elevated temperatures.

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546 **Table 1 – List of immune genes**
547

Gene name	Acronym	Accession number	PCR product length	Sequence of primer coding region	References
Reference gene					
Glyceraldehyde 3 phosphate dehydrogenase	*GAPDH*	EZ026309	187	F: AGGTGGAGCCAAGAAAGTCA R: TTAGCTAGAGGAGCCAGGCA	(Csaszar et al., 2009; Seneca et al., 2010)
Ribosomal protein S7	*RPS7*	EZ031290	197	F: CAGGCATGCTTACAACCAAA R: TCAACCTCCTTTGCTCCAGT	
Ribosomal protein L9	*RPL9*	EZ026324	292	F: CGTGTAAACGTGTGGTTTGC R: TTTGACACCTGAATTCGCAC	
Unknown transcript	*Ctg_1913*	EZ040581	280	F: GATTTAACCACCGGCAGTGT R: ATGGTAGGGAGGAGGCTGTT	
Toll-like receptor pathway					
Toll interleukin receptor	*TIR-1*	EF090256	137	F: AAAGCCGCAGTCATCAGTTT R: GAAATTGGCGTTGAATTCGT	(Miller et al., 2007)
TNF receptor associated factor 6	*TRAF-6*	DY583189	127	F: TGATGAATGTCCTTTCGCAG R: ACATGCTTTGCAAGCTGATG	
Mitogen-activated protein kinase/ERK kinase kinase-1	*MEKK-1*	DY581208 DY581138 DY582675	117	F: CTGCGGATATTTGGTCCTGT R: TTTCTTTGTCGGTTGATCCC	
Extracellular signal regulated kinase	*ERK-2*	EZ025389	217	F: CCAAAGGTTACAGCAAGGCT R: TGCGTGCCTTTTCATTCATA	
Mitogen-activated protein kinase p38	*MAPK p38*	EZ031759	237	F: AAAATCAGCAGTGAATCCGC R: TCGGGGTCTGAATACGTAGC	
Component of AP-1 transcription factor	*cFos*	EZ016042	177	F: CTGGAAAGAGAATTGCTGGC R: GACGATTGCACTTCGGACTT	
Component of AP-1 transcription factor	*cJun*	EZ020860	366	F: TCGATCGAAGGGACAGTTCT R: GTGCTAGTTGCGGTGTTCAA	
Activating transcription factor 4	*ATF4/5*	DY577805	247	F: GGCCAGAACGTATCACCAAT	

				R: TCTTCGAAATCAAACCCCTG	
Nuclear factor kappa B	*NF-kB*	G0000491 G0002043 DY582971 DY580118	152	R: CTCATATGCAGGTTGGTGGA F: GATGTTGCAGGCTCAGTTCA	
TRIF-related adaptor molecule	*TRAM*	EZ047194	157	F: AAGCTAACGGCTCACCAAGA R: TGTGCCATGCACAAGAAAAT	
Alternative complement pathway					
Complement factor B	*Bf*	GO001635	227	F: TTATCCATCCCGACGCTAAC R: AGGATCATCTTTTCCTGCGA	(Kimura et al., 2009)
Complement C3	*C3*	EF090257	167	F: CCGCTACACGCTAGACAACA R: CCGCAGAGTCGATGTACAAA	
Lectins					
Mannose-binding lectin	*millectin*	EU717895	257	F: AGCGAGTATCCACAACACCC R: GGCTTTTTCGATGTTTTCCA	(Kvennefors et al., 2008)
C type lectin-1	*CTL-1*	GO001638	312	F: GGGTTGTGTACAACGGCTTT R: CTTTCCATTCGGTTCTCCTG	(Grasso et al., 2008; Grasso et al., 2011)
C type lectin-2	*CTL-2*	GS01UH10	267	F: CAGGTCTGGATCGGACTCAT R: CATGTCCAGTGGTTGTACGC	
Hemolytic lectin-1	*HL-1*	EU863776 EU863777	335	F: TTCGCTCCAGAGGGAAACTA R: GCAGAAATGCCTTTGGTTGT	(Grasso et al., 2008; Grasso et al., 2011)
Hemolytic lectin-2	*HL-2*	EU863776	302	F: AACAGTTGAGATAACCGCCG R: TTGATTCCTGGTGCATTTGA	
Hemolytic lectin-3	*HL-3*	EU863777	379	F: TTCTGGAGATTGGGTAACGC R: TCGTTCTCAGCGTGTTGTTC	
Membrane attack complex / perforin					
Apical extracellular protein	*Apextrin*	EF091848	352	F: GGATTCGTACCAAAAAGGCA R: GAGGGGTCTGATATGGGGTT	(Miller et al., 2007)

	60 kDa proteinaceous toxin	*Tx60-A*	DY579588	207	F: TACTGCCCTTGAGGTTTGCT R: CTGAAAATCCCGCTGACTGT	

At the 5’-end, all primers contained universal fluorescent tags. Forward primer: AGGTGACACTATAGAATA . Reverse primer: GTACGACTCACTATAGGGA

551 **Table 2 – Overview of statistical analysis.** Statistically significant differences are indicated in ***bold and italics*** for the main ANOVA results.
552 The 95% confidence interval is given for pair-wise comparisons where the difference was considered statistically different (i.e. it did not contain
553 the value 0).
554

Gene	ANOVA	p-value	Tukey's HSD	95% Confidence Interval	
Apextrin	Temperature	***0.0074***	Ambient Healthy – 32°C Healthy	0.0147	0.9240
	Injury	***0.0234***	Ambient Injured – 32°C Healthy	0.1790	1.0400
	Temperature : Injury	0.2128			
ATF4/5	Temperature	0.3668			
	Injury	0.6372			
	Temperature : Injury	***0.0133***			
Bf	Temperature	***0.0323***			
	Injury	0.1765			
	Temperature : Injury	0.3414			
C3	Temperature	***0.0002***	Ambient Healthy – Ambient Injured	0.0971	1.7200
	Injury	***0.0007***	Ambient Healthy – 32°C Healthy	-1.7700	-0.0593
	Temperature : Injury	0.8464	Ambient Injured – 32°C Healthy	-2.6400	-1.0100
			Ambient Injured – 32°C Injured	-1.8100	-0.1830
cFos	Temperature	0.7165			
	Injury	***0.0196***			
	Temperature : Injury	0.3523			
cJun	Temperature	***0.0003***	Ambient Healthy – Ambient Injured	-0.4250	-0.0520
	Injury	***0.0001***	Ambient Healthy – 32°C Healthy	-0.4280	-0.0351
	Temperature : Injury	0.8737	Ambient Healthy – 32°C Injured	-0.6810	-0.2880
			Ambient Injured – 32°C Injured	-0.4330	-0.0601
			32°C Healthy – 32°C Injured	-0.4500	-0.0568
ERK-2	Temperature	0.5531			
	Injury	0.4315			
	Temperature : Injury	0.4086			
HL-2	Temperature	***0.0001***	Ambient Healthy – 32°C Healthy	-1.5700	-0.0754
	Injury	0.1499	Ambient Injured – 32°C Healthy	-1.9200	-0.5010
	Temperature : Injury	0.4899	Ambient Injured – 32°C Injured	-1.7900	-0.3640
MAPK p38	Temperature	0.8432			

	Injury	0.6897			
	Temperature : Injury	0.8290			
MEKK-1	Temperature	0.2473			
	Injury	0.1549			
	Temperature : Injury	0.7194			
Millectin	Temperature	< ***0.0001***	Ambient Healthy – 32°C Healthy	-2.8000	-0.7920
	Injury	0.1680	Ambient Healthy – 32°C Injured	-2.2500	-0.2410
	Temperature : Injury	0.4201	Ambient Injured – 32°C Healthy	-2.9100	-1.0000
			Ambient Injured – 32°C Injured	-2.3600	-0.4510
NFκB	Temperature	0.3367	Ambient Healthy – 32°C Injured	0.0266	1.2200
	Injury	***0.0060***			
	Temperature : Injury	0.7836			
TIR-1	Temperature	*0.0556*			
	Injury	0.3996			
	Temperature : Injury	0.5339			
TRAF6	Temperature	0.4919	Ambient Healthy – Ambient Injured	0.3880	1.2000
	Injury	***0.0003***	Ambient Healthy – 32°C Injured	0.1700	1.0300
	Temperature : Injury	***0.0086***			
Tx60	Temperature	***0.0003***	Ambient Healthy – 32°C Healthy	0.0673	0.5040
	Injury	0.5243	Ambient Injured – 32°C Healthy	0.0742	0.4890
	Temperature : Injury	0.4509			

555

Figure legends

Figure 1 – Toll-like receptor signalling pathways and the complement system. (I) Activation of Toll-like receptors result in the activation of various signal transduction pathways, including: 1) signalling via TRAM leads indirectly to the activation of TBK1, which relays the signal through to IRFs and NF-κB; 2) signalling via the MyD88-dependent pathway results in the activation of TRAF6 and ECSIT which activate the ERK-2, JNK and MAPK p38 pathways. Through activation of Tak1 and MEKK1, TRAF6 also indirectly activates the NF-κB pathway via the IKK complex. In addition, TRAF6 may induce apoptosis via a caspase-8-mediated pathway. NLRs indirectly activate MEKK1 thereby activating the ERK-2, JNK, MAPK p38 and NFκB pathways. (II) Signalling results in the transcription of immune response regulatory and effector genes as well as genes involved in cell survival and apoptosis. (III) The complement system is initiated following the binding of lectins to microbes, resulting in the conversion of C3 into C3b and C3a. In addition, Bf is activated and forms the C3b:Bb complex, an efficient C3 convertase. (IV) Lectins and C3b promote phagocytosis of the targeted microbe leading to its destruction. (V) C3b also induces the formation of the membrane attack complex (MAC) that is inserted into the membrane and cause lysis of the microbe. Abbreviations: TLR, Toll-like receptor; MyD88, myeloid differentiation primary response; IRAK, interleukin-1 receptor-associated kinase; TRAF, TNF receptor-associated factor; TNF, tumor necrosis factor; ECSIT, evolutionarily conserved signalling intermediate in Toll pathways; TAK1, transforming growth factor β-activated kinase-1; MEKK, MAPK/ERK kinase kinase; MKK, MAPK kinase; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; CREB, cAMP response element-binding protein; AP-1, activator protein-1; NLR, nucleotide-binding oligomerisation domain (NOD-like) receptor; RIP2, receptor-interacting serine/threonine-protein kinase-2; IKK, I-kappa-B kinase; NFκB, nuclear factor kappa B; TRAM, TRIF-related adaptor molecule; TRIF, TIR-domain-containing adapter-inducing interferon-β; TIR,

584 Toll/interleukin-1 receptor; TBK1, TANK-binding kinase; TANK, TRAF-family
585 member-associated NFκB activator; IRF, interferon regulatory factor. Bf, Factor B;
586 C3, Complement C3. Asterisk (*) indicates genes that are included in the gene
587 expression analysis in this study.
588
589 Figure 2 – Representative image of a branch of *Acropora aspera*. Injuries of
590 approximately 4 cm in length were artificially inflicted using an airgun.
591
592 Figure 3 – Relative expression of genes involved in the Toll-like receptor pathway in
593 injured and uninjured corals under ambient or elevated seawater temperatures. A)
594 *TIR-1*, B) *TRAF-6*, C) *MEKK-1*, D) *MAPK p38*, E) *ERK2*, F) *ATF4/5*, G) *cFos*, H) *cJun*
595 and I) *NFκB*. Capital letters (A, B, C) indicate experimental treatment groups that are
596 statistically similar. For figures without letters, no significant differences were
597 detected among the four treatment groups. The asterisk (*) identifies the genes for
598 which expression levels differed between the two health states (but not between the
599 two temperature treatments.
600
601 Figure 4 – Relative expression of genes involved in the complement system in
602 injured and uninjured corals under ambient or elevated seawater temperatures. A)
603 *HL-2*, B) *Millectin*, C) *Bf*, D) *complement C3*, E) *Apextrin* and F) *Tx60*. Capital letters
604 (A, B) indicate experimental treatment groups that are statistically similar. The
605 asterisk (*) identifies the gene for which expression levels differed between the two
606 temperature treatments (but not between the two health states).
607
608

Figure 1

Click here to download Figure: Figure 1 - Immune gene overview.eps

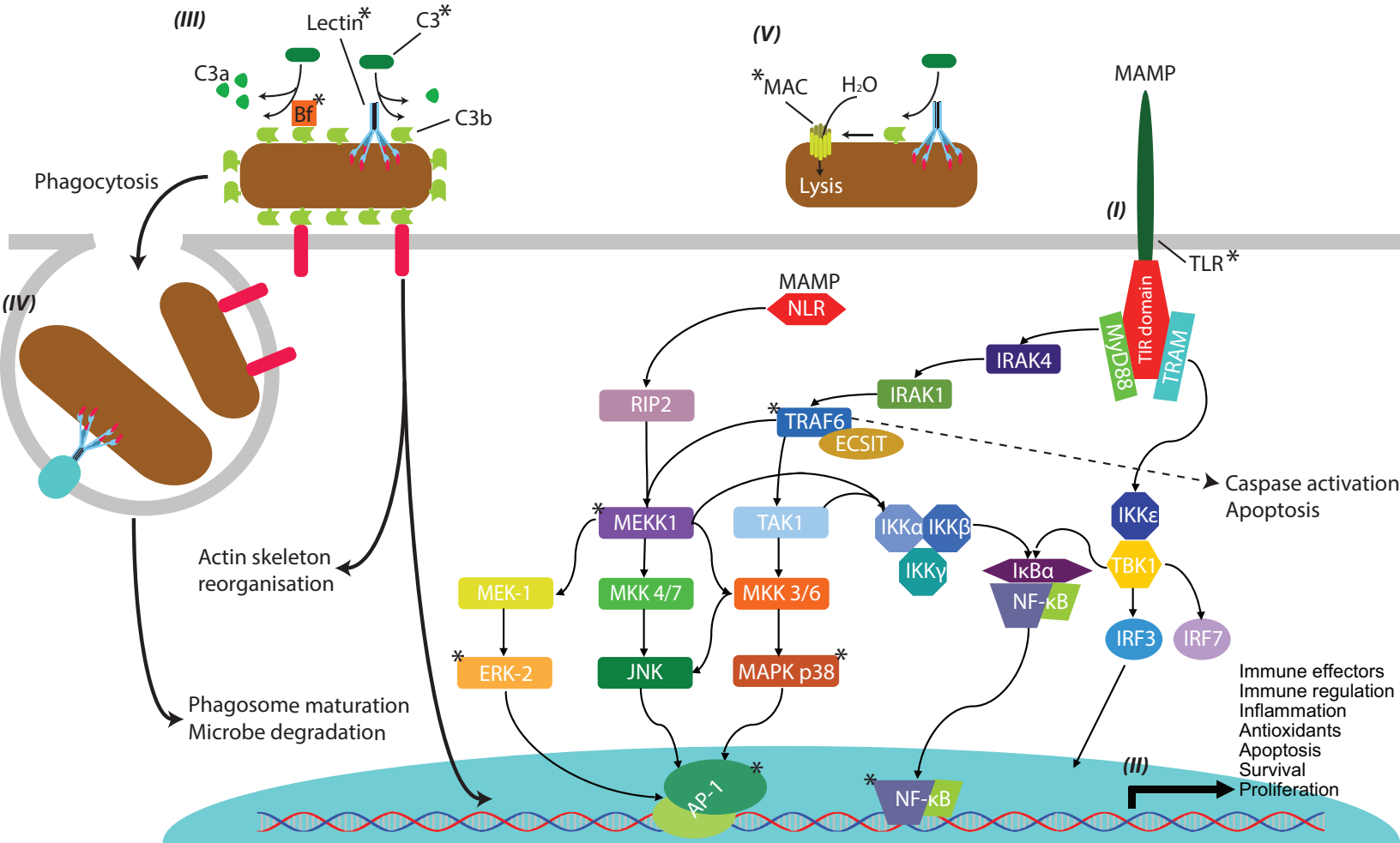

Figure 2
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Figure 3
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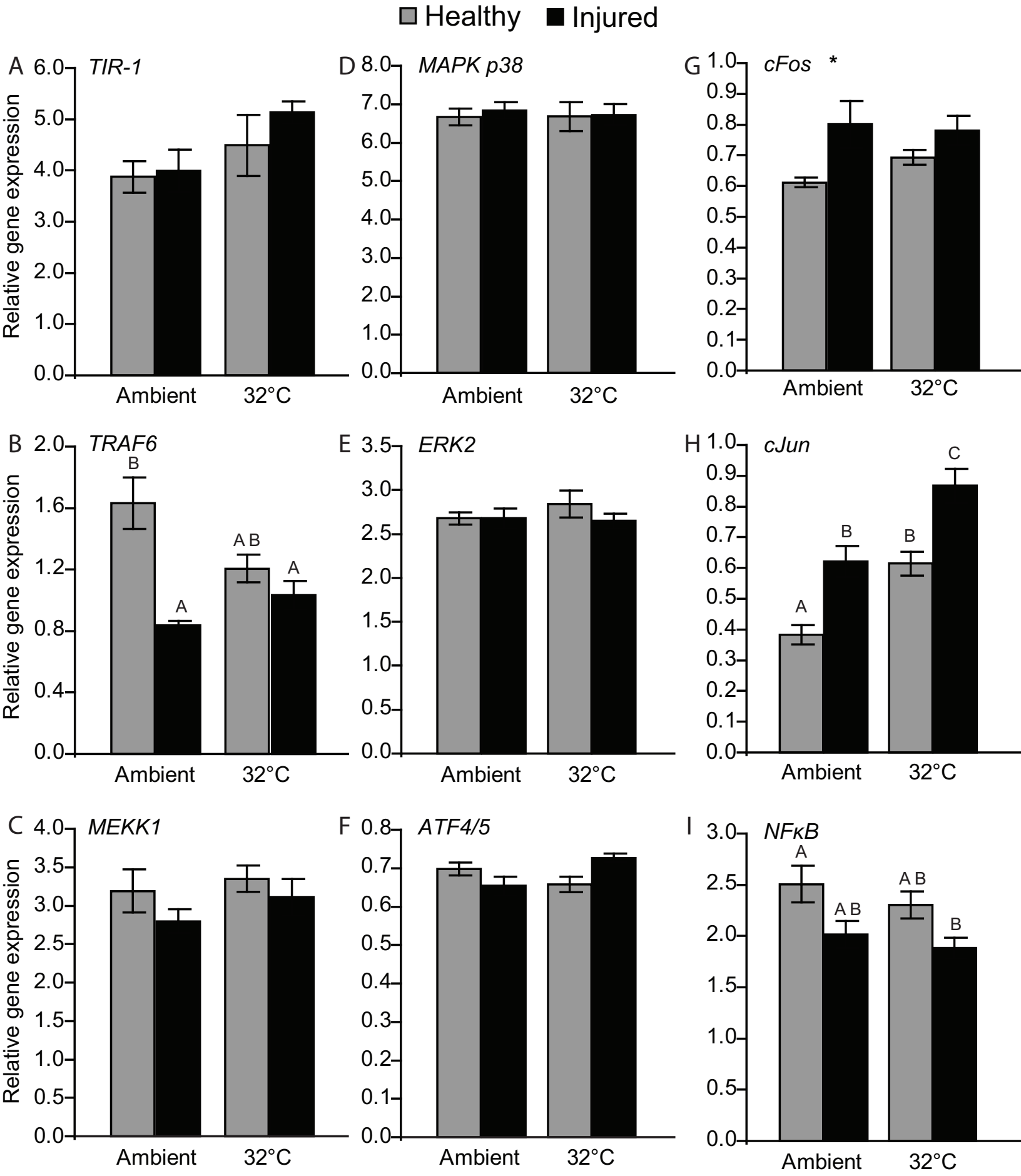
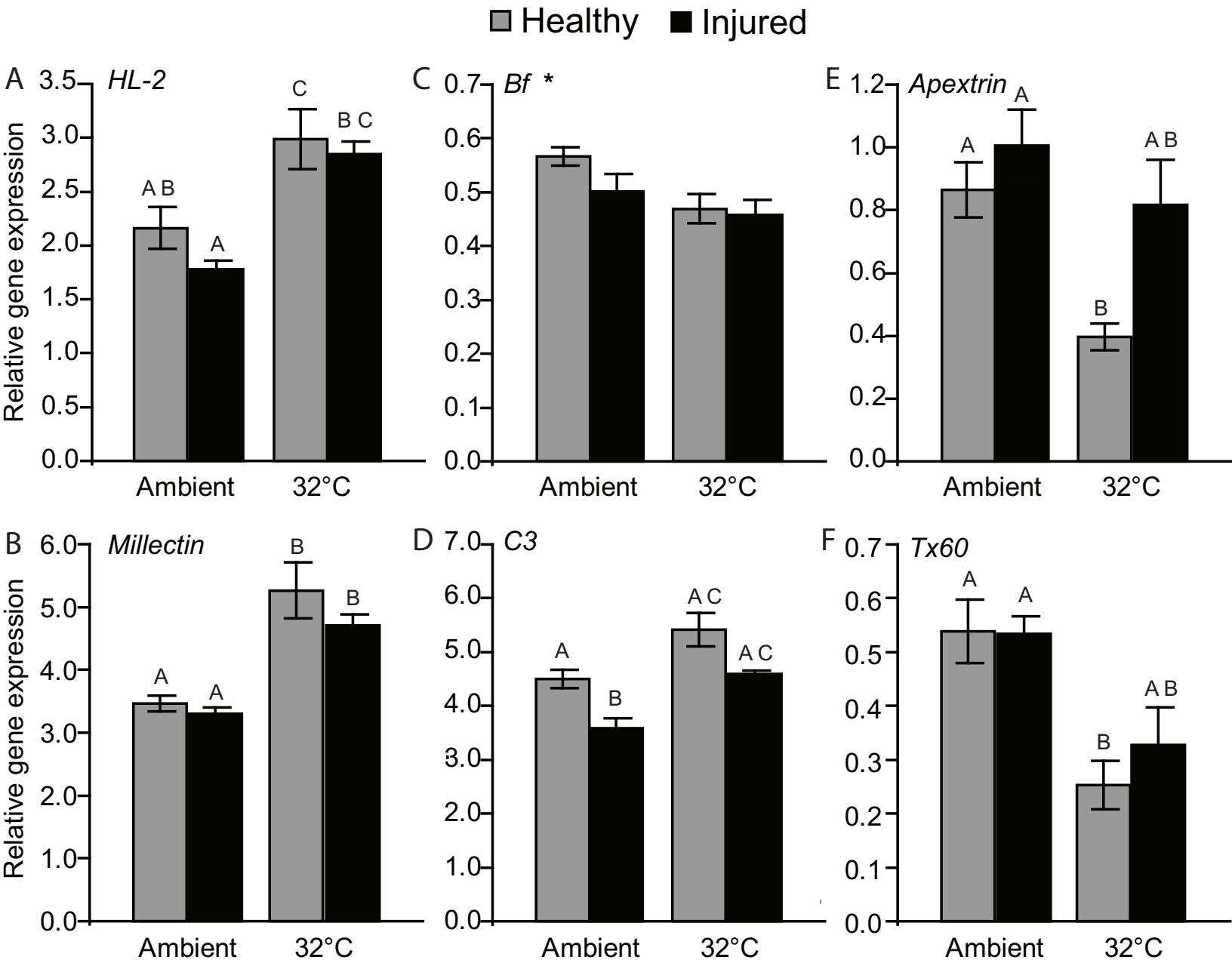


Figure 4
Click here to download Figure: Figure 4 - Gene Expression Complement.eps



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