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REVIEW

Transcriptomic responses to environmental temperature in eurythermal and stenothermal fishes

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ABSTRACT

Ectothermic species like fishes differ greatly in the thermal ranges they tolerate; some eurythermal species may encounter temperature ranges in excess of 25°C, whereas stenothermal species in polar and tropical waters live at essentially constant temperatures. Thermal specialization comes with fitness trade-offs and as temperature increases due to global warming, the physiological basis of specialization and thermal plasticity has become of great interest. Over the past 50 years, comparative physiologists have studied the physiological and molecular differences between stenothermal and eurythermal fishes. It is now well known that many stenothermal fishes have lost an inducible heat shock response (HSR). Recent advances in transcriptomics have now made it possible to examine genome-wide changes in gene expression (GE) in non-model ecologically important fish, broadening our view beyond the HSR to regulation of genes involved in hundreds of other cellular processes. Here, we review the major findings from transcriptomic studies of extreme eurythermal and stenothermal fishes in response to acute and long-term exposure to temperature, both time scales being critically important for predicting climate change responses. We consider possible molecular adaptations that underlie eurythermy and stenothermy in teleosts. Furthermore, we highlight the challenges that still face the field of comparative environmental genomics and suggest fruitful paths of future investigation.

Introduction

Characterizing the transcriptome – the entire complement of mRNA transcripts in a cell, tissue or organism – can be a useful approach for identifying specific genes that are responsive to a given stimulus or set of stimuli. During recent years, the rapid development of functional genomic technologies designed to explore changes in gene expression (GE) has increased our understanding of the genetic regulatory underpinnings of cellular and organismal responses to abiotic stressors. Linking transcriptomic responses to changes at higher levels of organization greatly enhances the power of functional genomics to explore organism–environment interactions on broad scales (Kassahn et al., 2009).

Given the ecological and economic importance of fishes, functional genomic approaches are being increasingly favored for exploration of the interaction between fishes and their abiotic environment through the modulation of GE (Cossins and Crawford, 2005; Goetz and MacKenzie, 2008; Prunet et al., 2008; Hook, 2010;

Tort and Teles, 2012). Fish are poikilotherms that often inhabit thermally variable environments and many aspects of their biology are acutely attuned to temperature. The purpose of this review is to revisit and summarize the body of literature focused on transcriptomic responses to acute and long-term temperature exposures in eurythermal and stenothermal fishes.

The cellular stress response

All organisms must respond to environmental stress, herein defined as exposure to external stimuli that negatively affect homeostasis. A major advance in comparative physiology that was enabled by functional genomics and related technologies is the characterization of a broad-scale, widely conserved cellular stress response (CSR) (Kültz, 2005). By comparing transcriptomic and proteomic data from diverse taxa, hypotheses concerning the CSR can be tested. To date, the cardinal hallmarks of the CSR include: (1) the protection of cellular macromolecules through molecular chaperoning, (2) the redistribution of metabolic resources away from 'house-keeping' functions and towards stress responses, (3) the reversible arrest of the cell cycle and, in cases of more profound stress, (4) programmed cell death through apoptosis.

The importance of molecular chaperoning of macromolecules during stress has been well established. In particular, the heat shock response (HSR), defined as the ability of the heat shock proteins (Hsps) to re-fold thermally damaged proteins and prevent their cyto-toxic aggregation, is a well-described phenomenon across taxa (Feder and Hofmann, 1999). This includes studies targeting the HSR in fishes (e.g. Dietz and Somero, 1992; Currie and Tufts, 1997; Iwama et al., 1998, 1999; Basu et al., 2002; Buckley and Hofmann, 2002, 2004; Fangue et al., 2006, 2011). In his review, Tomanek (2010) presents a thorough discussion of how the HSR varies among species from stable, moderately variable and extremely variable thermal environments and what biogeographical implications this might have. While not as extensively studied as the HSR, the ability of thermal stress to induce changes in cellular metabolism, interruption of cell cycle progression and initiation of apoptosis has been addressed through transcriptomic approaches in a variety of contexts.

The CSR can be considered a 'tiered' response, with early and later phase changes in GE occurring (e.g. Gracey et al., 2001; Buckley et al., 2006; Buckley and Somero, 2009; Logan and Somero, 2011; Windisch et al., 2014). The magnitude and duration of heat stress, for example, both directly influence the dynamics of expression of Hsp genes (Buckley and Hofmann, 2004; Logan and Somero, 2011). Therefore, when assessing GE datasets, it is important to note the time frame of the exposure and its magnitude.

Defining eurythermy and stenothermy

In comparative ecophysiology, the terms 'eurytherm' and 'stenotherm' are clearly defined, with the former representing poikilothermic species capable of tolerating a wide range of

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external, and therefore internal, body temperatures and the latter referring to poikilothermic species with a narrow range of thermal tolerance. However, the proper binning of a given species into each category can be subjective. The terms 'eurythermal' or 'stenothermal' often have the clearest meaning in relative terms among closely related species. However, in absolute terms, a specific definition can be more difficult, particularly because accurate data on the upper and lower thermal limits for many species are unavailable.

Both thermal generalist (eurythermal) and thermal specialist (stenothermal) strategies are thought to have costs and benefits. Eurytherms can access a wide range of habitats, with obvious benefits in the form of the potential for range expansion, increased prey availability, predator avoidance and tolerance of unpredictable or rapidly changing environmental temperatures. However, the cost of eurythermy is likely to be an energetic one, as the effects of temperature (Q_{10} effects) on metabolism, biosynthesis, membrane integrity and a host of other biological processes have to be dealt with in thermally variable habitats. This cost can largely be avoided in stenotherms living within a narrow temperature range, so long as the thermal environment does not change at a pace to which they cannot acclimatize or adapt. This is clearly a current concern in light of climate change in many aquatic environments, particularly in polar and tropical waters (Somero, 2010). Many tropical species, adapted to nearly constant warm water, are already living near their upper lethal thermal limits (Stillman, 2003; Somero, 2010, 2011), while many polar species lack the ability to tolerate rising water temperatures (Peck et al., 2014).

Studies focused on eurytherms and their transcriptomic responses to stress outnumber those on stenotherms by a considerable margin. For our purposes, we have elected to use the high latitude polar fishes as examples of cold-adapted stenotherms as they possess the lowest upper thermal threshold of any known vertebrates, and a tropical species, the damselfish, as an example of a relatively stenothermal warm-adapted species. Relatively speaking, all non-stenothermal fishes could be considered eurythermal. For comparison, we therefore focus on extreme eurytherms, defined here as those experiencing variation in habitat temperatures >20°C.

The current state of transcriptomics

Prior to the late 1990s, changes in GE were measured for only one or a few genes at a time using northern blot analysis or quantitative real-time PCR. Since then, technological advances from cDNA and oligonucleotide microarrays to RNA sequencing have made it relatively cheap and easy to examine transcriptome-wide changes in virtually any non-model species (Gracey and Cossins, 2003; Ekblom and Galindo, 2011). RNA sequencing is currently replacing microarray technology as the preferred method for transcriptomic analysis because it provides both sequence and abundance information for millions of reads (de Wit et al., 2012), whereas microarrays provide abundance information only and require up-front costs to construct a species-specific array (Cossins and Crawford, 2005). In the case of both technologies, however, the ability to generate data currently exceeds our ability to interpret the results. Limitations of analyzing large-scale genomic data include incomplete reference data and lack of bioinformatics expertise. Annotation of ecologically important genes still relies on inferring homology with genes characterized in model species. In the papers reviewed here, a typical annotation rate is 50% and many of these unannotatable genes exhibit differential expression in response to temperature stress. Future work will require understanding the function of unknown genes in ecologically important species. For

example, although most studies reviewed here do not focus on non-annotated differentially expressed genes, future data-mining from public data repositories across species may uncover non-annotated but highly temperature-responsive genes that are ripe for future investigation.

Do changes in GE regulate the proteome? Changes in GE may serve as one of the principal means of biological regulation, and regulation at the mRNA level is often correlated with activity at the protein level. A recent study showed modest correlation between gene and protein expression for >60% of all genes in mice and humans (Ghazalpour et al., 2011; Vogel et al., 2010); however, other studies have demonstrated lower levels of correlation (reviewed in Taniguchi et al., 2010) or a delayed response between mRNA and protein expression (Buckley et al., 2006; Fournier et al., 2010). This latter effect is critically important, because mRNA levels may increase quickly and, then, decrease rapidly, whereas the levels of the corresponding protein may rise slowly and persist for a much longer period than mRNA levels (see, for example, Buckley et al., 2006). Rates of protein turnover, post-translational modification, alternative splicing, translational efficiency and other processes can also act independently of transcription to alter the proteome (Vogel and Marcotte, 2012). Importantly, changes in mRNA expression may not be related to changes in protein activity per se (Gracey and Cossins, 2003). For example, if protein degradation or translation rates are affected by environmental stress, regulation of GE may be required to maintain constant protein levels. Likewise, highly labile transcripts may require constant transcription to maintain sufficient levels. In other cases, the ultimate product may be RNA itself (e.g. antisense RNA). Therefore, although gene and protein expression are tightly coupled in some cases, changes in GE that do not accompany changes at the protein level may still reflect important changes to cellular function.

Goals of this review

We set out to address the following questions. What are the molecular adaptations that underlie eurythermy and stenothermy in fishes? More generally, what molecular pathways underlie thermal plasticity of fishes? What are the similarities and differences in GE responses of eurytherms and stenotherms to acute versus long-term exposure to heat and cold stress? Somero (2005) began to explore these questions a decade ago. Although the number of studies has more than doubled since that time, the answers to these questions are far from clear.

At the time of writing, we identified 37 studies of transcriptomic responses to acute (<5 days) or long-term (>5 days) temperature exposure in fishes (Table 1). Beyond the technological differences among studies (e.g. platform, bioinformatics pipeline), other major differences include species and tissue type examined, thermal exposure regime (e.g. duration and magnitude; fluctuating versus steady) and data analysis pipeline. Furthermore, relative fold-changes are typically reported rather than absolute values, making comparisons difficult. Long-term exposure to different temperatures likely elicits GE changes underlying an acclimation or compensatory physiological response, whereas short-term exposures to temperature may elicit acute responses of varying degrees. However, a response time frame that elicits an acute response in one species may already induce signs of acclimation for another. Likewise, a long-term exposure that induces an acclimation response in one species may represent chronic stress in another (i.e. non-acclimation).

Thus, it is challenging to compare transcriptional responses among studies, particularly on a gene-by-gene basis. Therefore, we

Table 1. Transcriptomic studies on responses to temperature stress in eurythermal and stenothermal fishes

Study	Species	Eurytherm versus stenotherm	Tissues examined	Technique
Bilyk and Cheng, 2014	Antarctic cryopelagic nototheniid (*Pagothenia borchgrevinki*)	Stenotherm – extreme	Liver	RNAseq
Bilyk and Cheng, 2013	Antarctic cryopelagic nototheniid (*Pagothenia borchgrevinki*)	Stenotherm – extreme	Liver	454 Pyrosequencing
Buckley and Somero, 2009	Antarctic emerald notothenioid (*Trematomus bernacchii*)	Stenotherm – extreme	Gill	cDNA microarray*
Huth and Place, 2013	Antarctic emerald notothenioid (*Trematomus bernacchii*)	Stenotherm – extreme	Gill, liver, brain	454 pyrosequencing
Thorne et al., 2010	Antarctic plunderfish (*Harpagifer antarcticus*)	Stenotherm – extreme	Liver	cDNA microarray
Windisch et al., 2014	Antartic eelpout (*Pachycara brachycephalum*)	Stenotherm – extreme	Liver	cDNA microarray
Kassahn et al., 2007a	Damselfish (*Pomacentrus moluccensis*)	Stenotherm – warm water	Liver	cDNA microarray*
Kassahn et al., 2007b	Damselfish (*Pomacentrus moluccensis*)	Stenotherm – warm water	Liver	cDNA microarray*
Newton et al., 2013	Barramundi (*Lates calcarifer*)	Stenotherm – warm water	Muscle	RNAseq
Buckley et al., 2006	Long jaw mudsucker (*Gillichthys mirabilis*)	Eurytherm – extreme	Gill, white muscle	cDNA microarray
Gracey et al., 2004	Common carp (*Cyprinus carpio*)	Eurytherm – extreme	Gill, muscle, liver, kidney, brain, heart, intestine	cDNA microarray
Healy et al., 2010	Atlantic killifish (*Fundulus heteroclitus*)	Eurytherm – extreme	Gill, muscle	cDNA microarray*
Logan and Somero, 2010	Long jaw mudsucker (*Gillichthys mirabilis*)	Eurytherm – extreme	Gill	cDNA microarray
Logan and Somero, 2011	Long jaw mudsucker (*Gillichthys mirabilis*)	Eurytherm – extreme	Gill	cDNA microarray
Podrabsky and Somero, 2004	Annual killifish (*Austrofundulus limnaeus*)	Eurytherm – extreme	Liver	cDNA microarray
Anttila et al., 2014	Sockeye salmon (*Oncorhynchus nerka*)	Eurytherm	Heart (ventricle)	cDNA microarray
Chou et al., 2008	Zebrafish (*Danio rerio*)	Eurytherm	Gill	cDNA microarray
Evans et al., 2011	Sockeye salmon (*Oncorhynchus nerka*)	Eurytherm	Gill, liver	cDNA microarray*
Hori et al., 2010	Atlantic cod (*Gadus morhua*)	Eurytherm	Liver, kidney, skeletal muscle	SSH library characterization
Jeffries et al., 2012	Sockeye salmon (*Oncorhynchus nerka*)	Eurytherm	Gill	cDNA microarray
Jeffries et al., 2014	Sockeye (*Oncorhynchus nerka*) and pink salmon (*Oncorhynchus gorbuscha*)	Eurytherm	Gill	cDNA microarray
Ju et al., 2002	Channel catfish (*Ictalurus punctatus*)	Eurytherm	Brain	cDNA microarray
Lewis et al., 2010	Rainbow trout (*Oncorhynchus mykiss*)	Eurytherm	Red blood cells	cDNA microarray
Liu et al., 2013	Catfish (*Ictalurus* spp.)	Eurytherm	Liver, gill	RNAseq
Long et al., 2012	Zebrafish (*Danio rerio*)	Eurytherm	Whole larvae	cDNA microarray
Long et al., 2013	Zebrafish (*Danio rerio*)	Eurytherm	Whole larvae	RNAseq
Malek et al., 2004	Zebrafish (*Danio rerio*)	Eurytherm	Skeletal muscle	cDNA microarray
Meier et al., 2014	Brown trout (*Salmo trutta*)	Eurytherm	Whole fry	cDNA microarray*
Olsvik et al., 2013	Atlantic salmon (*Salmo salar*)	Eurytherm	Liver	454 pyrosequencing
Quinn et al., 2011	Artic charr (*Salvelinus alpinus*)	Eurytherm	Gill	cDNA microarray*
Scott and Johnston, 2012	Zebrafish (*Danio rerio*)	Eurytherm	Hypaxial fast muscle	RNAseq
Smith et al., 2013	Crimson spotted rainbowfish (*Melanotaenia duboulayi*)	Eurytherm	Liver	RNAseq
Tan et al., 2012	Rainbow trout (*Oncorhynchus mykiss*)	Eurytherm	Gill	RNAseq
Vergauwen et al., 2010	Zebrafish (*Danio rerio*)	Eurytherm	Liver	cDNA microarray
Vornanen et al., 2005	Rainbow trout (*Oncorhynchus mykiss*)	Eurytherm	Heart (atrium, ventricle)	cDNA microarray
Castilho et al., 2009	Pacific Bluefin tuna (*Thunnus orientalis*)	Eurytherm, partial endotherm	Heart (ventricle), muscle (white and red)	cDNA microarray*
Jayasundara et al., 2013	Pacific Bluefin tuna (*Thunnus orientalis*)	Eurytherm, partial endotherm	Heart (ventricle)	cDNA microarray

*Heterologous hybridization.

focus on summarizing and synthesizing key findings from studies of stenothermal versus extremely eurythermal fishes as these are likely to give us the clearest signals pointing to differences among species with different thermal tolerances, and suggest areas for future study. Much is also to be learned by looking at eurytherms from moderately variable environments (Tomanek, 2010), which we include in Table 1. However, a proper treatment of these papers is outside the scope of this review. Likewise, studies on the model zebrafish *Danio rerio* have used transcriptomic approaches to study the impact of temperature on GE and these are also listed in Table 1.

Responses to long-term heat exposure in eurytherms

The first study to examine transcriptome-wide changes in response to temperature stress in a non-model fish species was done in carp (*Cyprinus carpio*) (Gracey et al., 2004). A species-specific cDNA microarray was used to measure changes in GE across seven different tissues in response to cold treatment. Fish were held at 30°C (control), 23, 17 or 10°C for 22 days, and sampled at multiple time points during the exposure. This was the first study to show the magnitude and complexity of responses in GE to cold in a non-model fish species. Among all tissues, 260 genes (out of 13,440 cDNAs) shared significant responses to cold. Twenty-seven were

orthologous to yeast proteins also regulated in response to cold, showing that adaptive responses to cold may be conserved from single-celled eukaryotes to vertebrates. These included up-regulation of well-known mediators of cold adaptation [e.g. stearoyl-CoA desaturase, cold-inducible RNA binding protein (CIRBP), and high mobility group-like protein 2 (HMG)]. Tissue-specific responses revealed an additional 3201 differentially expressed cDNAs (with homology to 1728 previously described genes) with divergent patterns across tissues. For example, skeletal muscle exhibited a coordinated repression of genes that make up the structure of the sarcomere (e.g. actin, myosins, tropomyosin) and those involved in muscle contraction (e.g. parvalbumins and troponins) that may indicate structural remodeling of muscle tissue in the cold. In contrast, cardiac muscle did not show a similar response. The authors suggest that cold may have different effects on contractile tissues, with skeletal muscle exhibiting remodeling in response to cold perhaps to adapt locomotory activity and load. Other tissues showed divergent metabolic strategies. For example, brain tissue increased expression of glycolytic genes whereas skeletal muscle showed a consistent decrease of glycolytic genes. Thus, although there appears to be a conserved transcriptome phenotype across all tissues, individual tissues showed highly divergent responses that are likely related to their physiological role within the body.

Extreme eurytherms are often exposed to high variability in temperature on daily and seasonal time scales, but how organisms respond to constant versus fluctuating conditions remains understudied in fishes. One of the early transcriptomic studies examined the differential responses of the annual killifish (*Austrofundulus limnaeus*) following exposure to either a constant temperature of 20, 26 or 37°C, or daily fluctuations between 20 and 37°C for 14.5 days (Podrabsky and Somero, 2004). The fluctuating regime reflects variation in the ephemeral ponds where these fish live, characterized by acute and rapid changes in temperature on a daily basis. Liver tissue was examined from fish sampled every 24 h during the constant exposure to 20 and 37°C, and every 4 h in the control group (constant exposure to 26°C) and during diel fluctuation between 20 and 37°C. Different gene sets regulated the same biological processes in response to constant versus fluctuating temperature. For example, small Hsps played a bigger role in response to fluctuating temperatures whereas larger Hsps (Hsc70 and Hsp90) were strongly up-regulated during constant high temperature exposure. In addition, HMGB1 (a global regulator of transcription) exhibited one of the most striking expression patterns in the fluctuating regime. Expression was highly negatively correlated to daily temperature cycling with a 10-fold change in response. The authors suggest that HMGB1 may act as a global GE temperature sensor and function by adjusting transcription rates such that mRNA levels are maintained in the face of temperature change. Another key finding was that both temperature cycling and circadian rhythms regulated many of the same genes. Failure to take circadian patterns of GE into account could lead to inadequate sampling of the transcriptome and, therefore, erroneous conclusions about temperature effects on GE. Teasing apart GE differences due to temperature alone can be difficult if not appropriately accounted for in experimental design and statistical analyses (see Gracey et al., 2008). Finally, because high temperature variability can lead to higher thermal tolerance and increased thermal range (Tomanek, 2010; Schulte, 2014) and gene regulation appears to be different under stable versus variable thermal regimes, we highlight this as an important area for continued research.

Logan and Somero (2010) compared GE responses in a well-studied extreme eurytherm, the longjaw mudsucker, *Gillichthys*

mirablis, after exposure to ecologically relevant temperatures (9, 19 and 28°C) for 3 weeks. Acclimation to 9 versus 28°C results in an upper critical thermal maximum (CT_{max}) difference of 5°C (34.4 versus 39.7°C), an ecologically important difference. However, only 150 genes (of 1607 unique annotated genes on the array) differed in expression among acclimated groups, with relatively low fold changes. The highest temperature group at 28°C did not appear to induce an acute HSR; only one Hsp was upregulated (hsp90β). They suggested that this limited response might be a hallmark of extreme eurythermy. However, gene ontology categories over-represented at 28°C included protein biosynthesis and active ion transport, both energetically costly processes. Thus, although fish appeared to have largely remedied the effects of acute heat stress following acclimation to nearly a 20°C range, living in warmer water may result in higher energetic costs.

In summary, studies on long-term responses of extreme eurytherms to temperature have provided a number of important findings. First, although a conserved response to long-term cold exposure is observed across many tissue types in carp, tissue-specific patterns often show divergent or opposing transcriptome signatures (Gracey et al., 2004). Therefore, choice of tissue is of critical importance when comparing GE responses among populations and species. Second, annual killifish regulate a different set of genes in response to fluctuating versus constant temperatures (Podrabsky and Somero, 2004). Magnitude, duration and variability of temperature exposure in relation to an organism's natural habitat must be carefully considered in experimental design. Third, high mobility group proteins appear to be important in long-term exposure to both cold and warm temperature (Gracey et al., 2004; Podrabsky and Somero, 2004; Jayasundara et al., 2013), and may serve an important role in temperature compensation of global transcription rates. Fourth, circadian rhythms can either mask or inflate temperature effects on GE; therefore, experimental design must be carefully executed to avoid artifacts (see also Connor and Gracey, 2011). Fifth, limited changes in steady-state GE levels following long-term exposure to a wide range of temperatures and lack of an apparent HSR may be indicative of extreme eurythermy (Logan and Somero, 2010). As cautioned above, however, the turnover of mRNA for Hsps is rapid relative to the turnover of the protein itself (Buckley et al., 2006). Thus, failure to observe increases in mRNA for Hsps during long-term thermal acclimation may, in fact, be due to the completion of increased synthesis of the protein chaperones themselves. Even if warm acclimation leads to cessation of a CSR, maintenance costs for homeostasis at warmer temperatures may be high, resulting in a potential trade-off with more energy allocated toward homeostasis and less energy available for growth and reproduction.

Responses to acute heat exposure in eurytherms

Buckley et al. (2006) laid the groundwork for examining ecologically relevant acute heat stress responses in the eurythermal longjaw mudsucker, *G. mirabilis*. In their study, fish were exposed to an acute temperature increase from 18°C to 32°C and were held at 32°C for 2 h. One group of fish was then maintained at 32°C for three additional hours and the other 'recovery' group was ramped back down to 18°C and held at that temperature for the duration of the experiment. Gill and muscle tissues were sampled from the three groups (control, heat stress, recovery) throughout the experiment. In both tissues, acute heat stress induced a clear HSR with differential expression of all molecular chaperones present on the microarray (e.g. Hsp40, Hsp60, Hsp70, Hsc71, Hsp90, Hsp108). However, the majority of

responses were tissue specific. In gill, acute heat stress induced several genes encoding cytoskeleton components, whereas in muscle, the primary response was repression of genes involved in the cell cycle and proliferation. Importantly, this study also examined concomitant levels of protein abundance for six genes that were differentially regulated. Although GE was correlated with protein production, timing and magnitude of protein expression did not mirror mRNA concentration, as emphasized earlier in this review.

Logan and Somero (2011) expanded on this work by examining the acute HSR following long-term exposure to a range of temperatures to analyze how responses might vary across seasons or with thermal history. Fish were held at 9, 19 or 28°C for 3.5 weeks and then ramped at 4°C per hour up to a temperature that induced loss of equilibrium. Fish were sampled every hour during the heat ramp. Fifty-two genes shared a common acute heat stress response regardless of starting temperature, including strong HSR (HspA9, Hsp70, Hsp90β1, Hsp27, Hsp40, Hsp60, Hsp108) and CSR (e.g. genes involved in the ubiquitin-proteasome pathway, cell cycle and apoptosis) induction, illustrating a conserved response regardless of thermal history. However, the timing of this response differed, with an increase in acclimation temperature of 10°C resulting in a 2°C increase in temperature at which up-regulation of this battery of stress response genes turned on. Although this shift in 'T_{on}' had been described for two Hsps previously in *G. mirabilis* (Dietz and Somero, 1992; Buckley and Hofmann, 2002) and in other species as well (e.g. Buckley et al., 2001; Tomanek and Somero, 1999), this was the first study to show that this shift occurs for a whole suite of acute heat stress regulated genes. Acclimation also reduced the magnitude of the response for some of these conserved genes as a result of either constitutive up-regulation of genes during acclimation to warmer temperature or differential sensitivity of genes to the relative duration of heating versus cumulative heat stress (e.g. degree heating minutes). Finally, this study identified specific genes that were consistently regulated in response to mild (hsp70) versus extreme (CDKN1B) levels of heat stress and established these genes as potential biomarkers of stress level severity.

Studies comparing populations of fishes locally adapted to different environments have also been fruitful in identifying potential GE phenotypes correlated with thermal tolerance. For example, Healy et al. (2010) examined transcriptome responses of northern and southern populations of the killifish *Fundulus heteroclitus* during an acute heat stress. Fish were exposed to 34°C for 2 h followed by a recovery period (both subpopulations were previously held at 20°C for 4 weeks). The southern subpopulation was known to have a CT_{max} ~2°C higher than the northern subpopulation (Fangue et al., 2006). Using heterologous hybridization to the salmonid GRASP microarray combined with quantitative RT-PCR, they found that a subset of *hsps* were upregulated in the southern population (*hsp70-1*, *hsp27*, *hsp30*) in gill and muscle, whereas *hsp70-2* and *hsp90-alpha* showed the opposite response with up-regulation in the northern subpopulation in muscle. This study suggests that the role of Hsps in establishing thermal tolerance is complex and varies among different Hsp classes, with the possibility that up-regulation of smaller Hsps is better correlated with higher thermal tolerance than expression of larger Hsps.

In summary, acute and long-term temperature exposures result in very different transcriptome signatures in eurythermal fishes. First, in response to acute heat stress, common response genes across tissues are dominated by Hsps (Buckley et al., 2006; Healy et al.,

2010), whereas tissue-specific transcriptomes may be characterized by processes like structural remodeling and cell cycle regulation (Buckley et al., 2006; Healy et al., 2010). Second, extreme eurytherms have a strong HSR in response to acute heat stress (Buckley et al., 2006; Healy et al., 2010; Logan and Somero, 2011), but HSR and CSR timing and magnitude vary with thermal history (Logan and Somero, 2011). In contrast, based on transcriptional data, extreme eurytherms showed no HSR (Gracey et al., 2004; Logan and Somero, 2010) or a limited HSR (Podrabsky and Somero, 2004) following constant long-term exposure to cold or warm temperatures. Third, only one study has examined the relationship between gene and protein expression in response to acute heat stress in non-model fish (Buckley et al., 2006), and none have examined the relationship following temperature acclimation. It is unknown whether steady-state temperature acclimation GE levels mirror protein expression more closely than during acute heat stress. Finally, mild versus severe levels of acute stress result in different transcriptome profiles (Logan and Somero, 2011). Thus, knowing the 'severity' of the stress is critically important for making comparisons across species or populations and will vary with thermal history.

Responses to acute heat exposure in stenotherms
Cold-adapted polar fishes
The fishes of Antarctica possess some of the lowest upper lethal temperatures of any species, having evolved in the sub-zero waters of the Southern Ocean for 14–25 million years (Eastman, 2005). Early work on the role of molecular chaperoning of proteins in heat-stressed Antarctic fishes revealed that the HSR was missing in at least one of these cold-adapted species, the notothenioid *Trematomus bernacchii* (Hofmann et al., 2000). The initial hypothesis was that given their long history spent in the extreme cold, the HSR was lost in these fishes owing to its no longer being necessary in the absence of variable water temperatures. Follow-up studies demonstrated that mRNA for Hsp70 is present in *T. bernacchii* (Place and Hofmann, 2005) and that the transcriptional regulator of all Hsp genes, HSF-1, displays constitutive DNA-binding activity, even in field-caught specimens (Buckley et al., 2004). It has been conjectured that cold-denaturation of proteins is problematic for these species and that constitutive expression of otherwise stress-inducible proteins is required in at least some cold-adapted fishes (Place et al., 2004; Todgham et al., 2007). An alternative explanation for constitutive expression in the cold can be based on the retardation of protein folding/chaperoning processes at low temperature; higher titers of molecular chaperones may be required to support adequate rates of protein maturation along productive folding pathways. Whatever the mechanistic explanation for high constitutive levels of Hsps, the phenomenon seems taxonomically widespread: the findings have been repeated in other polar fishes, including the bald notothen, *Pagothenia borchgrevinki* (Place and Hofmann, 2005), the Antarctic plunderfish, *Harpagifer antarcticus* (Clark et al., 2008), the Arctic haddock, *Melanogrammus aeglefinus* (Afonso et al., 2008), an Antarctic ciliate *Euplotes focardii* (La Terza et al., 2007), as well as the larvae of the Antarctic midge *Belgica antarctica* (Rinehart et al., 2006).

As these initial investigations of the HSR were being done, environmental genomic methods were being applied in a variety of Antarctic species (Clark et al., 2004; Peck et al., 2005). In 2008, a reference transcriptome of the ecologically important, and increasingly economically exploited, Antarctic toothfish, *Dissostichus mawsoni*, was published (Chen et al., 2008). That study showed that genes encoding proteins involved with protein

biosynthesis, folding and degradation were over-represented in the cold-adapted transcriptome when compared with the transcriptome of a model species: the zebrafish *Danio rerio*. Likewise, genes involved in lipid metabolism, antioxidation, anti-apoptosis and immunity were also over-represented. Transcriptomes from the brains and livers of three other Antarctic fishes, *Notothenia corriceps*, *Chaenocephalus aceratus* and *Pleuragramma antarcticum*, have also been sequenced (Shin et al., 2012). The Antarctic transcriptomes were enriched with transcripts from genes involved in protein degradation via ubiquitination compared with temperate fishes. These transcriptomes were generated from un-treated fishes and this implies that, in keeping with constitutive expression of Hsps, routine protein homeostasis may be a significant cost in the extreme cold and that the ubiquitin-mediated proteo-degradative pathway may be of special importance.

Heterologous hybridization of reverse transcribed mRNA from heat-stressed *T. bernacchii* to an existing *G. mirabilis* microarray (Gracey et al., 2001; Buckley et al., 2006; Gracey, 2008) revealed that mRNAs from Hsp40, Hsp60, Hsp70, Hsp90 and Hsp108 are all present in *T. bernacchii* tissues. The same is true for *H. antarcticus* (Thorne et al., 2010) and *P. borchgrevinki* (Bilyk and Cheng, 2013).

Buckley and Somero (2009) also demonstrated that acute exposure to 4°C, despite being insufficient to induce Hsp expression, nevertheless caused over- and under-expression of hundreds of genes. These genes are involved in numerous biological processes related to the broader CSR, including cellular signaling, cytoskeletal restructuring, carbohydrate metabolism, fatty acid metabolism, cell growth inhibition, DNA repair and apoptosis. In that study, a modified CSR in Antarctic fish fauna was proposed. According to this model, in the absence of an inducible HSR, pathways leading to temporary cell cycle arrest, apoptosis or both may be promoted. Hsps can directly block both intrinsic and extrinsic apoptotic pathways (Beere, 2005), and it is possible that inability to induce Hsps above constitutive levels may prevent apoptosis. Evidence supporting this idea includes the heat-induction of the pro-apoptotic transcription factor CCAAT/Enhancer-binding Protein Delta (C/EBP-05) in heat-stressed *T. bernacchii* (Sleadd and Buckley, 2013) and the fact that exposure to temperatures as low as 2°C causes apoptosis in hepatocytes from the same species (Sleadd et al., 2014).

In another cDNA microarray-based study, the transcriptomic response in liver tissues from *H. antarcticum* to a 6°C exposure for 48 h included the up-regulation of genes involved in the classic inflammatory response (Thorne et al., 2010). A shift in metabolic output, another component of the CSR, may have also been reflected by induction of genes involved in the β-oxidation of fatty acids. Finally, genes related to the response to oxidative stress were also induced. These included targets of HIF-1α. This led the authors to conclude that oxygen may be a limiting factor to acute thermal challenge in this species.

Two recent studies have examined the effect of heat on the transcriptome of the bald notothen, *P. borchgrevinki* (Bilyk and Cheng, 2013, 2014). In the earlier study, a reference transcriptome was generated by combining mRNA from many individuals, including field-caught, warm-acclimated and acutely heat-stressed specimens. Depending on the treatment, samples were taken from between four and seven tissues. Sequencing of the transcriptome via 454 sequencing generated 42,620 contigs of which 17,591 were annotated. These were used to probe the GE profiles in liver and gill of field-caught specimens, which were compared with those of the zebrafish. Genes grouped into 58 gene ontology terms were enriched in the polar species. Gene ontology terms related to

transcriptional regulation, ubiquitin–protein ligase activity, ubiquitination of damaged proteins and protein binding, among others, occurred more frequently in the Antarctic fish. This lends yet more evidence that protein biosynthesis may be problematic in the cold and that the constitutive expression of Hsps is necessary but cannot prevent higher rates of protein ubiquitination than occurs in warmer climates. Genes involved in DNA repair were also prevalent, which may have signaled that specimens were experiencing aspects of a broader CSR.

Bilyk and Cheng (2014) used RNA-seq to characterize the liver transcriptome of *P. borchgrevinki* during exposure to 4°C for either 2 or 4 days. The patterns of GE were complex and in cases appear contradictory to patterns observed elsewhere. After 2 days at 4°C, a surprising down-regulation of all classes of molecular chaperones and polyubiquitins was detected. As stated above, many Antarctic fishes lack an inducible HSR, but the repression of the molecular chaperones during heat stress is certainly unusual. However, a similar repression of Hsps was observed in *H. antarcticus* exposed to heat stress (Clark et al., 2008), so this may represent a response specific to certain polar fishes and this certainly bears future study. The reduction in polyubiquitin transcripts is unexpected because in most cases, elevated expression of genes involved in the ubiquitin pathway is found in heat-stressed Antarctic species. GE profiles in *P. borchgrevinki* after 4 days of thermal stress suggest a cessation or slowing of cell proliferation, similar to what was observed in *T. bernacchii* (Buckley and Somero, 2009), and genes involved in transcription and translation were down-regulated, perhaps signaling a slowing of biosynthesis. The exposures used by Bilyk and Cheng (2014), while qualifying as 'acute' for the purposes of this review, were of the order of days while those employed in the Buckley and Somero (2009) study were of the order of hours. This may play a role in the observed differences in expression profiles.

Warm-adapted stenothermal fishes

There are fewer studies focused on transcriptomic responses to temperature in warm-adapted, tropical stenothermal fishes than exist for cold-adapted, polar fishes. However, Kassahn and colleagues used functional genomic approaches to measure heat-induced changes in GE in the stenothermal tropical coral reef fish *Pomacentrus moluccensis* (Kassahn et al., 2007a,b). This species inhabits a thermal window of only 6°C and likely exists near its upper lethal limit, like many tropical marine ectotherms. Specimens of *P. moluccensis*, held at 26°C for 2 days, were exposed to 34°C for 3 h. Heterologous hybridization of cDNA from the livers of the exposed and control fish to a zebrafish microarray revealed that 111 genes varied in their expression patterns in response to the treatment. Interestingly, no induction of molecular chaperones was observed, similar to the situation in many polar fishes. This suggests that a loss of an HSR is common among cold- and warm-adapted stenotherms as has been proposed previously (Tomanek, 2010). It is possible that Hsp genes are constitutively expressed in some tropical species in response to chronic heat stress in their environment.

While no induction of Hsps was observed, genes involved in protein processing, cell cycle regulation and cell growth, cytoskeletal structure, transcription and translation, cell communication, carbohydrate metabolism and response to stress (chaperonin containing TCP1), among others, varied in expression. However, out of 111 genes, only four of these were induced by heat stress while 109 were repressed. The authors summarize their findings by noting that GE profiles support hindered cell growth, the repression of transcriptional activity and increased protein breakdown – all

signatures of heat stress in other fishes. This study was followed by a broad-scale, multi-stressor analysis expanding on their heat stress studies (Kassahn et al., 2007b). After a 5 day exposure to 31°C, 324 genes were found to have varied their expression compared with controls. These included genes involved with protein turnover, metabolic shifts and response to oxidative stress. Again, these are all processes that have been identified as common in the cross-taxa CSR.

Responses to long-term heat exposure in stenotherms

Several studies have examined the effect of long-term exposure to warm temperature on the transcriptomes of stenotherms. In some cases, true acclimation may be occurring, marked by a return to homeostasis at the new temperature. However, in other cases the exposures may be more accurately described as chronic thermal stress.

Huth and Place (2013) generated a reference transcriptome for the Antarctic species *T. bernacchii* via 454 pyrosequencing and the *de novo* assembly of 30,107 unigenes. Libraries were generated from the gill, liver and brains of control individuals, held at either −1.5°C (control) or 4°C (heat stressed) for 28 days. Through comparison with existing databases, 13,003 genes were annotated. As in previous studies on this species (Buckley and Somero, 2009), no HSR was observed. However, numerous other genes differed in expression and many of these genes play a role in other aspects of the CSR. Two signals of an increase in metabolism were observed. Levels of apolipoprotein messages were elevated in the warm-acclimated livers, suggesting an increase in lipid metabolism and mirroring similar results from Chen et al. (2008) on the toothfish, *D. mawsoni*. In the gill, mRNA from genes encoding cytochrome oxidase C1 and CO were overexpressed as were those involved in lipid metabolism, suggesting a similar metabolic shift in this tissue as well. It has been previously shown that warm-acclimation leads to an increase in resting metabolic rate in this species and so this cellular signal correlates with whole-organismal measurements (Enzor et al., 2013).

An increase in metabolism at higher temperatures may create more ROS and therefore oxidative stress, as has been observed in other studies discussed above. Furthermore, even at ambient temperatures, the high oxygen content of polar waters and the increased mitochondrial densities of Antarctic fishes (O'Brien and Sidell, 2000) may create chronic oxidative stress in these species. Therefore, it may be that oxidative stress responses and redox regulation may be of critical importance in polar organisms. In concordance with this idea, expression of genes involved in redox regulation was significantly higher in warm-acclimated *T. bernacchii* (Huth and Place, 2013). Reductases, redoxins and dehydrogenases were all up-regulated in the warm-acclimated group.

There were dramatic differences in GE in the brain, where the cytoprotective genes ependymin-1 and S100β were strongly over-expressed in thermally challenged individuals. Also noteworthy was the finding that HMGB1 was up-regulated in the heat, in contrast to the findings in the annual killifish *A. limnaeus*, where a strong inverse correlation was found between HMGB1 expression and heat (Podrabsky and Somero, 2004). Huth and Place (2013) posit that their findings in *T. bernacchii* may be related to the role that HMGB1 can play in inflammation responses. Inflammation signals were also observed in heat-stressed *H. antarcticus* (Thorne et al., 2010) and in the Antarctic eelpout *Pachycara brachycephalum* (Windisch et al., 2014). Finally, many of the same genes that responded to a long-term thermal exposure also responded to an acute exposure to the same treatment temperature,

albeit in the opposite direction (Buckley and Somero, 2009). This underscores the importance of teasing apart short- versus long-term responses to environmental temperature.

Two studies focused on transcriptional responses to temperature in the Antarctic eelpout, *P. brachycephalum* (Windisch et al., 2011, 2014). Unlike some other fishes from cold habitats, this species possesses a typical HSR, similar to lower latitude species, can survive long term at 9°C, and has an optimal growth temperature of 3°C (Windisch et al., 2014). Expression of candidate genes that are associated with metabolism was measured in livers of eelpouts exposed to 5°C for up to 6 weeks (Windisch et al., 2011). Individuals were sampled at several time points, allowing for early and late responses to be characterized. The patterns of GE support a switch from lipid metabolism to carbohydrate metabolism during warm acclimation. As carbohydrate metabolism supports anaerobic metabolism, it is possible that this switch in metabolic fuel might be related to hypoxemia at warmer temperatures.

In a subsequent study, a species-specific microarray was used to compare GE profiles in eelpouts exposed to −1, 0, 3, 5, 7 or 9°C for 9 weeks (Windisch et al., 2014). Six-hundred and sixty-four genes (representing 4.3% of the 15,843 ESTs present on the array) varied in expression during thermal acclimation. These genes are involved in various cellular processes including signaling, cytoskeletal remodeling and metabolic shifts, processes also determined to be affected by acute heat stress in *T. bernacchii* (Buckley and Somero, 2009). Also affected were genes involved in post-translational modification, transcription and translation. Optimal growth was observed at 3°C, and this coincided with the fewest numbers of genes altering expression at the end of the time course, suggesting that this exposure was the least stressful; there are likely ecological reasons explaining why this species inhabits waters colder than its preferred temperature. Expression patterns again supported a switch from lipid to carbohydrate metabolism at elevated temperatures. Above 7°C, a HSR and an acute inflammatory response were observed, signaling cellular distress and making it likely that 7°C represents the 'pejus' temperature (*sensu* Pörtner, 2001) for this species. Likewise, a down-regulation in cell growth- and proliferation-associated genes occurred at warmer temperatures. Oxidative stress may have been occurring at cold temperatures, as glutathione S-transferase (GST) transcript levels were elevated in the cold. Windisch et al. (2014) suggest that oxidative stress at warmer temperatures may be mitigated through the activity of uncoupling proteins.

In summary, there is direct evidence that stenotherms from both cold and tropical environments lack an inducible HSR. At least in the Antarctic species, this is combined with the constitutive expression of Hsps, likely as a means of preserving protein homeostasis in the cold. GE studies on polar species further support this conjecture as clear patterns of expression of genes involved in protein degradation and ubiquitination were often observed. Other aspects of the CSR are observed in cold stenotherms. Oxidative stress responses were commonly measured in these studies in response to both acute and long-term heat exposure, highlighting the likely importance of oxidative stress in eliciting GE responses and perhaps establishing thermal limits. Likewise, inflammation responses were observed in both short- and long-term treatments. Metabolic shifts, a key aspect of the CSR, were commonly observed in the GE data. In some cases, specific genes have been identified as important temperature sensors, with the high mobility group proteins, for instance, being highly thermally responsive in both stenotherms and eurytherms. Finally, it appears that one fundamental difference between warm- and cold-water stenotherms

is that heat stress may lead to broad-scale repression of genes in tropical species, while causing at least an equal amount of induction in polar species.

Conclusions and suggestions for further study

The use of genomic approaches to investigate the impacts of environmental temperature on organismal function in eurytherms and stenotherms is a robust and growing field of research. In general, these studies further define the conserved CSR, while continuing to identify novel genetic pathways involved in this important response to abiotic stressors. These studies have also illuminated exceptions to the conserved nature of the CSR, with the lack of a HSR in some species of both cold- and warm-adapted stenotherms being the most notable. Oxidative stress responses and inflammation signals often accompanied heat treatment, underscoring the ways in which primary and secondary stressors can work in tandem to elicit complex GE patterns of response.

While a wealth of information has been obtained through the studies considered here, as well as those in moderate eurytherms such as the salmonids, endothermic fishes such as tunas and genetic models such as the zebrafish, we are only at the beginning of this transformative era. As the cost of high-throughput sequencing continues to decrease and species-specific tools continue to be developed, the utility of environmental genomics is expanding. As outlined above, cross-study comparisons are challenging for a variety of reasons. Efforts should be made when possible to examine GE changes on short-, medium- and long-term time scales as this is critical for describing molecular stages of thermal responses and how time scales for responses differ between stenotherms and eurytherms. Examining as broad a range of tissues as possible will also facilitate cross-study analyses. More studies are needed on additional species, with tropical stenotherms being the most currently understudied. The genomic era was inaugurated by powerful new tools that allowed us to move beyond the study of a handful of candidate genes. However, the promise of the post-genomic era is going to lie in a return to the study of single genes or genetic pathways that were discovered to be of likely importance through transcriptomics. Likewise, more integrative studies that combine transcriptomics, proteomics and higher-level physiological phenotypes are needed to push beyond the era of hypothesis generation.

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