Experimental Evolution in a Warming World: The *Omics* Era

Marta A. Santos ,[1,2] Ana Carromeu-Santos ,[2] Ana S. Quina ,[2,3] Marta A. Antunes ,[1,2]
Torsten N. Kristensen ,[4] Mauro Santos ,[1,5] Margarida Matos ,[1,2] Inês Fragata ,[1,2,†]
Pedro Simões [1,2,†,*]

[1]CE3C—Centre for Ecology, Evolution and Environmental Changes & CHANGE, Global Change and Sustainability Institute, Lisboa, Portugal

[2]Departamento de Biologia Animal, Faculdade de Ciências, Universidade de Lisboa, Lisboa, Portugal

[3]Egas Moniz Center for Interdisciplinary Research (CiiEM), Egas Moniz School of Health & Science, Almada, Portugal

[4]Department of Chemistry and Bioscience, Aalborg University, Aalborg, Denmark

[5]Departament de Genètica i de Microbiologia, Grup de Genòmica, Bioinformàtica i Biologia Evolutiva (GBBE), Universitat Autonòma de Barcelona, Bellaterra, Spain

[†]These authors contributed equally to this work.

***Corresponding author:** E-mail: pmsimoes@fc.ul.pt.

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Review

Abstract

A comprehensive understanding of the genetic mechanisms that shape species responses to thermal variation is essential for more accurate predictions of the impacts of climate change on biodiversity. Experimental evolution with high-throughput resequencing approaches (evolve and resequence) is a highly effective tool that has been increasingly employed to elucidate the genetic basis of adaptation. The number of thermal evolve and resequence studies is rising, yet there is a dearth of efforts to integrate this new wealth of knowledge. Here, we review this literature showing how these studies have contributed to increase our understanding on the genetic basis of thermal adaptation. We identify two major trends: highly polygenic basis of thermal adaptation and general lack of consistency in candidate targets of selection between studies. These findings indicate that the adaptive responses to specific environments are rather independent. A review of the literature reveals several gaps in the existing research. Firstly, there is a paucity of studies done with organisms of diverse taxa. Secondly, there is a need to apply more dynamic and ecologically relevant thermal environments. Thirdly, there is a lack of studies that integrate genomic changes with changes in life history and behavioral traits. Addressing these issues would allow a more in-depth understanding of the relationship between genotype and phenotype. We highlight key methodological aspects that can address some of the limitations and omissions identified. These include the need for greater standardization of methodologies and the utilization of new technologies focusing on the integration of genomic and phenotypic variation in the context of thermal adaptation.

Key words: **climate change, thermal adaptation, experimental evolution, evolve and resequence, genomics, transcriptomics.**

Introduction

Climate Change and Thermal Adaptation

The Earth's global mean temperature has increased by roughly 1 °C since the start of the industrial era, and the average rate of increase has been approximately 0.18 °C per decade in the last 40 years. This increase has been accompanied by a higher incidence of extreme climate events, including heat waves and the associated risks of extreme droughts, forest fires, and floods. Additionally, the climate has become more unpredictable (IPCC 2023). Temperature is a key determining environmental factor for biodiversity and the distribution of species, given its high impact on the physiology of organisms, resounding across different levels of biological organization (e.g. Huey et al. 2012; Kellermann et al. 2012; Somero 2012; Araújo et al. 2013; Sunday et al. 2019; Walsh et al. 2019). The multitude of consequences of climate change and global warming is already having strong effects on the abundance and distribution of biodiversity, namely, by changing ecosystem functioning and species distribution and abundance (Somero 2012; Pecl et al. 2017). In light of the current climate scenario, an average of 8% of species is

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predicted to face the risk of extinction (Urban 2015). Recent evidence suggests that the negative impact of global warming may be underestimated. Even moderate temperature increases will present major metabolic challenges to many terrestrial and aquatic ectotherms, both in tropical and temperate regions (Deutsch et al. 2008; Dillon et al. 2010; Huey et al. 2012; Overgaard et al. 2014; Jørgensen et al. 2022).

Populations may respond to deteriorating environments in different ways (Kristensen et al. 2020). These include seeking microhabitats with suitable temperatures in their current geographical distribution areas (Scheffers et al. 2014), dispersing to less affected altitudes or latitudes (Parmesan 2006; Wellenreuther et al. 2022), mounting adaptive plastic responses (Diamond and Martin 2016; Gibert et al. 2019), or genetically adapting to the changing conditions (McGaughran et al. 2021; Edelsparre et al. 2024). The ability to genetically adapt to temperature changes—thermal adaptation—may prove crucial in enabling organisms to cope with the effects of global warming in the long run. Indeed, a number of studies have identified instances of rapid thermal evolution, suggesting that adaptation may be sufficiently rapid to enable organisms to respond to some degree of environmental change (Hoffmann et al. 2003; Parmesan 2006; Hoffmann and Sgrò 2011; Urban et al. 2016; Bell 2017). On the other hand, the evolution of physiological traits, including the ability to cope with high stressful temperatures, appears to be evolutionarily constrained in some populations (Kellermann et al. 2009, 2012; Araújo et al. 2013; Schou et al. 2014), which may hinder adaptation to warming climates. This alarming scenario calls for a more comprehensive understanding of the evolutionary potential of populations, including an assessment of the speed and genetic basis of adaptation. We argue that it is of particular importance to quantify patterns of genetic variation and covariation associated with key physiological and life history traits, as well as to understand the link between genotype, phenotype, and ultimately fitness. This knowledge will assist in determining the resilience of species to climate change and global warming. It will also provide fundamental insights that can inform mechanistic species distribution models, which in turn can facilitate the prediction of future biodiversity patterns and assist in the evaluation of solutions to combat the ongoing biodiversity crisis (Urban et al. 2016; Waldvogel et al. 2020; Aguirre-Liguori et al. 2021).

Experimental Evolution as a Tool to Study Thermal Adaptation

A variety of methodologies have been employed to investigate thermal adaptation (reviewed in Hoffmann and Sgrò 2011; Franks and Hoffmann 2012; Waldvogel et al. 2020). The comparative approach is a commonly used method in evolutionary biology (Harvey and Pagel 1991), and e.g. spatial studies across environmental gradients, known as clinal studies, have provided initial insight into thermal adaptation (reviewed in Rodrigues and Cogni 2021). Another approach that is widely used focuses on genetic or phenotypic changes in natural populations through time, where environmental conditions have varied (Rudman et al. 2022).

Experimental evolution has been gaining momentum in the study of adaptive evolutionary responses (Long et al. 2015; Remigi et al. 2019; Malusare et al. 2023). This research framework can be defined as the study of the real-time evolutionary changes of experimental populations due to experimenter-imposed conditions. Populations are here studied across several generations under defined and reproducible conditions that are most readily achieved in the laboratory. Having replicate populations is a fundamental aspect to consider as it allows to disentangle selection from genetic drift in the populations (Garland and Rose 2009). The experimental evolution approach is extremely powerful because, by knowing (and not inferring) ancestral states (Magalhães and Matos 2012), it allows to directly estimate evolutionary rates, reveal evolutionary patterns, establish causal relations, control for potentially confounding factors, and distinguish differentiation due to deterministic mechanisms from more stochastic effects (Simões et al. 2008; Kawecki et al. 2012; Fragata et al. 2014; Lenski 2017). By imposing contrasting selection treatments, experimental evolution can generate contrasting genotypes and phenotypes through divergent selection which allow to study the biological mechanisms underlying population differentiation. Experimental evolution can thus provide valuable insights into the context of adaptation to climate change, offering direct evidence for adaptation (or lack of it) to diverse thermal environments in different key traits (Bubliy and Loeschcke 2005; Ketola et al. 2013; Tobler et al. 2015; Manenti et al. 2016; Santos et al. 2021, 2023). This approach also enables the linking of phenotypic and genetic changes (e.g. Tobler et al. 2014; Michalak et al. 2019) and the investigation of the predictability of thermal evolution (e.g. Tenaillon et al. 2012; Kellermann et al. 2015; Deatherage et al. 2017; Batarseh et al. 2023).

It is of paramount importance to address the underlying genetic changes associated with thermal adaptation, regardless of whether comparative or experimental evolution approaches are employed. This is essential for the prediction of future responses to climate change (Bay et al. 2018; Aguirre-Liguori et al. 2021). Classical techniques for investigating such genetic variation include quantitative genetic breeding designs, mapping of quantitative trait loci (QTL) and the study of candidate genes for thermal adaptation. Indeed, prior to the advent of next-generation sequencing (NGS) techniques, the combination of experimental evolution with other molecular techniques represented a fruitful avenue of research (see Box 1).

Those pre-omics approaches yielded only a limited amount of information on candidate genes or gene regions that could explain phenotypic variation. Consequently, they did not permit an integrated view of the genetic response of populations. In particular, the low resolution

Box 1. Pre-omics thermal experimental evolution

The search for the genetic basis of adaptation to thermal changes has been a long-term pursuit, starting in the second half of the 20th century (reviewed in Hoffmann et al. 2003). Thermal experimental evolution studies, mainly using *Drosophila* as a model system, have addressed the issue (i) through selection of the most resistant individuals to either cold or heat stress (e.g. Stephanou et al. 1982; Quintana and Prevosti 1991; McColl et al. 1996; Feder et al. 2002) and (ii) with populations evolving for extended periods of time at different, nonextreme temperatures (e.g. Wright and Dobzhansky 1946; Van Delden and Kamping 1989; Cavicchi et al. 1995).

These studies typically found a clear response to the imposed thermal regimes, strongly suggesting the existence of genetic variation for thermal adaptation. Specific variants, such as heat-shock proteins (HSP) and chromosomal inversions, were targeted in a candidate approach, based on previous studies in natural populations (Hoffmann et al. 2003; Sørensen et al. 2003). In the pioneering work of Wright and Dobzhansky (1946), differences in the patterns of inversion polymorphism change were observed in lab populations of *Drosophila pseudoobscura* evolving at different temperatures, suggesting a role of this structural variation in thermal adaptation (see also Parsons 1973). Additional evidence for this role was later found in experimental studies of thermal selection in *D. melanogaster* (Van Delden and Kamping 1989; Quintana and Prevosti 1991). Consistent chromosomal polymorphism changes were observed in *Drosophila subobscura* populations in response to different thermal selection regimes though not the expected patterns considering clinal and seasonal variation of inversions (Santos et al. 2005). The potential role of HSP in the thermal adaptive response was more thoroughly addressed in these pre-omics studies, with evidence for associations between functional phenotypic changes and both HSP expression (Stephanou et al. 1982; Sørensen et al. 1999; Feder et al. 2002; Ketola et al. 2004) and allele frequency changes (McColl et al. 1996; Bettencourt et al. 2002). The HSP expression mechanisms associated with thermal response were complex, suggesting the existence of underlying trade-offs (see Sørensen et al. 1999; Feder et al. 2002 for evidence in Hsp70).

Several thermal selection experiments used molecular markers (such as microsatellites) to generate linkage maps and target candidate QTLs (e.g. Norry et al. 2004; Rand et al. 2010) to better understand the genetic basis of the adaptive response. For instance, a concordant candidate variant for thermal adaptation (*shaggy* gene region in *D. melanogaster*) was obtained from two independent selection experiments and was also consistent with patterns of variation in nature (Rand et al. 2010).

Overall, these experimental evolution studies showed the existence of available genetic variation allowing adaptive responses to thermal challenges, although heritability estimates for knockdown resistance from selected lines seem to be relatively low (McColl et al. 1996; Bubli et al. 1998; Gilchrist and Huey 1999; Hoffmann et al. 2003). These pre-omics experiments were very relevant to the field, as they shed light on potential genetic variants underlying thermal adaptation that were later analyzed with higher detail in larger scans. However, several questions remained related to how adaptation to new thermal regimes shapes the genotype to phenotype link and different regions of the genome (see main text).

of the genetic scans was insufficient to address, with the necessary depth, classical questions concerning the genetic basis of thermal adaptation such as the following: Are there many genes of small effect involved or few genes of major effect? Do laboratory selection experiments uncover the same target genes responsible for putative thermal adaptation in nature? Are the same genes/pathways involved in adaptation in different species? What is the relative impact of regulatory and structural molecular changes? The emergence of high-throughput sequencing techniques has filled some of these gaps (see Kulski 2016 for a historical overview). Indeed, significant advances in NGS technology are enabling genomic and transcriptomic scans with much higher resolution in a large number of individuals (e.g. by performing pool analysis) and also to study both model and nonmodel organisms (Long et al. 2015; Porcelli et al. 2015; Schlötterer et al. 2015).

Such sequencing approaches hold the promise of an integrative view of the responses to thermal adaptation and climate change, by bridging different levels of molecular variation, namely, the genome, the transcriptome, the epigenome, and even the holobiome. This, together with other *omic* techniques, such as proteomics and metabolomics, will provide a clearer picture of the pathways involved in thermal responses. Since the advent of NGS, our understanding of the genetic underpinnings of thermal adaptation has increased considerably. Comparative studies on the genetics of natural populations have identified genes and gene regulatory elements that constitute targets of climatic selection (Franks and Hoffmann 2012; Somero 2012; Waldvogel et al. 2020). The high number of candidate genes obtained from these studies (Franks and Hoffmann 2012; Porcelli et al. 2015) unsurprisingly suggests a polygenic basis for thermal adaptation. The expanded understanding of the genetic architecture of traits important for coping with high (and variable) temperatures has thus provided a more nuanced picture. While the focus was previously centered on heat-shock protein (HSP) genes (Sørensen et al. 2003; Franks and Hoffmann 2012; Chen et al. 2018), the current understanding of the genetic basis of coping with high temperatures is more nuanced. It is now evident that a multitude of other genes, often interacting with each other, contributes to this process (Porcelli et al. 2015; Waldvogel et al. 2020). Other significant evidence indicates that genomic variants respond to seasonal variation (Bergland et al. 2014; Rudman et al. 2022) and that structural variants contribute to the geographical differentiation of populations from distinct climates (Kapun et al. 2016; Bogaerts-Márquez et al. 2021).

Despite the recent accumulation of knowledge within this research field, several relevant issues need further in-depth research. This includes a more detailed understanding of the rate of adaptive genetic changes and whether thermal tolerance candidate genes/specific selected genetic variants are general or environment specific, the roles of regulatory heritable epigenetic changes in promoting evolution, and the impact of distinct genetic backgrounds on thermal adaptation (Franks and Hoffmann 2012; Porcelli et al. 2015; Stajic et al. 2019). Moreover, a more detailed analysis of the functional relationship between molecular variants and adaptive traits is required. This should include for instance a more comprehensive understanding of the significance of host microbiomes in coping with thermal stress, particularly in ectotherms (Moghadam et al. 2018; Jaramillo and Castañeda 2021). Expanded knowledge on these questions will allow for better predictions of evolutionary responses under climate change scenarios (Waldvogel et al. 2020; Aguirre-Liguori et al. 2021). The combination of experimental evolution with NGS technologies (evolve and resequence [E&R]) (Turner et al.

Box 2. Experimental designs in thermal E&R research

In our literature review, we were able to identify four major experimental design setups applied in E&R thermal studies (see Fig. 1). We considered as experimental evolution, studies applying three different approaches: "laboratory natural selection," "laboratory culling," and "artificial selection," as defined in Garland and Rose (2009). From our survey, we found that most of the variation in design was associated with the model organism used. Indeed, in bacterial studies, the main design focuses on the use of lab-adapted populations and the possibility of performing direct comparisons of replicated lines evolving under new thermal conditions with the ancestral populations (after suspended animation) (Fig. 1c). On the other hand, studies in *Drosophila* mostly use recently introduced populations and compare evolved replicate populations after imposition of thermal regimes to the ancestral populations or between each other (when subjected to different thermal regimes) (Fig. 1a). Bacterial studies typically entail populations with very large sizes and high number of replicate lines derived from initially single asexual clones, tracking the evolution through several hundreds or thousands of generations of de novo beneficial mutations (e.g. Lenski 2017). *Drosophila* experiments, on the other hand, mostly involve starting populations with high standing genetic variation with moderate sizes and typically analyze the spread of low to intermediate variants in the population through dozens to at most a few hundred generations (see Long et al. 2015 for a review). In addition, two other designs were applied in the E&R literature (although much seldomly): one consists of the imposition of new thermal regimes after populations are adapted to the laboratorial conditions, with such populations evolving under the new thermal regime being compared to those maintained under ancestral conditions, that serve as controls (Fig. 1b); another design involves the comparison between populations evolving under new thermal regime vs. those under control conditions with no available information on the ancestry of the populations (Fig. 1d).

Even though the different experimental designs reported share several similarities, they also present distinct features and limitations. First and foremost, it is important to stress here the need for proper controls which are vital to relate phenotypic and genotypic variation. One type of control is the ancestral population itself, a possibility that is available in bacterial studies resorting to the freezing of the ancestral strains (Fig. 1c). Comparisons of evolved replicated lines with the ancestral strain will allow to directly assess the genetic changes arising due to evolution under the newly imposed thermal conditions (e.g. Bennett et al. 1992; Lenski 2017). But ideally, there should be at least another source of control (as used in the design in Fig. 1b) that evolves in parallel with the new regimes, to account for other sources of changes in the lab environment, that could otherwise be wrongly interpreted as due to the new regime imposed. This should also be the single source of control when the design in Fig. 1c is impossible to implement (i.e. due to limitations of cryobiology). Preferably, these control (ancestral) populations should already be adapted to the laboratory conditions at the start of the experiment, so that the experimental populations in study respond only to the newly imposed conditions of the selection regime. In fact, confounding effects could arise from the simultaneous occurrence of selection in response to changes in humidity, photoperiod, food, and other environmental variables associated with laboratory adaptation. This may obscure the causal link to temperature, limit the interpretation of the environmental factors underlying genomic changes, and result in higher heterogeneity across studies, thus contributing to a low overlap between sets of candidate variants in genomic studies. This caveat is present in the experimental designs in Fig. 1a and d. In fact, a large number of studies with the design shown in Fig. 1a (95%) may not be able to effectively separate genomic changes due to thermal selection from those due to laboratory adaptation, especially in short-term studies. While starting with lab-adapted populations (as in designs in Fig. 1b and c) is advisable, too many generations of lab adaptation before the starting of the thermal selective regimes may entail a loss of genetic variability. Thus, the best should be a compromise between these two. The Design in Figure 1d has an additional important caveat, which is the lack of replicated lines generated from a similar genetic background. This limits our ability to quantify the importance of genetic drift, considering possible interactions with the distinct backgrounds. In fact, this design includes an additional level of (unknown) variation between populations, associated with possible differences in the ancestral genetic background. Thus, this design requires a high number of populations studied under both control and new thermal conditions, to reduce this bias. In fact, strictly speaking, the design in Fig. 1d does not comply to the more demanding conditions of experimental evolution design, which define replication as mandatory (Garland and Rose 2009; Kawecki et al. 2012).

More complex experimental designs can also be envisioned, namely, those aiming at understanding the effects of multiple environmental stressors. These should be orthogonal in order to account for the independent as well as the combined effect of the environmental stressors under study. To our knowledge, this kind of experimental setup is missing from the E&R literature, with the notable exception of the study by Brennan et al. (2022) that analyzes the effects of ocean warming and acidification in the marine copepod *Acartia tonsa*.

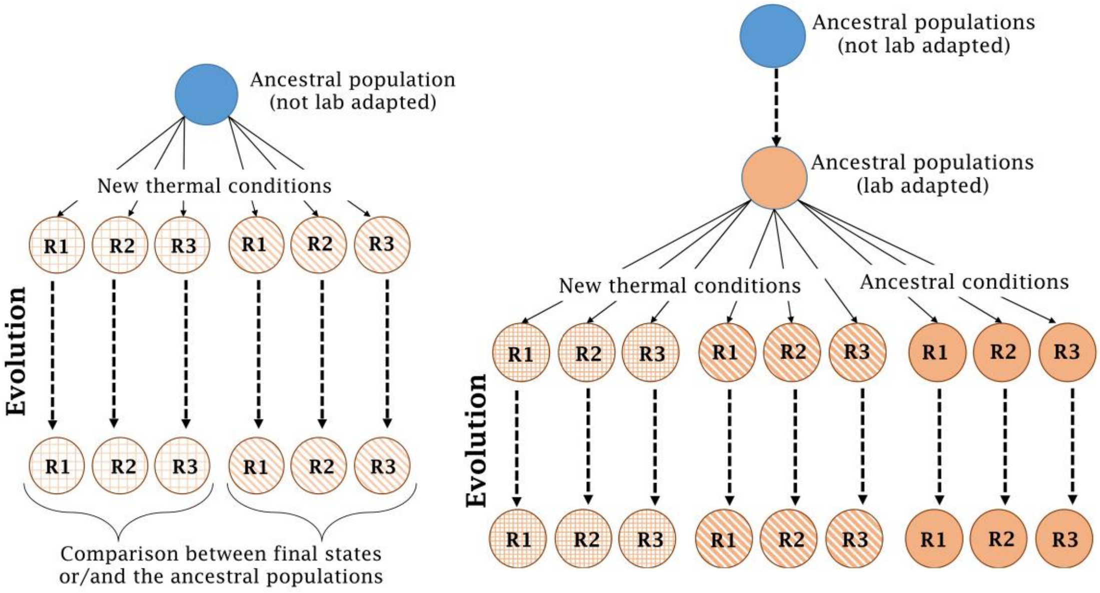
2011) has the potential to address these knowledge gaps. Below, we will review the literature on E&R studies addressing thermal adaptation and show how these have contributed to increase our knowledge on the genetic basis of thermal adaptation. The present study will further illustrate thermal E&R research by focusing on three case studies, each representing a different model organism. In addition, we analyze the overlap in major candidate genes and functions, as well as highlight the open questions, the challenges that lie ahead and suggest some routes of action to allow for more impactful research.

Methods Applied in the Literature Review

Literature Search

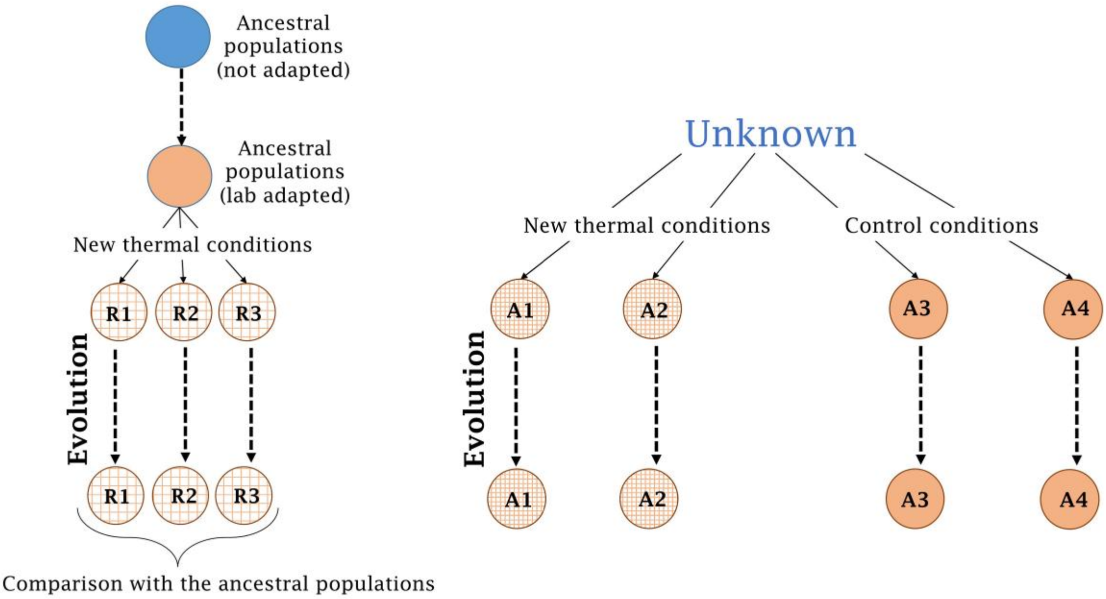
We accessed "Web of Science" (https://www.webofscience.com/wos/woscc/basic-search) and "PubMed" (https://pubmed.ncbi.nlm.nih.gov/advanced/) repositories to identify experimental evolution studies addressing thermal evolution coupled with genomic and/or transcriptomic analyses. These searches were done until 2022 August 31. We used several search strings including different combinations of the following key words: "Genomics", "Genetics", "Transcriptomics", "Gene Expression", "Evolution", "Experimental Evolution", "Adaptation", "Laboratory Natural Selection", "Artificial Selection", "Directed Evolution", "Adaptive Laboratory Evolution", "Laboratory selection", "Temperature", "Heat", "Cold", "Thermal Tolerance", "Thermotolerance", and "Knockdown"—see more details in the Supplementary Material.

These search criteria returned 144 studies. From this initial list, we excluded (i) review and theoretical articles; (ii) papers analyzing previously reported data; (iii) studies not performing thermal experimental evolution—or that do not separately assess the impact of temperature from other environmental stressors and/or other sources of evolutionary change (e.g. effects of laboratory adaptation; see also below and Box 2); (iv) studies that neither performed genomic nor transcriptomic analyses; (v) studies that did not include at least two replicates of the experimental populations; and (vi) thermal evolution studies in viruses. Studies on "directed evolution" were not included as they fell outside the scope of the review since they do not target evolutionary questions at the population level. This includes studies focusing on protocols aimed at increasing the thermal stability of specific enzymes and/or using engineered genetic backgrounds within an evolutionary context. "Adaptive laboratory evolution" studies—mostly in bacteria and yeast—were also excluded when replication at the population level was absent and/or the experimental design



(a) – Non lab adapted ancestral population

(b) – Lab adapted ancestral population (evolving in synchrony)



(c) – Lab adapted ancestral population (suspended animation)

(d) – Unknown ancestral population

Fig. 1. Overview of the different experimental designs that are used in thermal experimental evolution (see also Table 1). a) Nonlab-adapted ancestral population, where there may be confounding effects of lab adaptation during adaptation to a new thermal regime. b) Lab-adapted ancestral population (evolving in synchrony), at the time of foundation of new thermal regimes; the ancestral population is already lab-adapted and is maintained throughout the duration of the experiment. This allows comparisons between thermal regimes and control regimes. c) Lab-adapted ancestral population (suspended animation), at the time of foundation of new thermal regimes; the ancestral population is already lab-adapted. However, it is maintained in suspended animation and revived for comparisons, a typical design used in bacteria. d) The ancestral population is unknown, several lines/populations from the same ancestral population. Populations are kept at control and new thermal conditions.

did not allow to disentangle the effects of temperature from other environmental factors.

Using these criteria, we obtained a total of 53 papers that investigated genomic and/or transcriptomic changes during adaptation to new thermal conditions, using experimental evolution (see Table 1).

Search for Common Candidate Genes/Functions Involved in Thermal Adaptation

We estimated the overlap of most relevant candidate genes/functions highlighted in E&R studies reported in our review for bacteria, yeast, and *Drosophila*. These organisms represent the majority of organisms studied in E&R

Table 1 Overview of the targeted 53 thermal experimental evolution studies that focus on disentangling genomic or transcriptomic changes associated with different types of thermal regimes

Higher taxon	Species	Selection temperature	Timing	Experimental design	Type of selection					Genomic or transcriptomic		Phenotypic traits				Evolutionary time frame	More than one generation assayed?	References
					Alternate	Constant	Fluctuating	Increasing	Other	Genomic	Transcriptomic	Life history	Physiological	Morphological	Behavior			
Bacteria	*Escherichia coli*	Heat	Lifelong	Lab-adapted ancestral (suspended animation)	...	X	...	...	...	...	Microarrays	...	X	...	...	Long	No	Riehle et al. (2003)
					...	X	...	...	...	GWAS	...	X	...	...	...	Long	No	Rodríguez-Verdugo et al. (2014)
					...	X	...	...	...	Whole genome	...	X	...	...	...	Long	No	Tenaillon et al. (2012)
					...	X	...	...	...	Whole genome	Microarrays	X	...	...	...	Long	No	Riehle et al. (2001)
					...	X	...	...	...	Whole genome	Whole transcriptome	X	X	...	...	Long	No	Sandberg et al. (2014)
					...	X	...	...	...	Whole genome	...	X	...	...	...	Long	No	Hug and Gaut (2015)
					...	X	...	...	...	Whole genome	Whole transcriptome	X	...	...	...	Short	No	Batarseh et al. (2020)
					...	X	...	...	...	...	Microarrays	X	...	...	...	Long	No	Riehle et al. (2005)
					...	X	...	...	...	...	Candidate gene approach	X	...	...	...	Long	No	Rodrıguez-Verdugo et al. (2016)
					...	...	...	X	...	Whole genome	...	X	...	...	...	Long	Yes	Kishimoto et al. (2015)
		Cold and heat			...	...	X	...	...	Whole genome	...	X	...	...	...	Long	NA	Lambros et al. (2021)
				Non lab-adapted ancestral	...	X	...	...	...	Whole genome	...	X	...	...	...	Long	No	Wein and Dagan (2019)
				Lab-adapted ancestral (evolving in synchrony)	X	X	...	...	...	Whole genome	...	X	...	...	...	Long	No	Deatherage et al. (2017)
	Bartonella spp.	Cold and heat		Non lab-adapted ancestral	X	X	...	...	...	Whole genome	...	...	...	...	...	Long	No	Gutiérrez et al. (2018)
	Desulfovibrio vulgaris	Heat		Non lab-adapted ancestral	...	X	...	...	...	Whole genome	...	X	...	...	...	Long	No	Kempher et al. (2020)
	Serratia marcescens			Lab-adapted ancestral (suspended animation)	...	X	...	...	...	Whole genome / Epigenome	...	X	...	...	...	Short	No	Bruneaux et al. (2022)
	Zymomonas mobilis and *E. coli*			Lab-adapted ancestral (suspended animation)	...	...	...	X	...	Whole genome	...	X	X	...	...	Long	No	Kosaka et al. (2019)
Drosophila	*D. melanogaster*	Cold and heat	Stage specific	Non lab-adapted ancestral	X	X	...	...	Knockdown and shock	Whole genome	...	X	X	X	...	Long	Yes	Michalak et al. (2019)
					X	X	...	...	Knockdown and shock	...	Microarrays	X	X	X	...	Short	No	Sørensen et al. (2007)
			Lifelong	Non lab-adapted ancestral	...	X	...	...	...	Whole genome	...	...	...	...	...	Short	No	Tobler et al. (2014)
					...	X	...	...	...	...	Whole transcriptome	...	...	...	...	Long	No	Hsu et al. (2021)
					X	X	...	...	...	Whole genome	...	...	...	...	...	Long	No	Winbush and Singh (2021)
					...	...	X	...	...	Whole genome	...	...	...	...	...	Long	Yes	Mazzucco and Schlötterer (2021)
				Unknown ancestral	...	X	...	...	...	Whole genome	...	...	...	...	...	Short	No	Lajbner et al. (2018)
		Cold	Stage specific	Non lab-adapted ancestral	X	...	...	...	Shock	...	Microarrays	X	X	...	...	Short	No	Telonis-Scott et al. (2009)
		Heat	Stage specific	Non lab-adapted ancestral	X	...	...	X	Shock	...	Microarrays	X	X	X	...	Short	No	Nielsen et al. (2006)
					X	...	...	X	Shock	...	Microarrays	X	X	X	...	Short	No	Sørensen et al. (2005)
			Lifelong	Non lab-adapted ancestral	...	X	...	X	...	Whole genome	...	X	X	...	...	Short	Yes	Schou et al. (2014)
	D. melanogaster with Wolbachia	Cold and heat	Lifelong	Non lab-adapted ancestral	...	X	...	...	Reverse	Whole genome	...	X	...	...	...	Short	Yes	Versace et al. (2014)
					...	X	...	...	...	Whole genome	...	...	...	...	...	Long	Yes	Mazzucco et al. (2020)
	D. simulans	Cold and heat	Lifelong	Non lab-adapted ancestral	...	X	...	...	...	Whole genome	Whole transcriptome	X	X	...	...	Long	No	Mallard et al. (2018)
					...	X	...	...	...	...	Whole transcriptome	...	X	...	X	Long	No	Jakšić et al. (2020)
					...	X	...	...	...	...	Whole transcriptome	X	X	...	...	Long	No	Mallard et al. (2020)

(continued)

Table 1 (continued)

Higher taxon	Species	Selection temperature	Timing	Experimental design	Type of selection					Genomic or transcriptomic		Phenotypic traits				Evolutionary time frame	More than one generation assayed?	References
					Alternate	Constant	Fluctuating	Increasing	Other	Genomic	Transcriptomic	Life history	Physiological	Morphological	Behavior			
					...	X	...	...	...	Whole genome	...	X	X	...	...	Long	Yes	Langmüller et al. (2020)
					...	X	X	...	...	...	Microarrays	...	X	...	...	Short	No	Manenti et al. (2018)
					...	X	X	...	...	Whole genome	...	X	X	...	...	Short	No	Sørensen et al. (2020)
					...	X	...	...	...	Whole genome	...	...	...	...	...	Long	Yes	Otte et al. (2021)
	D. subobscura	Cold and heat	Lifelong	Non lab-adapted ancestral	...	X	...	...	...	...	Microarrays	X	...	...	...	Short	No	Laayouni et al. (2007)
Fungi	*Saccharomyces cerevisiae*	Cold	Lifelong	Lab-adapted ancestral (suspended animation)	...	X	...	...	...	...	Microarrays	X	X	...	...	Long	Yes	López-Malo et al. (2015)
		Heat	Lifelong	Lab-adapted ancestral (suspended animation)	...	...	...	X	...	Whole genome	...	X	...	...	...	Long	Yes	Huang et al. (2018)
					...	X	...	...	...	Whole genome	Microarrays	X	X	...	...	Long	No	Caspeta et al. (2014)
					...	X	...	...	...	...	Microarrays	X	X	...	...	Long	No	Caspeta and Nielsen (2015)
					...	X	...	...	...	...	Microarrays	X	X	...	...	Long	No	Caspeta et al. (2016)
				Non lab-adapted ancestral	...	X	...	X	Relaxed	...	Microarrays	X	X	...	...	Long	No	Yona et al. (2012)
	S. cerevisiae, *S. uvarum*, and hybrids	Cold	Lifelong	Unknown ancestral	...	X	...	...	...	Whole genome	...	X	...	...	...	Long	No	Smukowski Heil et al. (2019)
	Zymoseptoria tritici	Heat	Lifelong	Lab-adapted ancestral (evolving in synchrony)	...	X	...	...	...	Whole genome	...	X	...	...	...	Short	No	Möller et al. (2018)
Other	*Caenorhabditis remanei*	Heat	Stage specific	Non lab-adapted ancestral	X	...	...	X	Shock	...	Whole transcriptome	...	X	...	...	Short	No	Sikkink et al. (2014)
	Callosobruchus maculatus	Cold and heat	Lifelong	Non lab-adapted ancestral	...	X	...	...	...	mtDNA haplotypes	...	X	...	...	...	Short	No	Immonen et al. (2020)
	Chironomus riparius	Cold and heat	Lifelong	Non lab-adapted ancestral	...	X	...	...	...	Whole genome	...	X	X	...	...	Long	No	Pfenninger and Foucault (2020)
	Cladocopium goreaui	Heat	Lifelong	Lab-adapted ancestral (evolving in synchrony)	...	X	...	X	...	...	Whole transcriptome	X	X	...	...	Long	No	Chakravarti et al. (2020)
	Thalassiosira pseudonana	Heat	Lifelong	Lab-adapted ancestral (evolving in synchrony)	X	X	...	...	...	Whole genome	...	X	X	...	...	Long	No	Schaum et al. (2018)
	Tigriopus californicus	Heat	Stage specific	Unknown ancestral	...	...	...	...	Knockdown	...	Whole transcriptome	X	X	...	...	Short	No	Kelly et al. (2017)
	Tribolium castaneum	Heat	Lifelong	Lab-adapted ancestral (evolving in synchrony)	...	X	...	...	...	...	Whole transcriptome	X	...	...	...	Short	No	Koch and Guillaume (2020)

The different types of experimental designs are further described in "Box 2—Experimental designs in thermal E&R research." The column "Evolutionary time frame" refers to the duration of the experimental evolution study ("short": lower or equal to 50 generations; "long": more than 50 generations). GWAS, Genome-Wide Association Study.

research, thus providing a reasonable sample size (see supplementary table S1, Supplementary Material online, a subset of Table 1 only including studies used in the overlap analysis). Specifically, we screened the relevant papers for the candidate genes for thermal adaptation highlighted by the authors; i.e. they were either mentioned directly in the text and/or in the main tables and figures. We chose this approach as it would be impractical to analyze all the vast data sets of candidate genes of the different studies, particularly in *Drosophila* studies, where the thermal response is highly polygenic. From this list, we retrieved for each gene the gene ontology (GO) categories associated with "biological processes" and "molecular function" to have a measure of the overlap at the functional level. This was done by searching for the GO terms associated with each specific gene and species (or the phylogenetically closest model species) in GO, FlyBase, and Saccharomyces Genome Database (SDB), depending on the study organism. We applied several criteria for data inclusion: (i) Genes that were not listed or for which functional information was not present were excluded. (ii) Follow-up studies that analyze a small fraction of genes already reported in selected thermal E&R studies were removed to avoid artificial inflation of the overlapping results (see supplementary table S2, Supplementary Material online). Additionally, we focused on the analysis of genes involved in thermal adaptation in general, rather than splitting our data set into genes responding to either heat or cold adaptation, given the low sample size. We considered data obtained from both genomic and transcriptomic studies as they provide information about important genes and functions involved in thermal adaptation. These two levels were not treated separately in the analysis to increase sample size and thus the possibility of finding common trends. For each organism type, we estimated the percentage of overlap per candidate gene, by calculating the ratio between the number of studies that reported a specific gene, relative to the total number of studies. We also estimated the total number of times that a gene function was reported for each organism type to highlight the most common features within and between organism types.

Trends in Thermal E&R Methodologies

From our literature review (see Table 1 and Fig. 2a to d), we highlight the following trends in the research of the genomics of thermal experimental evolution:

The Majority of Studies Were Conducted in Bacteria and *Drosophila*

Figure 2a shows that the literature on this topic is heavily biased toward studies on bacteria and Drosophila (bacteria 32% and Drosophila 70% of studies), with much fewer studies in fungi (15%) and other organisms (13%). These organisms differ in their evolutionary genetic dynamics, with evolutionary responses in Drosophila relying mainly on standing genetic variation and with new mutations being much more important in bacteria. Assays in these organisms presented contrasting experimental setups (see Box 2). Additional differences between the two model systems were observed in the selection protocols applied: Studies in bacteria mostly focused on heat selection at constant temperatures while in Drosophila, thermal protocols were more variable and also addressed cold selection (see Table 1 and case studies below). Unsurprisingly, the majority of studies in bacteria (88%) were long-term studies (defined as >50 generations). In contrast, the number of short- and long-term studies in Drosophila is almost even.

The rather low diversity of taxa used in E&R studies is a limitation that is perhaps inherent to experimental (laboratory) evolution studies, namely, the consideration of practical aspects such as suitability for lab rearing (Burke et al. 2014; Long et al. 2015; Lenski 2017). While this limits our ability to generalize findings, the knowledge generated can be used as a toolbox of selected variants from which to infer the potential for thermal adaptation in other species (e.g. see Brennan et al. 2022 for a nice exception in a marine copepod).

The Majority of Studies Focused on Heat Stress in Non Dynamic Environments

The studies reviewed varied in the methodologies used, either differing in the type of thermal selection (cold vs. heat selection), the nature of thermal variation (constant vs. varying temperatures) and (in multicellular organisms) on the life stage targeted (see Fig. 2b). There is a clear focus on heat selection, with populations mainly exposed to constant stress throughout the life cycle. However, temperature changes due to global warming may also entail temperature reductions and/or increased thermal variation across days or seasons. In this sense, we highlight the paucity of studies involving dynamic thermal environments. The use of more "ecologically relevant" environmental scenarios—i.e. attempting to capture some of the complexity and range of environmental variation, namely through fluctuating and/or rising temperatures as an alternative to more acute protocols of selection—will also be an important step in reducing "unwanted" methodological noise and generating more relevant insights adaptation to climate change. Furthermore, due to the extremely low effective sizes they generate, severe heat selection protocols may negatively affect the ability to link phenotypic and genomic variation due to extensive linkage disequilibrium.

Phenotypic Variation Was Assessed in a Small Number of Traits

The majority of studies include phenotypic data from two categories: life history and physiological traits (see Fig. 2c), in most cases by referring to previous papers from the same team. On a negative note, there are a strikingly low number of studies assessing changes in morphological (four studies) or behavioral (one study) traits. Behavioral

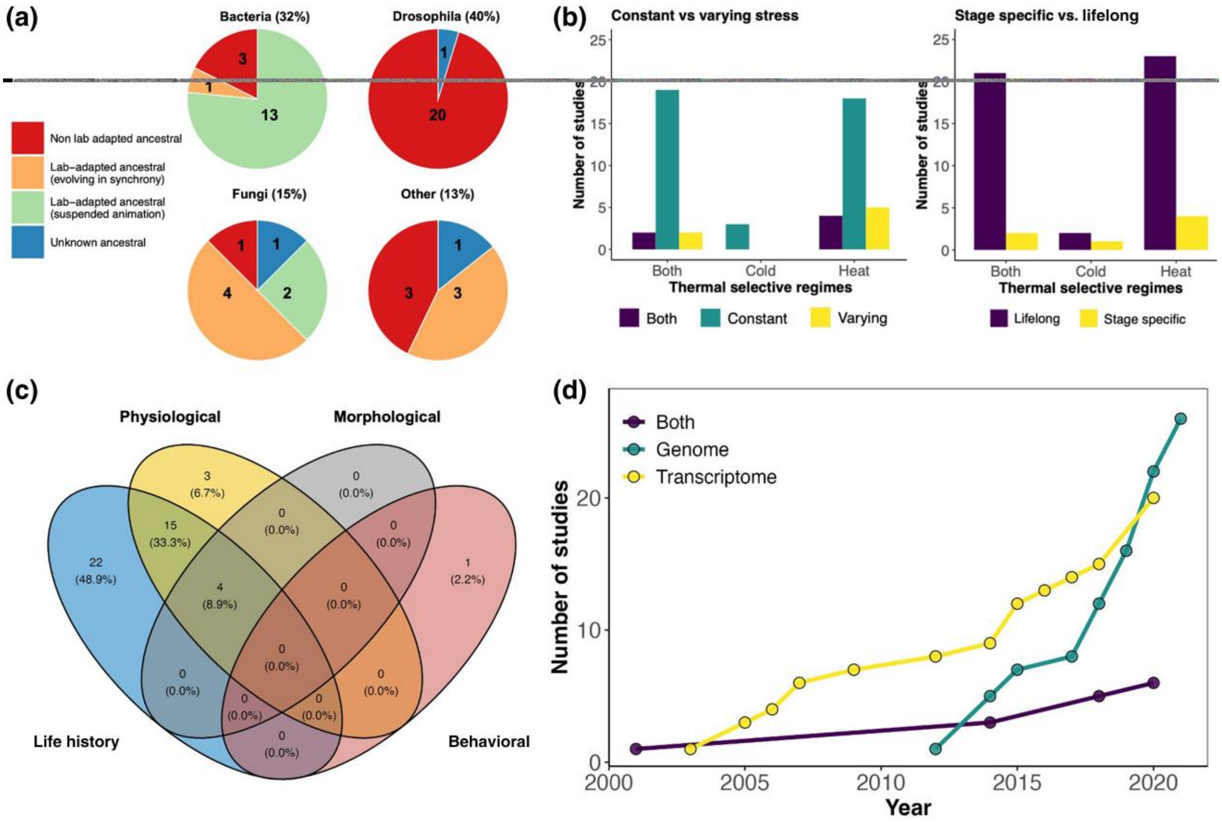


Fig. 2. Major trends in thermal E&R research. a) Proportion of studies that apply one of the four types of experimental design explored in this review for four categories of organisms. Number of studies for each category of organism and type of experimental design are indicated within pie charts. There is a clear polarization of the type of experimental applied according to the organism studied, with *Drosophila* using mostly nonlab-adapted ancestral and bacteria using mostly comparisons to the lab-adapted ancestral. In fungi, there are a similar number of studies for each experimental design category. b) Number of studies that test the impact of constant or varying (left panel) and lifelong or stage specific (right panel) thermal selection. Most studies focus on testing the impact of constant temperature at the genomic level (see left panel). Some of those studies test both constant cold and heat selection in the same experiment ("both"). However, very few studies test the impact of constant versus varying selection. In the right panel, the same trend continues, with most studies focusing on applying lifelong selection and very few studies applying selection on specific stages only. c) Venn diagram showing how many studies quantify changes in life history, physiological, morphological, and behavioral traits, in addition to genomic changes. Most studies focus on fitness or fitness-related traits, some of which also analyze physiological traits. d) Cumulative number of studies that use genomic, transcriptomic or both approaches to study thermal experimental evolution in different types of organisms. The initial transcriptomic studies are mostly quantifying gene expression using microarrays. Whole transcriptome studies started to appear only after 2014. Overall, there has been an increase in the number of studies that use genomic or transcriptomic approaches, especially in the last 10 years. However, studies combining both approaches are only starting to emerge.

thermoregulation plays an important role in the ability of species to respond to changes in temperature (Dillon et al. 2009; Sunday et al. 2014; Muñoz 2022). Nonetheless, these are also traits that are difficult to assay. Morphological traits would also be important to consider in the context of thermal responses, as for example, larger organisms may be better able to withstand temperature fluctuations, but on the other hand, smaller organisms may have a thermodynamic advantage in warmer environments in the longer term (Angilletta 2009). A better understanding of the overall response to thermal selection will be achieved by integrating data from different types of traits including reproduction and other life history traits, which are likely to be more relevant than lethal thermal endpoints, such as the commonly used critical thermal maximum (Kristensen et al. 2015; Walsh et al. 2019; van Heerwaarden and Sgrò 2021).

The Combination of Genomic and Transcriptomic Information Is an Important but Underutilized Approach in E&R Studies

In Fig. 2d, we present a breakdown of the NGS approaches that have been used to tackle thermal adaptation in the context of E&R studies. Transcriptomic studies using microarray techniques were used prior to whole-genome sequencing, but genomic studies have become relatively more frequent in the last 10 years (Fig. 2d). Typically, a reasonable number of candidate genes were uncovered by these microarray scans (Laayouni et al. 2007; Sørensen et al. 2007; Telonis-Scott et al. 2009). However, repeatability in the lists of candidate genes obtained across studies and populations was very low (see below) (Sørensen et al. 2007; Telonis-Scott et al. 2009; Sarup et al. 2011), and resolution power of such scans is inferior to that of whole transcriptome (e.g. analysis with RNAseq) making the latter a better approach.

Gene expression variation can be used as a bridge between the genomic level and that of a higher-order phenotypic level, i.e. that of phenotypic traits. By integrating the information from DNA and RNA and other omics layers, it is possible to gain a deeper understanding of the importance of regulatory variation and how it shapes the physiology and metabolic pathways involved in the adaptive response to different environments (see Macaulay et al. 2015; Li et al. 2019; Layton and Bradbury 2022). This approach might be particularly useful to tackle the genetic basis of genotype by environment interactions and thus characterize the evolution of thermal plasticity in the context of climate change (Oomen and Hutchings 2022). However, few E&R studies of thermal adaptation use a complementary approach of genomic/transcriptomic analysis (see Riehle et al. 2001 for an exception), although this trend appears to be changing in recent years (Fig. 2d).

More Focus on Functional Validation Is Needed

In our literature review, we found a limited number of studies performing functional validation of observed candidate genes, almost exclusively limited to *Escherichia coli* (e.g. Sandberg et al. 2014) and *Saccharomyces* (e.g. Huang et al. 2018). It may be the case that some follow-up studies performing functional validation were not included in our review, possibly reinforced by a more recent tendency for such studies to appear. Still, it would be important to increase the number of studies performing functional validation in order to estimate the relative role of each gene in the response to thermal stress. Functional validation can be performed using genomic resources such as knockin or knockout/knockdown lines which are available for model systems (Jagdish and Nguyen Ba 2022; see also tools for emerging models in Gudmunds et al. 2022). There is also a potential to investigate the importance and functionality of epigenetic variation in E&R studies (Bruneaux et al. 2022), which is relevant to understanding the molecular mechanisms underlying phenotypic variation during climate change (Layton and Bradbury 2022). Emerging RNA interference technology, using synthetic structurally well-defined short double-stranded RNA (small interfering RNA), has recently advanced rapidly and offers a way to silencing posttranscriptional expression of specific genes (Bartel 2018). This approach can also be used to functionally validate genes of interest.

Case Studies

We will now provide an overview of the main biological findings in thermal E&R research by focusing on the three model organisms with a higher representation.

Genomics of Heat Stress Response in Experimentally Evolved *Drosophila*

Drosophila spp. are the most widely used sexual multicellular eukaryote models in thermal experimental evolution. For example, Michalak et al. (2019) used the E&R approach to analyze, after 31 generations of selection, the genomic response in replicate *Drosophila melanogaster* lines selected in different ways for increased heat tolerance (heat-shock [HS] and heat-knockdown [KD]). Despite the different heat stress intensity applied by these two types of heat selection (hardening followed by HS at 38 °C vs. KD at 40 °C), both regimes increased in heat tolerance, suggesting strong correlated selection responses. The authors found a relatively large number of candidate single nucleotide polymorphisms (SNPs) for both selection regimes, although higher for the HS regime (1918 vs. 255 in KD). Multiple candidate variants in these regimes were also uncovered in transcriptomic and proteomic analyses (Sørensen et al. 2007, 2017b), which is consistent with a strong directional selection at the sequence level. Despite the large number of candidate SNPs, Michalak et al. (2019) found a low overlap (20) in the selected variants between the HS and KD protocols, again mirroring previous gene expression results (Sørensen et al. 2007). Interestingly, E&R studies have also shown that adaptation to different thermally stressful environments (hot and cold, in this case) also relies on distinct genomic targets (Tobler et al. 2014; Otte et al. 2021).

A deeper understanding of the molecular pathways of heat stress response can be achieved by combining data on phenotypic traits with that on structural DNA variation and gene/protein expression patterns. However, these levels of biological organization can be difficult to reconcile under a coherent stress response, given the effects of cis-, trans-, and posttranscriptional regulation (see Sørensen et al. 2007, 2017b; Michalak et al. 2019) as well as G × E and G × G interactions. Mallard et al. (2018) used a comprehensive approach including phenotypic, genomic, and gene expression profiling to address the genetic basis of thermal adaptation to fluctuating temperature (18 to 28 °C) in experimentally evolved lines of *Drosophila simulans*. The authors detected few candidate genomic regions, highlighting two interacting genes—*Sestrin* and *SNF4Aγ* as major contributors to the functional response of a general downregulation in energy production. Given the relatively small number of candidate variants, the authors were able to establish a direct link between genomic and transcriptomic changes. The reported downregulation of metabolism as the main response to thermal stress contrasts with the findings of the abovementioned study of gene expression in the KD and HS lines, which gives more emphasis on the upregulation of different metabolic pathways (e.g. protein biosynthesis and phototransduction) in the stress response (Nielsen et al. 2006; Sørensen et al. 2017b).

Overall, these *Drosophila* studies point to a polygenic, largely independent response to thermal stress(es)—see also the "Overlap between candidate variants/functions from thermal E&R studies" section. However, studies performing functional validation of candidate genes in this genus are needed to better assess the relationship between genomic and phenotypic variation.

Genomics of Heat Stress Response in *Escherichia coli*

The bacterium *E. coli* is the most widely used asexual model in experimental evolution studies and accounts for some of the earliest studies on genome-wide response to thermal stress. Using the classic *E. coli* populations from Bennett et al. (1992), the Long lab studied the structural and gene expression changes following evolution at 41.5 °C for 2,000 generations (Riehle et al. 2001, 2003, 2005). Using high-density DNA arrays, Riehle et al. (2001) reported repeatability of thermal adaptation, e.g. through a duplication involving a chromosomal region containing four genes (*rpoS*, *nlpD*, *pcm*, and *surE*) with roles in thermal stress responses and starvation resistance. Replicated parallel changes between populations were observed in follow-up studies on the genome-wide changes at the transcription level (Riehle et al. 2003, 2005). Despite the low replication (3 to 6-fold) and the limited genomic scope of the high-density arrays, similar findings across replicates and the strong selective signature in several functional categories suggest robustness of the observed patterns.

More recently, Tenaillon et al. (2012) analyzed over 100 lines of *E. coli* after 2,000 generations of evolution at 42.2 °C and also found strong mutational convergence at the level of genes, operons, and functional units (Tenaillon et al. 2012), similar to Riehle et al. (2005). They highlighted several possible mutations leading to similar adaptive outcomes, but also the extensive epistasis that generated at least two distinct adaptive trajectories involving mutations in the RNA polymerase complex (namely, *rpoB*) or in the *rho* termination factor. In a follow-up study, Rodriguez-Verdugo et al. (2016) characterized gene expression profiles of 12 of these populations to check the effect of three mutations in the *rpoB* gene in global transcriptional levels. The authors found that *rpoB* mutations conferred fitness advantages through a broad restoration in the gene expression patterns of hundreds of genes from stressed toward a prestressed state. Restoration was also observed at the phenotypic level in a larger set of the same lines (Hug and Gaut 2015) and in *E. coli* B *rho* mutant strains (González-González et al. 2017), suggesting that early mutations in genes coding for transcriptional regulators may be under positive selection in lines adapting to sustained heat stress (see also Sandberg et al. 2014).

Deatherage et al. (2017) addressed the specificity of adaptation to different temperatures in a genomic study of 30 populations of *E. coli* B that evolved for 2,000 generations under 5 thermal regimes (ranging from 20 °C to 42 °C). While some mutations where shared between pairs of regimes, but five genes showed thermal specificity (*nadR*, *hslU*, *mrdA*, *gltB*, and *iclR*), none directly involved in global regulatory processes. Interestingly, Batarseh et al. (2023) found that adaptation to cold temperature was contingent on the type of adaptation to high temperatures (i.e. whether it involved mutations in *rpoB* or *rho* genetic pathways).

A different, very important question concerns whether fitness trade-offs underlie thermal adaptation in *E. coli* and whether adaptation to high temperatures is controlled by the same genes/pathways. Interestingly, Rodríguez-Verdugo et al. (2014) found, in *E. coli* lines evolving at 42.2 °C for 2,000 generations, that some populations adapted with fitness trade-offs at low temperatures due to antagonistic pleiotropy involving the *rpoB* adaptive pathway, whereas others—hosting a mutation in the *rho* adaptive pathway—did not (Rodríguez-Verdugo et al. 2014). Collectively, these *E. coli* studies provide an integrative and mechanistic view of thermal adaptation, complementing fitness estimates with changes at the transcriptomic and genomic levels and highlighting the effects of structural variation.

Genomics of Heat Stress Response in *Saccharomyces cerevisiae*

S. cerevisiae is increasingly used in experimental evolution as a unicellular eukaryotic organism with possibility of sexual and asexual reproduction. Despite this potential, there are still relatively few papers addressing the genomics of thermal responses in this species (Table 1). Yona et al. (2012) used experimental evolution to test the effect of chromosomal duplications and associated gene expression levels in thermal adaptation. The authors studied four replicate populations of diploid yeast cells in three environments: constant 30 °C, a gradual increase of temperature until 39 °C, and constant 39 °C. After 450 generations, a duplication of chromosome III was found in all 39 °C evolved strains, suggesting that it played an important role in adaptation to extreme thermal environments, although it was transient in two of the replicates. This suggests that chromosomal duplications may contribute to a rapid response to short-term stress, by eliminating the need for upregulation of HS genes but may not be beneficial in the long term.

Huang et al. (2018) conducted an experimental evolution study by applying stepwise temperature increases during 46 generations (from 37 °C to 42 °C) and used whole-genome sequencing to address the genomic basis of prolonged thermotolerance adaptation. The six evolved strains (haploid state) showed a significant improvement in prolonged thermotolerance adaptation at 40 °C. While some mutations only occurred in a subset of the analyzed populations, similar changes in independent strains (i.e. parallelism) were observed in other genes, such as *CDC25*. These mutations suggest that changes in the RAS-cAMP signaling pathway, which is critical for cell growth in *S. cerevisiae*, contribute to the thermotolerant phenotype. As detailed in Yona et al. (2012), previous studies have shown rapid adaptation through chromosome duplication (see also Caspeta et al. 2014 for a similar finding). Huang et al. (2018) also found 12 chromosomal regions duplicated or deleted with potential impact on thermal adaptation. Another interesting result was the parallel loss of mitochondrial genomes in all six terminal strains, reinforcing the idea that loss of response to oxidative stress increases heat tolerance.

These studies highlight the importance of investigating different timescales, as they may reveal fundamentally different evolutionary changes and dynamics. Additionally, they emphasize the role of structural variants, such as duplications, in enhancing thermal adaptation. Studies should also explore the potential to compare thermal evolution under sexual vs. asexual reproduction, possible in this organism.

Overlap Between Candidate Variants/Functions From Thermal E&R Studies

We searched for an overlap between candidate variants and functions within the studies comprising our literature review, focusing on bacteria, *Drosophila*, and yeast (see supplementary table S1, Supplementary Material online). We observed a striking pattern of low overlap between candidate variants from the different studies using the same organism (supplementary table S2, Supplementary Material online). In *Drosophila*, of the 137 genes enlisted in 14 studies, only 18 were reported in 2 studies, all others being mentioned only once. In yeast, of the 97 genes reported in a total of 7 studies, 8 appeared in 2 of the studies, whereas for bacteria, 21 genes (of the 112 genes mentioned in total) appeared in 2 or more studies (in a total of the 12). The lack of overlap at the genomic level in *Drosophila* thermal experimental studies was previously highlighted by Michalak et al. (2019), with few genes overlapping between the aforementioned study and the Tobler et al. (2014) study. Both studies also showed a low overlap with findings in Mallard et al. (2018). The lack of common genes is also observed at the gene expression level in *D. melanogaster* (Sarup et al. 2011). In *E. coli*, Deatherage et al. (2017) and Tenaillon et al. (2012) also found a low overlap in candidate loci for common heat selection to 42 °C, although both studies started from the same REL1206 *E. coli* strain.

There are several possible explanations for the generally low overlap in the genomic and transcriptomic thermal responses, namely, (i) absence of proper controls or adequate replication to account for the confounding effects of laboratory adaptation or other sources of evolution (inadequate experimental design); (ii) different thermal stress protocols applied; (iii) the different genetic backgrounds of the experimental populations; (iv) the high rate of false positives due to the large number of candidate SNPs (reviewed in Long et al. 2015; Schlötterer et al. 2015) and/or long-range linkage disequilibrium (Franssen et al. 2015; Barghi and Schlötterer 2019); and (v) the lack of adequate controls to account for the confounding effects of laboratory adaptation. In the future, it will be important to increase efforts to reduce the number of false-positive variants, as well as streamline the diversity of thermal selection protocols (and associated target phenotypes) and methodologies used for the detection of candidate SNPs (e.g. definition of the critical threshold used). A possible approach to minimize false positives is to use the analysis of haplotype blocks/reconstruction to deal with regions of low recombination, also associated with inversions or hitchhiking with low-frequency selected alleles (Franssen et al. 2015, 2017; Schlötterer 2023). Better discrimination of the effects of drift and selection as drivers of genomic change can also strengthen the consistency across studies particularly in populations with low initial standing genetic variation where "soft sweeps" are likely to occur. This is often the case for traits with a large mutational target size and high levels of genetic redundancy among loci (Höllinger et al. 2019; Barghi et al. 2020). To mitigate these potential limitations, studies can use simulations to distinguish between drift and selection (Baldwin-Brown et al. 2014) or temporal covariance analyses (Buffalo and Coop 2020). We would like to emphasize that our overlap analysis was not intended to be an exhaustive comparative analysis of lists of candidate genes/functions across studies, but rather an approach to extract common features identified as relevant for understanding thermal adaptation at the genomic level. Nevertheless, our finding of low gene overlap between studies should be rather conservative, considering a possible upward bias resulting from authors focusing on genes highlighted in previous studies.

On a positive note, there are some concordant findings between E&R studies. For instance, studies in bacteria have revealed relevant de novo mutations and transcriptional regulation processes underlying stress responses as well as variants involved in trade-offs—namely, highlighting the roles of *rpo* (*rpoB*, *rpoC*, and *rpoS*) and *rho* pathways (see supplementary table S2, Supplementary Material online). Interestingly, studies in yeast show that genes involved in ATP synthesis and cell wall membrane are the common targets in heat tolerance. In *Drosophila*, an interesting overlap occurred for the SNF4Aγ gene, highlighted as a major genomic target associated with energy homeostasis in Mallard et al. (2018), also signaled as relevant in the Michalak et al. (2019) study—see supplementary table S2, Supplementary Material online. Gene expression studies in *Drosophila* also show some overlap between candidate genes detected in *D. melanogaster* (Hsu et al. 2021) and *D. simulans* (Manenti et al. 2018). Despite the different species and discrepant number of generations, both Hsu et al. (2021) and Manenti et al. (2018) highlighted the upregulation of genes associated with cellular response to stress such as the *Turandot* genes as an evolutionary response to thermal fluctuations.

A higher concordance between studies is observed at the functional level with some features being recurrently involved in the thermal response (see supplementary table S3 fig. S1, Supplementary Material online). We highlight the role of DNA, protein, and ATP binding processes, which probably reflect the importance of transcription factors and protein interactions; "response to heat stress," likely involving chaperone activity in both bacteria and *Drosophila*; and structural changes in cell wall in both bacteria and yeast.

Moving Forward

The cumulative data generated by E&R studies have provided important insights into the molecular mechanisms of thermal adaptation in several taxa. For instance, E&R

studies in asexual populations have revealed relevant de novo mutations and transcriptional regulatory processes underlying stress responses, as well as variants involved in trade-offs. Furthermore, structural variation—such as duplications and deletions—has also been shown to play an important role in shaping genetic responses in the context of thermal change. Unsurprisingly, when populations harbor initial standing genetic variation, there is a general polygenic evolutionary response, with different variants being selected depending on the populations and thermal regimes.

A key issue that needs to drive future thermal E&R research is the need for a deeper understanding of the link between the phenotypic traits and the underlying molecular variation. Here, it is essential to characterize the traits relevant for adaptive thermal responses. One possible avenue is to shift the focus toward relevant fitness-related traits, such as fertility and behavior (e.g. thermoregulation) and away from lethal endpoints that may have less ecological relevance (Walsh et al. 2019; Bretman et al. 2024; Dougherty et al. 2024). In addition, thermal plasticity can be considered as a main trait in itself, as a major component of thermal evolution (Huang et al. 2020; Oomen and Hutchings 2022). This means that studies addressing thermal response in just one environment or at one time point might miss important variation underlying thermal response (e.g. see Ørsted et al. 2019; Noer et al. 2024).

The bridge between the phenotype and the genome in the context of thermal adaptation can be further strengthened by exploiting recent developments in genome engineering to gain insight into the function of specific molecular variants (Rohde et al. 2018). These include the genomic toolbox available for model organisms (knockin, knockout, CRISPR, and RNAi), knowledge that can then be available to validate the functional role of candidate variants revealed in studies on other organisms, even beyond the scope of E&R. These tools are already being used in the context of experimental evolution in *E. coli* and *Saccharomyces* (see Sandberg et al. 2014; Rodriguez-Verdugo et al. 2016; Huang et al. 2018, in Table 1). Its application to a wider range of species is a promising prospect (see Gudmunds et al. 2022 and Jakšić et al. (2020) for a notable exception in *Drosophila*), although there are technical challenges, namely, the need to cover a large number of candidate genes/regions generated by most studies. There is also the potential to gain further insight into the association between phenotype and genotype through the development and use of bioinformatic and available statistical tools, such as GWAS and gBLUP (Sørensen et al. 2017a; Exposito-Alonso et al. 2019; Lecheta et al. 2020), which are rarely used in E&R studies. Thus, there is an untapped potential for collaboration and development here.

E&R studies have enormous potential to unravel the genetic architecture of traits under thermal selection, to assess evolutionary potentials and the tempo of evolution, and to advance our knowledge of how and whether species can adapt to global warming. The next step is to integrate the knowledge gained from these studies into a broader perspective on thermal adaptation. In this context, one option could be to combine information gathered from scans of natural populations from different thermal environments with genes/variants that show evidence of adaptive response under more controlled conditions (Porcelli et al. 2015; Hsu et al. 2021; Thorhölludottir et al. 2023). This approach has led to some discrepancies. On the one hand, a recent study by Hsu et al. (2021) found a significant overlap between adaptive variants responding to temperature in the laboratory and those associated with clines in natural *D. melanogaster* populations (Hutter et al. 2008; Zhao et al. 2015). On the other hand, Porcelli et al. (2015) found very little overlap between a genomic study of natural *D. melanogaster* populations from different regions (Reinhardt et al. 2014) and the thermal E&R study of Tobler et al. (2014). Improving functional annotation and increasing methodological repeatability (both at the experimental and bioinformatic level) will be crucial to improve the overlap between studies. A possible approach may be to define functional categories of genes with a higher significance in terms of their potential role within the targeted evolutionary scenarios. These could include genes with common functions and closer (phylogenetic) origin, even if under different designations. This is an ambitious task that may be achieved progressively as more studies of NGS are conducted within a thermal experimental evolution framework.

In addition, E&R experiments can provide relevant insights into processes that may affect climate change adaptation in nature, for instance by addressing how different levels of hybridization/gene flow shape the genomic background of populations/species and their ability to respond to thermal shifts. The combined wealth of information from different methodological approaches on thermal adaptation should be incorporated into mechanistic models of future species distributions under climate change (Urban et al. 2016; Bay et al. 2018; Waldvogel et al. 2020; Aguirre-Liguori et al. 2021). This will be an important step toward better predictions of species responses to climate change and ultimately help to identify species or regions that are more vulnerable to increased thermal variation, providing valuable input to managers and decision-makers in developing better conservation strategies. In the near future, crosstalk between different fields that study climate change, from different angles, will be crucial to guide and develop the next steps of E&R studies. Such studies should include ecologically relevant experimental designs and studies performed in nature as exemplified by multifactorial global change setups with e.g. warming of experimental plots (Guo et al. 2019; Köhler et al. 2021), allowing for the implementation of an E&R strategy.

Supplementary Material

Supplementary material is available at *Molecular Biology and Evolution* online.

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Conflict of Interest

The authors declare no competing interests.

Data Availability

No new data were generated in this review article.

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