REVIEW

Gene expression biomarkers of heat stress in scleractinian corals: Promises and limitations

Yohan D. Louis[1], Ranjeet Bhagooli[2]*, Carly D. Kenkel[3], Andrew C. Baker[4], and Sabrina D. Dyall[1]

[1]Department of Biosciences, Faculty of Science, University of Mauritius, Réduit 80837, Republic of Mauritius
[2]Department of Marine & Ocean Science, Fisheries & Mariculture, Faculty of Ocean Studies, University of Mauritius, Réduit 80837, Republic of Mauritius
[3]Australian Institute of Marine Science, PMB No. 3, Townsville MC, QLD 4810, Australia
[4]Department of Marine Biology and Ecology, Rosenstiel School of Marine and Atmospheric Science, University of Miami, 4600 Rickenbacker Cswy., Miami, Florida, USA
*Corresponding author E-mail address: r.bhagooli@uom.ac.mu; Tel. +230-4037916; Fax. +230-4676744

Abstract

Gene expression biomarkers (GEBs) are emerging as powerful diagnostic tools for identifying and characterizing coral stress. Their capacity to detect sublethal stress prior to the onset of signs at the organismal level that might already indicate significant damage makes them more precise and proactive compared to traditional monitoring techniques. A high number of candidate GEBs, including certain heat shock protein genes, metabolic genes, oxidative stress genes, immune response genes, ion transport genes, and structural genes have been investigated, and some genes, including *hsp16, Cacna1, MnSOD, SLC26*, and *Nf-kB,* are already showing excellent potential as reliable indicators of thermal stress in corals. In this mini-review, we synthesize the current state of knowledge of scleractinian coral GEBs and highlight gaps in our understanding that identify directions for future work. We also address the underlying sources of variation that have sometimes led to contrasting results between studies, such as differences in experimental set-up and approach, intrinsic variation in the expression profiles of different experimental organisms (such as between different colonies or their algal symbionts), diel cycles, varying thermal history, and different expression thresholds. Despite advances in our understanding there is still no universally accepted biomarker of thermal stress, the molecular response of corals to heat stress is still unclear, and biomarker research in *Symbiodinium* still lags behind that of the host. These gaps should be addressed in future work.

Keywords: coral, gaps, gene expression biomarkers, thermal stress, variations

1. Introduction

Scleractinian corals are the principal habitat builders of modern coral reefs. As such, they are critical components of one of the most diverse ecosystems on earth, harboring 32 of the 34 recognized animal phyla, including 800 hard coral species and more than 4,000 species of fish (Birkeland, 1997; Spalding *et al.,* 2001). Corals are delicate symbioses between an animal host and diverse dinoflagellate algae in the genus *Symbiodinium*, also commonly referred to as 'zooxanthellae' (Wells, 1957). Climate change, overfishing, nutrient pollution, disease, ocean acidification, and coastal development are among the escalating direct and indirect human pressures contributing to reef decline (Brown, 1997; Hughes, 2003), and many of these varied stressors can result in coral bleaching (the expulsion of algal symbionts, or a reduction in their per-cell pigment concentrations) (Coles and Jokiel, 1977; Falkowski and Muscatine 1981; Lesser *et al.,* 1990; Dove *et al.,* 2000). The breakdown of the cnidarian-*Symbiodinium* partnership results in a significant energy loss for the animal host, leading to reduced growth and reproduction, and increasing the risk of disease and starvation (Bruno *et al.,* 2007; Hoegh-Guldberg *et al.,* 2007). Mass coral bleaching events occur when bleaching affects the majority of the zooxanthellate ("symbiont-bearing") hosts on a reef, and typically occurs over large spatial scales (1000s of km^2) (Hoegh-Guldberg, 1998). The occurrence of natural disturbances, such as rising sea surface temperature (SST) and ocean acidification, is increasing as a result of climate change (Hoegh-Guldberg *et al.,* 2007). Sustained periods of elevated SSTs, usually in shallow areas where the incident solar irradiance is also high, are now recognized as the principal factor driving contemporary mass coral bleaching events. Severe episodes of mass coral bleaching usually result in high coral mortality and decreases in coral cover. They also commonly lead to changes in species composition, local extirpation of some reef species, and reductions in species richness (Wilkinson *et al.,* 2008; Alemu and Clement, 2014). Ecological extinction of corals reefs in some regions has been forecast to occur within the next 20 to 50 years if corals are unable to adapt and/or acclimatize sufficiently rapidly to keep pace with warming, and if effective reef management strategies are not quickly implemented (Sheppard, 2003; Hoegh-Guldberg, 1999; Baird *et al.,* 2009; Bhagooli and Sheppard, 2012).

Conservation of coral reefs is a global environmental concern, however the tools for implementing proactive management solutions are currently lacking, particularly for evaluating and predicting the health of corals *in situ* (Aswani *et al.,* 2015). The advent of molecular tools and resources for corals has highlighted the possibility for gene expression biomarker (GEB) development as a means of detecting and quantifying coral stress even before the onset of symptoms (Kenkel et al., 2011; Traylor-Knowles and Palumbi, 2014; Kenkel *et al.,* 2014). Biomarkers are critical tools in biomedical research and clinical practice, where they are used to determine whether patients will benefit from particular treatments (predictive biomarkers), monitor the progression of a disease or efficacy of a prescribed treatment (monitoring biomarkers) and even to predict survival (prognostic biomarkers; Oldenhuis *et al.,* 2008). Such a molecular toolkit for corals could help reef managers identify reefs under stress, pinpoint the causative stressors, and target resilient individuals for restoration. For example, corals from a reef showing stress response biomarkers could be transplanted to a healthier site, or corals showing heat resistance biomarkers could be transplanted or selected for adaptive breeding programs (van Oppen *et al.,* 2015) to prevent collapse of vulnerable reefs.

However, despite more than a decade of research, it is unclear how accurately can we predict the occurrence of stress factors based on changes in the expression of coral and symbiont genes. This is primarily the result of substantial variation in the stress tolerance of different species (Rowan, 2004) and species combinations (Rocker *et al.,* 2012) as well as in gene expression patterns (Granados-Cifuentes *et al.,* 2013). Research into these areas, as well as into ontogenetic changes in gene expression, are emerging as frontiers in the field of GEB development.

This review synthesizes the current state of knowledge in the field of coral GEBs, addresses the potential drivers of variation between studies in results, and highlights gaps in our knowledge to outline a framework for the direction of future research in this area.

2. Gene expression biomarkers of heat stress

In predictive medicine, the term biomarker refers to biological measurements used in the prediction of disease risk and early detection of disease to improve treatment selection and monitor the outcome of therapeutic interventions (Simon, 2011). A "Genomic Biomarker" is therefore a DNA or RNA sequence with similar properties. A gene expression biomarker should reflect the expression of a gene, the function of a gene, and the regulation of a gene (Novelli *et al.,* 2008). In the field of coral biology and conservation, the application of gene expression biomarkers to diagnose heat stress in corals has raised a great deal of interest. Suitable GEB candidates should be able to assess the heat stress of corals rapidly, before onset of visible signs such as bleaching. Expression of genes can be immediate and early, where genes which are expressed immediately after stimulation by external factors and are then downregulated, such as *hsp* 70. Genes can also show delayed and late expression relative to the timing of the stimulus. Expression of 'late' genes is normally induced by early genes (Chambers *et al.,* 1999).

Research on gene expression patterns in coral, with the ultimate aim of informing conservation efforts, started in the early 2000s. During this 'discovery' phase, some genes rose to scientific prominence as they were repeatedly reported to be differentially expressed when the cnidarian host and/or the symbiont were subjected to thermal and/or irradiance stress, well before the onset of visible signs of stress, such as bleaching (Rosic *et al.,* 2014a). These genes included those involved in heat shock response, metabolism, oxidative stress, immune response, and ion transport, among others (Table 1, 2). These studies provided an initial impression of the molecular stress response in corals and laid the foundation for the further development of biomarkers (Figure 1). Below, we consider the most studied genes in corals and *Symbiodinium* from each of these categories in turn and evaluate their potential applicability as gene expression biomarkers.

Figure 1. Schematic representation of the expression of genes involved in thermal stress, how they are involved in homeostasis, and the use of these genes as early biomarkers of heat stress in corals

2.1 Heat shock genes

The most studied candidate genes in coral and *Symbiodinum* transcriptomic responses to thermal stress are those encoding the heat shock proteins (HSPs) (Rodriguez-Lanetty *et al.* 2009; Kenkel *et al.,* 2011; Leggat *et al.,* 2011; Meyer *et al.,* 2011; Rosic *et al.,* 2011) , particularly *hsp70* and *hsp90*. HSPs are conserved proteins whose expression is triggered by a wide range of stressors (Schmitt *et al.* 2006). HSPs are molecular chaperones and have vital cytoprotective functions. They are involved in protein folding, unfolding, sorting transport, and assembly of complexes. They also protect cells from apoptosis and stress (Li and Srivastava, 2004). During a stress event, such as exposure to elevated temperature, events such as protein misfolding, aggregation or disruption of regulation and disassembly of multiprotein complexes may occur, leading to subsequent activation of signaling pathways. Through their cytoprotective functions, HSPs are thought to restore proteolitic homeostasis. Upregulation of HSPs occurs through the heat shock response (HSR) which is launched when the transcription factor HSF1 is activated and/or de-repressed during a stress event. HSF1 ultimately binds to promoters of heat shock genes (Pirkkata *et al*., 2001; Jolly and Morimoto 2000). Therefore, following heat stress, upregulation of both coral and *Symbiodinium* HSPs is expected.

2.1.1 *hsp70* is an early responder to general stress

In stony coral hosts, expression of *hsp70* has been reported to be upregulated during laboratory-induced thermal stress experiments. In a long-term thermal stress experiment, adult colonies of *Acropora aspera* were exposed to a 1°C increase in temperature for 6 days and maintained at the maximum experimental temperature (34°C) for two additional days. Significant up-regulation of *hsp70* was reported on days 7 (6.4-fold increase compared to day 1) and 8 (8.2-fold) when the temperature was 4°C above the control transcript levels of hsp70 (Leggat *et al.,* 2011). Using microarrays, Rodriguez-Lanetty *et al.* (2009) detected rapid upregulation of *hsp70* in aposymbiotic larvae of *Acropora millepora* after 3 h of exposure to 28°C (~2-fold increase) and 31°C (~4-fold increase) relative to 24°C controls. However, after 10 h of exposure, expression levels dropped at 28°C (~2-fold decrease) and 31°C (~2.5-fold decrease). However, after10 h at 31°C, transcript levels of *hsp70* remained significantly higher relative to controls.

In *Symbiodinium*, *hsp70* gene expression levels rose when adult *A. millepora* colonies harboring *Symbiodinium* clade C3 were exposed to both rapid (+ 8°C over 18 h) and gradual (+ 7°C over 5 d) thermal stress (Rosic *et al.,* 2011). A 0.39-fold increase in symbiont *hsp70* transcript abundance was reported after 18 h during the gradual thermal stress experiment when temperature was 29°C (3°C above the control). However, after reaching the target maximum experimental temperature of 32°C, *hsp70* expression levels then dropped (by 0.59-fold after 18 h

in the rapid ramp treatment and by 0.69-fold after 120 h in the gradual ramp treatment). The different responses could be due to different ramping and sampling times. Leggat *et al.* (2011) also reported a much more limited increase in *hsp70* gene expression of the symbiont (only a 1.2-fold increase on day 5 of the gradual ramping experiment, when temperatures reached 32ºC). Although the data is limited, the differences in the expression responses of *hsp70* between the host and symbiont suggest that *Symbiodinium* may be less capable of transcriptional acclimatization than their hosts.

Expression of *hsp70* is also altered by other stresses. When the coral *Montastraea* (*Orbicella*) *franksi* was exposed to copper, *hsp70* was upregulated by ~5 fold after 4 h of exposure to 100 ppb and ~3.5 fold after 8 h of exposure to both 30 and 100 ppb. Exposure to oil dispersant (Corexit TM9527) at concentrations of 5, 10 and 50ppm, caused the expression of hsp70 to increase by 3 -3.5 fold after 8 h (Venn *et al.,* 2009).

Non-symbiotic dinoflagellates have also exhibited upregulation of *hsp70*. For example, *Prorocentrum minimum* showed upregulation by ~7-fold in response to 24 h exposure to copper (0.50 mg L^{-1}) and by 1.4 and 1.8 fold when exposed to 0.1 mg L^{-1} and 0.2 mg L^{-1} bisphenol A, a component of plastics and epoxy resin (Guo *et al.*, 2012).

Further experiments are required to confirm this trend across taxa, determine the relative timescale of responses and range of detectability of fold-changes.

2.1.2 *hsp90* is an early responder to general stress

Microarray analyses on adult *Montastraea* (*Orbicella*) *faveolata* colonies revealed that host *hsp90* was slightly up-regulated after thermal stress, with a 1.28-fold increase in abundance after ~11 days of abrupt exposure to 32ºC, compared to controls at ~29ºC. (Desalvo *et al.,* 2008). Another long-term thermal stress study reported up-regulation of *hsp90* in adult *Acropora aspera* nubbins when gradually exposed to 32ºC, compared to controls at 28ºC. In this case, *hsp90* transcript levels increased with exposure time with 1.5, 4.9 and 10.5-fold up-regulation on days 5, day 7 and day 8, respectively, as determined by quantitative PCR (Leggat *et al.,* 2011) . Similarly, aposymbiotic larvae of *A. millepora* showed ~1.5-fold upregulation of *hsp90* after 3 h abrupt exposure to 28ºC (compared to controls at 24ºC). The extent of up-regulation was even higher (3-fold) when they were abruptly shocked at 31ºC (Rodriguez-Lanetty *et al.,* 2009). In the genus *Porites*, a comparable pattern of *hsp90* expression, assayed by qPCR, was observed in adult colonies of *P. astreoides* when exposed to heat stress. In a laboratory-induced heat/light stress experiment, *hsp90* expression was up-regulated by approximately 6-fold after 3 h of exposure to 35–36ºC (7-8ºC warmer than controls and with 10-fold higher light intensity; Kenkel *et al.,* 2011). In a similar study, it was noted that when exposure time to thermal stress (31ºC) was extended to 6 weeks, down-regulation of *hsp90* gene expression occurred, as assayed by qPCR. Colonies that paled in the study exhibited a 2.4-fold decrease in *hsp90* expression, while

colonies that bleached showed a 1.6-fold down-regulation (Kenkel *et al.,* 2013). This sustained stressed possibly caused irreversible cellular damage. The observed down-regulation might also be the consequence of the decrease in cell density.

In contrast, in the symbiont, the *hsp90* gene is reported to be downregulated following thermal stress. After 18 h of thermal stress, *hsp90* gene expression was significantly downregulated by 0.57-fold at 26°C and by 0.43-fold at 32°C (compared to controls at 24ºC). Prolonged thermal stress led to further declines, i.e., by 0.23-fold after 72 h at 29°C and 0.22-fold after 120 h at 32°C. The same expression patterns were observed in freshly isolated and cultured *Symbiodinium* cells in a control experiment (Rosic *et al.,* 2011). Leggat *et al.* (2011) also showed that *Symbiodinium hsp90* gene expression decreased following 32°C thermal stress over 7 d (by 0.26-fold) and 8 d (by 0.23-fold).

The expression of coral *hsp90* is also altered by other stresses. When *M. franksi* was exposed to copper at concentrations of 30 and 100 ppb, the expression of coral *hsp90* was upregulated by 2.5 and 2.3 fold respectively after 8 h. The specificity of *hsp90* to other stress factors remains to be evaluated (Venn *et al.,* 2009).

Why *Symbiodinium* and the coral host exhibit opposite patterns of *hsp90* gene expression upon exposure to thermal stress is still not understood. A host-cnidarian buffering system might be involved, partially protecting the symbiont from physiological stress by assisting in specific cellular processes (Barshis *et al.,* 2014; Richier, 2005).

2.1.3 *hsp16*

Small HSP (smHSPs), such as *hsp16*, generally assist other chaperones in the refolding of denatured polypeptides and prevent their aggregation (Veinger *et al.,* 1998). *Hsp16* is one of the most responsive genes to heat stress reported to date in corals. When adult colonies of *Porites astreoides* were subjected to lab-induced irradiance (100 times higher than control) and thermal stress (7-8ºC above controls), a ~700-fold and ~800-fold upregulation of *hsp16* was observed in two different experiments (Kenkel *et al.,* 2011). In a follow-up study investigating gene expression at lower stress intensity (no irradiance stress and heat stress of +4ºC), Kenkel *et al.* (2014) reported 10 times less upregulation of *hsp16* indicating a large dynamic range in this gene. This makes it a good candidate as a biomarker of heat stress, but gene expression has been investigated only in genus *Porites* and no studies have targeted the response of *hsp16* in *Symbiodinium* with respect to thermal stress. In addition, the specificity of hsp16 expression response to thermal stress alone remains to be determined. The potential shown by this gene, as marker of heat stress in coral, warrants future research. *Hsp16* expression during heat stress has not been studied in *Symbiodinium.* Such a promising GEB definitely deserves attention in the symbiont as well.

2.1.4 *hsp60*

Hsp60, also known as chaperonin 60 or cpn 60, is a Group I chaperonin found in mitochondria but also in cytosol, vesicles, extracellular space, cell membrane and blood (Cappello *et al.,* 2008). In corals, a ~4-fold up regulation was observed in adult colonies of *Porites astreoides* after exposure to heat stress (Kenkel *et al.,* 2011). Expression also tended to be upregulated in response to temperatures 2°C above ambient (29°C to 31°C) and showed a ~2.2 fold increase in transcript abundance between 31°C and 33°C, suggesting expression is graded in response to the level of stress experienced (Kenkel *et al.,* 2014). Western blot analysis of *hsp60* protein expression showed comparable patterns when *Seriatopora hystrix, Montipora monasteriata and Acropora echinata* were heat shocked at 34°C. An initial increase in *hsp60* protein expression was noted but sustained stress brought about a downregulation of the protein expression (Seveso *et al.,* 2014). Consistent early upregulation of *hsp60* following heat stress in corals has been observed in all these studies, but different temporal responses have not been studied. No studies have yet investigated the response of *hsp60* gene in *Symbiodinium*, or tested the specificity of *hsp60* to thermal stress.

2.1.5 *Tcp-1*

T complex polypeptide is an *hsp60* family member (Wagner *et al.,* 2004) playing an important role in the folding of various proteins including actin and tubulin. *Tcp-1* seems to exhibit a relatively delayed response to heat stress after other HSPs have responded. In the larvae of *A. millepora* no response to thermal stress was detected for this gene in the first 10 h of exposure to 28°C or 32°C (Rodriguez-Lanetty *et al.,* 2009). In the coral *Orbicella faveolata* *Tcp-1* was upregulated 1.36-fold after 24 hours at 32°C (Desalvo *et al.,* 2008). Future work should further investigate the response of *Tcp-1* to thermal stress as, in contrast to other HSPs, *Tcp-1* could be involved in a delayed heat stress response. No studies have investigated gene expression patterns of *Tcp-1* in *Symbiodinium.*

2.2 Oxidative stress genes are late responders to general stress

Reactive oxygen species (ROS) production is a key element in the cellular pathology of bleaching, regardless of stressor (Baker & Cunning 2016). During thermal stress, photosynthetic dysfunction leads to the accumulation of ROS and is highly damaging to cells. ROS denature proteins, damage nucleic acids and oxidize membranes (Lesser, 2006; Weis, 2008). The first line of defense against ROS involves induction of superoxide dismutase (SoD), manganese superoxide dismutase (MnSoD), glutathione peroxidase (Gpx1), peroxidasin homologue precursor (Pxdn) and thioredoxin (Txn). These enzymes convert ROS to hydrogen peroxide (H_2O_2) and water. Catalase (Cat) activity then regulates the increasing amount of H_2O_2 in host cells (Merle *et al.,* 2007). Upregulation of these oxidative stress genes is expected following heat stress as a direct consequence of increased oxidative stress.

Seneca *et al.* (2010) sampled tagged *A. millepora* colonies in the field during a bleaching event in 2002. They reported 1.81-fold upregulation of a catalase homolog (AmCaT) in thermally

stressed (bleached) samples exposed to naturally elevated temperature of 32ºC compared to expression in the same corals under normal environmental conditions (< 29ºC) one year earlier. Souter *et al*. (2011) suggested MnSoD as a useful bioindicator of bleaching stress in corals as they observed significant and consistent upregulation by 1-fold in adult *A. millepora* after 9 d of exposure to 32ºC. An increase in oxidative stress genes was also observed in adult *M. faveolata,* with glutathione-s-transferase sigma and thioredoxin reductase 1(*TR-1*) being upregulated by 1.26 and 1.36-fold following thermal stress of 32ºC for ~11 days (Desalvo *et al.,* 2008). Interestingly, in aposymbiotic larvae of *A. millepora* subjected to heat stress of 31ºC, no increase in oxidative stress genes was observed (Rodriguez-Lanetty *et al.,* 2009). These results support existing evidence indicating *Symbiodinium* photosystem II as the principal source of ROS in host cells (Downs *et al.,* 2000; Weis, 2008, Jones *et al.,* 1998). However, in a similar experiment where embryos of *M. faveolata* where subjected to heat stress, upregulation of some oxidative stress related genes (cytochrome p450, soma ferritin, catalase and peroxidasin-like protein) by 1-2-fold was observed after 48 h of exposure to 29°C and 31.5°C (Voolstra *et al.,* 2009). The peroxidasin-like protein showed the highest susceptibility to heat stress among the candidate oxidative stress, with 12.4-fold upregulation after 48 h at 31.5°C (Voolstra *et al.,* 2009). However, when adult colonies of *M. faveolata* were subjected to longer exposure times (~11 days) at 32ºC, peroxidasin-like protein was the most downregulated gene (3.45-fold, Desalvo *et al.,* 2008). However, hypersaline stress has also been reported to increase expression of thioredoxin by 2-fold (46 ppt) and 1.7-fold (43 ppt).

In *Symbiodinium*, *cytochrome P450* (*CYP*) genes have been reported to be upregulated by 2.5 to 4-fold at moderately elevated temperatures (+ 3ºC and +6ºC above ambient) (Rosic *et al.,* 2010). However, expression of these genes decreases to initial levels at +9ºC above ambient, possibly as a result of impairment of photosynthesis, cellular damage and decrease in cell density at temperatures above 30 ºC. Future research need to be done to confirm if cytochrome *CYP* genes show consistent upregulation under heat stress.

Expression of ROS genes is not necessarily specific to thermal stress alone. Exposure of *M. franksi* to the copper (30 μg L^{-1}) for 48 h resulted in upregulation of the oxidative genes glutathione S transferase (1.2-fold), peroxidasin-homolog-like (3.2-fold), catalase (1-fold) and cathepsin B (0.5-fold; Schwarz *et al.,* 2013). Furthermore, induction of an oxidative stress-responsive protein was also observed when *A. tenius* was exposed to tributylin chloride, an antifouling agent (Yuyama *et al.,* 2012). The soft coral *Scleronephthya gracillimum* also exhibited upregulation of the antioxidant gene ferritin following exposure to a polycyclic aromatic hydrocarbon, Benzo(a)pyrene (Woo *et al.,* 2012).

Overall, certain oxidative stress response genes may be good candidates as late heat stress gene expression biomarkers, particularly peroxidasin-like proteins (Voolstra et *al.,* 2009). However, the broad response of some of these genes to multiple stressors suggests they are not specific to temperature stress alone.

2.3 Immune response genes respond to general stress

Several studies have highlighted the correlation between bleaching events and subsequent disease outbreaks (Muller *et al.,* 2008; Cróquer and Weil, 2009; Rogers *et al.,* 2009). Rodriguez-Lanetty *et al*. (2009) hypothesized that high temperature may have a detrimental effect on the host innate immune system, based on their observation that a c-type mannose-binding lectin gene, was downregulated by 3-fold in *A. millepora* following 10 h exposure to thermal stress (31ºC). In *Porites*, complement C3, another key player in innate immunity, was also downregulated by 6-fold following heat (7-8ºC above ambient) and light (100x higher than ambient) stress (Kenkel *et al.,* 2011*)*. However, there was no significant change in expression of complement C3 following short-term temperature exposure (29-33ºC), and no differential regulation was observed among bleached and healthy corals collected during a natural bleaching event (Kenkel *et al.,* 2014). Conversely, the expression of a major transcription factor involved in regulating immune response, *Nf-kβ (Nf-kβ$_1$, Nf-kβ$_2$)* increases by 1-2-fold as a result of 9-days heat stress at 32ºC (Souter *et al.,* 2011). Results from these different studies demonstrate that immune response genes are potentially valuable direct biomarkers of heat stress, but may also be indirectly influenced by disease pathology. Complement factor C3-like protein (C3-Am) was upregulated by ~0.5-fold following physical injury. The mannose binding lectin, Millectin, was upregulated by ~0.3-fold and ~0.15-fold after 45 and 360 minutes, respectively, following lipopolysaccharide (25g) injection. C3-like protein (C3-Am) was also upregulated by ~0.15 and 0.8-fold following peptidoglycan (5g) injection after 6 and 12 h, respectively. These injections mimicked events occurring during infection by pathogens (Kvennefors *et al.,* 2010). Other genes involve in the immune response (e.g., astacin and cathepsin L alpha-macroglobulin and serine proteinase inhibitor) were also shown to be 1-3 fold upregulated in disease tissue associated with white syndrome, compared to healthy tissue (Wright *et al.,* 2015). Additional studies are needed to confirm the direction of regulation, as well as the specificity and timing of expression response. The response of immune genes in the symbiont has not been studied.

2.4 Genes involved in calcium ion (Ca^{2+}) signaling respond to general stress

Transport of ions across cellular membranes is vital for cellular homeostasis and transepithelial transport or neuronal signal transduction. In addition, transport of calcium ions is a key process in coral calcification (i.e., growth, Al-Horani *et al.,* 2003; Furla *et al.,* 2000; Marshall *et al.,* 2007), which is known to be affected by heat stress (Huang *et al.,* 1998). A calcium transporter, *Cacna1s* was 5-fold upregulated following thermal stress (~4ºC above ambient) in *A. millepora* larvae (Meyer *et al.,* 2011). The high responsiveness of *Cacna1s* makes it an interesting potential biomarker. A member of the *SLC26* family, a putative bicarbonate/chloride exchanger, is among the most differentially expressed genes following heat stress (Kenkel *et al.,* 2013). In *Porites astreoides,* 92-fold downregulation of this gene has been reported following exposure to 30.9ºC (Kenkel *et al.,* 2013).

Calcium-modulated protein, also known as *Calmodulin* or *CaM*, is a calcium binding protein. Ca^{2+} binds to CaM, which acts as an intermediate messenger protein, and in turn regulates target proteins to bring about various responses (Stevens, 1983). When adult colonies of *M. faveolata* were exposed to sudden heat stress at 32°C for 24 h, a downregulation of *CaM* was observed (DeSalvo *et al.,* 2008). A slight decrease in *CaM* transcript abundance (1.38-fold) was also observed following thermal stress (3°C above ambient for 10 d) in adult colonies of *Acropora palmata* (DeSalvo *et al.,* 2010a). Yet, when aposymbiotic larva of *Acropora millepora* were exposed to abrupt thermal stress (7°C above ambient) for 3 and 10 h, stable expression of *CaM* was observed (Rodriguez-Lanetty *et al.,* 2009). These authors suggested that differential expression of *CaM* in other studies was influenced by the presence of stressed symbionts. Further experiments are needed to confirm either of these two trends.

Genes involved in calcium ion transport processes also show differential regulation under elevated pCO_2, or ocean acidification scenarios. Upregulation of genes involved in calcium and carbonate transport, conversion of CO_2 into HCO_3^- and organic matrix proteins was reported in the coral *Pocillopora damicornis* after gradual exposure to decreased pH of 7.2-7.8 for three weeks (Vidal-Dupiol *et al.,* 2013). This suggests that regulation of ion transport is modified by acidification stress in addition to thermal stress. Studies have yet to target ion transport genes in *Symbiodinium*.

2.5. Most genes involved in central metabolism tend to be poor biomarkers of heat stress

Metabolic genes include candidates involved in pathways such as glycolysis, the tricarboxylic acid cycle (TCA cycle), gluconeogenesis, and fatty acid synthesis. Early microarray work on *Orbicella faveolata* coral embryos concluded that metabolic genes were more downregulated than upregulated after 12-48 h of heat stress at 2-4°C above ambient (Voolstra *et al.,* 2009). Of the 14 candidate metabolic genes studied, five were upregulated and the remaining was all down regulated (Table 1). These results are consistent with those of Desalvo *et al.* (2008), who reported downregulation of all six metabolic genes assayed in their microarray analysis in adult colonies of *Orbicella faveolata* subjected to thermal stress 3°C above ambient (Table 1). Modest changes were observed in both studies, ranging from 1.11- to 1.8-fold down regulation. GAPDH is a commonly used control gene for qPCR-based studies (Kenkel *et al.,* 2011; Souter *et al.,* 2011; Seneca *et al.,* 2010), but it also exhibits differential expression patterns in response to temperature stress. In adult *Acropora aspera*, upregulation by 1.7, 1.9 and 4.4-fold was observed after 5, 7, and 8 days, respectively, at 4-6°C above ambient (Leggat *et al.,* 2011). Conversely, this gene was shown to be downregulated in naturally bleached *P. astreoides* (Kenkel *et al.,* 2014). Similar to GAPDH, adenosine kinase was used as internal control gene in one RT-qPCR experiment (Kenkel *et al.,* 2011) but showed 2.0-2.1 downregulation as candidate gene in a RNA-Seq experiment (Kenkel *et al.,* 2013). Significant upregulation of other metabolic candidates including α-ketoglutarate (1.2, 2, and 1.3-fold, after 3, 7, and 8 days, respectively, at 4-6°C above ambient), glycogen synthase (1.7-fold after 8 days at 6°C above ambient) and glycogen phosphorylase (1.5, 1.8, and 3.6-fold after 5, 7, and 8 days, respectively, at 4-6°C above ambient) has also been observed in response to heat stress (Leggat *et al.,* 2011). While phosphoenolpyruvate carboxykinase *(PEPCK)* was upregulated ~2-fold when measured after six

weeks of thermal stress at 3ºC above ambient (Kenkel *et al.,* 2013). *Symbiodinium* metabolic genes have not been investigated to any great extent. Transcript abundance of *Symbiodinium GAPDH* and α-ketoglutarate slightly increased during thermal stress (4-6ºC above ambient) by 1.3-fold and 1.2-fold increase respectively (Leggat *et al.,* 2011).

By themselves, metabolic genes tend to be poor candidates of biomarkers of heat stress in corals due to their variable response and low magnitude change in expression. Possibly, as discussed by Leggat *et al.* (2011), metabolic genes encode key metabolic proteins that are not solely regulated by transcription but are also subject to post-translational and allosteric modifications. The importance of these post-translational mechanisms can only be assessed by proteomics analyses. However, *PEPCK* may be worth further investigation, because expression of this gene is believed to be link to increase host gluconeogenesis to compensate for symbiont loss. Several metabolic genes have been studied but still have not received enough attention as most of the genes have been targeted by one study till now.

2.6 Structural genes are possibly heat stress specific

Structural genes encode proteins whose primary function is to form part of a physical structure within a cell. Although commonly used as internal control genes in many studies (Pagarigan and Takabayashi, 2008; Vandesompele *et al.,* 2002), coral actin genes were highly responsive to acute thermal stress (3 h at 3-6ºC above ambient), with consistent ~4-fold downregulation in *Porites* spp. (Kenkel *et al*. 2011, 2014). Further evidence of differential expression of structural genes following heat stress were also reported in *Montastraea (Orbicella) faveolata and Acropora palmata.* In *M. faveolata,* differential expression of five genes associated with the actin cytoskeleton were observed following 10 days of abrupt exposure to heat stress of ~32ºC. Gelsolin , lethal giant larvae homologue 2 , and tropomyosin were downregulated whereas myosin 7 A (MYO7A) and myosin 9 A (MYO9A) are slightly upregulated (Desalvo *et al.* 2008). Similarly, in *A. palmata,* Gelsolin, tropomyosin, Tropomyosin-2, Myosin-2 essential light chain and L-Actin were downregulated by 1- to 2-fold after 1 day of gradual heat stress of ~32.7ºC.Only Myosin-10 was upregulated by approx 3-fold (Desalvo et al. 2010a). Moreover, when the coral *Stylophora pistillata* was exposed to the pollutant Aroclor 1254, there was no change in the expression of an actin-related protein 2/3 complex (Chen *et al.,* 2012), suggesting these genes may be specific to heat stress. No studies have yet targeted structural genes in *Symbiodinium* for a heat stress study, although actin has been used as a housekeeping gene (Leggat *et al.,* 2011).

2.7 Other candidate genes

Exocyst complex component 4 (EXOC4) is a component of the exocyst complex, a multiple protein complex essential for targeting exocytic vesicles to specific docking sites on the plasma membrane (Terbush *et al.,* 1996). *EXOC4* is known to interact with the actin cytoskeletal remodelling and vesicle transport machinery, hence *EXOC4* is thought to be linked to the process of symbiont expulsion. Upregulation of this gene in paling, but not fully bleached or healthy

corals, provides support for this proposed role (Kenkel *et al.,* 2013). However, further experiments are needed to determine specificity of this expression and confirm the pattern.

2.8 Internal controls for GEB assays

Genes whose expression does not vary in the tissues or cells under investigation, or in response to experimental treatment are normally used as internal control genes. Internal control genes, also referred to as housekeeping or reference genes, help in normalization of gene expression assays to eliminate between-samples variations (Vandesompele et al., 2002). However, depending on the design of the assay (e.g. the 'double-gene assays' developed by Kenkel et al. 2011, 2013) or the statistical method of analysis (e.g. Bayesian analysis can be control-gene independent, Matz *et al.,* 2013), internal control genes may not always be necessary.

In studies on heat stress in corals, genes showing the most stable expression in response to the selected stress should be identified and/or verified for each focal stressor and species as part of the study. Typical internal control gene candidates in coral hosts include ribosomal proteins, e.g. host Ribosomal protein S7 (Rp-S7; Leggat *et al.,* 2011; Souter 2011). Ribosomal protein L11 (RPL11) and the elongation initiation factor 3H (EIF3H) and have proven to be the most stable in *P. astreoides* (Kenkel *et al.,* 2011; 2014). In *Symbiodinium,* ribosomal protein S4 (Rp-S4; Rosic *et al.,* 2010; 2011; 2014), and S-adenosyl-L-methionine synthetase (SAM; Rosic *et al.,* 2010; 2011; 2014a) are commonly used, among others.

Table 1. List of candidate genes studied to date with potential use as biomarkers of thermal stress. Duration of exposure, d=days, h=hours. Treatment type, The non-preconditioned =NPC, preconditioned =PC. Ramped thermal stress =†, immediate thermal stress =*.

Table 2. List of differentially expressed *Symbiodinium* genes following heat stress that may be potential biomarkers of thermal stress.

3. Source of variability between studies and potential solutions

For some of the candidate biomarker genes in host and symbionts, consistent trends of regulation during heat stress have been observed in several studies. However, many other genes differ in the magnitude of change or their direction of expression (Fig. 2). Differences in experimental procedures such as acclimation conditions, acclimation time, initial ramping rate, sampling time points, water quality, light exposure, and gene expression quantification method, as well as differences in host and symbiont biology (Rodriguez-Lanetty *et al.,* 2009; Voolstra *et al.,* 2009; DeSalvo *et al.,* 2010b; Rosic *et al.,* 2010; 2014a; 2014b; Leggat *et al.,* 2011; Meyer *et al.,* 2011; Kenkel *et al.,* 2011, 2013; Barshis *et al.* 2014; Parkinson et al., 2016) may account for differences in results across studies (Table 3). In addition, variation in gene expression also occurs at different life stages (Hill *et al.*, 2000). Therefore, direct comparison between studies using adult and larval stages may be problematic.

Figure 2 (A) Number of studies reporting upregulation or downregulation of gene expression biomarkers in adult corals (N=24 studies). Corals are grouped according to coral morphology. *Symbiodinium* are *in hospite*, except in culture where noted. (B) Number of studies reporting upregulation or downregulation of gene expression biomarkers in coral larvae (N=11 studies).

Table 3. Summary of sources of variation between studies

3.1 Differences in experimental procedures

Most studies have attempted to simulate a thermal stress event in the lab but differences in experimental design (such as pre-acclimation time and conditions, initial ramping rate, sampling time points, water quality, light exposure) may result in different responses. Furthermore, different studies used different gene isoforms, which may have different biological roles and expression patterns, this can also account for variation in results (Table 1). Certain genes, e.g *hsp90,* are considered "hub genes" due to their involvement in multiple pathways (Lehner *et al.* 2006). Differential expression may depend on the pathway being more solicited during heat stress.

3.2 Comparing field studies to lab-induced thermal stress

At the current early stage of biomarker development, both field studies and laboratory experiments have studied expression of candidate genes. Results from these studies cannot be directly compared, as laboratory experiments test the specific effects of one or two factors whereas in field studies multiple factors vary naturally, potentially influencing expression of

genes. Rather than direct comparisons, the specificity and expression range of biomarkers should be tested under controlled laboratory conditions. Similar to human clinical trials, after biomarkers are validated in the laboratory broader field applicability testing is warranted.

3.3 High natural variation in gene expression

Evidence of high variability in gene expression between different colonies has been commonly reported. Granados-Cifuentes *et al.* (2013) reported that 17% of genes in their microarray were differentially expressed in six *A. millepora* colonies after four weeks of acclimatization in a common garden experiment with similar environmental conditions. Among those differentially expressed were genes involved in oxidation/reduction, apoptosis, transport, translation and response to general stimuli. These results support a previous study of *A. millepora* where two candidate genes (AmSw, DY585805; AmTrib, DY587605), and an internal control gene, (Ctg1913) showed inter-colony variation during a brief bleaching event (Seneca *et al.* 2010). Császár *et al*. (2010) also observed high inter- and intra-colony variation in antioxidant genes, ferritin, mnSOD, Zn^{2+}-met and *hsp70* in different colonies of the same coral species after exposure to thermal stress in the laboratory. Variation between *A. hyacinthus* colonies were reported in an laboratory experiment mimicking extreme temperatures in the lagoon of Ofu island, American Samoa (Seneca and Palumbi, 2015). Variation in gene expression also occurs between different parts of the same coral colony. RNA-seq reveals that between coral tip and base, genes involved in developmental pathways like Notch, Wnt, and BMP, extracellular matrix production were differentially expressed (Hemond *et al.,* 2014).

Also, the same coral colony might be harbouring different *Symbiodinium.* The algal symbionts vary in their thermotolerance (Rowan, 2004) and variation in gene expression has been documented between different symbiont taxa (Parkinson *et al.,* 2016; Rosic *et al.,* 2014b). The observed variation in such studies can be attributable to the fact that the *Symbiodinium* under investigation may belong to different species or different individual strains. These differences may be compounded under heat stress. Barshis *et al*. (2014) reported natural gene expression variation between *Symbiodinium* lineages while studying the transcriptional profiles of different *Symbiodinium* following heat stress, with 35% of candidate genes showing significant variation attributable solely to *Symbiodinium* type. Hence intraspecific variation in coral expression seen within colonies might also be the result of the fact that corals are responding to heat stress on different *Symbiodinium* harboured in their gastrodermal cells.

Intercolony variation in gene expression might also occur as a result of allelic variation in the host occurring between microclimates differing in environmental conditions such as temperature. Bay and Palumbi (2014) demonstrated that colonies of *A. hyacinthus from* different pools of a back reef lagoon in American Samoa differed in genotype. *A. hyacinthus* in the warmer pool had, on average, almost twice as many alleles at selected loci as coral in the cooler pool. Genetically

diverse populations of *Porites astreoides* from inshore and offshore reefs of Florida Keys differing in environmental conditions, with inshore reefs being subject to higher temperatures, have been reported, (Kenkel *et al.,* 2013). It is argued that coral host genotype might play an important role in holobiont capacity to resist heat stress and hence might be involved inter-colony gene expression variability.

The expression of some genes in corals also changes naturally in the field over time. Edge *et al.* (2008) followed the expression of a panel of 32 selected genes in a field population of *M. faveolata.* The selected genes included those involved in key processors such as respiration, oxidative stress, maintenance of cellular integrity, apoptosis, post-translational processing and response to xenobiotic exposure. Most of these genes showed little variation from their average level of expression during spring and early summer. Yet, in late summer, the variation in expression of these genes was higher. Triggers of this natural variation in the field were suspected to be environmental changes such as changes in temperature, salinity and light intensity but might also be related to physiological events such as spawning.

3.4 Thermal history

Another factor affecting transcriptional profiles of corals in response to heat stress is their thermal history. The influence of prior thermal exposure on coral response to subsequent heat stress is known to affect response of corals to future stress. The transcriptional effect of pre-conditioning corals to sub-lethal temperature was studied by Bellantuono *et al.* (2012). The study revealed nine differentially expressed genes between pre-conditioned (PC) and non-conditioned (NC) colonies when exposed to the same bleaching temperature for 10 days. Differences in transcriptional profiles included both the magnitude of change in expression, and its direction. Lectin, tyrosine kinase receptor, and follistatin showed consistent upregulation even after preconditioning. These genes may be good candidate biomarkers of heat stress. Natural variation in thermal history can also be a factor explaining contradictory results in the direction of regulation of certain genes following heat stress between studies. Barshis *et al*. (2013) thermally stressed colonies of *A. hyacinthus* from backreef environments with different thermal profiles, and found that corals from highly variable sampling environments (summer maximum $\geq$ 34^{o}C and daily variation of 6^{o}C) had higher expression of 60 genes under non-stressful conditions compared to colonies from moderately variable environments. These 'frontloaded' genes were less up-regulated in these 'resilient' corals when exposed to heat stress. Among these frontloaded genes were heat shock proteins, and antioxidant enzymes, as well as genes involved in innate immunity, cell adhesion, apoptosis and tumor suppression. Short-term pre-conditioning can also elicit a frontloading response: when the coral *Acropora nana* was subjected to three different acclimatization treatments, significant differences were observed in gene expression response following heat stress. Corals acclimated to higher temperatures (29-31^{o}C) did not show changes in gene expression compared to corals preconditioned to lower temperatures (less than 29^{o}C) and they also had higher physiological tolerance to bleaching (Bay and Palumbi, 2015).

3.5 *Symbiodinium* identity

Different *Symbiodinium* harbored can also account for discrepancies between similar studies. DeSalvo *et al.* (2010b) found a positive relationship between symbiont and host transcriptomic state when comparing gene expression profiles of *M. faveolata* colonies after acclimatization, heat stress and recovery. They reported that transcriptomic profiles were similar for colonies harboring the same *Symbiodinium* genotype, rather than colonies subjected to similar experimental conditions. Similarly, Rocker *et al.* (2012) studied the transcriptional response of juvenile *A. millepora* inoculated with different *Symbiodinium*. Juveniles harboring mixed communities of *Symbiodinium* in clades C and D initially showed higher upregulation of *hsp70* and *hsp90* genes following exposure to 32ºC, compared to juveniles harboring only one *Symbiodinium* type, but these genes were subsequently downregulated over the course of the experiment. Conversely, juveniles harboring only a single *Symbiodinium* type showed no change in expression during the experiment. Differential expression of genes based on symbiont genotype was also shown in a laboratory thermal stress experiment, in which two photosynthetic genes, psb A and psa A, were downregulated 2-3-fold only in heat sensitive *Symbiodinium* A13 and C1b-c (McGinley *et al.* 2012).

3.6 Diel cycle

As in other animals, gene expression in scleractinian corals can be affected by circadian rhythms. In *A. hyacinthus,* up to 100-fold changes in gene expression were reported when comparing response at noon vs. midnight. These genes included highly responsive genes, such as transcription factors associated with cryptochromes, thyrotroph embryonic factor, and D site-binding protein, as well as genes involved glucose transport and glycogen storage (Ruiz-Jones and Palumbi, 2015). Brady *et al*. (2011) observed that the gene expression of certain genes in aposymbiotic larvae and adult colonies of *A. millepora* was also influenced by a diel cycle. Thousands of contig reads were differentially expressed between day and night samples. Further investigation of six candidate genes using qPCR showed significant changes in gene expression between day and night. Levy *et al*. (2011) reported that stress-related genes and antioxidant genes in corals are under the control of an endogenous clock in anticipation of oxidative stress originating from symbiont photosynthesis during the day.

In *Symbiodinium*, genes involved in a circadian clock have also been reported but research in this area is still in its infancy (Sorek *et al.,* 2014). Oxygen-evolving enhancer 1 (OEE1) a component of PSII, showed decreased expression during the day, in both free living and *in hospite Symbiodinium,* compared to night measurements (Sorek *et al.,* 2013).

3.7 Host buffering system

A host cnidarian buffering system (Barshis *et al.,* 2014; Richier, 2005) might be involved in dampening *Symbiodinium* expression. Richier (2005) reported the expression of novel proteins

when *Symbiodinium* were grown in culture compared to *in hospite* ones, suggesting the existence of a host buffering system. Parkinson *et al.* (2015) shed further light on this system by subjecting *Acropora palmata* fragments to cold-stress of 20ºC for 3 days. Hosts which showed the greatest change in gene expression (184 genes differentially expressed) had less stressed *Symbiodinium* which showed less fluctuation and lower impairment of photochemical efficiency compared to hosts showing relatively stable gene expression (only 14 genes differentially expressed). They suggested that host identity and expression pattern affects *Symbiodinium* stress response. By the same argument, changes in *Symbiodinium* gene expression might also affect expression of host genes. However, the relatively small fold changes in *Symbiodinium* compared to coral hosts (Leggat *et al*., 2011) suggests this may be less common.

3.8 Expression of host gene may be graded and regulated by thresholds

The graded expression response of some genes in proportion to the level of stress experienced may explain differences in fold-changes observed across studies. Regulation of gene expression by stimuli/stress thresholds have been reported in many animal systems. For example, the expression of the proto oncogene *fos* in gerbils proportionally increased with photon exposure (Dkhissi-Benyahya *et al.,* 2000). A mathematical model predicted the existence of temporal regulation of gene expression in cyanobacteria for the gene IsiA (iron stress induced protein A) which is transcriptionally induced in response to iron depletion or oxidative stress (Legewie *et al.,* 2008). Such regulation systems are believed to help ensure that energetically expensive proteins are only expressed when stress exceeds a critical threshold limit, limiting the production of these proteins in response to short-term exposures when they may not be needed (Legewie *et al.,* 2008). A similar response was reported in the coral *Porites astreoides*, where the heat shock protein genes, *hsp16* and *hsp90*, and *actin* showed a graded expression response when the host was exposed to a linear increase in temperature from 29ºC to 33ºC. Based on previous studies, it was hypothesized that 33ºC represented a critical threshold triggering more extreme gene expression response (Kenkel *et al*., 2014).

4. Future directions

Reviewing recent studies of gene expression biomarkers of coral heat and light stress (Table 1, 2), reveals several areas that are poorly understood, and which need further attention. One of the major research gaps remains the lack of a universally accepted biomarker(s) of heat stress. To date, most potential GEBs have been studied in only one coral species, or mostly in the genus *Acropora* (Fig. 1). The most studied genes (*hsp*90 and *hsp70*) have been tested in only six of the 800 known hard coral species. We propose that a shortlist of potential GEBs of thermal stress should be analyzed in a suite of different representative coral species from different regions of the world. This will help to determine consistency of GEBs within and between coral species. Potential GEBs of heat stress also showed differential regulation when subjected to other stresses like heavy metals (Venn *et al.,* 2009), pollutants, and changes in pH. To test for specificity of the biomarkers under investigation we suggest that including other stressors when designing future experiment might be an effective way of testing for specificity of the candidate heat stress biomarker. Additionally, more transcriptomic studies of heat stress in corals and *Symbiodinium* is

essential. Given the high variation seen across individuals, particular emphasis should be laid on including more individuals in future studies. Sufficient studies are needed to draw an accurate general regulation trend for most of the potential GEBs. If future research still fails to identify universally accepted GEBs of heat stress, we believe that research can be focused on combined expression of several genes. A suite of GEBs can prove efficient, similar to the double gene assay that showed robust reciprocity in two *Porites* species and across studies (Kenkel *et al.,* 2011; 2014). The basis of the double gene assay is the difference in the expression levels of two genes showing antagonistic responses. This difference is then used as a stress index. Interesting results have been reported so far as the assay has been able to distinguish between unstressed and heat/light stressed samples. The assay has also proved to be transferable across species of the genus *Porites*. We propose that this assay can be incorporated in future research or researchers might design similar type assays using two or more genes. While comparatively most studies have focused on the animal host, the search of GEBs in *Symbiodinium* is also needed. A universally accepted GEB might also come from Symbiodinium transcriptomics studies. We suggest that research on gene expression biomarkers of heat and light stress in *Symbiodinium* should be broadened. Equal consideration of *Symbiodinium* should be given in future transcriptomics studies.

Another key avenue for future research is to decipher the precise molecular mechanisms involved in the thermal stress response of corals, which would help to better situate the role of the targeted biomarker in any particular pathway(s). Thereby, increasing our understanding of the observed expression patterns of GEBs**.** Most transcriptomics experiments on coral response to heat stress have been done under laboratory conditions. Due to complex natural interactions in the field, transcriptomic response of coral might not be similar to those observed under control conditions. We suggest that future work will also need to be focused on validating gene expression responses of coral *in situ*. The ultimate goal of GEBs is to find cosmopolitan biomarkers as well as develop simple routine assays to assess coral heat and light stress status. Developing standard reproducible transcriptomic assay protocols that can be used anywhere around the world, particularly portable diagnostic kits, should be a research priority. Such a kit would be very practical for reef managers to take rapid decisions in the field. In Table 4, we summarize future directions to aid in development of consistent GEBs of thermal and light stress in corals.

Table 4. Research gaps in the development of gene expression biomarkers of heat stress in scleractinian corals

5. Concluding remarks: The future of gene expression biomarkers as indicators of coral heat and light stress status

Gene expression biomarkers of coral health promises proactive management of coral reefs. Questions pertaining to reproducibility across species, stress-specificity, temporal variation, thermal history, life stages, and worldwide reproduction of the technique are now emerging. We propose *hsp16, Cacna1, MnSOD, SLC26,* peroxidasin-like protein, *CaM and NF-ĸ*B as having high potential as heat stress biomarkers for coral hosts, and cytochrome P450 as a potential heat stress biomarker in *Symbiodinium.* However, we recognize that since different individuals might respond in different ways (Granados-Cifuentes *et al.,* 2013; Kenkel *et al*., 2013; Bay and Palumbi, 2014) the use of a single universal GEB might be insufficient, and instead rather a suite of GEBs might be needed to assess heat stress. Among the identified gaps, stress specificity is a priority research gap that needs to be filled for these genes. Future work needs to establish whether expression patterns of these GEBs can indeed be correlated with a specific stressor and if their expression is consistent across coral taxa. If gene expression biomarkers are to be useful, this issue must be addressed in the development of suitable markers. One solution may be to focus not on the absolute change but on the consistent relative change of genes in response to different conditions while accounting for random effects of different coral genotypes in statistical models. Expression patterns could be then used to differentiate between stressors. Future work should focus on these interrogations so that we can rapidly translate acquired knowledge of coral GEBs into a practical approach that could be used by reef managers around the world.

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Figure 1

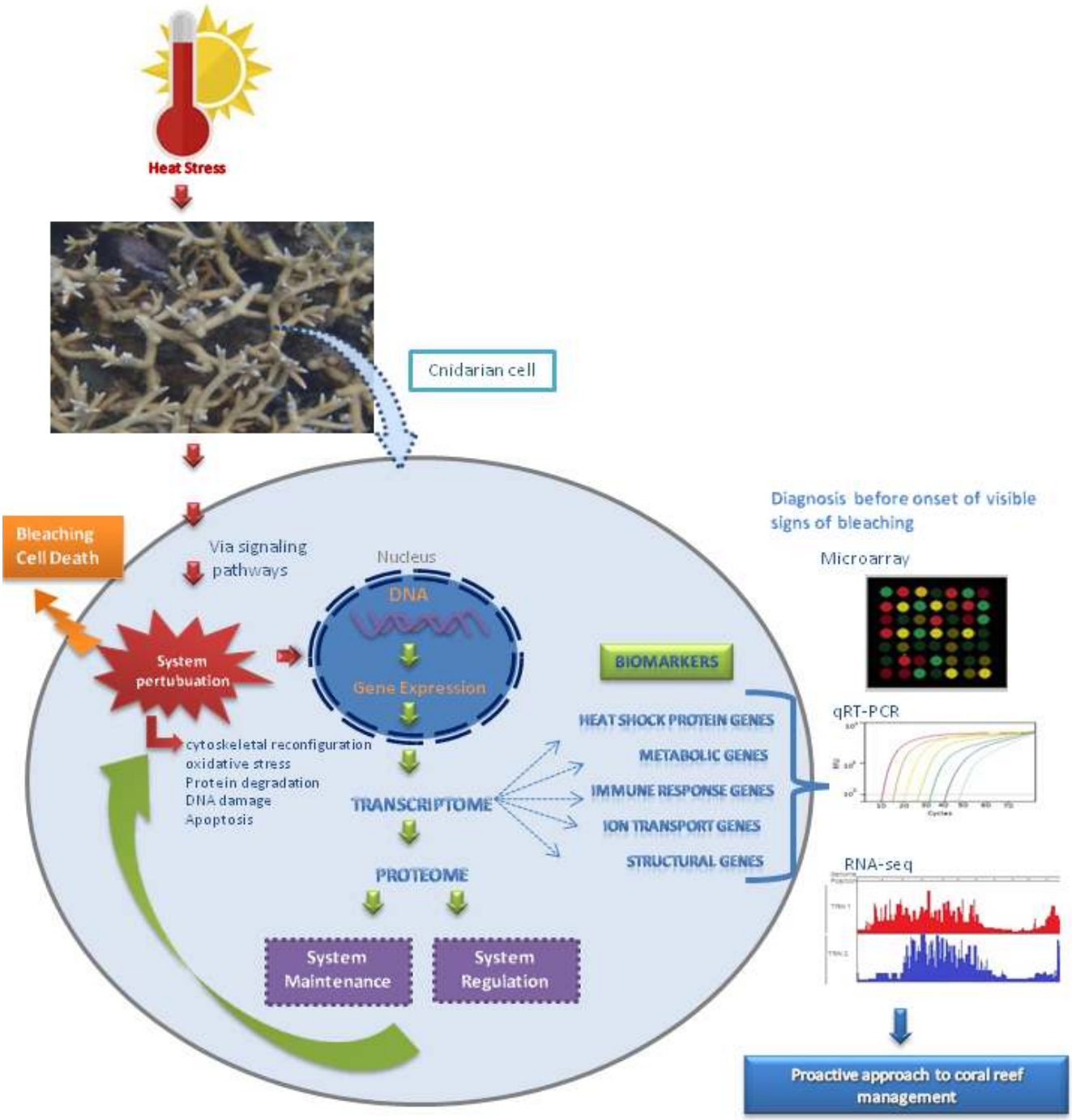

Heat Stress
Cnidarian cell
Bleaching
Cell Death
Via signaling
pathways
Nucleus
DNA
Gene Expression
System
pertubuation
cytoskeletal reconfiguration
oxidative stress
Protein degradation
DNA damage
Apoptosis
TRANSCRIPTOME
PROTEOME
System
Maintenance
System
Regulation
BIOMARKERS
HEAT SHOCK PROTEIN GENES
METABOLIC GENES
IMMUNE RESPONSE GENES
ION TRANSPORT GENES
STRUCTURAL GENES
Diagnosis before onset of visible
signs of bleaching
Microarray
qRT-PCR
RNA-seq
Proactive approach to coral reef
management

Figure 2a

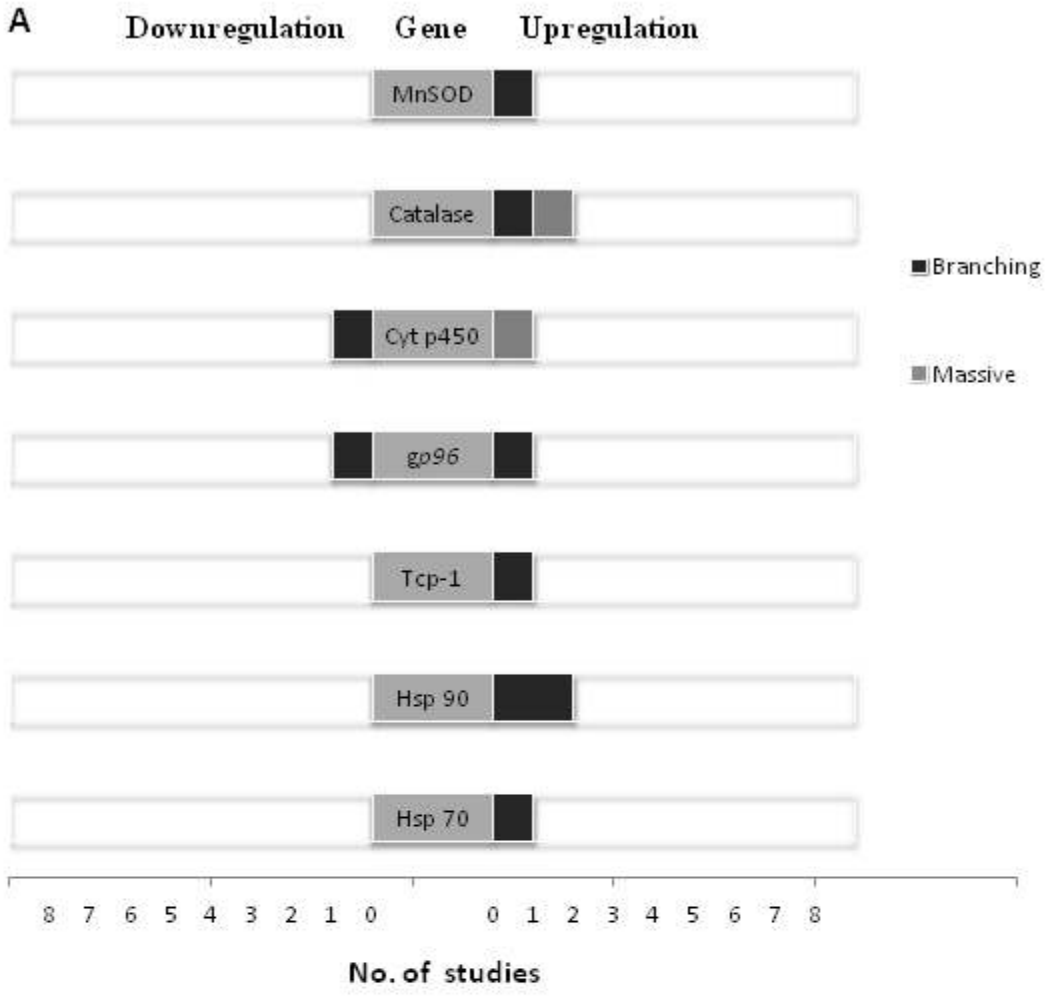
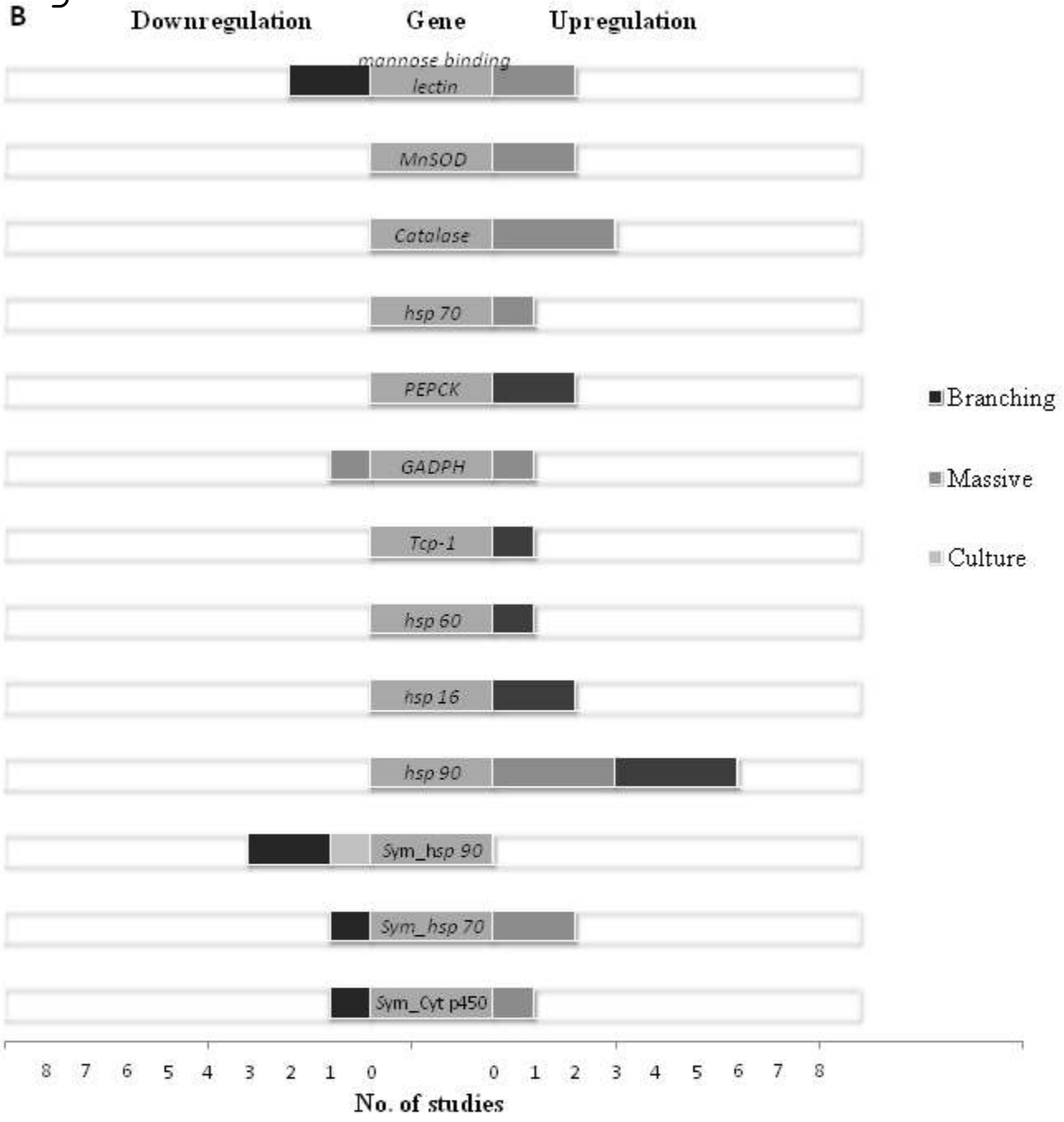

A
Downregulation
Gene
Upregulation
MnSOD
Catalase
Cyt p450
gp96
Tcp-1
Hsp 90
Hsp 70
Branching
Massive
8 7 6 5 4 3 2 1 0
0 1 2 3 4 5 6 7 8
No. of studies

Figure 2b


B
Downregulation
Gene
Upregulation
mannose binding lectin
MnSOD
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hsp 70
PEPCK
GADPH
Tcp-1
hsp 60
hsp 16
hsp 90
Sym_hsp 90
Sym_hsp 70
Sym_Cyt p450
Branching
Massive
Culture
8 7 6 5 4 3 2 1 0 0 1 2 3 4 5 6 7 8
No. of studies

Table 1. List of candidate genes studied to date with potential use as biomarkers of thermal stress. Duration of exposure, d=days, h=hours. Treatment type, non-preconditioned =NPC, preconditioned =PC. Ramped thermal stress =†, immediate thermal stress =*.

Genes of interest	Accession No.	Host organism	Host life stage	Stressor	Temperature (°C)		Direction of regulation	Duration of exposure	Fold Change	Technique	Reference
					Control/ Ambient	Stress					
Heat Shock Response											
hsp90	DY584045.1	*A.aspera*	Adult	Heat	28	32†	↑	5,7,8 d	1.5, 4.9,10.5	qRT-PCR	Leggat *et al.,* 2011
	DR988373	*M.faveolata*	Adult	Heat	29.2	32*	↑	10 d	1.28	microarray	DeSalvo *et al.,* 2008
	AOSF1451	*M.faveolata*	Larvae	Heat	31.5	31*	–	12 h	No change -	microarray	Voolstra *et al.,* 2009
	D016-C6	*A.millepora*	Larvae	Heat	24	31*	↑ ↑ ↓ ↓	3h 3h 10 h 10 h	3 - - 5.74	microarray	Rodriguez-Lanetty *et al.,* 2009
	DC999947	*A.tenuis*	Adult	Heat Chem (DCMU) Chem (TBT-Cl)	24	32*	↑ ↑ ↑	27h 13d 11d	1.54 1.99 6	HiCEP &RT-PCR	Yuyama *et al.,* 2012

	-	*P.astreoides*	Adult	Heat & light	27.8	30.9*	↑	3 h	1.6 -2. 5	qRT-PCR	Kenkel et al,. 2011
	-	*P.astreoides*	Adult	Heat	27.2 °C	30.9*	↓	6 weeks	2.4	RNA-seq	Kenkel et al,. 2013
		P.astreoides	Adult	Heat	27.8	31* 33*	↑ ↑	3 h	1.5 2.4	qRT-PCR	Kenkel et al,. 2014
	-	*P.damicornis*	Adult	pCO2	-	-	↑	3	1.6-2.9	RNA-seq	Moya *et al.,* 2015
hsp70	GO000475.1	*A.aspera*	Adult	Heat	28	32†	↑	7,8 d	6.4, 8.2	qRT-PCR	Leggat *et al.,* 2011
	A017-C4	*A.millepora*	Larvae	Heat	24	31*	↑	3, 10 h	3	Microarray	Rodriguez-Lanetty *et al.,* 2009
		A.millepora	Adult	Heat	27	32†	↑	9 d	0.12	qRT-PCR	Császár *et al.,* 2010
hsp16		*P.astreoides*	Adult	Heat & light	27.8	35-36*	↑	4d	700 -800	qRT-PCR	Kenkel *et al,.* 2011

		P.astreoides	Adult	Heat Heat	27.8	31* 33*	↑ ↑	3-4 h	4.5 10.6	qRT-PCR	Kenkel *et al.,* 2014
hsp60		*P.astreoides*	Adult	Heat & light	27.8	30.9*	↑	3 h	4	qRT-PCR	Kenkel *et al,.* 2011
		P.astreoides	Adult	Heat	27.8	31* 33*	↑ ↑	3 h	1.3 2.2	qRT-PCR	Kenkel *et al.,* 2014
Metabolism											
Glyceraldehyde-3-phosphate dehydrogenase	EZ026309.1	*A.aspera*	Adult	Heat	28	32†	↑	7,8 d	1.9, 4.4	qRT-PCR	Leggat *et al.,* 2011
		P.astreoides	Adult	Natural bleaching event	29-30	-	↓	-	11	qRT-PCR	Kenkel *et al.,* 2014
Oxidative stress											
Cytochrome p450	CAON1879	*M. faveolata*	Larvae	Heat	31.5°C (incubation)	29* 31.5*	↑ ↑		1.26 1.35	microarray	Voolstra *et al.,* 2009
Catalase	AOSF550	*M. faveolata*	Larvae	Heat	31.5°C (incubation)	29* 31*	↑ ↑	2d	2.0 2.3	microarray	Voolstra *et al.,* 2009
		A. millepora	Larvae	Heat	24	31*	-	3, 10 h	No change	microarray	Rodriguez-Lanetty *et al.,* 2009
	-	*A.aspera*	Adult	Heat	24	30†	↑	24 h	Data not available	RNA-seq	Rosic *et al.,* 2014a

		A.aspera	Adult	Ammonium enrichment			↑	24 h	Data not available	RNA-seq	Rosic *et al.,* 2014a
Catalase homolog (AmCat)	DY586920	*A. millepora*	Adult	Natural bleaching event	24	-	↑	-	1.8	qRT-PCR	Seneca *et al.,* 2010 - field
Manganese superoxide dismutase (MnSoD)	EZ027843	*A. millepora*	Adult	Heat	27	32*	↑	9 d	Data not available	qRT-PCR	Souter et al. 2011
	-	*A. millepora*	Larvae	Heat	24	31*	-	3, 10 h	No change	microarray	Rodriguez-Lanetty *et al.,* 2009
	DY581262	*A. millepora*	Adult	Heat	27	32†	↑	9 d	0.20	qRT-PCR	Császár et al. 2010
glutathione-s-transferase sigma-like (GST-S)	DR987062	*M. faveolata*	Adult	Heat	29.23	32†	↓	10 d	1.29	microarray	Desalvo *et al.*,2008
glutathione-s-transferase mu (GST-M)	DR988371	*M. faveolata*	Adult	Heat	29.23	32†	↑	10 d	1.26	microarray	Desalvo *et al.,* 2008

glutathione-s-transferase		*A. millepora*	Larvae	Heat	24	31*	-	3, 10 h	No change	microarray	Rodriguez-Lanetty *et al.,* 2009
glutathione-s-transferase	Q9N1F5, Q3T100	*A.aspera*	Adult	Heat	24	30†	↑	24 h	Data not available	RNA-seq	Rosic *et al.,* 2014a
	Q3T100			Ammonium enrichment	24	-	↑	24 h	Data not available	RNA-seq	Rosic *et al.,* 2014a
Immunity											
c-type mannose-binding lectin	EU863781.1	*A. millepora*	Larvae	Heat	24	28* 31*	↓ ↓ ↓	3 h 3 h 10 h	Data not available Data not available 3	microarray	Rodriguez-Lanetty *et al.,* 2009
mannose-binding lectin		*A.hyacinthus*	Adult	Heat	29	32.9*	↓	3 d	27.2	RNA-seq	Barshis *et al*. 2014
mannose-binding lectin					28	31†	↑	2 d	1.59 (NPC)	microarray	

mannose-binding lectin		*A. millepora*	Adult	Heat	28	31†	↓		0.93 (NPC)	microarray	Bellantuono *et al.,* 2012
mannose-binding lectin					28	31†	↓ ↑		2.16 (NPC) 0.42 (PC)	microarray	
Ion transport											
Calmodulin (CaM)	DR987178	*M. faveolata*	Adult	Heat	29.23	32†	↓	10 d	−1.38	microarray	Desalvo *et al.,* 2008
		A. millepora	Larvae	Heat	24	31*	-	3, 10 h	No change	microarray	Rodriguez-Lanetty *et al.,* 2009
Cytoskeleton											
Actin		*P. astreoides*	Adult	Heat	27.8	30.9 *	↓	3 h	4	qRT-PCR	Kenkel *et al.,* 2011
		P. astreoides	Adult	Heat	27.8	33*	↓	3 h	4	qRT-PCR	Kenkel *et al.,* 2014

Table ΠΠ List of differentially expressed *Symbiodinium* genes following heat stress that may be potential biomarkers of thermal stress.

Gene of interest	Accession No.	*Symbiodinim* ITS2 Type	Host organism	Stressor	Temp/^{o}C		Direction of regulation	Duration of exposure	Fold change	Technique	Reference
hsp90	*EH038163.1*	*C3*	*A. aspera*	Heat	28	32†	↓	7, 8 days	0.77, 0.78	qRT-PCR	Leggat *et al.*, 2011
	EH038163.1	C3	*A. millepora*	Heat	23-24	26†	↓	18 hours	0.57	qRT-PCR	Rosic *et al.*, 2011
						32†	↓	18 hours	0.43		
						29†	↓	3 days	0.23		
						29†	↓	24 hours	0.20		
		C1	Cultured			32†	↓	24 hours	0.25		
						32†	↓	5 days	0.22		
	-	C3	*A. aspera*	Heat	24	30†	↑	24 hours	1.6	RNA- seq	Rosic *et al.*, 2014a
				Ammonium enrichment	-	-	↑	24 hours	1.4		
hsp70	EH037708.1	C3	*A. aspera*	Heat	28	32*	↑	5 days	1.2	qRT-PCR	Leggat *et al.*, 2011
	EH038080.1	C3		Heat	23-24	26†	↑	18 hours	0.39		

						29†	↑	18 hours	0.57		
						32†	↓	18 hours	0.60		
						32†	↓	5 days	0.70		
		C1	Cultured	Heat	23-24	29†	↑	24 hours	0.25		
						32†	↓	24 hours	0.87	qRT-PCR	Rosic *et al.*, 2011

Table ㅎ. Summary of sources of variation between studies

Source	Reference
Differences in experimental design	-
Comparing field studies to lab-induced thermal stress	Leggat *et al,* (2011), Kenkel *et al.,* (2014)
Natural intercolony variation in coral host gene expression as a result of acclimatization to different conditions or differences in *Symbiodinium*	DeSalvo *et al.* (2010b), Granados-Cifuentes *et al.* (2013), Rocker *et al.* (2012), McGinley *et al.* (2012). Barshis *et al.* (2014), Seneca and Palumbi, (2015), Bellantuono *et al.* (2012), Bay and Palumbi (2015)
Variation in the time of sampling (e.g., diel patterns in expression)	Brady *et al*. (2011), Levy *et al*. (2011), Ruiz-Jones and Palumbi (2015)
Expression of host genes may be graded and regulated by thresholds	Kenkel *et al,* (2014)
Existence of a host buffering effect	Richier, (2005), Barshis *et al.* (2014), Parkinson *et al.* (2015)

Table 4. Research gaps in the development of gene expression biomarkers of heat stress in scleractinian corals

Gap	Description	Proposed future work
No universally accepted biomarker of thermal stress in hard corals	Most potential GEBs have been studied in only one coral species, or mostly in the genus *Acropora* (Fig. 1). The most studied genes (hsp90 and *hsp70*) have been studied in only 6 coral species.	A shortlist of potential GEBs of thermal stress should be analyzed in a suite of different coral species in different reef regions.
Specificity of response	Potential GEBs of heat stress also showed differential regulation when subjected to other stresses like heavy metals, pollutants, and changes in pH.	Include other stressors in future experiment to test for specificity of the candidate heat stress biomarker.
General regulation trend	General regulation trends have been reported for few GEBs due to contrasting results of different studies or a single study reporting differential expression of a gene.	More transcriptomic studies of heat stress in corals and *Symbiodinium,* with particular emphasis on including more individuals, should be done to establish general regulation trends.
Limited understanding of the molecular responses of coral and symbiont to heat stress	Understanding the precise molecular pathways involved in response of corals and *Symbiodinium* would help increase knowledge regarding the diagnostic potential of gene expression responses.	Future research should focus on elucidating links between molecular responses and higher order phenotypes of coral and its symbiont.
Only a few genes studied in *Symbiodinium*	Symbiont transcriptomics may help identify GEBs of heat stress if coral transcriptomics fails to yield a universal GEB.	Broaden research on gene expression biomarkers of heat and light stress in *Symbiodinium.*
Variation in expression of single genes	Significant variation in gene expression levels between studies, between species, location, and even within colonies has been reported.	Research should focus on combined expression of several genes. A suite of GEB can prove efficient, similar to the double gene assay that showed robust reciprocity in two *Porites* species and across studies (Kenkel *et al.,* 2011; 2014).

Field studies	Only one study (of 24 recent studies on GEBs of heat and light stress) was carried out in the field.	Due to complex natural interactions in the field, future work should focus on validating gene expression response of coral *in situ*. This could prove to be more informative.
Application of biomarkers in field by reef managers	To find cosmopolitan biomarkers as well as develop simple routine assays to assess coral heat and light stress status.	Development of a reproducible qRT-PCR protocol be used anywhere in world or simple portable kits that could provide instantaneous data in the field may be a feasible concept.