The transcriptional landscape of adaptive thermal plasticity within and across generations: a counterbalance in gene expression induced by parental acclimation

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July 16, 2024

Abstract

There is increasing evidence for the co-occurrence of adaptive within-generation (WGP) and transgenerational (TGP) plasticity and the ecological scenarios driving both types of plasticity. However, some aspects of their transcriptional mechanisms, such as the role of alternative splicing and the gene regulation involved in the compensatory effect of parental acclimation on the offspring's fitness in relation to life stages, have remained elusive. We explore these fundamental questions by considering the desert endemic Drosophila mojavensis for which prior evidence indicates adaptive thermal acclimation within and across generations. We implement a full factorial design to estimate genome-wide patterns of differential gene expression (DE) and alternative splicing (AS) in response to acclimation treatments performed in the parental and offspring generations, as well as considering larva and adult stages. Our results demonstrate that mechanisms of alternative splicing represent a substantial difference between WGP and TGP. These mechanisms contribute substantially to transcriptional plasticity within generations but not across generations. We found a great number of genes associated with transcriptional TGP, which is exclusive to larva stages and not adult samples. Finally, we provide evidence demonstrating that parental acclimation in TGP triggers a great number of the same genes normally down-regulated in WGP. Thus, parental acclimation appears to compensate for the down-regulation of genes during thermal stress in the offspring generation. This result might be one of the mechanisms explaining the compensatory effect of parental acclimation in the offspring generation.

Introduction

Thermal acclimation allows organisms to overcome periods of extreme climatic conditions (Hoffmann and Sgró, 2011; Overgaard et al., 2011; Anderson et al., 2012), which is essential for maximizing fitness in fluctuating environments (Lande, 2009; Chevin et al., 2010). These types of plastic responses not only occur within the lifespan of an individual through within-generation plasticity (WGP) but also across multiple generations by transgenerational plasticity (TGP) (Mousseau and Fox, 1998; Uller, 2008; Nelson and Nadeau, 2010; Bonduriansky et al., 2011; Heard and Martienssen, 2014). WGP has long been investigated across a diverse number of taxa and a wide range of traits (Hoffmann et al., 2005; Dillon et al., 2007; Bowler and Terblanche, 2008; Fusco and Minelli, 2010). However, the adaptive significance and underlying transcriptional bases of TGP when co-occurring with WGP are less understood.

The interaction between WGP and TGP and their co-occurrence differ across taxa (Walsh et al., 2015, 2016), with some studies reporting the decoupling of WGP and TGP. However, evidence for the co-occurrence of WGP and TGP is rapidly increasing in different organisms (Jablonka et al., 1995; Molinier et al., 2006; Carone et al., 2010; Herman and Sultan, 2011; Herman et al., 2013; Uller et al., 2013; Heckwolf et al., 2018). When co-existing, there is extensive variation in the magnitude and direction of plasticity within and across generations (Galloway and Etterson, 2007; Uller et al., 2013; Walsh et al., 2015; Gillis and Walsh, 2018;

Diaz et al., 2020; Rösvik et al., 2020), which reflects different degrees of adaptability, generating cases of silver spoon (Walsh et al., 2024), bet-hedging (Joschinski and Bonte, 2020), or negative carry-over effects (Waite and Sorte, 2022). From these, scenarios of co-existing adaptive plasticity are particularly interesting to understand how evolution simultaneously shapes the transcriptional landscape of these two types of plasticity. For example, current models predict the evolution of adaptive TGP as an anticipatory response to overcome periods of otherwise unfavorable conditions in the offspring when the parent-to-offspring environmental predictability increases (Uller, 2008; Badyaev and Uller, 2009; Bonduriansky et al., 2011; Hoyle and Ezard, 2012; Kuijper and Hoyle, 2015; Proulx and Teotónio, 2017). This hypothesis has been formally tested using thermal tolerance data (Diaz et al., 2020). However, its predictions at the transcriptional level are currently lacking evidence.

When evolving simultaneously, the outcome from adaptive WGP and TGP allows organisms to overcome unfavorable thermal conditions due to a predictive acclimation period in the same or the parental generation (Mousseau and Fox, 1998; Uller, 2008; Nelson and Nadeau, 2010; Bonduriansky et al., 2011; Heard and Martienssen, 2014; Clark et al., 2019). Despite the recent increase in studies comparing multiple acclimation responses, it is still unclear to what extent selection targets the same regulatory networks during the acclimation process in both cases. Selection may modulate the level and direction of expression on different sets of genes during WGP and TGP or the same genes but inducing divergent transcriptional paths (Bell and Stein, 2017; Hales et al., 2017). Only a few studies have addressed this question, and even fewer have considered cases where both WGP and TGP are adaptive. So far, studies of predator-induced plasticity in *Daphnia* (Bell and Stein, 2017; Hales et al., 2017) and extended Dauer diapause in *C. elegans (Webster et al., 2018)* suggest independent transcriptional mechanisms for WGP and TGP. Similarly, recent evidence from thermal acclimation in sticklebacks (Shama et al., 2016), sea urchins (Clark et al., 2019), and coral reef fish (Bernal et al., 2022) suggests the decoupling of transcriptional plasticity within and across generations. Another fundamental gap in these studies is the role played by mechanisms of alternative splicing (AS) (Telonis-Scott et al., 2009) or intron retention (IR). Although these mechanisms of transcriptional change are often overlooked, there is substantial evidence connecting AS with thermal plasticity in animals (Anduaga et al., 2019; Steward et al., 2022) and plants (Dikaya et al., 2021; John et al., 2021), including a specific role of IR in the control of gene expression (Yablonovitch et al., 2017; Anduaga et al., 2019). The contribution of these mechanisms to TGP is rarely considered, but there is evidence from the coral reef fish suggesting a small but complementary role of AS in transgenerational acclimation (Ryu et al., 2018).

In this study, we address these fundamental questions by considering a scenario with previous phenotypic evidence of adaptive thermal acclimation within and across generations in the desert endemic *Drosophila mojavensis (Diaz et al., 2020).* In our previous study, we compared larval and adult plasticity, which allowed us to test predictions on the level of TGP between life stages. We demonstrate that the parental environment is more likely to reflect that of the offspring in the early stages than adulthood, which correlates with differences in TGP between life stages. Here, we take advantage of this scenario to investigate how evolution shapes the transcriptional landscape when adaptive WGP and TGP evolve simultaneously. First, we expand our scope to a more complete view of transcriptional plasticity by investigating the relative contributions of differential expression (DE) and alternative splicing (AS) mechanisms to WGP and TGP. Second, we test if patterns of transcriptional plasticity within and across generations reflect our predictions from heat tolerance data between life stages, where major transcriptional changes are expected in larval TGP due to their higher parent-to-offspring environmental predictability. Third, we estimate the number and transcriptional changes of genes responding to WGP and TGP to investigate differences in the level, direction, and splicing of gene expression associated with thermal acclimation within and across generations. Our results contribute to expanding the understanding of transcriptional evolution when multiple sources of acclimation co-exist and how organisms may adapt to climate change scenarios (Hoffmann and Sgró, 2011; Sgrò et al., 2016; Donelson et al., 2018; Bonamour et al., 2019).

Results

Experimental design and RNA-Seq

Previous studies have demonstrated adaptive within- (WGP) and transgenerational plasticity (TGP) of heat tolerance following acclimation treatments at 36 °C in parents and offspring of *D. mojavensis* (Diaz et al., 2020). Here, we investigate the underlying transcriptional bases of these types of plasticity by using the same experimental design, which allows us to address fundamental questions on how selection drives adaptive WGP and TGP while simultaneously considering larval and adult stages of *D. mojavensis* . Our approach involves a full factorial design where parents and offspring were exposed to either an acclimation temperature of 36 °C or a control treatment at 25 °C (Figure 1). RNA-Seq libraries were sequenced from larva and adult samples collected in the offspring generation, with three biological replicates for each of the four combinations of acclimation treatments (Figure 1).

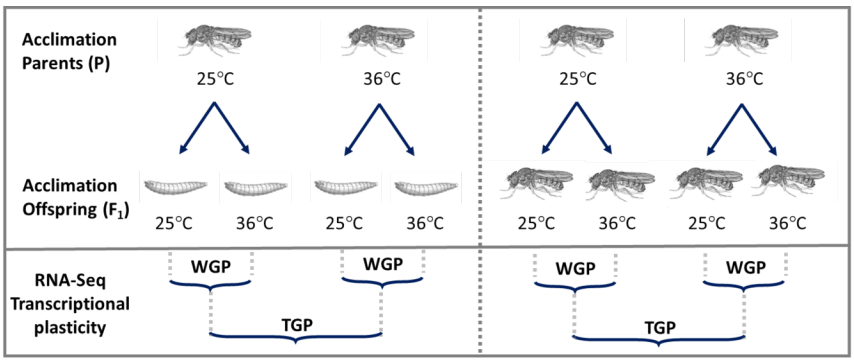

Figure 1. Experimental design used to investigate the effect of acclimation at 36 °C vs. control treatments at 25 °C in parents (P) and F_1 offspring. Acclimation treatments in the parental generations were performed on adult stages, while offspring acclimation was performed on either larva or adult samples. All samples for RNA-Seq were collected in the offspring generation as a result of the four different combinations of acclimation treatments. The parental effect was used to assess transcriptional transgenerational plasticity (TGP), while the offspring acclimation was used to estimate within-generation plasticity (WGP).

We sequenced nearly 400 million paired-end Illumina reads across the 24 RNA-seq libraries. Of these, an average of 17 million reads per library mapped to the reference genome **following** trimming and filtering of sequence reads. An independent read-count matrix for each gene feature was generated (*i.e.,* exons, junctions, introns, and gene-wide). All relative changes of transcriptional plasticity were estimated by comparing read counts between acclimated samples at 36 °C and control samples at 25 °C as performed in the parental or offspring generations (Figure 1).

Differentially expressed (DE) and alternatively spliced (AS) genes are associated with transcriptional plasticity

We start by expanding our scope of transcriptional change to characterize genome-wide patterns of differential gene expression (DE) and differential alternative splicing (AS) significantly associated with transcriptional plasticity in larva and adult stages of *D. mojavensis* . We observed a wide spectrum of genes associated with both types of plasticities and life stages (Figure 2a). Overall, we found that more than twice as many genes were significantly associated with transcriptional plasticity in larvae compared to adult samples (2719 vs. 1096 genes, respectively, Figure 2a). In addition, we found similar (*e.g.,* adults) or even higher numbers (*e.g.,* larvae) of AS genes, relative to DE genes, associated with acclimation performed in the offspring generation (WGP) (Figure 2a). Interestingly, AS genes are particularly associated with WGP, with no AS genes significantly responding to TGP (Figure 2a).

Figure 2. Overall results of transcriptional within- (WGP) and transgenerational plasticity (TGP) in larvae and adults of *D. mojavensis*. All comparisons were performed between acclimated samples at 36 °C and control samples at 25 °C using three biological replicates following FDR corrections.

a) Barplots show the number of genes detected with significant WGP (only), TGP (only), and their overlap (WGP and TGP). Each bar shows the number of detected genes with significant DE-only, AS-only and their overlap (DE and AS). **b)** Error bars show the intron retention (IR) change estimated for up- and down-regulated genes following WGP and TGP. IR change was estimated as the Euclidian distance between the IR rates of acclimated samples at 36 °C vs control samples at 25 °C. Significant comparisons ($\alpha < 0.05$) following GLM analysis are indicated with *. **c)** Venn diagram shows the number of DE genes detected with significant WGP in larvae (only), adults (only), and their overlap (larvae and adults).**d)** Venn diagram shows the number of AS genes detected with significant WGP in larvae (only), adults (only), and their overlap (larvae and adults). **e)** Venn diagram shows the number of DE genes detected in larvae with significant WGP (only), TGP (only), and their overlap (WGP and TGP).

The role of intron retention (IR) in transcriptional plasticity

Since alternatively spliced genes significantly associated with transcriptional plasticity are WGP-specific, we next investigated a more general role of splicing in the regulation of gene expression by estimating rates of intron retention (IR). Transcripts with retained introns often contain premature stop codons, and these transcripts are degraded by the nonsense-mediated decay (NMD) pathway (Farlow et al., 2010)**. This mechanism has also been associated with the control of gene expression, as an increase in the rate of retained introns results in higher transcript degradation (Jacob and Smith, 2017; Hadar et al., 2022). We tested this hypothesis as a possible mechanism of gene regulation in transcriptional plasticity by estimating IR changes between acclimated vs. control samples (IR change) and then compared the IR change between up and down-regulated genes (Figure 2b). IR changes were consistently higher for down-regulated genes when compared to up-regulated genes in both WGP and TGP (Figure 2b). This finding implicates IR as one mechanism for the control of gene expression in thermal plasticity and is consistent with increased IR rates in response to stress conditions in other species (Jacob and Smith, 2017; Hadar et al., 2022)** .

Transcriptional WGP involves DE and AS mechanisms

Since significant DE genes and AS genes show different transcriptional patterns in acclimation treatments and life stages, we next explored the relative contributions of AS and DE genes as well as their associated ontology functions independently for the effects of acclimation performed in the offspring (WGP) and the parental generation (TGP). For WGP, we found that in addition to DE genes, AS genes account for a great part of transcriptional plasticity. In larva samples, we found 1028 DE genes and 1609 AS genes associated with WGP, while 611 DE genes and 507 AS genes are associated with WGP in adults (Figure 2c, 2d, and 3a). The majority of these genes differ between mechanisms of transcriptional change, with less than 4 % overlapping genes between DE and AS (Figure 2a). Similarly, the majority of genes responding to WGP differ between life stages, as only 8 % of the genes responding to WGP overlapped between larvae and adults (Figures 2c, 2d, and 3a).

Functional analysis of DE and AS genes in transcriptional WGP

DE genes in larval WGP show significant enrichment for 14 gene ontology categories (GO), while 21 are associated with WGP in adults (Figure S1). Of these, only four categories are common to both stages (Figure S1), although some of them are related to similar biological functions. Most DE genes in larvae are associated with i) proteolysis and ii) several additional pathways with structural/cell/metabolic functions. Moreover, DE genes in adults are associated with i) proteolysis, ii) chaperone activity and heat-shock response, iii) other stresses, iv) Egg development, and v) several additional structural/cell/metabolic functions (Figure S1). The overlapping genes that respond to acclimation in both larvae and adults are associated with ten GO categories, eight of which are already present in the two independent analyses (Figure S1). As expected, most overlapping categories between the two stages are associated with the heat-shock response (Figure S1), including proteolysis and chaperone activity.

The molecular pathways associated with AS genes in WGP differ substantially from those of DE genes and show functional differences between larvae and adults. We detected 19 GO functions associated with

WGP in larvae, while 27 are associated with WGP in adults (Figure S2). Of these, only three categories are common to both stages (Figure S2). AS genes in larva and adult samples are associated with multiple types of structural/cell/metabolic functions, and, unlike DE genes, none of them are part of the heat-shock response (Figure S2). Interestingly, larva samples are associated with alternative splicing via spliceosome, and both larva and adult samples are associated with molecular functions of gene regulation, such as chromatin remodeling and the regulation of translation. The overlapping AS genes between larvae and adults are enriched by nine GO categories, eight of which are already present in the two independent analyses (Figure S2). The majority of these overlapping categories between the two life stages are associated with muscle activity, in addition to chromatin remodeling and protein binding (Figure S2).

Figure 3. Transcriptional landscape comparing differential gene expression (DE) with significant within-generation plasticity (WGP) between larvae and adults of *D. mojavensis*. All comparisons were performed between acclimated samples at 36 °C and control treatments at 36 °C using three biological replicates following FDR corrections. **a)** Scatterplot shows the transcriptional landscape with relative expression levels (log2-fold-change) for DE genes with significant WGP in larva vs adult samples. **b)** Boxplots show the level of differential gene expression (log2-fold-change) for genes of the heat-shock response (*i.e.*, proteolysis and Hsps) with significant WGP in larva and adult samples.

Heat-shock DE genes in transcriptional WGP differ between larvae and adults

Our GO analyses provide a broad characterization of the functions associated with transcriptional plasticity. However, these analyses might also defuse the role of the most important functions in the heat-shock response, which involves a massive up-regulation of genes associated with molecular chaperones (HSPs) and proteolysis genes (Sørensen et al., 2005; Mahat et al., 2016). Thus, we next investigated the number and direction of gene expression in these gene categories (Figure 3b). We found that the number of Hsp genes and their relative level of up-regulation are substantially higher in adults (11 up-regulated genes) than in larva samples (5 upregulated genes and 2 down-regulated genes) (X^2 , $p = 0.002$, Figure 3b). On the other hand, while the number of up-regulated proteolysis genes is substantially higher in larvae (56 genes) than in adults (17 genes), the relative level of up-regulation seems to be higher in adults (X^2 , $p < 0.001$, Figure 3b). These results demonstrate functional differences in the heat-shock response between life stages, suggesting that larvae rely primarily on proteolysis genes. In contrast, adults rely more on Hsp genes during acclimation in WGP to prepare for upcoming thermal stress.

Transcriptional TGP is exclusive to larva stages and does not involve AS

Based on the results of our previous study, where TGP of heat tolerance was exclusively detected in larvae, we tested whether the transcriptional landscape of plasticity reflects our observation at the phenotypic level *(Diaz et al., 2020)* . Our previous phenotype data correlated with a higher level of parent-to-offspring environmental predictability in the early stages, which is a hypothesis that has not been formally tested using transcriptional data. As predicted, we found substantial transcriptional responses associated with parental acclimation in larvae, with 419 DE genes, while only one DE gene was detected in adult samples (Figure 2a). On the other hand, no genes responding through AS were detected with significant TGP in larvae (Figure 2a).

Great overlap of DE genes associated with WGP and TGP

We found 1028 DE genes associated with WGP, while 419 DE genes were associated with TGP in larvae (Figure 2e). From these, 56 % or 234 genes overlapped the two types of acclimation treatments (Figure 2e). This result initially suggests a similar effect of thermal plasticity within and across generations. However, the majority of the overlapping genes between WGP and TGP are located in the quadrant II of the scatterplot that represents the transcriptional landscape of thermal plasticity (Figure 4a). This means that most of these genes are up-regulated in TGP but down-regulated in WGP.

Functional analysis of DE genes associated with WGP and TGP

DE genes with transcriptional TGP are enriched by 11 GO categories, five of which are shared with WGP

(Figure S3). Overlapping genes between WGP and TGP are significantly associated with 11 categories, six of them already present in the independent analyses (Figure S3). Thus, transcriptional TGP is associated with some GO categories found in WGP, such as i) proteolysis, in addition to ii) DNA replication, and ii) several additional structural/cell/metabolic functions (Figure S3). The overlapping genes between WGP and TGP are significantly linked to different pathways of structural development, such as i) apical construction, ii) embryonic cell shape, iii) body morphogenesis, and iv) cuticle development, as well as pathways of v) collagen production, vi) oxidoreductase, and vii) endopeptidase activity (Figure S3).

Figure 4. Transcriptional landscape comparing differential gene expression (DE) between WGP and TGP in the larva of *D. mojavensis*. All comparisons were performed between acclimated samples at 36 °C and control treatments at 36 °C using three biological replicates following FDR corrections. **a)** Scatterplot shows the transcriptional landscape with relative expression levels (log2-fold-change) for DE genes with significant WGP vs. TGP. **b)** Boxplots show the level of differential gene expression (log2-fold-change) for genes of the heat-shock response (*i.e.*, proteolysis and Hsps) with significant WGP and TGP.

Heat-shock DE genes with larval WGP and TGP

Although the proteolysis genes are common to both acclimation treatments, the lack of enrichment for molecular chaperons in TGP (Figure S3) suggests that the parental acclimation might differ functionally from the canonical heat-shock response that results from the offspring acclimation in WGP. To better understand how the heat-shock response differs between WGP and TGP, we investigated the number of genes and direction of gene expression associated with proteolysis and molecular chaperones (HSPs) (Figure 4b). We found that the number of proteolysis and Hsp genes that are up-regulated in larvae is lower in TGP than in WGP. While 56 proteolysis genes and 5 Hsp genes are up-regulated in WGP, 25 proteolysis genes and 2 Hsp genes are up-regulated in TGP (Figure 4b). These differences in the direction of expression are not significant for Hsp genes due to low numbers (X^2 , $p = 0.129$) but are significant for proteolysis genes (X^2 , $p < 0.001$). Interestingly, only one of these genes is down-regulated in TGP, while WGP shows patterns of up- and downregulation. Although the total number of genes is higher in WGP than in TGP, the relative level of up-regulation is higher in TGP than in WGP (Figure 4b).

Transcriptional counterbalance between WGP and TGP

Our results suggest that selection for adaptive TGP targets similar genes and functions when evolving simultaneously with WGP, with 56 % or 234 overlapping genes between the two types of plasticity. However, the direction of gene expression in the majority of these genes follows opposing transcriptional trajectories in the two types of plasticity (Figure 4a). Of the 234 overlapping genes between WGP and TGP, 215 are up-regulated in TGP. Of these, 204 genes are also down-regulated in WGP (Figures 5a and 5b).

During the heat-shock response in WGP, a large portion of the cellular resources is used to induce the molecular chaperones and proteolysis genes needed to overcome periods of thermal stress at the expense of a massive down-regulation in other genes. We hypothesize that the parental acclimation in TGP represents a transcriptional counterbalance, which seems to compensate for the future decrease in gene expression during thermal stress caused by the environment in the offspring. To understand better this transcriptional counterbalance provided by parental acclimation, we followed the transcriptional trajectories of the overlapping genes between WGP and TGP across all combinations of acclimation treatments (Figure 5c). We found a strong interaction effect between the parental and offpring acclimation treatments. As expected, on average, the gene expression level in these genes is significantly down-regulated by the acclimation treatment performed in the offspring. However, it is up-regulated by the acclimation treatment performed in the parental generation (Figure 5d). More interestingly, this interaction leads to a transcriptional compensation where acclimated larvae at 36 °C whose parents were also acclimated at 36 °C show no significant differences in the level of the gene expression compared to larvae that did not receive any acclimation treatments in the parental or offspring generations (Figure 5c). This pattern of gene expression is not detected when considering non-overlapping DE genes between WGP and TGP (*i.e.* , WGP-only and TGP-only genes, Figure S4).

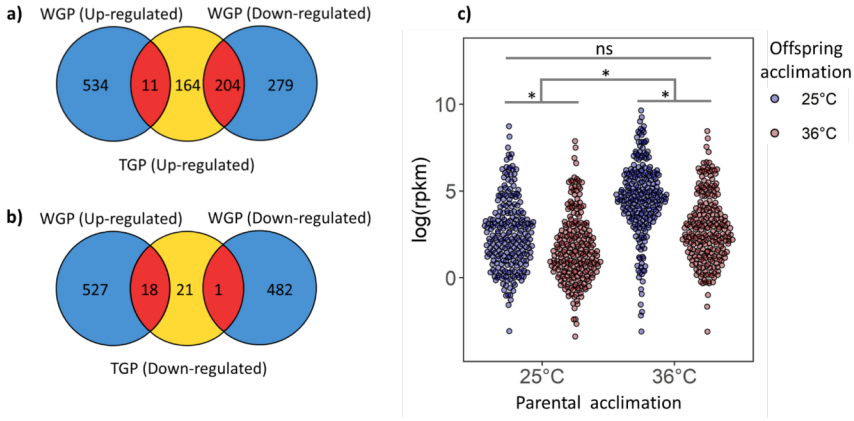

Figure 5. Relationship between DE genes with transcriptional within- (WGP) and transgenerational plasticity (TGP) in the larva of*D. mojavensis*. All comparisons were performed between acclimated samples at 36 °C and control treatments at 36 °C using three biological replicates following FDR corrections. The Venn diagrams show **a)**the number of up and down-regulated genes with WGP vs. up-regulated genes with TGP; **b)** the number of up and down-regulated genes with WGP vs. down-regulated genes with TGP; and **c)** Boxplots showing the transcriptional trajectories of the overlapping DE genes significantly associated WGP and TGP, across the acclimation treatments performed in the parental and offspring generations. Significant comparisons ($\alpha < 0.05$) following GLM analysis are indicated with *.

Discussion

Although differences in the level of predictability of environmental cues within and across generations might result in the decoupling of WGP and TGP, there is transcriptional evidence in some organisms where both types of plasticity co-exist (Shama et al., 2016; Bell and Stein, 2017; Hales et al., 2017; Webster et al., 2018; Clark et al., 2019; Bernal et al., 2022). We consider the case of the xeric-adapted *D. mojavensis* where we have previously detected adaptive thermal plasticity within and across generations to address fundamental questions on how selection shapes the transcriptional landscape of these plastic responses. By implementing a full factorial design, we expand our scope of transcriptional plasticity to consider the role of alternatively spliced genes (Venables et al., 2012). With this approach, we provide compelling evidence demonstrating substantial transcriptional differences between WGP and TGP, where alternative splicing mechanisms play a major role in WGP but not in TGP. We demonstrate that transcriptional TGP is more prevalent in the larva stage than in adults, as expected from the level of parent-to-offspring environmental predictability (Diaz et al., 2020). Finally, we provide evidence for a transcriptional counterbalance in TGP, where parental acclimation seems to compensate for the low gene expression caused by thermal stress in the offspring generation.

The role of DE and AS in transcriptional plasticity

In addition to differential gene expression (DE), we found evidence for differential alternative splicing with substantial consequences for transcriptional plasticity not reflected in DE. Moreover, we detected the functional specialization of alternatively spliced genes (AS) relative to DE genes in response to thermal acclimation. AS genes are only linked to WGP, while DE genes are associated with WGP and TGP.**Our results of transcriptional WGP show that the** number of AS genes often exceeded that of the DE genes, demonstrating that AS is an important mechanism of transcriptional regulation in response to thermal acclimation in *D. mojavensis* . These mechanisms appear mutually exclusive, with only 4 % of overlapping genes between DE and AS. The role of AS in transcriptional plasticity following acclimation is well supported in multiple organisms (Anduaga et al., 2019; Dikaya et al., 2021; John et al., 2021; Steward et al., 2022). The great majority of this evidence comes from studies of WGP, while the evidence for TGP is more limited. Our

results suggest that differential AS may have little or no role in TGP (Ryu et al., 2018). However, we provide evidence for a more general role of intron retention (IR), which is a particular case of AS, being likely linked to negative gene regulation (Farlow et al., 2010). An increased rate of retained introns, which often contain premature stop codons, has been associated with higher transcript degradation that might result from the nonsense-mediated mRNA decay (NMD) pathway (Jacob and Smith, 2017; Hadar et al., 2022). We found that IR rates are significantly higher for down-regulated genes than for up-regulated genes, suggesting IR is a mechanism for regulating gene expression in response to thermal acclimation within and across generations. This finding is consistet with increased IR rates in response to stress conditions in other species (Jacob and Smith, 2017; Hadar et al., 2022).

The heat-shock response and transcriptional WGP

The acclimation effect within a generation, commonly known as heat hardening or heat-shock response, has been widely investigated across several organisms for decades (Krebs, 1999; Krebs and Bettencourt, 1999; Hoffmann et al., 2003; Sgrò et al., 2010; Kellermann and Sgrò, 2018; Diaz et al., 2020). We found that DE genes are linked to **functions that are typical of heat hardening, including the expression of proteolytic pathways, heat-shock proteins (HSPs), and other molecular functions that might protect tissues from the damage caused by high thermal exposures** (Dahlgaard et al., 1998; Sørensen et al., 2005; Bahrndorff et al., 2010; Diaz et al., 2015; Mahat et al., 2016; Cai et al., 2017). In contrast, AS genes are involved in multiple structural, cell, and metabolic functions, including muscle assembly, as well as in different mechanisms of gene regulation, such as spliceosome activity, chromatin remodeling, and translation regulation.

We detected substantial differences in transcriptional WGP between larvae and adults. The number of genes showing transcriptional WGP in larvae was approximately twice and four times higher in larvae than in adults for DE and AS genes, respectively. Similarly, the number and level of expression of genes associated with the heat-shock response differed substantially between life stages. This result is not surprising as it also matches our expectations from heat tolerance data in *D. mojavensis*, where *WGP* had a higher contribution to larval tolerance when compared to adult tolerance (Diaz et al., 2020). This is consistent with the literature on thermal tolerance in several organisms, reporting a greater thermal tolerance at early life stages than in adults (Sørensen and Loeschcke, 2002; Zizzari and Ellers, 2014). Larvae are more bound to the fluctuations of their environment since they are constrained to their substrate, while flying adults can seek more suitable thermal microclimates (Krebs and Loeschcke, 1995; Feder et al., 1997). Our results suggest that larvae may cope with thermal acclimation in WGP by inducing the expression of genes associated with proteolysis and/or inducing the alternative splicing of genes that are not directly related to the more energetically expensive heat-shock response.

Transcriptional counterbalance of parental acclimation in TGP

Transcriptional studies show that when WGP and TGP co-exist, these plastic responses tend to follow different trajectories and are influenced by different sets of genes and molecular functions (Shama et al., 2016; Bell and Stein, 2017; Hales et al., 2017; Webster et al., 2018; Bernal et al., 2022). However, this question has rarely been considered when both plastic responses are adaptive. We address this question in *D. mojavensis (Diaz et al., 2020)* , where we have previously analyzed the level of parent-to-offspring environmental predictability linked to TGP when comparing life stages. As expected from previous thermal tolerance data, transcriptional TGP was only detected in larva stages. Our results also support the hypothesis of diverging transcriptional trajectories between plastic responses within and across generations. However, in contrast to previous findings in other organisms (Bell and Stein, 2017; Hales et al., 2017), we found that such diverging trajectories involve many of the same genes normally expressed in WGP. Approximately 56 % of DE genes associated with TGP overlapped with WGP. Interestingly, most genes significantly associated with TGP are up-regulated, as opposed to transcriptional WGP, where many more genes are down-regulated in response to acclimation in the offspring.

We suggest that this up-regulation caused by the parental acclimation in TGP represents a transcriptional counterbalance of gene expression that helps explain the molecular bases of the adaptive component previously detected from heat tolerance data in TGP (Diaz et al., 2020). Thus, the offspring might benefit from parental acclimation by restoring the expression of genes that will be down-regulated by the heat-shock response when they are themselves acclimated. To understand their role in thermal tolerance, we identify different types of these genes triggered by parental acclimation, which can be classified into two groups. First, parental acclimation activates some genes clearly associated with the heat shock response, such as proteolysis, and only two Hsp genes (Sørensen et al., 2005; Mahat et al., 2016). One of these, Hsp83, is down-regulated in WGP, suggesting a transcriptional constraint in the activation of this gene in the larva stage, which is compensated by parental acclimation. Interestingly, this gene has been associated with maternal transfer to early embryos in *D. melanogaster* (Ding et al., 1993), being expressed during oogenesis and embryogenesis.

We also identified genes activated by parental acclimation that might be associated with heat tolerance but are not part of the heat-shock response. Genes associated with GO categories of collagen modifications are particularly interesting, such as procollagen-proline 4-dioxygenase activity and peptidyl-proline hydroxylation to 4-hydroxyl-L-proline. We identified four such genes, which encode enzymes that catalyze the formation of hydroxyproline (Myllyharju, 2008; Gorres and Raines, 2010). Collagens are extracellular matrix proteins that contribute to tissue structure and remodeling (Myllyharju and Kivirikko, 2004). This hydroxylation increases the melting temperature of helical collagen, which allows these proteins to be stable at body temperatures in mammals (Rappu et al., 2019). In addition, the down-regulation of these enzymes has been previously associated with thermal sensitivity in low-tolerant *D. melanogaster* lines (Vermeulen et al., 2014). As a major connective tissue, more thermally stable hydroxylated collagen in the offspring larvae of acclimated parents might allow them to tolerate the direct effect of heat stress without necessarily accessing the energetically expensive heat-shock response (Vermeulen et al., 2014). The fact that these pathways are not directly activated during WGP suggests that such genes are constrained or negatively affected by the massive up-regulation of chaperones during the heat-shock response in WGP acclimation.

We provide compelling evidence demonstrating substantial and distinct differences between adaptive WGP and TGP that contribute to explaining how selection shapes their transcriptional evolution. The first difference is evidenced in the role played by the mechanisms of alternative splicing in transcriptional plasticity, which is linked to acclimation within generations but not to parental acclimation. Moreover, our results demonstrate the importance of exploring alternative splicing in plasticity studies, as these mechanisms involve distinct genes and functions from those detected in differential expression analyses. The second difference between WGP and TGP was detected in the direction of gene expression. Most differentially expressed genes in response to thermal acclimation are common to both types of plasticity but are primarily down-regulated in WGP and up-regulated in TGP. We propose that this pattern might be a transcriptional counterbalance, where parental acclimation compensates for the negative effects of thermal stress on the expression level of some genes despite their potential role in thermal tolerance. Instead of enhancing the expression of the more energetically expensive molecular chaperones that characterize acclimation in WGP, parental acclimation counteracts the negative effects of the heat-shock response. How much of this pattern can be extended to other organisms or traits remains to be seen. However, we believe our findings help understand the molecular bases of adaptive TGP and how the offspring benefit from parental acclimation to increase their fitness compared to unacclimated parents.

Methods

Samples and experimental design

The source population for thermal experiments was generated by pooling four isofemale lines of *D. mojavensis* originally collected in Santa Catalina Island, California. This population was maintained at 25°C, under a 12:12 h light:dark cycle, and controlled density conditions in 8-dram glass vials with banana-molasses media for four generations before experiments (Coleman et al., 2018). The experimental design to assess transcriptional within- (WGP) and transgenerational plasticity (TGP) was carried out as described in (Diaz

et al., 2020) and involved acclimation treatments at 36 °C and a control treatment of 25 °C in the parental and offspring generations (Figure 1). The parental treatments were performed in adult samples, while the offspring treatments were performed independently for larvae and adults. For parental acclimation, 10-12 days-old adults were placed in cages with banana-molasses food plates at 36 °C or 25 °C for 24 h (Figure 1). Then, a new food plate was placed in each cage for flies to oviposit at 25°C for another 24 h. Half of these plates containing offspring eggs were immediately placed at 36 °C or 25 °C for 36 h to evaluate WGP in offspring larva. The second half of plates were transferred to vials at 25°C until adult flies eclosed to evaluate WGP by acclimating offspring adults at 36 °C or 25 °C for 24 h (Figure 1). The chosen temperatures and periods correspond to the maximum treatment that triggers a heat-shock response without killing individuals in the process. Groups of 50 individual larva or ten female adults were collected and pooled for each sample in the offspring generation. Three biological replicates were considered per each of the four combinations of parental and offspring acclimation treatments. This generated 12 pooled samples per stage and 24 samples overall. All samples were placed immediately in TRIzol and kept at -80 °C until RNA extractions.

RNA extraction, cDNA library construction, and sequencing

Total RNA was extracted using Direct-zol RNA kit (Zymo Research). Both RNA quality and quantity were inspected on a Bioanalyzer (Applied Biosystems/Ambion). cDNA libraries were created using KAPA Stranded mRNA-Seq Kit according to the manufacturer's instructions. The 24 RNA-seq libraries were sequenced at Novogene Inc. using the HiSeq SBS v4 High Output Kit on Illumina platform flow cells with runs of 2 x 150 bp paired-end reads. Illumina's HiSeq Control Software and CASAVA software (Illumina, Inc.) were used for base calling and sample demultiplexing.

Sequence trimming and mapping

Paired-end reads were trimmed for quality, and adapter sequences were removed with a minimum quality base of Q = 20 and a minimum read length of 50 bp using the software Trimmomatic (Bolger et al., 2014). Trimmed reads were then mapped to the *D. mojavensis* reference genome (Allan and Matzkin, 2019) (SRP190536) using the splice-aware mapper *GSNAP (Wu and Nacu, 2010)* with the option of new splice events detection. Generated *sam* files were converted to *bam* format after indexing and filtering for a minimum mapping quality of MQ = 20 using **SAMtools (Li et al., 2009). These mapping results were then used for all differential expression and alternative splicing downstream pipelines.**

Differential Expression (DE)

We created a gene-level read count matrix for all samples using *featureCounts* (Liao et al., 2014). The read count matrix was filtered for a minimum count cutoff = 3 cpm over at least two replicates per comparable group. All DE analyses were performed using the R package *edgeR (Robinson et al., 2009)* after TMM library normalization. Normalized read counts were analyzed by Generalized Linear Models (GLM) assuming a negative binomial model of read counts. All comparisons were performed using a full factorial design that included parental and offspring acclimation treatments using three biological replicates. Transcriptional TGP was assessed by comparing samples whose parents were acclimated at 36 °C vs 25 °C, while WGP was detected by comparing relative expression changes between offspring samples acclimated at 36 °C vs 25 °C. Genes with a false-discovery rate (FDR) corrected p-value of < 0.05 (Benjamini and Hochberg, 1995) and a $\log_2$-fold-change threshold of > 1.0 were considered significant. **A correlation matrix comparing relative expression levels of significant DE genes was then generated in order to investigate the relationship between transcriptional WGP and TGP.**

Alternative splicing (AS)

We used the *JunctionSeq* (Hartley and Mullikin, 2016)**pipeline in order to detect genome-wide patterns of alternative spliced genes. AS is defined as the relative regulation of isoforms belonging to a multi-isoform gene with respect to a given biological condition (Hartley and Mullikin, 2015). The pipeline is based on differential usage calculated from both exon and junction feature coverages. The pipeline relies on the originally implemented method in *DEXSeq (An-***

***ders et al., 2012)*, which tested differential usage of annotated exons, but extended to splice junction usage and both annotated and non-annotated splicing events. A new flattened GTF annotation file where overlapping features are not allowed was first generated using *QoRTs* (Hartley and Mullikin, 2015). All overlapping genes were merged as composed by a flat set of non-overlapping exons and splice junctions with unique identifiers. *QoRTs* was also used to generate a read count matrix for AS analysis, including three types of read counts per gene as estimated by exons, junction and gene level counts. The generated count matrix was then used by *JunctionSeq* R package** (Hartley and Mullikin, 2016)**to estimate differential exon and junction usage with respect to gene-wide expression. No read was counted more than once in the model since exon and junction dispersions are fitted independently.** As for DE analyses, AS genes were detected if at least one exon or splice junction was differentially used as a result of parental or offspring acclimation at 36 °C vs 25 °C for TGP and WGP, respectively, using three biological replicates. Only features with p-values of < 0.05 after FDR correction were considered significant.

Intron retention rates (IR)

Intron retention is a specific type of AS that is not necessarily captured by *JunctionSeq* and can have different biological implications for the control of gene expression. An intron can be retained in the final mature *mRNA*, coding for a new function (Jacob and Smith, 2017; Monteuuis et al., 2019) or a nonfunctional transcript that is degraded by the nonsense-mediated decay (NMD) (Farlow et al., 2010)**. We investigated whether DE changes due to WGP and TGP involve mechanisms of IR using the *IRFinder* pipeline (Middleton et al., 2017). A new reference annotation was built by removing all overlapping features present in the same strain sense of individual introns and then unique identifiers were assigned to each flattened exon. Only regions with high mapping scores as estimated through simulated reads across the genome were identified and included in the flattened annotation file. A read count matrix with all reads overlapping splice junctions was generated and IR rates were estimated as: IR rate = *junction reads* / (*junction reads* +*intronic reads*) for each sample using the *IRFinder* R package (Middleton et al., 2017).**

Because IR changes are more likely linked to mechanisms of down-regulation by transcript degradation, we tested this hypothesis by estimating IR changes between acclimated samples at 36 °C vs 25 °C for WGP and TGP. We compared IR changes for significantly up- vs down-regulated DE genes. A GLM analysis was performed using categories of up- and down-regulation as independent variables and the level of IR change as the dependent variable for each comparison. GLM analysis was performed following square root transformation to normalize the error distribution and to achieve homoscedasticity.

Functional and evolutionary analyses

Overrepresentation of specific categories of biological functions was investigated using the *GOseq* R package framework (Young et al., 2010) after extracting orthologous genes from the *D. melanogaster* reference genome. This analysis was performed for significant DE and AS genes with transcriptional WGP and TGP in larvae and adults and for the overlaps between the different gene sets.

Acknowledgments

We would like to thank Carson Allan, Joshua M. Coleman, and Nathaniel Talamantes for their assistance in the fly work of this project. This work was supported by the University of Arizona, and an NSF grant (IOS-1557697) to L.M.M. We thank the Catalina Island Conservancy and the United States National Park Service at Organ Pipe National Monument for allowing the original collection of the Drosophila utilized to establish the stocks used in this study.

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Data Accessibility Statement

RNAs-seq reads have been deposited in the Sequence Read Archive under the accession number (BioProject link will be available upon acceptance).

Authors' contributions

FD and LMM conceived the initial idea. The project was designed by FD and LMM. FD performed most of the fly work, library construction and transcriptomic analyses. All authors participated in the data analysis and the writing of the manuscript. All authors read and approved the final manuscript.