Tropical high-altitude insects show limited capacity to handle high temperatures

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Abstract

Growing summer season, increased anthropogenic activities poses a continual challenge to resident species Ectotherms like insects are especially vulnerable to rapid climatic changes. High-altitude tropical insect populations have been rarely examined for their responses to high-temperature. We exposed a tropical highland out-bred population of *Drosophila melanogaster* from the Himalayas to growing summer conditions in outdoor mesocosm units. Population response to thermal changes was tracked over ninety days at phenotypic and genotypic level. Whole genomic resequencing data suggested a clear seasonal allelic shift. Interestingly, the general heat responsive genes were missing in the summer due to monsoon allele shift; an atypical response noted for high-altitude populations. Instead, candidates involved in kinases and phosphorylation emerged as key players. Heat-knockdown time decreased over time indicating a limited ability to handle increasing temperature. Merging data from both allelic shifts and heat-knockdown time indicated a limited capacity for high-altitude insects in handling climate warming.

Keywords: *Drosophila*, Heat, Climate change, Rapid adaptation, High altitudes

1. Introduction

Natural habitats are heterogeneous and organisms come across diurnal and seasonal temperature changes. Understanding how populations respond to environmental variations is a fundamental question in ecological and evolutionary physiology. While it is known that temperature influences the physiology and reproductive rates in a single generation, temperature fluctuation across generations also influences thermo-sensitive life history traits (Klepsatel, Girish, and Gáliková 2020) In particular, *Drosophila* and indigenous species in certain native conditions thrive in a short timeframe and may have compromised the ecosystem (Soto-Yéber et al. 2018). To check this, several hypotheses exist in understanding the phenotypic plasticity that these species show in changing environmental conditions.

Adaptive selection occurs at segregating allelic variants and requires time. There is an alternative mechanism which comes without the cost of selection. Often a single genotype could demonstrate different phenotypes in different environments and this property is known as phenotypic plasticity (Bradshaw 1965). While it is a response to short-term environmental change which could be over seasonal timescales (Bradford and Roff 1993; Brakefield and Reitsma 1991), it has been well documented across insect taxa. For example, dung fly *Sepsis cynipsea* shows body size variation throughout the summer (Blanckenhorn et al. 1999). A seasonal change in color morphs' frequency has been observed in *Adalia bipunctata* beetle (Brakefield 1985). Seasonal changes in color morphs of *Drosophila ananassae* have been found associated with drought tolerance (Parkash et al. 2012). But the genetic basis of phenotypic plasticity and its interplay with seasonal dynamics is poorly understood. Secondly, adaptive tracking has not been given a required importance as it was considered that adaptive evolutionary

change is a very slow process and hard to expect any change in ecological time-scale (Stone, Erickson, and Bergland 2020; Slobodkin 1980).

On the other hand, temporal variations are geographically widespread and are cyclic. Many insect species reproduce multiple times during a growing season and pass through several generations, which could shape the population ecology and trigger rapid adaptations. To understand temporal variations, studies are required which undergo phenotypic plasticity at various timescales (Siepielski, DiBattista, and Carlson 2009). The one basic assumption is that traits which increase fitness in a given environment will be favored. A considerable work has been done along these lines to understand the season dynamics (Rudman et al. 2022a; Dempster 1955) . Unfortunately, all of these studies come from temperate regions where climatic conditions and environmental stress pressure is significantly lower when compared to tropical hot conditions, which are on the threshold side. Furthermore, during fall, temperate regions show more severe environmental conditions when compared to spring, whereas in the tropics, peak summer season is a major determiner to population dynamics. For example, diapause is a temperate phenomenon and never evolved in tropical populations (Schmidt and Conde 2006). There are indeed changes in allele frequencies associated with thermal tolerance and desiccation as some of the genes induce pleiotropic effects.

In the tropics, temperatures often cross permissible physiological limits for insects. Studying thermo-sensitive genomic regions will shed light on molecular mechanisms behind temperature sensation besides understanding insects' physiological responses to future climate warming scenarios. The role of temperature responsive genes (TRGs) in ectothermic physiology is not known much; our understandings are limited and have never been explored under natural conditions and particularly not in high-altitude tropical mountain populations. The powerful

genetic tractability of the *Drosophila* model system has been instrumental in elucidating TRG functions (Venkatachalam 2007). Noxious temperature sensing has evolved at both larval (Daniel Tracey Jr, Wilson, and Laurent 2003) and adult (Xu et al. 2006) stages of Drosophilids.. Since *Drosophila* piggybacks between these thermal preferences, particularly in the early life stages, state of relative conservation among species and unrelated temperature changes at species origin was felt necessary to understand the TRGs oscillations.

In this work we collected a *Drosophila melanogaster* population from a high-altitude locality in Western Himalayas, outcrossed it, released it into the mesocosm units in a tropical orchard set-up with overlapping generations, and sub-sampled it at three different time points (TPs) spanninga duration of ninety days. We aim to demonstrate that direct observation of evolution in ecological time frames could resolve basic questions about adaptation. Therefore we sampled the populations at different time-points (i.e. growing tropical summer season) for whole genome re-sequencing and attempted to address fundamental questions around pace, temporal dynamics, and underlying genomic architecture of populations under heat selection. We attempt to discuss the underlying results of TRGs at different TPs and the raison d'etre behind phenotypic plasticity in the insects for understanding their adaptive evolution. The dynamic responses in these traits and variations at the plasticity level are complex and active areas of investigation to understand how fast organisms are responding to these stresses (Maurya et al. 2021; Rudman et al. 2022b).

2. Materials and Methods

2.1 *Drosophila* collection and maintenance

We used a highland population of *D. melanogaster* which was collected from Western Himalayas (Rohru, Himachal Pradesh, India; 31.2046^{0}N, 77.7524^{0}E, Altitude 1554 meters). The flies were collected using banana baits in the year 2018. Each collected female was placed in a separate tube at the site of collection. Almost 270 such lines were made in the field using females captured during the field trip. Upon 10 to15hr arrival to the lab, vials were placed at room temperature (25^{0}C). The successful lines were examined based on the taxonomic keys. Overall, 40 lines of *D. melanogaster* were successful and were maintained in the laboratory, on standard 'agar-jaggery-yeast-maize' media at 25^{0}C, with a 12/ 12light/dark cycle. All the 40 lines were used in this work.

2.2 *Out-crossing*

Forty isofemale lines of *D. melanogaster* were outcrossed (founding numbers 1000 adults: 500 males + 500 females)in a 0.61m X 0.61m X 0.61m dimension cages made up of plexiglas acrylic sheet. For this, 10 males and 10 females each from 40 isofemale lines were used. Each generation of eggs was collected in twenty half-pint size culture bottles. As soon as the pupae became darker and ready to enclose, bottles were moved to the plexiglass cage and plugs were removed. This allowed random mating in the founding base population. Once all the flies had enclosed in the cage, eggs were collected and transferred to fresh food bottles for the next generation. These lines were outcrossed for 5 generations keeping populations' size between 5-6 thousand individuals every generation.

2.3 *Mesocosm Cages*

To track adaptive changes in real time, and whether or not changes in temperature (increasing temperature over growing tropical summer) lead to changes in allelic frequencies, we tracked populations (3-fold replication) in outdoor mesocosm cages for over the period of 90 days (Fig. 1). For this, we used three outdoor cages (Ahmedabad University Experimental Evolution Station). Each mesocosm was a 1.5x1.5x1.5 meters in dimension (custom designed at Ahmedabad University Fabrication Shop) outdoor insect rearing enclosure surrounding a mature (dwarf) sapota tree. Three cages were used for this experiment (MESO-1, MESO-2, and MESO-3). Each cage was founded with 1000 males and 1000 females collected from the 4th generation of laboratory cage (i.e. outcrossed population). Every morning at 09.00 am fresh food (50 ml of standard 'agar-jaggery-yeast-maize') in a half-pint bottle was placed in each enclosure for the entire duration of the experiment (15th April 2020 to 15th July 2020). Flies were allowed to oviposit on the fresh food for 24 hours. Each morning, bottles with eggs laid in the last 24 hours, were sealed with cotton plugs and larvae were allowed to develop (inside the same cages); upon eclosion, bottles were opened and adults were released into the cages. Thus populations were cultured under a natural regime of overlapping generations. Temperature and RH in the cages were recorded using HOBO U23 Pro v2 data loggers (Onset Computer Corp., Bourne, MA, USA).

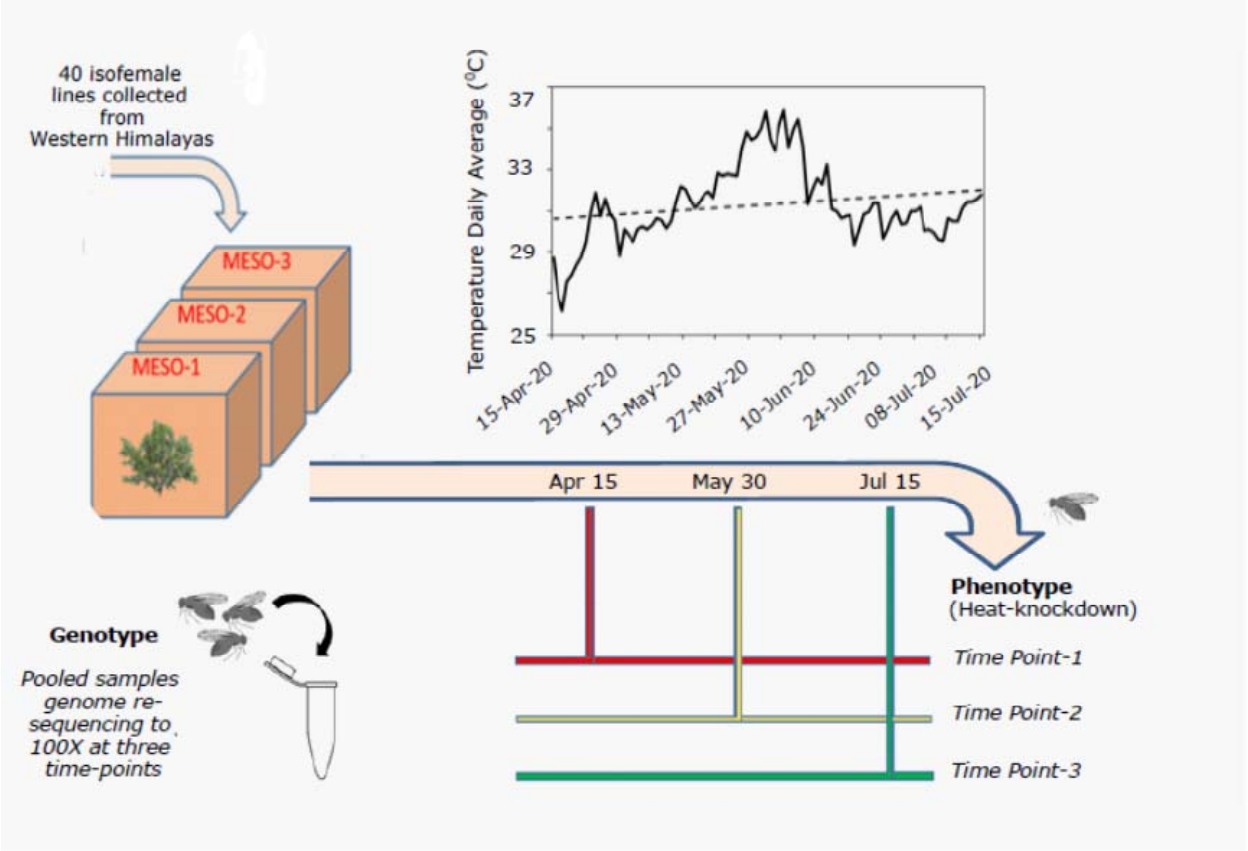

Figure 1. Outdoor mescocosm experimental set-up. Forty *D. melanogaster* isofemale lines were pooled to generate an outbred population. Lines were outcrossed for 5 generations in a 24x24x24 inch dimension cages under laboratory conditions (25^0C). From this outcrossed population (maintaining the size close to 5000-6000 individuals every generation) we released 500 males and 500 females in each outdoor mesocosm cages (MESO-1, MESO-2, and MESO-3). For the next 90 days food was changed every day and the previous bottle with eggs was capped and placed in the cage itself. Upon eclosion adults were released in the existing cage and bottles were discarded. Cages were sampled at three time-points (TP) for genomic and phenotypic analysis: at the beginning (TP1), in the middle (TP2) and at the end (TP3).

2.4 *Adaptive tracking of populations*

Samples for genomic and phenotypic measurements were collected at three time-points for adaptive tracking. Samples for TP1 were collected from the founding adults while the TP2 and TP3 samples were collected on the 45th and 90th day, respectively. Eggs were collected in 30bottles at lower density (10 bottles for each replicate cage). These bottles were allowed to develop under a constant temperature (25°C) in the laboratory and phenotypic data was collected using F3 individuals.

2.5 *DNA sample preparation for genomic analysis*

Pooled genome resequencing was performed with 100 *D. melanogaster* females in triplicate from each outdoor cage, a total of nine samples were collected for whole genome sequencing analysis. The DNA extraction procedures involved homogenizing the flies (n = 100) in 200 µL of lysis buffer (100 mM Tris-Cl, 100 mM EDTA, 100 mM NaCL, 0.5% SDS). The homogenate was incubated at 65°C for 30 minutes. Following this, proteins were precipitated by adding 800 µL of 2 parts 5M potassium acetate, 5 parts of 6M lithium chloride solution and 15 minutes ice incubation. The supernatant was collected after centrifugation of the homogenate at 12K RPM for 15 minutes at room temperature. DNA was precipitated by adding 800 µL of isopropanol and centrifugation at 12K RPM for 15 minutes. The pellet was collected and washed with 70% ethanol. After removing the ethanol, the pellet was allowed to dry at room temperature and was re-suspended in 100 µL of TE buffer.

2.6 *Library construction and quality control*

1µg of DNA was used as input material for the DNA sample preparations. Sequencing libraries were generated using NEBNext® DNA Library Prep Kit following manufacturer's recommendations and indices were added to each sample. The genomic DNA is randomly

fragmented to a size of 350bp by shearing, then DNA fragments were end polished, A-tailed, and ligated with the NEBNext adapter for Illumina sequencing, and further PCR enriched by P5 and indexed P7 oligos. The PCR products were purified (AMPure XP system) and resulting libraries were analyzed for size distribution by Agilent 2100 Bioanalyzer and quantified using real-time PCR.

2.7 *Next Generation Sequencing*

The whole genome sequencing (WGS) of natural populations was done to check the role of mutations in functional candidates. Library preparation and tagmentation was done using DNA-350 bp by default, while the sequencing was performed on a HiSeq PE150. The samples were mixed for library construction using a PCR-free library preparation guide. The downstream analysis and annotation was done with raw reads run through an in-house benchmarked pipeline (Meena et al. 2018), tweaked for a whole exome pipeline (https://github.com/prashbio/WES).

After the allele frequencies were mapped, we read all mutation positions in MESO-1, MESO-2 and MESO-3 for all the three time point scales (T1|T2|T3) and created a data structure which contained three types of positions: single, double and triple mutations. For example, single types of mutations are those that are positioned only once across the 3 different TPs, double- any two TP scales while triple – across all three of them. This was done for MESO-1, MESO-2 and MESO-3 (experimental scale) and the resulting files were checked for interpreting the possible SNPs**.** However, we asked whether mutations that are identical are present at least in 2 time points at the same position and finally a composite matrix containing mutational positions of MESO-1, MESO-2, and MESO-3 was done.

2.8. Heat-knockdown assay

We tested F3 females (ca. 3 days old) for their thermal tolerance to an increasing temperature using the dynamic heat ramp assay. In this assay, temperatures ecologically relevant to the species are the base-point after which they are steadily increased till the fly is knocked down (Lutterschmidt and Hutchison 1997). Individual flies were collected in glass tubes of $0.05m^3$ volume. The tubes were immersed in a water bath set at room temperature (ca. 25°C). Temperature was increased at the rate of 1°C/ minute with the upper thermal limit being 43°C. We measured three technical replicates with 24 flies each from three cages across three time points. Nested ANOVA (experimental cages within time-points) was used to assess the variation in knock-down time. Post-hoc analysis was performed using the *glht* () function from the "multcomp" package (Hothorn, Bretz, and Westfall 2015). For genomic analysis, the flies were aspirated from the cages itself.

3. Results

3.1. Allele frequencies

The MESO-1, 2, & 3 replicates were run through the whole genome sequencing (WGS) pipeline where we inferred causal SNPs across these sub-population (Table 1). The minor allele frequencies were tabulated from the depth with number of reads showing variation (DP4) with 1) forward ref alleles; 2) reverse ref; 3) forward alt; 4) reverse alt alleles, used in variant calling using the formula, variant allele frequency(VAF) = (forward alt + reverse alt alleles) / (forward ref alleles + reverse ref + forward alt+ reverse alt alleles). After setting up the MAF<=0.15 assuming that 15% of the SNPs would have this MAF, we further retained heterozygous SNPs. All 9 raw reads yielded 9,963,857 SNPs per sample. The common most SNPs between the MESO-1, 2, & 3 cages and the outliers were tabulated by mapping the chromosomal positions to

flybase reference genome (flybase.org last accessed date: September 22, 2021) (Table 1/Fig. 2). By removing those specific minor allele frequencies (MAFs) above 0.15, we could assume that there is a clear distribution of contamination. We therefore sought to assess the possibility of observed TRG with MAF <0.15 which gave us an average of 240 SNPs per sample (Supplementary Table 1). This raises the possibility that allele frequency could be generated by *D. melanogaster* alleles consistently at various time point scales. We further assessed the frequency of SNPs that are fixed for alternate alleles and cross checked this with the previously identified SNPs associated with seasonal changes (Temperature) and tabulated all SNPs for time points (Table 1).

Table 1: Average number of SNPs after filtering

Total identified SNPs/	89,674,715/9,963,857
Average SNPs per TP sample	
>=10 DP <=400	5,015,244
Total number of SNPs with MAF <0.15/	3,573,280/397,031
Average MAF <0.15/sample	
Total used in analysis/	5,015,244/ 240
Average bona fide SNPs per TRG	

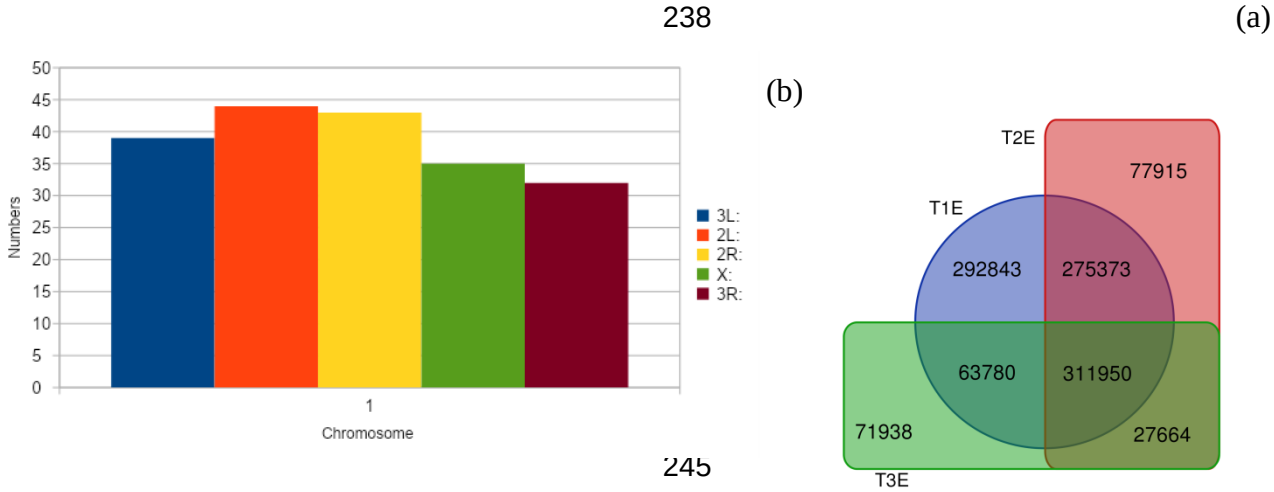

Figure 2. (a) Flybase SNPs mapped to our experimental candidates across all chromosomes (b) The SNPs mapped across the three time point experimental pools. The numbers indicate the individual time point SNPs apart from the most common SNPs at the intersection.

3.2. Positional mapping with Flybase SNPs

The downstream annotation was processed with chromosomal alias obtaining 1870 total number of scaffolds with an average length of 17,195,935 per chromosome. The chromosomal distribution of SNPS is largely at the interface of 2L and X (Data not shown; Supplementary Table 1, Fig. 3). We also mapped the SNPs to that of reported 193 SNPs from flybase to check if TRG mutations exist. We found Single TP SNPs: (n=noPOS: 4,666), Double TP SNPs: POS: (n=2,536) and no Triple TP SNPs (n=POS: 0).The final list of protein coding genes were mapped across various chromosomes (Supplementary Table 1). As the current analysis represented

identifying common mutations, we further looked into mapping the existing known SNPs (for heat stress).

3.3. Population comparisons

The time point samples and replicates mapped to the temperature regime were treated as T1_MESO-1, T2_MESO-1 and T3_MESO-1 which we call them as populations. As we obtain the data, the coverage across these three sub-populations were filtered for common mutations or SNPs that have been retained from T1_MESO-1,2&3 through T3_MESO-1,2&3 or the unique set of SNPs through them (Supplementary Table 1). While removing the SNPs with total counts with the same allelic proportion, we deem that they have not undergone any changes through the three experiments. On the contrary, those SNPs that have an overlap with changes in SNPs across the same positions were filtered and we therefore limited the downstream analysis to such overlapping SNPs from three sub-populations or experiments. As we combined all the reads, we have checked the significant differences between the experiments for overlapping SNPs using the chi-square test as a standard practice. The *p*-value heuristics resulting from the analysis based on three population comparisons were in agreement. Another approach was to check the SNP mean density which could be done using an existing method (proposed by Burke et al 2010 (Burke et al. 2010); Winbush & Singh 2021 (Winbush and Singh 2021)) which measures the quantile score but our study is not a measure of nucleotide diversity from a threshold value instead the number of SNPs emerging from a whole genome sequencing study.

While we have found SNPs that have been deleted from T1 to T3 (Supplementary File 2), the MAFs in earlier cases were set to as big as MAF<0.89 which is 89%. Our stringent cutoff (Winbush& Singh, 2021) indicates that highly sensitive SNPs emerge from our analysis and

when these were tried to map them to flySNP base, none or not many of them are mapped indicating that these are novel (data not shown). Nevertheless, we lacked sequencing data on similar scales where whole genome sequencing (WGS) was done so that we could have identified convergent or divergent regions contributing between the three sub-populations. For each comparison, we observed that 77,915 (24.97%), 71,938 (23.06%) and 27,664 (8.8%) SNPs in the respective three time points existed indicating that the temperature affects the sensitivity of the flies. We then mapped these SNPs to summarize the genes that have escaped the mutations in T3_MESO-1, 2 &3. When we map them to the table depicting counts for genes associated with these SNPs for T3_MESO-1, 2 & 3, we observe that a large number of diseased phenotypes are associated with these conditions. However, despite consistent read depth of coverage and SNPs, we observe a partial overlap suggesting that the accorded convergence can be attributed to slightly reduced heterozygosity which may invite a bias. However, this was reconfirmed from our validation set of founder experiments.

As we identified the genes with diseased phenotypes we were interested to see whether or not the candidate loci of these genes have an influence on the pathways. To check this, our panther gene ontology annotation derived enriched genes unique to temperature. In summary, our analysis identified a large number of candidate genes and SNPs associated with heat handling wiring machinery. Rather, our approaches indicate the variation across different subpopulations, as we argue that several diseased phenotypes have emerged from our analysis. Taken together, temperature sensitivity plays a very important role and serves as a determinant to mesocosm experimental study. Although genetic drift augurs well for taking temperature as a context, functional validation for observational variation is presumptive for maintaining such

selection pressure. It may be argued that the function of the genes stemming from allelic variation has allowed us to determine candidate TRGs.

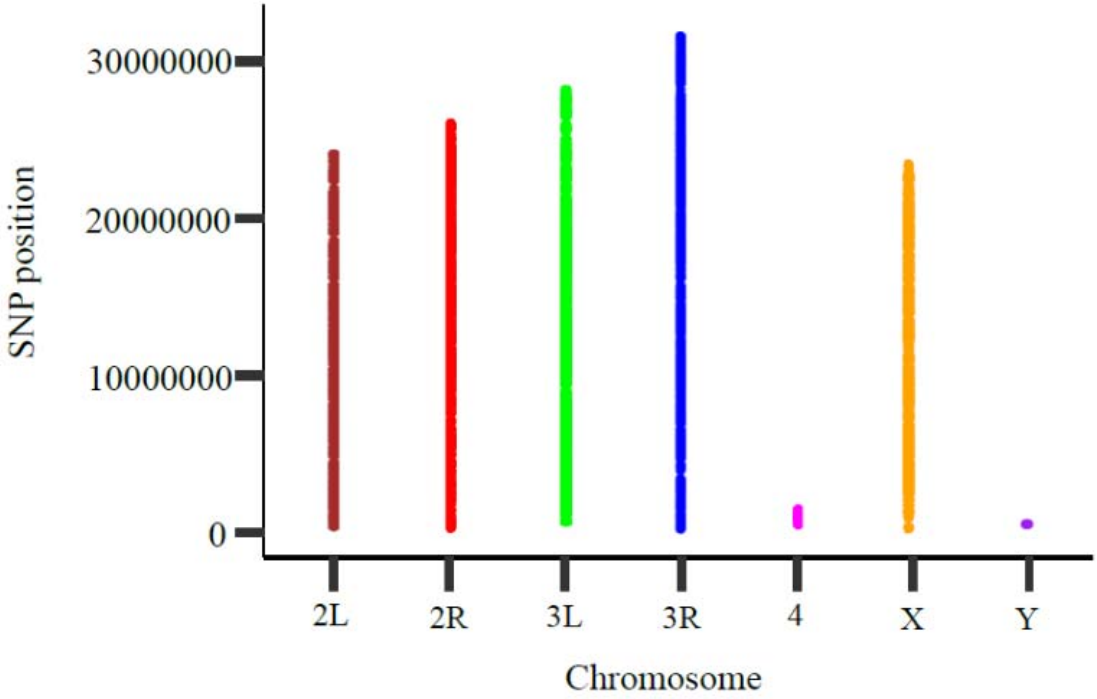

Figure 3. Variable polymorphisms shown to have a large deviation from time point scale 1 to time point 3 taking into account read depth and the number of sampled chromosomes. The X-axis shows the chromosomes with the Y-axis showing the single nucleotide polymorphism (SNP) positions.

To check genomic signatures affected by seasonal fluctuations, we pooled sequencing data containing the SNPs by estimating the allele frequencies (AF). From each population and time point, the three replicate cages were merged and the files were segregated to average read depth of 20–200× coverage (Supplementary Table 1). We observed that these estimated AFs

have been shown to be accurate for understanding the magnitude of genetic variation through the time point scales. From Fig. 1, we reason that variable polymorphisms had a large deviation from time point scale 1 to time point 3 taking into account read depth and the number of sampled chromosomes. Of the 3,119,540 common SNPs tested, we identified substantial numbers falling between the statistical values, meaning they are called integrated thermal responsive SNPs. However, the AFs corresponding to selection coefficients per generation were not done with this statistical power as they are variable. Our rationale was to assess evolutionary features underlying rapid adaptive states in response to selection pressure. As the data is variable with the random disturbance and is different across elements of the vector, we asked whether the SNPs are enriched among functional genetic elements. To find out whether or not the SNPs are genic, we screened total SNPs present on specific genes common to these time points. A putative interaction map indicates that the genes are largely spread across with the key pathways attributing to these factors are MAP kinases and other important signaling pathways (Fig. 4). In general, we find four important genes and a few un-characterized genes which could be attributed to pleiotropy and linkage disequilibrium largely due to TRGs/seasonal variation.

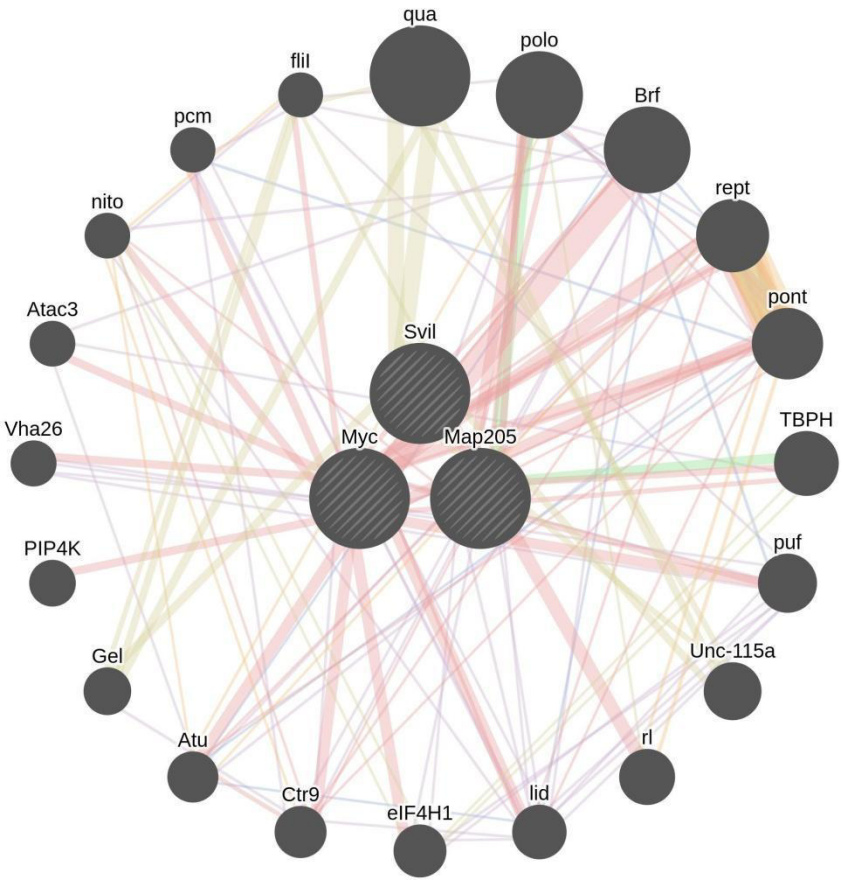

Figure 4. A putative interaction map of Svil, Myc and MAP205 with interacting partners connected through edges (lines). The pink edges indicate that they are known to be physically interacting with each other while other edge colors denote pathways, domain association, co-localization, neighborhood and genetic interactions if any.

3.4. Heat tolerance

Knock-down times of flies significantly differed among all three time-points (df = 2, F= 966.30, P < 0.0001) (Supplementary Table 2). Heat knockdown time was significantly lower for flies from T2 (middle time-point) (median = 60.38 minutes, interquartile range (IQR) = 4.55, N = 72) in comparison to early (T1) (median = 73.22 minutes, IQR = 3.07, N = 72) (estimate = -

13.151, t = -27.115, P < 0.0001) and late (T3) (median = 65.39 minutes, IQR = 3.71, N = 72) (estimate = 4.275, t = 8.814, P < 0.0001) time-points. Variation in heat knockdown response is partially explained by variation in experimental cages too (df = 6, F= 42.12, P< 0.0001) (Fig. 5).

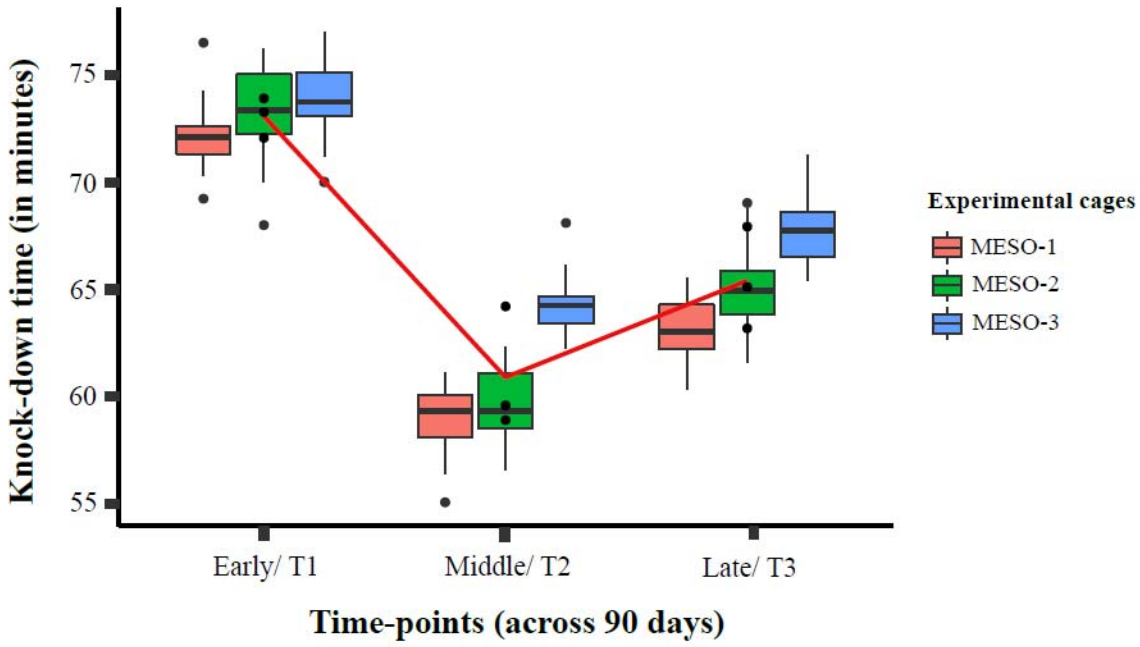

Figure 5: Box-plots representing variation in knock-down times in response to heat ramping assays, across three time-points (early, middle and late) from three replicate experimental cages. Flies from the middle time-point (45th day) display the significantly lowest tolerance to increase in temperatures compared to the early (day 0) and late (90th day) time-points.

4. Discussion

An organisms' response to changing environmental conditions determines its fitness. Adaptations to rapid environmental fluctuations are known (Franks and Hoffmann 2012).

Responses to rapid environmental change could be micro-evolutionary or mediated through phenotypic plasticity (Sgrò, Terblanche, and Hoffmann 2016). Trait plasticity in response to climate change has been documented across diverse taxa, e.g. in birds (Charmantier et al. 2008), mammals (Nussey, Wilson, and Brommer 2007), and insects (Ragland and Kingsolver 2008). Still, climate-mediated-response studies display a temperate geographical bias (Feeley, Stroud, and Perez 2017). For example, a "global" study (Seebacher, White, and Franklin 2015) found physiological rates (Q_{10}) of key metabolic enzymes to be on the rising trend in the past few years (calculated from 1990-2010). The authors however admit biased conclusions owing to a greater representation of sampled studies from North America and Europe in contrast to tropical regions. Again, while temperature is the central focus of most of these climate-mediated-response studies, the confounding effects of another strong seasonal predictor – photoperiod -- are often overlooked (Bradshaw and Holzapfel 2008). Diapause, an adaptation to survive freezing winter temperatures but sensed through shorter photoperiods (Manenti, Sten, and Loeschcke 2021; Danks 1987) is common for temperate rather than tropical species (Denlinger 1986).

Contrarily, tropics exhibit lesser variation in photoperiod compared to temperate regions. Climate in the tropics is generally described as warm and humid, although higher altitudes experience cooler temperatures. Besides, tropical landscapes span diverse ecosystems ranging from desert, scrub woodlands to moist deciduous rainforests. Seasons across these diverse landscapes are largely under the influence of both temperature and precipitation changes in the tropics. Temperature fluctuations throughout the year are generally less pronounced in the tropics in comparison to temperate regions (Janzen 1967). However, the warming and cooling phases of El Nino and La Nina, respectively could affect intra-annual climate variability in the

tropics (Malhi and Wright 2004). Further, since tropical species have evolutionarily faced low temperature fluctuations (Sheldon et al. 2018), a small degree of temperature rise could pose challenges in terms of survival and fitness (Deutsch et al. 2008). Thus, climate mediated temperature responses may strikingly differ in the lower latitudinal tropics versus temperate regions at higher latitudes (Sheldon 2019).

Global warming is predicted to induce habitat shifts and lower altitude/ tropical populations are less likely to be buffered to thermal extremes unless they shift poleward (Kellermann et al. 2012) (but see (Overgaard, Kearney, and Hoffmann 2014)). The question of how tropical organisms with narrowly adapted thermal ranges will cope with temperature fluctuations imposed by rapid climatic change needs inputs from multiple perspectives. Our study reports the genetic variation linked to TRGs in a tropical *Drosophila* species and thus paves way for further research into thermal acclimation in tropical species.

While temperature may be the proximate cause influencing thermal acclimation, the ultimate modulators are molecules sensing temperature changes. While adaptive response studies test phenotypic outcomes (e.g. through thermal performance curves), studies correlating this ability to adapt to the underlying genetic variation are still far and few. In recent years adaptive responses to thermal changes have been reflected through the gene expression profiles too (Kelly, 2019). Thus, selecting candidate genes responding putatively to thermal stress could provide crucial cues in understanding thermal acclimation (Sørensen and Loeschcke 2007). We therefore aimed at targeting TRGs as candidates to check allelic variation across the growing season under tropical conditions.

We used whole genome sequencing and filtered common SNPs for TRGs across the three time-points (T1 to T3). Results demonstrate considerable allelic variation across the three time-points. Temperature would increase from T1 to T2 and then decrease from T2 to T3. We therefore surmise that allelic variation could execute through physiological plasticity in response to temperature extremes in this tropical species. Higher allelic variation was observed in the first two time-points in comparison to the third (T3), where temperature could plateau due to the onset of monsoon. Further studies could determine if the observed allelic variation corroborates positively with survival and fitness in the peak hot season. In temperate orchards, considerable allelic variation with respect to seasons (winter versus fall) has been noted (Bergland et al. 2014) maintained through balancing selection. However, in this case, considerable allelic variation for temperature responsive genes was seen within a span of 90 days (corresponding to peak summer season). It therefore remains to be analyzed if selection is indeed strong, relaxed or balancing through seasonal oscillations like temperate counterparts.

Plasticity may incur costs (Krebs and Loeschcke 1994). These costs along with other constraints (e.g. biochemical) may limit thermal acclimation (Seebacher, White, and Franklin 2015) and should be further investigated. While the mechanistic basis of temperature regulating TRGs is still being explored (Singh et al. 2019), our study which reveals novel SNP's in *Drosophila* TRGs opens up new avenues for testing thermal acclimation hypotheses. The pleiotropic effect of temperature sensation on other functions can be seen through the association of different pathways integrated through the protein interaction map. Nonetheless, genes associated are novel and not typical of those associated with thermal acclimation, e.g. heat shock proteins (Sørensen and Loeschcke 2007).

We found two independent lines of evidence: First, a large number of kinases and phosphorylases are associated with stress, possibly linked to heat/temperature as evident from the protein interaction map (Fig. 4). Second, the large number of key regulators and transcription factors that are associated with cell proliferation and growth. Underlying these, *Myc* emerged as a key gene with the interactants possibly associated with genetic arrangements, and most importantly reducing the mutational load or burden. This is also in agreement with the fact that the *Myc* plays a key role in insufficiency of haplotypes reducing the mutation load which further is the key factor for extended lifespan or pro-aging (Greer et al. 2013; Morrison, Murakami, and Cleghon 2000). On the other hand, phosphatidylinositol 5-phosphate 4-kinase (PIP4K) is known to regulate the growth during fly development (Gupta et al. 2013). While its activity is implicated in cellular responses, regulating the growth factors, the protein interaction networks clearly reveal that there are transient mutations underpinning the changes in heat/temperature stress as these changes could be because of the activity of downstream kinases. We envisage that the pathways associated with PIP4K switch signals modulating the strength of a signal. Taken together, the kinases and phosphatases contribute to the outcome or ability to acclimate of the flies in these conditions. As they adapt, essential regulatory processes and changes mediating phosphorylation and kinases are known. Further, our genomic sequencing has emerged as an approach to understand such “adaptive loci" influencing these signaling pathways.

Mesocosm experiments as ours closely resemble natural conditions are hitherto reported only in temperate populations (e.g. (Rudman et al. 2022b)) but not for tropical species. Ours is therefore the first study to track genetic variation at more realistic and natural time-scales in a tropical fruit fly species. We considered a time frame where summer temperature steadily increases, peaks and then wanes. Our results from heat ramp assay corroborate this, in that

increase in temperatures of the external environment (cages) also altered the thermal sensitivity. Thus, the peak in the temperature during T2 (Figure 1) also corresponded with flies from T2 being the most sensitive to thermal stress and hence the faster knock-down time in T2 compared to T1 and T3 (Fig. 5). However, further studies could ascertain if seasonal variation in alleles related to TRGs are connected/ pleiotropic with fitness-related traits. Thus, in the temperate regions, diapause associated genes are known to be up and down regulated corresponding to winter and fall seasonal variation, respectively (Zhao et al. 2015) with outcomes in fecundity (ovariole size (Schmidt et al. 2005)). In tropical scenarios, where adaptations like diapause are less known, one could expect adaptivity to thermal extremes to evolve through alternate gene regulatory networks. Since *Drosophila melanogaster* originated from Africa (David and Capy 1988) and then dispersed to other continents, reaction norms for temperature tolerance could be expected to be broadly similar across the tropics than across temperate regions. Surprisingly, our study populations from higher altitudes (Rohru, western Himalayas) exhibited limited tolerance to higher temperatures, thus re-affirming the speculation that tropical species are indeed sensitive to higher temperature thresholds. It remains to be tested if differential response to temperature extremes does exist across altitudinal populations. Given clinal variation for desiccation resistance of Drosophilids from the Indian subcontinent (Rajpurohit et al., 2013), exploring thermal tolerance and associated genomic variation is an interesting research avenue.

Seasonal changes in the tropics are also linked to rainfall. Compared to temperate regions, tropics exhibit striking changes in humidity from ca. 50% (summer) to ca. 90% (rains) across seasons. Few studies report the impact of temperature-humidity interactions on life history traits in the tropics. Thus, high (not moderate) temperatures in conjunction with low relative humidity have been shown to lower fecundity in fruit flies (Maurya et al. 2021). Higher

temperatures trigger up-regulation of genes related to desiccation tolerance (Rajpurohit et al. 2013). Flies under high desiccation stress displayed a lag in mating initiation time (Arya et al., 2021). It is possible that the lowered fecundity of females at high temperatures could be due to effect of temperature individually in the male sex (low sperm count) (David et al. 2005). Temperature-humidity interactions could thus affect both mating behaviour and gametogenesis, crucial for reproductive fitness. Sexually dimorphic role of TRGs through temperature is therefore an interesting avenue for future work.

Conclusions

Tropical species are likely to be affected by global increase in temperature since the thermal range to which they could respond is narrow compared to their temperate counterparts. Our study hints that the narrow temperature tolerance of tropical species could be linked with alternate molecular pathways for thermal sensitivity. We found a considerable number of newer candidate alleles whose frequencies are significantly shifting as temperature increases over the growing season. Our study highlights that genomic signatures could be corroborated with field based studies to understand organismal responses to changing environmental conditions. Our findings of allelic variation have implications not only in tracking fitness outcomes across seasons but also in pest management. Future work could combine molecular and field based approaches for a comprehensive understanding of climate mediated changes.

Competing interests: None to declare.

Authors' contribution

SR: Conceived the idea, Experiment design, Experimentation, Managing resources, and Writing the manuscript.

PSS: Raw data processing, Reads collection, and Writing code

HM: Analyzing data, Writing the manuscript

HA: Data collection

RA: Figures, Reads collection

PS: Raw data processing, Reads collection, Writing code, Writing the manuscript

VL: Conceived the idea, Managing Resources, Writing manuscript

Data Availability

All supplementary files are deposited in the Dryad repository https://doi.org/10.5061/dryad.7pvmcvdwr .

Raw sequence reads of the genome will be made available through NCBI upon manuscript acceptance or upon reviewer request

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