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Tânia Alexandra Pereira Dias

Insights on the Heat Resistance of *Drosophila melanogaster*

Contributos sobre a resistência ao calor de *Drosophila melanogaster*

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Contributos sobre a resistência ao calor de *Drosophila melanogaster*

Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biologia Molecular e Celular, realizada sob a orientação científica do Doutor Jorge Vieira do I3S - Instituto de Investigação e Inovação em Saúde e do Doutor António Calado do Departamento de Biologia da Universidade de Aveiro

“Everything is theoretically impossible until it is done”

Robert A. Heinlein

o júri

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Palavras-chave *Drosophila melanogaster*, resistência ao calor, *CG2224, CG6553, CG1146, GluRIA*, adaptação local, gradientes geográficos

Resumo

Compreender as bases da adaptação local tem sido um dos maiores problemas das ciências da vida. Neste estudo procurou-se compreender os mecanismos por de trás da resistência ao calor da mosca cosmopolita, *Drosophila melanogaster*.

Ao olhar para 168 estirpes, pertencentes a nove populações da Europa de *D. melanogaster*, foi possível caracterizar a variação local do fenótipo resistência ao calor, e relacioná-lo com gradientes geográficos assim com variáveis bioclimáticas. No entanto, apenas os machos parecem mostrar uma correlação com a latitude. Aparenta também haver forças seletivas diferentes a agir sobre os dois sexos, com machos a serem impactados pela temperatura e fêmeas pelo efeito combinado de temperatura e precipitação.

E ao olhar para SNPs nas regiões 5' não codificantes de genes com altos coeficientes de correlação com a latitude e longitude, foi possível identificar genes candidatos à resistência ao calor, utilizando estirpes RNAi para testar o efeito da supressão do gene no fenótipo. Estabeleceu-se, assim, um protocolo que com sucesso identificou genes com impacto fenotípico a partir de variação local.

Keywords

Drosophila melanogaster, heat resistance*, CG2224, CG6553, CG1146, GluRIA,* local adaptation, geographical gradients

Abstract

Understanding the basis of local adaptation has been one of the most challenging problems of life sciences. Here, an effort was made to try to better understand the molecular mechanism implicated in the heat resistance of the cosmopolitan fly, *Drosophila melanogaster*.

By phenotyping 168 strains, from nine European populations of *D. melanogaster* it was possible to characterize local variation of the heat resistance phenotype. As well as correlate that variation with geographical gradients, such as latitude and longitude, and bioclimatic variables. However, males appear to be the only ones differentiating under a latitudinal cline in Europe. At the same time, both sexes seem to be under different selective forces, with males being influenced mainly by temperature, while females are under the combined effect of temperature and rainfall.

By looking at SNPs in the 5'- non-coding regions of genes with high coefficients of correlation with latitude or longitude, it was possible to identify novel candidate genes to heat resistance, using RNAi strains to suppress gene expression. A protocol was established that successfully identifies genes with phenotypical impact, by looking at local variation.

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List of Abbreviations

%CV – Percentage of coefficient of variance

ATP – Adenosine Triphosphate

ADAR *- adenosine deaminase acting on RNA*

bp – base pair

cDNA – complementary DNA

cpo *– couch potato*

CT_{max} – Critical thermal maximum

CT_{min} – Critical thermal minimum

DNA – desoxyribonucleic acid

dNTPs - Nucleoside triphosphate

DTT – Dithiothreitol

ER – endoplasmic reticulum

HS – Heat Shock

Hsc70 *– heat shock constitutive protein 70*

HSF *– Heat Shock Factor*

Hsp *– Heat Shock Protein*

Hsp22 *– Heat shock protein 22*

Hsp23 *– Heat shock protein 23*

Hsp26 *– Heat shock protein 26*

Hsp27 *– Heat Shock protein 27*

Hsp33 *– Heat shock protein 33*

Hsp40 *– Heat shock protein 40*

Hsp60 *– Heat shock protein 60*

Hsp70 *– Heat shock protein 70*

Hsp90 *– Heat shock protein 90*

HspB8 *– Heat shock protein B8*

LDL – low density lipoprotein

max – maximum

mth *– methuselah*

$MgCl_2$ – Magnesium chloride

min – minimum

miRNA – micro RNA

mRNA – messenger RNA

PCR – Polymerase chain reaction

RNA – Ribonucleic acid

RNAse – Ribonuclease

RNAse OUT - Recombinant Ribonuclease Inhibitor

RPL32 - *Ribosomal protein L32*

rpm - revolutions per minute

RT buffer - Reverse Transcription buffer

sHsp – *small Heat Shock Protein*

SNP – Single Nucleotide Polymorphism

tim – *timeless*

T_{max} – Maximum temperature

T_{min} – Minimum temperature

Topt – Optimal Temperature

U – unit

ULT – Upper lethal temperature

Chapter 1 – *Introduction*

1. *Drosophila melanogaster* – an optimal model system

Drosophila melanogaster, commonly known as the fruit fly, has been used in laboratory experiments for almost 100 years. Although, it is not easy to pinpoint the exact time of its transition from the fields to the laboratory, its increased popularity begun with the works of Thomas Hunt Morgan, the Nobel Prize winner of 1933 with the finding that allowed him to prove the theory of inheritance by showing that the *white* gene resided on the X chromosome[1].

Besides its small size and short life cycle, which make it easier to manipulate and achieve large numbers of individuals in a small-time scale, at a relatively low cost, its genome is also much smaller than the average mammalian, with only 3 autosomal pairs of chromosomes, plus one heterosomal pair, all very well documented. Moreover, the continued interest and investment in *Drosophila* has allowed the development of a wide range of tools that facilitates work in this organism[1, 2].

Although it is now known as a cosmopolitan organism, present in every continent and most islands, except for extreme environment regions, there is ample evidence suggesting that *D. melanogaster* originated from the sub-Saharan Africa. It is currently believed that around 10,000 to 15,000 years ago, a climatically suitable window was created that allowed the flies to migrate northward into the Mediterranean region and further into Europe and Asia[3, 4] were it suffered a severe population bottleneck[5].

The expansion from the tropics, required from *D. melanogaster* an adaptation to the seasonality of temperate habitats in order for it to successfully colonize all types of environments (Figure 1). The need to respond to this change had been showed many times through the presence of single-gene polymorphism and quantitative trait variation[1, 6]. Today, *D. melanogaster* populations extend as far south as Tasmania and as far north as Finland[5].

Recent evidence has suggested that colonization of North America and Australia happened later, likely facilitated by human travels in the last 150 years, with the former being the convergence of flies originating from both Africa and Europe[5, 7, 8].

With such a wide distribution, *D. melanogaster* has become a model organism for studies of local adaptation.

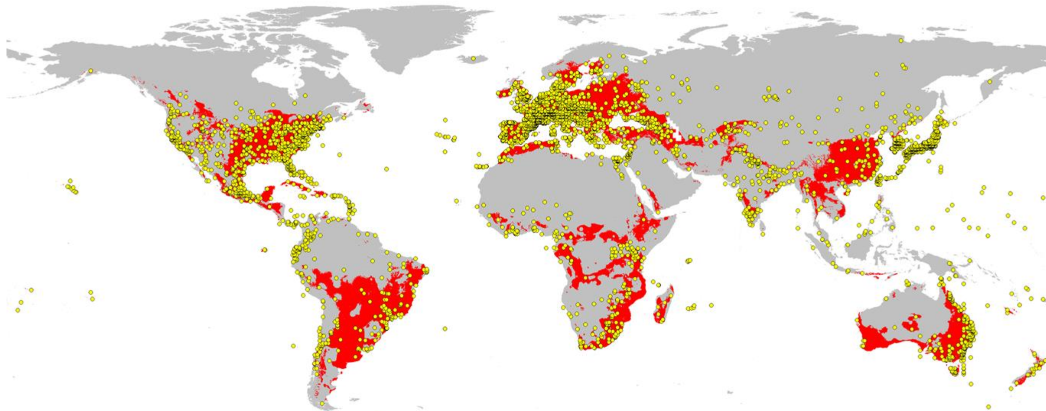

Figure 1 - D. melanogaster distribution worldwide (from http://evolution.ibmc.up.pt/) In red is represented the predicted distribution based on bioclimatic variables; in yellow the sites where the species have been sampled.

2. Environmental variables impacting dispersion

When a species moves to a new environment, it must adapt to a panoply of potentially stress factors ranging from food supply, predation, to environmental variables such as temperature, precipitation, solar radiation, winds, daylight hours and several others.

Since all these variables cause stress upon the organism, it is possible that in nature they act as selective forces for both the genotype and phenotype. Several studies have been done on the strength of these variables as selective forces driving local adaptation.

Below, some climatic variables of interest are investigated in their importance as selective forces.

2.1 Temperature

Because temperature changes not only spatially, but daily and with a seasonal pattern, it requires from an individual the ability to survive and adapt to constant changes in the temperature of its environment throughout the year. Furthermore, since temperature affects virtually all biochemical and physiological processes of an organism, it rises as one of the most relevant environmental factors dictating both survival and dispersal of species[9].

In fact, in a recent study of European and North American populations of *D. melanogaster,* temperature was implicated as the most important environmental variable, creating a selective force over those populations in conjunction with wind and rainfall[10].

Another study in populations of *D. melanogaster* from Maine and Florida in North America, found that 67% of total variation in fly dormancy was due to temperature[11].

Interestingly, studies on fly's body size have also reported the phenotype as under a selective force driven mainly by temperature. With high temperatures favouring small bodied flies, and lower temperatures favouring larger ones[12, 13]. Likewise, fecundity, despite not showing a direct correlation with body size, has been reported as differentiating under a temperature selective force[13].

Desiccation tolerance was also found to be strongly associated with the minimal temperature the populations are exposed to[14].

Moreover, in the Indian subcontinent, temperature was also found to be one of the most important forces acting selectively over many of the studied phenotypes[15].

2.2 Humidity

The concentration of water vapour present in the air of a given environment can also severely impact the performance of the fly. Low humidity can lead to rapid desiccation by the fly, whose water content is relatively small, which will induce stress and can possibly lead to death.

A study on the humidity effects of the environment on *D. melanogaster*'s ability to survive exposure to high temperatures showed that it significantly impacts the fly's ability to cope with changes in the environment[16]. It appears that high humidities are not limiting to the fly's distribution, since they can shelter themselves to avoid the stress, but drier conditions create a more stressful heat environment[17].

Taken together, humidity will act as a constraint to species dispersal.

2.3 Precipitation

Because precipitation can lower overall body temperature, irrespective of the environmental temperature, it has also been investigated in its role as a natural selective agent for stress resistance phenotypes.

In 2012, a study on 94 *Drosophila* species, found a negative correlation between annual precipitation and the upper limit of permissive temperatures. Here, species from hot and dry regions, needed a higher heat resistance, while in wetter regions, species distribution was mainly unrelated with temperature[18].

Furthermore, a recent analysis performed on data collected on European populations of *D. melanogaster* found rainfall to be the second most important selective agent defining species distribution[10].

2.4 Daylight hours

Circadian clocks of animals have evolved to a circannual clock which anticipates not only daily changes but yearly environmental ones, in order for the organism to time specific activities.

It is important, especially for small animals whose ability to produce and retain heat is reduced or non-existent, to be able to predict such changes and prepare for them. For example, it may be more favourable for insects to be more active during the warmer periods of cold days, while in hot days, activity might be more favourable in the early morning or late afternoon to avoid heat stress. In fact, this is such an important effect that behaviour in *Drosophila* is heavily affect by the number of daylight hours[10].

Moreover, multiple associations between geographic gradients and genes known to affect the circadian rhythm of the fly have been identified[19] which suggests relevance of this variable in local adaptation.

3. Thermal Resistance

Thermal tolerance is considered a life-history trait, or fitness component, that can be defined as a part of a phenotype for which the increase of value on a trait will lead to an overall better fitness if all other traits remain unchanged. Its importance is also related with its dependency from other life-history traits such as developmental rate, size at reproductive maturity, number of offspring, physiological and even genetic level, meaning that the improvement of one trait, could lead to the undermining of another[20].

Insects are often seen as ectoderms, which means they rely heavily on their environment to regulate their own body temperature. However, in reality, flies can modulate their physiology as a way to withstand unfavourable temperatures. For example,

by ventilatory mechanisms via the tracheal system, insects can achieve a lower body temperature than that of their environment, or cool themselves by increasing water evaporation[21]. Furthermore, they can also alter the sensitivity of certain traits to allow them to survive in the new thermal environment[21]. At the same time, ectoderms also display behavioural changes when confronted with temperatures they cannot support. When in high temperatures adult *Drosophila* will often move to micro-environment with more tolerable conditions in order to regulate their body temperature[22], and when unable to do so, the fly will vary its positions, alternating which body parts are exposed to the heat or cold source, or even flap appendages as a way to force cool air to circulate and help with its cooling[21].

The thermal performance of the fly is most often described by thermal performance curves, which are defined by parameters of biological relevance such as maximum performance temperature, i.e., temperature at which performance is maximal, the optimal temperature (T_{opt}) and the critical thermal limits at which performance is permitted but not optimal, CT_{min} and CT_{max}.. Considering that CT_{min} is the minimum temperature where normal functions are permitted, and CT_{max} the maximum temperature, with the optimal temperature somewhere in the middle, the thermal performance curve will have a somewhat bell shape, with an exponential increase as temperature rises towards the fly's optimal window, followed by a rapid decrease in performance as it moves higher than the permitted temperatures[23, 24]. Furthermore, the upper lethal temperature (ULT) is often very close to the CT_{max}[25]. In short, although life is permitted in a broad range of temperatures, most species will have their maximum performance restricted to a narrower range.

3.1 The challenges of high temperatures

When an organism comes in contact with a sudden increase in temperature, they must adapt quickly or risk suffering cellular and physiological damage. As a major stressor, heat presents itself as a limiting factor to life's distribution.

Amazingly, only a few degrees above optimal growth temperature (~25ºC for *D. melanogaster*[26]) represents a challenge for the organisms' survival and will trigger a heat shock response. This happens mainly because throughout evolution, proteins have been optimized to be stable only at the individual's growth temperatures. The increase in temperature, as small as it might be, will immediately cause proteins to lose their 3-

dimensional structure and, consequently, stop working. In fact, this phenomenon is so relevant, that many of the morphological and phenotypic effects of heat-induced stress can be explained by imbalance of protein homeostasis[27].

Furthermore, beyond the scope of unfolding proteins, high temperatures will cause internal disorganization of the cell. One of the major consequences being that damage is incurred to the cytoskeleton leading to loss of function of several organelles, for example, both the Golgi system and the endoplasmic reticulum become fragmented. Severe heat leads to aggregation filament-forming proteins, which results in the collapse of intermediary actin and tubulin networks. There is also a significant loss of mitochondria, which has been associated with the drop of ATP levels in response to exposure to heat[27, 28].

However, high temperatures will also disrupt RNA splicing and the sites of ribosome assembly will swell, causing large granular depositions composed of incorrectly processed ribosomal RNAs and aggregation of ribosomal proteins[27, 29]. There is also an accumulation of nontranslated mRNAs, translation initiation components and proteins related with mRNA function in large RNA-protein structures[27, 30]. In addition, the lipid bilayer also suffers changes, including alteration in membrane fluidity, higher permeability, that results in the acidification of the cytosol as well as the compromise of ion homeostasis within the cell, and changes in the ratio of protein to lipids with a decrease in lipid stores[27, 28, 31].

Depending on the duration and severity of the heat-induced stress, these effects can result in arrest of the cell cycle or even cell death [27, 32].

4. Heat Resistance

Tolerance to high temperature, i.e., heat resistance, varies greatly both among individuals and within populations. And it is affected by a multitude of factors such as fly age and life stage, wing morphology, body size, body colour, food and oxygen availability, previous history of acclimation by the parents and even photoperiod[21].

When *D. melanogaster* is first exposed to temperatures above its optimal maximum, thermal receptor neurons from the peripheral nervous system, present in the antennas are activated. In each antenna, *D. melanogaster* flies possess three heat neurons, as well as three cold ones. The former being activated by heat and inhibited by cold, while the latter is activated by low temperatures and inhibited by high ones[33].

As soon as the thermal receptors are activated, there is a release of neurotransmitters, neuromodulators and neurohormones in order to activate the central nervous system. Neural projector neurons which project to the protocerebrum, the lateral horn and the mushroom bodies, all together, create an internal image of the environmental temperature in order to build an appropriate response. This can be an induction of changes in activity of the flight muscles, behavioural changes, an increase on the water evaporative loss through spiracles, changes in the metabolic rate and the anaerobic metabolism or, at the small scale the induction of heat shock genes[21, 33].

4.1 The Stress Response

Heat shock (*HS*) genes, also known as stress genes, are activated as the first defence of the organism against various stress factors. This molecular response acts as a transient gene-expression program which is very rapidly activated.

Although, all *HS* genes are upregulated when the fly is under stress, they can generally be divided into two categories, constitutive, i.e., genes that under normal conditions are transcribed at relatively high levels and suffer only a small increase when temperature rises; and inducible, i.e., genes normally expressed at extremely low levels, with an extremely high induction once under stress[26].

HS genes are activated very quickly in order to allow the cell to adapt before suffering irreversible damage [26, 34].

Transcription of *HS* genes is regulated by the *heat shock factor* (*HSF*) family. *HSF* can be activated by a variety of distress signs from the cell, such as the increase of denatured proteins.

HSF is normally kept in its inactive state in the cytoplasm in association with the chaperone Hsp70/Hsp40 and Hsp90, but the sudden accumulation of unfolded proteins and subsequent proteotoxic stress results in the separation of this complex. At this time the free HSF suffers homotrimerization and is transported to the nucleus. Here, it can suffer hyperphosphorylation by a number of kinases, which either promotes or inhibits the HSF transcriptional activity depending on the kinase acting: sumoylation, which negatively affects the binding of HSF to the DNA strand, or even acetylation as a way of regulating its activity. Lastly, it binds to the DNA at the *Heat Shock Element* (*HSE*) site and allows transcription of *HS* genes[26, 27, 35]

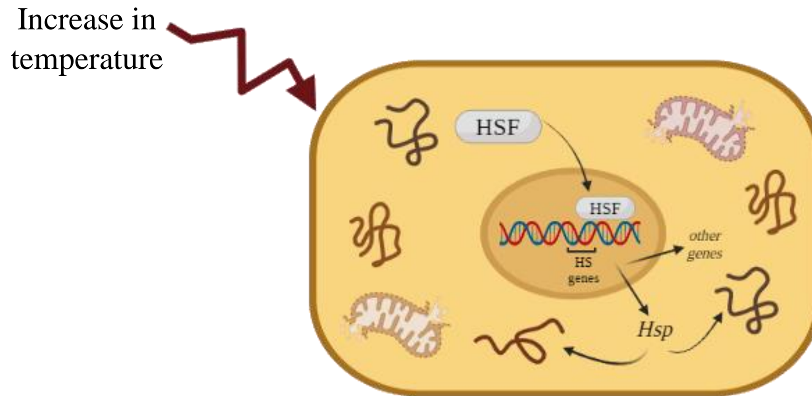

Figure 2 - Schematic representation of the HSF activation with its migration to the nucleus and activation of *HS* genes. Image created with http://biorender.com

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Although, it has been proposed that HSF regulates more than HS genes[36, 37], *Heat Shock Proteins* (*HSP*) remains the largest and most well studied family of heat resistance genes under the control of HSF.

Heat Shock Proteins were first described in *Drosophila* by the puffing pattern of polytene chromosomes in the salivary glands in response to an increase in the environmental temperature[34, 38] and are commonly known as chaperones, for their ability to bend denatured proteins and cause alterations in the protein structure [26, 39]. However, they also serve the important role of compartmentalizing and facilitating the entry of abnormal proteins in degradation pathways such as the lysosome, autophagic vesicles and proteasome pathways. Because keeping proteostasis is essential for the performance of the cell, and ultimately of the whole individual, Hsps are usually present in the cell at all times in varying quantities since there is a constant need for chaperone assistance in *de novo* protein folding, as proteins aren't entirely stable and aggregation competes with folding processed even at physiological temperatures[27, 40].

Since Hsps need to be kept in stoichiometric ratios relative to the unfolded proteins in the cell, as to the avoid the increase of non-native proteins and subsequent aggregation of proteins, when a stress factor is induced, such as a raise in environmental temperature, which causes an increment of denatured proteins, *Hsp*s are transcribed in high quantities and can quickly become a prominent constituent of cytosolic proteins[27]. Thus, the accumulation of Hsps is directly correlated to heat stress.

4.2 Heat Shock Proteins

Heat Shock Proteins (*Hsps*) although first described in *Drosophila*[38] in 1962, have currently been characterized in archaea, bacteria and eukarya with similar roles across different organisms[21, 41]. For example, *Hsp70* is the most highly conserved *Hsp* with 60% sequence identity between prokaryotes and eukaryotes[27].

These proteins interact with a broad range of non-native proteins by recognizing exposed hydrophobic amino acids. They then proceed to bind with the protein either directly on the exposed hydrophobic patches or by recognizing peptide sequences or structural elements of the non-native protein; however, they are mostly dependent on ATP to be able to perform[27]. It is important to note that Hsps do not alter structural information, but rather are used to prevent molecular interaction that lead to aggregation.

Often, these proteins will need the assistance of co-factors, commonly named co-chaperones, in order to work properly.

Broadly, they are divided into 5 categories according to their molecular weight and sequence ancestry, *Hsp90/100, Hsp70, Hsp60, Hsp40* and the *small Heat Shock Proteins* (*sHsp*s). However, there are some *Hsps* that do not fall into one of these categories, such as *Hsp33*[27].

From these groups, *Hsp70,* the most commonly described in the heat response, is composed of five protein encoding genes and can further be divided into the constitutive proteins, i.e, those always present in the cell, namely *Hsc70*, whose role is focused mainly on protein folding at the ribosome, trafficking and transport of those proteins; and inducible genes, i.e, those whose expression increase ten-fold when the cell is under stress. *Hsc70* has been reported as especially important for cell-growth with a role on the suppression of p53 and tumour dissemination[42]. On the contrary, *Hsp70* can inhibit cell division and its main role is related to *de novo* folding of proteins, refolding of aggregated proteins as well as prevention of aggregation[39]. Moreover, Hsp70 is mainly found on the fly in the endoplasmic reticulum (ER) and cytosol[27].

Hsp90 is a of constitutive gene, displaying high transcript levels under normal conditions, as well as being upregulated under stress. However, *Hsp90* can only bind to native proteins and has a very limited binding spectrum. Furthermore, it works with a vast array of co-chaperones, in a very well defined manner, to create the most sophisticated response of all *heat shock proteins*[27].

sHsps are a highly heterogeneous group in both sequence and size[27]; however, all of them possess a C-terminal alpha crystalline domain as well as a oligomeric structure. Moreover, their dispersal within the cell also varies widely[39], for example, Hsp22 is present in the mitochondrial matrix, Hsp23 and Hsp26 in the cytosol and Hsp27 in the nucleus. From this group the most ancient member is likely *HspB8* which is present in the X chromosome. Contrary to the other *heat shock proteins*, *sHsps* cannot form soluble complexes with denatured proteins, instead, they act as passive holdases, or storage deposits, for unfolded proteins to prevent their aggregation until other chaperones, like Hsp70 or Hsp100 arrive[39]. Because they cannot change the structure of proteins, sHsps is the only chaperone family which is ATP-independent. Moreover, they often interact with partially folded proteins to prevent their aggregation after stress. All together *sHsps* are the most upregulated of the *Hsps* since they work to maintain protein homeostasis in an ATP-independent matter[43].

Interestingly, although the upregulation of *Hsp*s is an integral part of the response to heat exposure, it has been observed that in cases of prolonged exposure, *Hsps* expression will decline with time, most likely because of the energetic demand that the continued use of these proteins calls for[21].

4.3 Other Candidate Genes for Heat Resistance

Although much of the heat resistance response can be accounted for by the action of *heat shock proteins*, the entirety of the process cannot be justified by looking only at *Hsp*s because the time course of the entire resistance response matches poorly with the expression profile of Hsps[44].

In fact, after the stabilization of protein homeostasis, which is mainly achieved by the action of Hsps, there is still a need for clearing misfolded proteins and irreversibly aggregated proteins from the cell, which is carried out by components of the proteolytic system. Any deformation of covalent bonds on nucleic acids such as heat induced methylation of ribosomal RNA and/or damaged occurred to the DNA must be correct by the action of RNA and DNA-modifying enzymes, so that normal transcription may be resumed. Furthermore, because most mitochondria will be damaged, the supply of energy within the cell must be restored and stabilized. There is also a need for an increase in membrane-modulating proteins that permit a restoration of membrane stability and function[27].

It has been reported that 50-200 genes are significantly induced under heat shock from archaea to human cell lines, which implies a much more complex mechanism behind the response to heat shock from the flies.

Currently, there are 83 genes reported in *D. melanogaster*[45] as being involved in the heat response, from which 15 pertain to the previously addressed family of *heat shock proteins*. From the remaining 68 genes, there is one miRNA, *mir-980*, with a role in memory formation and muscle function, whose expression under hyperthermia is downregulated and is thought to help preserve muscle activity after exposure to temperatures as high as 33ºC[46, 47].

All other 67 genes show a variety of functions although most can be separated into five main molecular functions, being that the two main categories are genes encoding for proteins involved in binding and catalytic activity, followed by molecular functions as regulators, structural molecule activity and lastly transporter activity.

Genes involved in heat resistance, can further be divided into those encoding for components of the cell, those encoding for intracellular components and lastly, those encoding for part of a protein complex.

When looking to the protein class that these genes are inserted in, there is more variety. Heat resistance genes can be chaperones, much like the *Hsps* family, transcriptional regulators, membrane trafficking proteins, enzymes of metabolite interconversion, nucleic acid metabolism protein, protein modifying enzymes, protein-binding activity modulators, scaffold proteins, transmembrane signal receptors or transporters.

With such a wide diversity of challenges imposed by heat resistance, it is not surprising that so many genes, with such widely varying functions, need to be recruited in order to minimize the effects of high temperature and confer a degree of resistance to the fly.

5. Identifying local variation

Natural populations will encounter a multitude of stress factors that diminish their fitness, both across space as well as time. Because of this, no fixed genotype will grant the highest fitness for all variables and thus, across different environments, different alleles will be selected for maximum fitness, which maintains polymorphism levels in the population[6].

However, identifying the molecular basis of local adaptation is a long-standing challenge of life sciences that has yet to yield an answer. Currently, a successful approach has been sampling individuals along latitudinal, longitudinal or altitudinal transects to find variation both at the phenotypic and genotypic level.

Since clines are often predictable along vast geographical regions in both biotic factors such as biodiversity, and abiotic factors like temperature and precipitation, sampling along such transects has enabled the identification of measurable phenotypes that are defined by geographical gradients and that are often replicable through similar climatic regions[5].

This parallel variation as already been found, for example between the Australian continent and North America. Sampling along coastal clines of those continents provided information on similar conditions to which the flies are exposed. In both continents, higher latitude flies experience greater variations of temperature throughout the year as well as lower mean temperatures from their low-latitude counterparts[5].

At the same time, this technique allows the attenuation of difficult to control factors, such as gene flow in natural populations, which is theoretically more predictable along defined clines[5].

One excellent example of a phenotypic pattern along latitudinal clines is the body size of flies from different locations with high latitudinal populations displaying an overall larger body than those of lower latitudes[5, 20]. However, it is not only in body size that this correlation can be found, typically high-latitude flies have lower fecundity but live longer lives and are more stress-resistant than flies from tropical or subtropical climates, i.e., low-latitude populations. This same pattern has been found in North America[5, 20], Australia, and in populations spanning from Africa to Europe[5, 20, 24].

Desiccation tolerance, i.e, the ability of an organism to withstand or endure drought conditions, also demonstrated a positive cline in the populations of the east coast of the U.S. and on the Indian subcontinent, with temperate populations withstanding desiccation better than tropical ones[14].

These results seem to suggest that high latitudes, maybe because of harsher seasonal variation, demand from the flies increased stress-resistance and metabolic reserves even at the cost of a loss of fecundity, while populations in warm climates, with more food availability and less fluctuation of climacteric conditions, favour high levels

of fecundity at the cost of body size[20, 48]. Furthermore, these patterns seem to be largely due to temperature[10, 13, 20].

For thermal tolerance, populations from higher latitudes in North America seem to display better cold tolerance[5, 48] and recover quicker from cold exposure[20]. Similarly, high-latitude populations in Australia display the same pattern of cold tolerance[5]. Thus, cold tolerance appears to be differentiating in parallel in both continents. Another study, performed in North American populations, revealed a latitudinal pattern with increase heat shock resistance towards high-latitude populations, associated with a longer life span[48], which is not surprising since this positive association has been described before in *Drosophila* between an increase in Hsps and life span[49, 50]. However, other studies report this correlation as the opposite, with low-latitude flies displaying better heat resistance than those of high-latitude populations, which better conforms with the known trade-offs of cold and heat resistance, i.e, when populations have better heat resistance, they will display less resistance to cold; the contrary is also observed. This difference is likely due to different phenotyping approaches[51].

In Australia, the heat hardening capacity of the flies, i.e, the ability of after exposure and survival to a given temperature to survive and sometimes surpass that temperature, showed a significant geographic variation[52]. Heat knockdown resistance also showed a clear latitudinal cline in this continent, with a decrease in resistance on populations from higher latitudes[53].

In southern Asia, studies in the Indian sub-continent performed on the *Drosophila* genus of this area allowed the detection of opposing clines for desiccation and starvation resistance, with starvation resistance decreasing in high latitude populations while desiccation resistance increased[15, 54].

While phenotypes have been identified to be differentiating along clines, so has genetic variation been found to follow the same trends. Despite this, researchers have had a difficult time connecting this variation to changes in the fitness of the flies. One successful example is the *couch potato* (*cpo)* required for processes such as synaptic transmission, climate adaptation, olfaction, diapause and behavioural responses[55]. Changes in *cpo* expression correlate with latitude in North America populations, as well as changes in diapause phenotype in the same populations. This reportedly allows the populations to better deal with the seasonality of the climate[5, 6]. Flies from the North American east coast at high latitude show a greater dormancy inducibility that those in the subtropical or tropical regions of the same cline[20, 56]. A similar, although weaker

pattern can be found in Europe[56]. However, this same trend could not be found in Australia east coast[5] unless the cline had a latitudinal range similar to that of the North American populations[20].

Another interesting example, also related to diapause, is the gene *timeless* (*tim*). *Tim* has been reported as a key component of the circadian rhythm of the fly, as well as involved in the mating behaviour, DNA replication and larval phototaxis[57]. A polymorphism of this gene emerged in the south-eastern Italy (*ls-tim)* relatively recently (~ 300 years) which is a slightly longer form of the ancestral (*s-tim)* with 23 additional residues. Sampling along this region showed that this polymorphism was present in high frequencies, of 80%. However, the frequency of the former falls linearly as the sampled populations move from the south to northern Italy[56, 58]. Interestingly, in Northern America populations there is also a cline present, but it is the opposite of that found in Italy, with around 30% frequency of *ls-tim* in the north and only about 15% frequency on the south[56].

Inversions have also been found to be differentiating under climatic patterns, for example the ln(3R)Payne inversion polymorphism is typically found in intermediate frequencies in warm regions and in lower frequencies towards the temperate climates. This inversion has been connected to smaller bodies and decreased stress resistance, which is consistent with previously established data on both phenotypes[20]. Thus, it is likely that this inversion affects both phenotypes to some degree.

Chapter 2 - *Objective*

1. Understanding heat resistance variation on natural European populations

Despite the best efforts of researchers to understand local adaptation, and the results gathered throughout the years, much still remains to be understood on how phenotypes correlate with climatic variables. Especially on European populations, that have not often been used for such assays.

By characterizing the heat knockdown time of 168 strains from 9 European populations each one from a different country, this dissertation aims to add to the effort of characterizing the heat resistance phenotype in Europe at the continental scale. Contrary to many of the previous studies, here populations will be studied across a gradient for both latitude and longitude.

This study is a part of a much larger study performed by the European Drosophila Population Genomics Consortium (DrosEU) which is a collaborative consortium of scientists and laboratories interested in evolutionary genetics and genomics of *Drosophila melanogaster* with the aim of collecting, generating and analysing genomic, phenotypic, and environmental data from *Drosophila* populations across Europe (https://droseu.net)

2. Identifying novel genes for heat resistance

Both phenotypes and genotypes have previously been found to be under selection along clines.

Many studies have pointed to temperature acting as the major driving force along such gradients to create locally adapted phenotypes. Thus, by looking at genes with single nucleotide polymorphisms (SNP) on their 5' non-coding region with high correlation coefficient with either latitude or longitude, it will be possible to identify candidate genes for heat resistance.

To test the involvement of candidate genes in heat resistance, RNAi strains were used to suppress gene expression and heat knockdown time was evaluated for each strain in relation to the control.

Chapter 3 – *Methodology*

1. Fly Stocks

1.1 Natural Populations

One hundred and sixty-eight strains of *D. melanogaster* were established from nine European populations (Figure 3). Each stock was established from one isofemale line and acclimated to laboratory conditions for two generations before beginning the experiment. This allowed a standardization of both environmental and trans-generational effects.

Figure 3 - Location from where the nine populations were collected. Coordinates are the following (latitude(°), longitude(°), altitude (when applicable) (m)): Portugal (41,15;-8,41;175), Spain (41,62;0,62;173), Turkey (40,23;32,26;680), Germany (48,18;11,61;520), Austria (48,38; 15,56), Ukraine (48,75;30,22), Denmark (55,94; 10,21), Russia (57,98;33,24;217) and Finland (61,1;23,52;88)

Moreover, for each population 13-20 strains were established.

Drosophila was kept under standard conditions of 25ºC, 60% humidity and standard food medium[20], and those were the conditions used for fly stocks during the experiment. Furthermore, to avoid crowding effects, also previously well documented[20],

vials for the phenotyping experiments were required to have a density of less than 200 flies. Preliminary tests asserted that for this condition to be met, a maximum of 65 flies, chosen randomly from the stock, must be transferred to a new vial with fresh food medium. These flies were allowed 24-48h to oviposit before parental flies were discarded.

Afterwards, eggs were allowed to develop. The development from egg to adult takes approximately 9-10 days; however, some variation was found between strains which extended the period up to 12 days. This has also been previously reported in literature[20]. Only flies born during the two days of hatching peak were considered for heat resistance experiments.

After collection flies were anesthetised under general CO_2 anaesthesia which allowed them to be separated by sex and stored into new, smaller vials, with a density of 15-20 flies per tube. Three replicates for each sex were done for heat resistance experiments. Lastly, flies were allowed to mature at 25ºC for seven days, including the day they were born, before being submitted to heat treatment.

To ensure that flies were uninjured and there was no misidentification of flies, all vials were double checked under general CO_2 anaesthesia 48h before the experiment and flies were transferred to new vials of food.

1.2 RNAi strains

Through the joint effort of several research groups, 48 European *D. melanogaster* populations were sequenced across 32 locations, in a total of 13 countries. Samples were collected in the spring, summer and fall. And findings compiled into a vcf file[59].

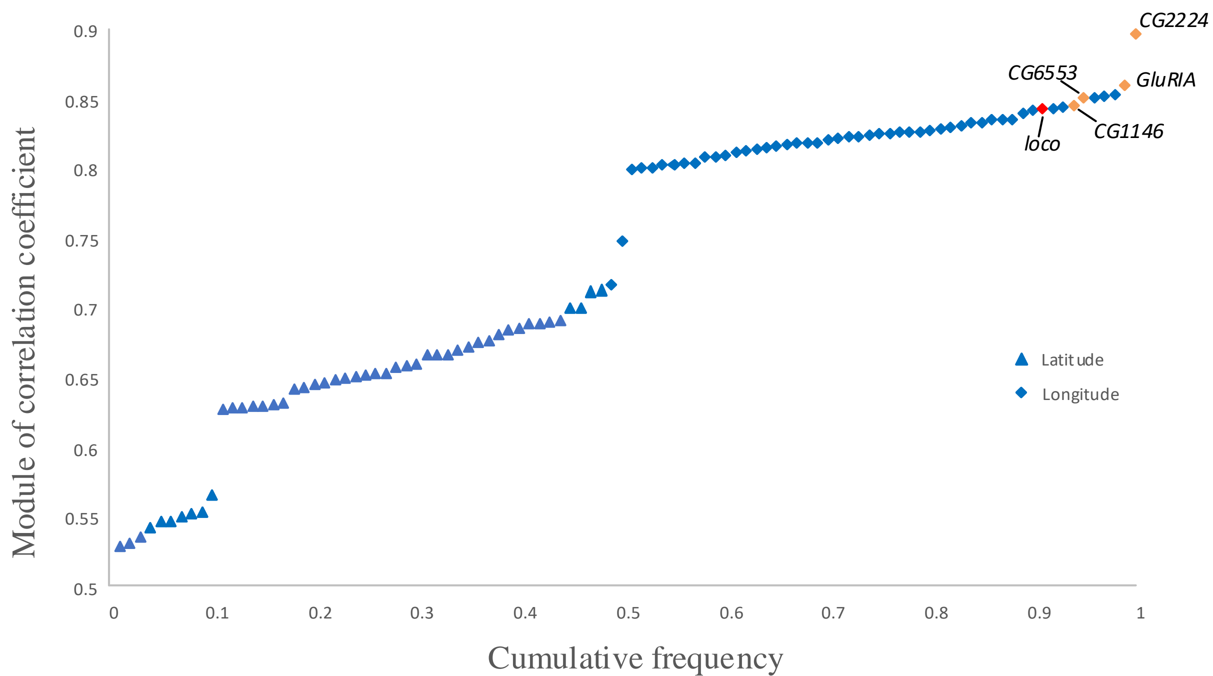


Figure 4 - The top scoring module of the correlation coefficient values between the frequency of 5' non-coding SNP and longitude/latitude. In red genes previously annotated as involved in heat resistance and in orange those selected in this study. Only genes showing a correlation coefficient above 0,5 are shown.

From this file, it was possible to establish a correlation between latitude or longitude and the presence of Single Nucleotide Polymorphisms (SNP) in the 5'- non-coding region of genes (Figure 4).

Table 1 - Characterization of the four genes that show high correlation coefficients between the frequency of 5' non-coding SNP and longitude

Gene Name	Chromosome Location	Function
CG2224	3R	Deubiquitinase activity
GluRIA	3L	Glutamate receptor activity
CG6553	2R	Unknown
CG1146	3L	Unknown

Since most genes currently implied in thermal resistance displayed selection along clines, it was possible to establish such correlations with unannotated genes, or less well studied ones, and infer a possible role in heat resistance. For this, only genes with a SNP which explained more than 25% of population variation were considered.

From the top scoring modules of correlation coefficient value (Figure 4), four genes were selected as candidate genes for heat resistance, all of them varying along a longitudinal cline: *CG2224* (R= 0,853), *GluRIA* (R= -0,850), *CG6553* (R= -0,835), *CG1146* (R= 0,832) (Table 1).

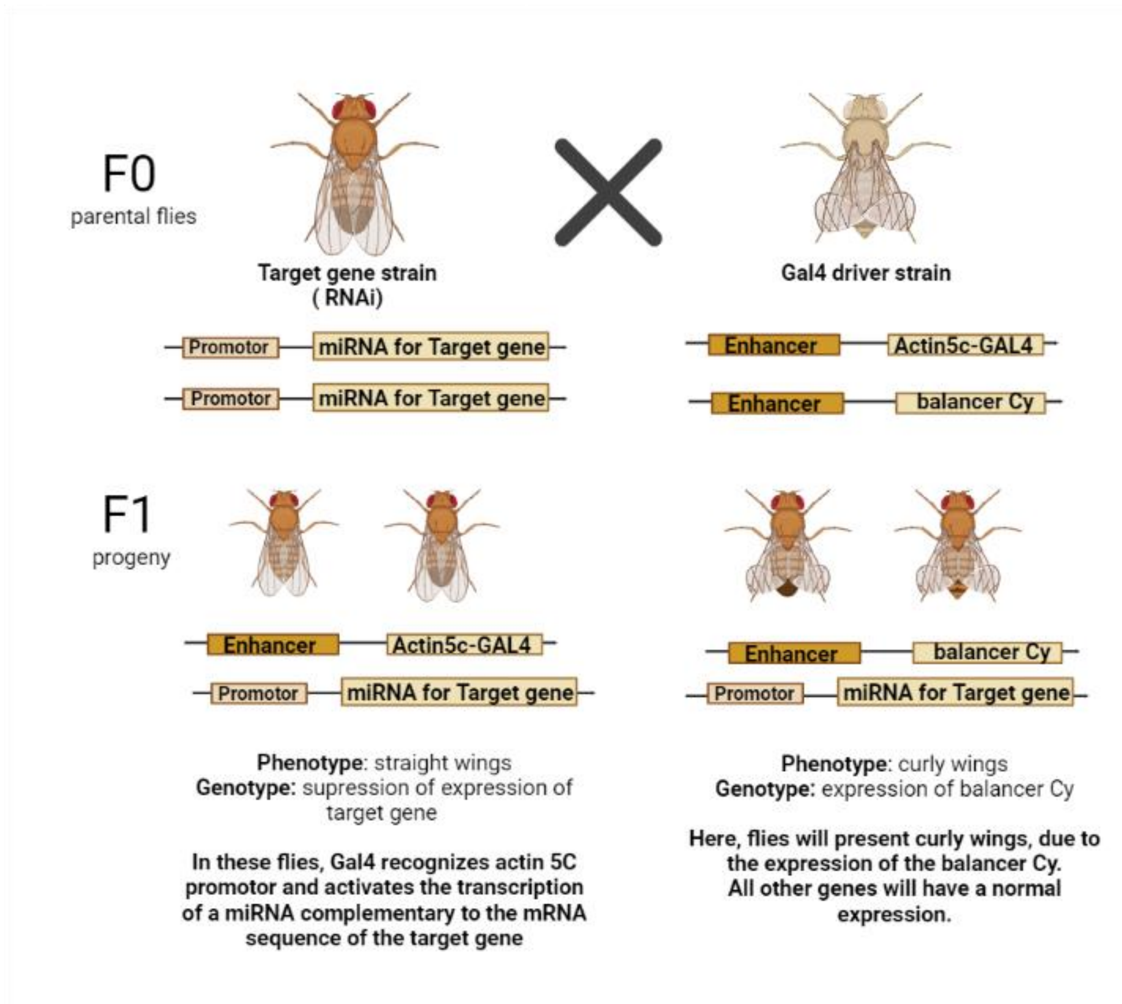


Figure 5 - Schematic representation of the Actin5c-GAL4 system. Image created using https://biorender.com

Transgenic RNAi flies targeting each candidate gene were obtained from Vienna Drosophila RNAi Centre [VDRC, Vienna, Austria[60]], stocks were: 108622 (*CG2224)*, 108019 (*GluRIA*), 100855 (*CG6553)*, 107857 (*CG1146)*. As well as the Actin5c-GAL4 system, stock 25374, used to induce gene expression suppression.

The Gal4 system functions by Gal4 binding to specific promotor which activates the transcription of the desired gene. In this case, a micro RNA (miRNA) which will bind to its complementary mRNA (the gene whose expression needs to be suppressed)[61]. It is possible to use this system to induce suppression only in certain tissues; however, in this study expression was suppressed in all tissues by using the *actin 5C* promotor.

Stock flies were acclimated to 25ºC, 60% humidity and raised on standard food medium for one generation before the beginning of experiments.

For density control, 65 flies were randomly selected and transferred to new vials with standard food medium. Flies were then allowed to oviposit for 48h before parental flies were discarded. This new vial was maintained under controlled conditions (25ºC, 60% humidity) until hatching occurred.

Females reach sexual maturity somewhere between their 1st and 4th day but are only sexually unreceptive for the first 8-12h after eclosion[20]. Therefore, female flies from these vials were captured and sexed within the first 6 hours after eclosion. This process ensured virginity.

Twenty females and male virgin flies were collected and stored into small vials. To ensure no mistakes were made when separating females from males, flies were allowed to mature for a few days (2-3 days) before being rechecked. Vials containing females and males were discarded.

Crosses were done in two directions: Actin5C-Gal4 ♂ x RNAi ♀ and Actin5C-Gal4 ♀ x RNAi ♂ to ensure no parental effect on the results.

To obtain generation F1, 20 flies from Actin5C-Gal4 line were crossed with 20 RNAi flies in vials with standard food medium.

As described previously, parental flies were discarded after 48 hours and F1 flies were only collected during the two days of hatching peak.

After collection, flies were anaesthetized under CO_2 to allow the identification of their phenotype. Flies were separated and stored into small vials with food into 4 groups: straight winged females, straight winged males, females with curly wings and males with curly wings.

Straight winged flies represent the individuals with the expression suppression induced and, therefore, represent the test group in the heat treatment, while curly winged individuals are flies with a wild-type genotype which will serve as the control group in the heat treatment.

For heat treatment, nine vials of 15-20 individuals of each group were prepared and confirmed up to 48h before the experiment begun. To control for age effects, heat treatment was performed on the 7th day including that in which the flies were born.

2. Heat Treatment

For knockdown time due to heat stress, 15-20 flies were placed into transparent vials, that were sealed with cotton at the top and placed inside an incubator at 37ºC.

Flies were checked every 30 minutes for a period of 7 uninterrupted hours, and the number of deaths were registered.

At the end of the protocol, flies were transferred to 1,5 mL Eppendorf and stored at -80ºC for future RNA extraction.

3. RNA Extraction

To confirm the suppression of gene expression from the RNAi crosses, 3 to 6 female flies from each cross were randomly selected from the flies submitted to heat treatment.

Total RNA was isolated using TRIzol™ Reagent (Invitrogen, Spain) according to the manufacturer's instructions. Flies were homogenised thoroughly in 1,5 mL Eppendorf tubes in TRIzol™ Reagent. After a few minutes at environmental temperature, to allow stabilization of cellular compounds by the reagent, lysates were centrifuged for 10 minutes at 5000 rpm at 4ºC. The resulting clear supernatant was transferred to a new tube and homogenised with chloroform. Next the mix was incubated and centrifuged for 15 minutes at 5000 rpm at 4ºC. Supernatant was transferred to a new tube and the previous step is repeated two more times. Washing the sample with chloroform allows a better separation of protein and DNA from the desired RNA sample.

Glycogen and isopropanol were then added to the aqueous phase and incubated, followed by centrifugation (10 minutes at 7000 rpm at 4ºC) to allow RNA precipitation. Since the precipitate appeared as a gel-like pellet at the bottom of the tube, supernatant was discarded carefully with the assistance of a micropipette.

Next, RNA was washed by adding ethanol 75% to the tube and centrifuged for 5 minutes at 5000 rpm. Supernatant was discarded with the help of a micropipette. This step was repeated twice, before allowing the pellet to dry for 10 minutes on ice. To solubilize the RNA, the pellet was re-suspended in RNAse-free water by pipetting up and down.

Afterwards, to ensure the sample was not contaminated with DNA, digestion with Turbo DNA-free™ Kit (Invitrogen, Spain) was performed according to instructions from the manufacturer. Samples were incubated at 37ºC for 30 minutes.

Finally, samples were stored at -80ºC until being needed.

RNA integrity and concentration was asserted with Experion™ Automated Electrophoresis System. This approach utilizes an algorithm which compares only three regions of an electrophoretic profile to a series of degradation standards.

All samples considered for cDNA synthesis were required to have more than 120 μg/μL and an RQI above 7.

4. cDNA synthesis

RNA samples extracted previously were reversely transcribed into cDNA by using the SuperScript™ II Reverse Transcriptase (Invitrogen, Spain) and following the manufacturer protocol.

RNA was added in order to obtain a final concentration of 1ng/μL, followed by random primers and dNTPs as well as sterile and RNase-free, water. The mixture was then heated to 65ºC for 5 minutes before quickly chilling on ice. To the mix it was added RT Buffer (10x), $MgCl_2$ (25mM), DTT (0,1M) and RNase OUT (40 units/μL) and SuperScript™ II RT. The tubes were centrifuged before being incubated under a program of 10 min. at 25ºC, followed by 50 min. at 50ºC and 5 min. at 85ºC.

Lastly, RNaseH was added to the samples and they were incubated at 37ºC before being stored at -20ºC.

5. Real-Time PCR

Real-time PCR was performed on an Applied Biosystem Real time PCR CFX96 (BioRad), using SYBR Green PCR Master Mix (Thermo Fisher Scientific).

The final volume of the reaction was 20 μL with the following components: 1 μL of cDNA, 1 μL of each primer (forward and reverse at 10 mM), 10 μL of SYBR Green PCR Master Mix and 7 μL of H_20 RNase-free. Every experiment was done in duplicates and with the following program: 3 min at 95°C; 40 cycles of 30 s at 94°C, 30 s at 56°C and 30 s at 72°C.

For primer design, the program OLIGO 4.0 (Molecular Biology Insights, Inc. (DBA Oligo, Inc.)) was used, except for the two references genes which already had primers with good efficiency described in the literature[62, 63]

Messenger RNA levels were normalized by utilizing two reference genes: *RPL32* and *Actin 42A*.

All primers used can be found below on table 2.

Table 2 - Amplified genes and primers used for Real-Time PCR

Gene	Product length (bp)		Tm (ºC)	Sequence
	cDNA	Genomic DNA		
CG1146	231	231	59	F: CGGAATGGCTACCTTACCTC R: GCTGACGCTTGGGATACACC
CG6553	120	176	50	F: AGGCGAGAACCAGAACATTT R: ACCACGATTAGGAAGCAGAA
GluRIA	175	242	52	F: CGAGTATGACGAGCGATTGG R: TTGACCCGAGAGTTGATTGC
CG31949	241	319	59	F: GGTCCTATGTTTGGCTCTCC R: CTTTCTTATGTCCCGTTTTA
CG2224	235	293	54	F: TACTGGCTGGTCATCTGTCC R: CAGATTATGATGCCCGAAGC
RPL32	101	101	58	F: CGTTTACTGCGGCGAGAT R: CGTTGGGGTTGGTGAGGC
Actin 42A	138	292	60	F: GCGTCGGTCAATTCAATCTT R: AAGCTGCAACCTCTTCGTCA

6. Statistical Analysis

Non-parametric tests were used to infer possible phenotypic differences on the RNAi crosses, as well as differences between populations. Pearson Correlation was used in order to find associations between two variables, such as latitude, longitude and bioclimatic variables with the experimental group. Mann-Whitney test was used to find statistically significant differences between groups.

This was performed using the software SPSS Statistics 27 (SPSS Inc., Chicago, Illinois).

Chapter 4 – *Results*

1. Evaluating Heat Resistance in Natural Populations

1.1 Heat knockdown time

To characterize the patterns of differentiation of heat resistance on the studied populations, each population was represented by 13-20 strains, and each strain was phenotyped three times independently for each sex.

Heat knockdown time was registered every 30 minutes for a period of 7 hours. This implies that populations with higher heat knockdown times survive better under heat stress conditions, while lower values entail less resistance.

Not surprisingly, females and males perform very differently under the experimental conditions (Mann-Whitney test; N=504; $P < 0{,}001$), with females surviving longer than males.

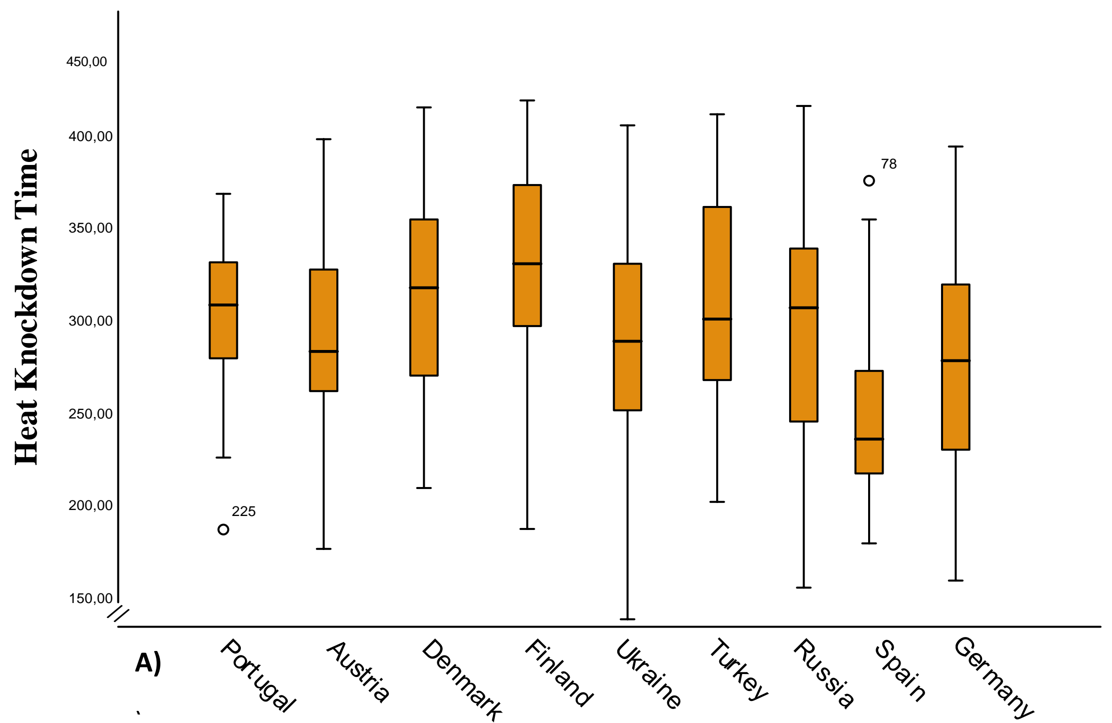
Interestingly, the most resistant populations for both groups (females, males) is not consistent (Table 3). For females, the population with the highest average knockdown time is the one from Portugal, while for males it is the Finnish population. Furthermore, the less resistant population is also different, for females being the German population, while for males it is the Spanish one. However, the Spanish population is also one of the least resistant ones for the females.

Overall, for the 420 minutes of the treatment, females survived until the end (out of 9 populations, all of them had at least one surviving female at the last reading). Furthermore, all populations survived 37ºC up until the 3-hour mark, at which point they slowly succumbed to the heat stress. On the contrary, males usually started dying before 3 hours had passed (5 out of 9 populations) and all of them had deaths registered before 3,5 hours. Moreover, contrary to the females some populations did not resist the entirety of the treatment (4 out of 9, almost half, died around 6,5 hours), and none could survive until the 420 minutes (7 hours).

Table 3 - Mean, standard error, minimum and maximum in minutes separated by sex and population. Portugal is the population with stronger females, while Finland has the strongest males. German and Spanish populations are the one with less resistant flies.

		Females			**Males**		
Population	N	Mean ± Standard Deviation	Minimum	Maximum	Mean ± Standard Deviation	Minimum	Maximum
Portugal	48	393,08 ± 21,68	339,47	420,00	304,10 ± 38,78	186,00	367,89
Austria	60	387,04 ± 28,62	284,21	420,00	291,28 ± 49,62	175,50	397,50
Denmark	60	386,32 ± 34,65	268,50	420,00	311,87 ± 54,85	208,50	414,71
Finland	60	376,10 ± 45,72	211,58	420,00	329,63 ± 54,84	186,32	418,50
Ukraine	57	372,28 ± 43,63	248,33	420,00	288,77 ± 57,39	137,37	405,00
Turkey	60	363,63 ± 41,23	273,16	420,00	307,55 ± 57,08	201,00	411,00
Russia	60	352,465 ± 49,96	192,00	418,50	293,65 ± 62,66	154,50	415,50
Spain	39	328,64 ± 50,23	233,68	417,00	248,37 ± 49,37	178,50	375,00
Germany	60	321,63 ± 44,80	231,00	400,00	275,50 ± 57,62	158,33	393,53

When looking at the box plot of the variation of the population (Figure 6), females appear to have more outliers. This is, however, not worrying as the number of outliers is relatively small.



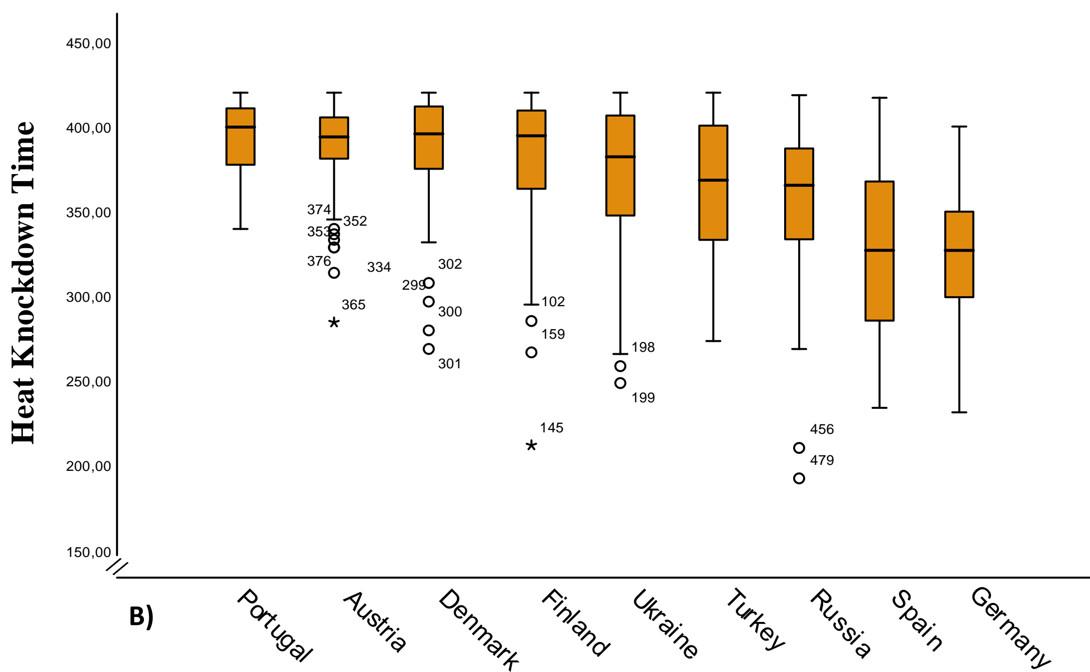


Figure 6 - Box plot of heat knockdown time (in minutes) for (A) females and (B) males. Overall, females are more heat resistant than their male counterparts.

At the end of the experiment, overall mortality per population was analysed by dividing the number of deaths at the last reading by the total number of individuals of a replicate. Interestingly, but not unsurprising, males display less variation amongst populations and an overall higher mortality rate, with 8 out of 9 population displaying more than 85% mortality by the end of the experiment (Table 4). Whereas for females

only 3 out of 9 populations have a mean mortality rate at the end of the experiment of over 70%.

Table 4 - Mean mortality rate at the end of the 37ºC seven-hour exposure. Mortality in the males is over 85% for almost all populations, while females only have one population with mortality over 80%.

Population	Mean Mortality Rate	
	Female	Male
Portugal	36,58%	96,56%
Austria	41,56%	94,21%
Denmark	38,15%	86,37%
Finland	46,07%	80,73%
Ukraine	51,29%	93,59%
Turkey	56,47%	87,36%
Russia	72,54%	92,67%
Spain	72,79%	97,81%
Germany	81,85%	95,81%

1.2 Reliability of the established protocol

To understand the difficulty of estimating heat resistance with the established protocol it is necessary to have an accuracy indicator which can relay information on the consistency and replicability of the data.

With that purpose, the percentage of the coefficient of variation (%CV) for each strain was calculated. The coefficient of variation is the ratio between the standard deviation to the mean, multiplied by 100 to get the percentage.

Since this measure is unitless, it allows the comparison of data sets with different means. Furthermore, it shows the extent of variability in relation to the mean of the population. In sum, the higher the CV, the greater the dispersion. For females, the average %CV is 7,00, while for males its 13,00 (Mann-Whitney test; N=336; $P < 0{,}001$) (

Table 5).

Table 5 – Mean, median and standard deviation pertaining to the percentage of the coefficient of variance (%CV) when considering all strains.

	N	Mean	Median	Standard Deviation
Female	168	7,00	5,25	6,12
Male	168	13,00	12,06	7,23

Moreover, the cumulative distribution of %CV (Figure 7) shows that while for females 76,8% of the %CV values are below 10%, which is a reasonable value, for %CV, for males only 41,7% of the %CV values are below 10%.

Thus, using the established protocol, it is more difficult to get a reliable estimate of heat resistance for males than for females. Since males are less heat resistance than females (the average survival time at 37ºC, across all populations is 365,5 minutes for females, but only 296,3 for males, a difference of 69,2 minutes, i.e., females survive on average more than one hour more than males) it is possible that the conditions used to survey heat resistance are too extreme for the males, which could lead to a high variance between same strain replicas.

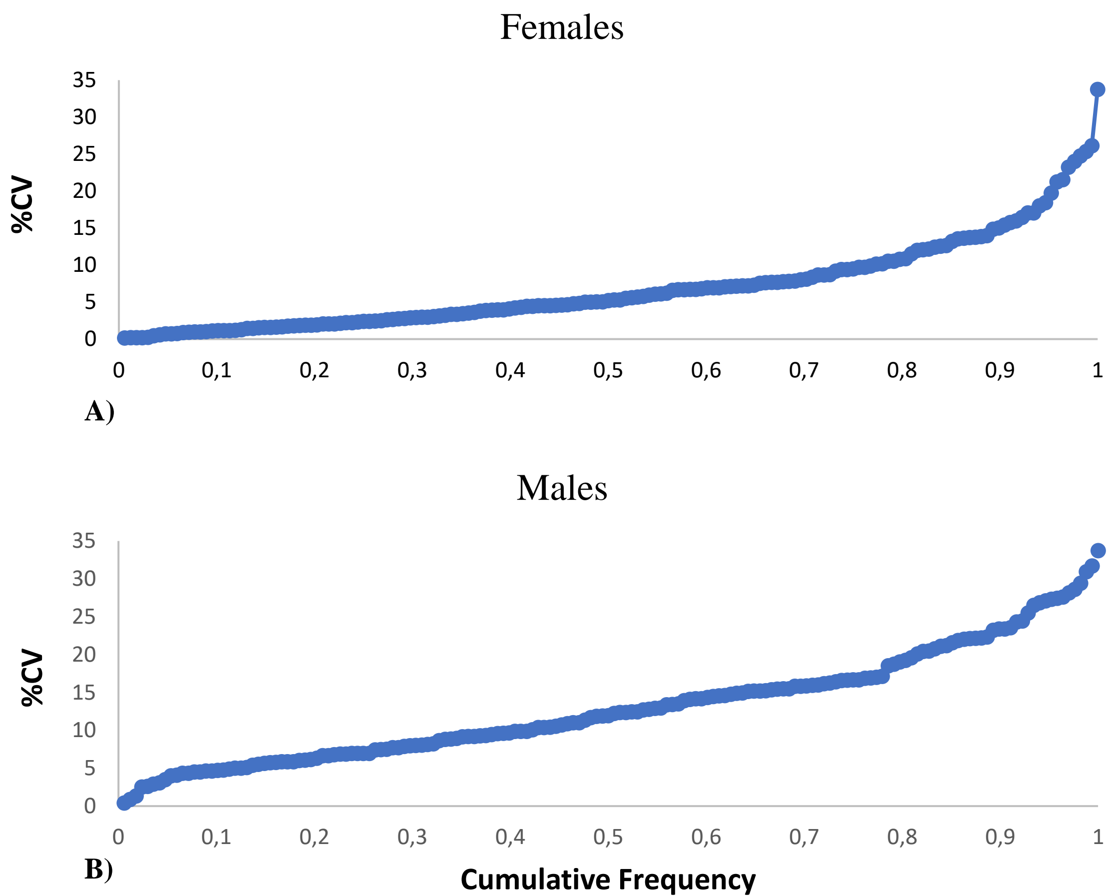


Figure 7 - The cumulative distribution of the percentage of the coefficient of variance of the 168 strains tested for both (A) females and (B) males. Males display a greater variance between strains than females.

Nevertheless, there is a fraction of the %CV variation that can be attributed to strains, since there is a highly significant correlation between the %CV of females and males (Pearson's R=0,33; N=168, $P < 0{,}01$).

1.3 Geographical patterns of variation

A significant correlation was found between male heat resistance and latitude (N=168; Pearson's R=0,21; $P < 0{,}01$), but not for females and latitude (N=168; Pearson's R=0,06; $P > 0{,}05$). This is unexpected, given that, as shown above, measurements are more reliable for females than males.

For longitude, no significant correlation was found in neither females or males (N=168; Person's R= -0,040 for females and R= 0,120 for males, $P > 0{,}05$ for both).

However, it should be noted, that climatic variables (such as temperature, precipitation, daylight hours) do not show perfect correlations with either latitude or longitude. As such, correlations could still be observed between female heat resistance and climatic variables.

In order to address this issue, correlations between the heat resistance of each individual strain and the 19 WorldClim Bioclimatic variables[64] were assessed. These variables were derived from the monthly temperature and rainfall values to generate more biologically meaningful variables that capture trends and limiting factors. Also note that a quarter entails ¼ of a year.

Curiously, all significant correlations with the heat resistance of males are related to temperature, whereas the heat resistance of females appears to be correlated with variables capturing both temperature and rainfall (Table 6).

Correlation coefficients can be quite different between males and females, depending on the variable considered. For instance, when contemplating the mean temperature of the warmest quarter, the correlation coefficient for males is -0,23, but only -0,09 for females; when looking at the temperature annual range, the correlation coefficient is -0,02 for males and -0,20 for females. Although in some cases they can be quite similar, for example, mean diurnal range and mean temperature of the wettest quarter have similar correlation coefficients for both male and females' heat resistance.

Table 6 - Pearson Correlation between females and males and 19 bioclimatic variables, significant correlations were found for both sexes.

	Females			Males		
	Pearson Correlation	Sig. (2-tailed)	N	Pearson Correlation	Sig. (2-tailed)	N
Mean Diurnal Range	-,181*	0,019	168	-,184*	0,017	168
Isothermality	0,039	0,613	168	-0,125	0,107	168
Temperature Seasonality	-,154*	0,046	168	0,022	0,775	168
Max. Temperature of Warmest Month	-0,136	0,078	168	-,222**	0,004	168
Min. Temperature of Coldest Month	0,072	0,354	168	-0,132	0,087	168
Temperature Annual Range	-,201**	0,009	168	-0,019	0,804	168
Mean Temperature of Wettest Quarter	-,212**	0,006	168	-,248**	0,001	168
Mean Temperature of Driest Quarter	0,127	0,101	168	0,008	0,919	168
Mean Temperature of Warmest Quarter	-0,092	0,236	168	-,232**	0,003	168
Mean Temperature of Coldest Quarter	0,068	0,382	168	-0,131	0,090	168
Annual Precipitation	0,049	0,527	168	0,001	0,994	168
Precipitation of Wettest Month	0,039	0,620	168	-0,018	0,815	168

Precipitation of Driest Month	-,236**	0,002	168	-0,096	0,215	168
Precipitation Seasonality	0,117	0,131	168	0,025	0,744	168
Precipitation of Wettest Quarter	0,057	0,462	168	-0,007	0,928	168
Precipitation of Driest Quarter	-,214**	0,005	168	-0,133	0,085	168
Precipitation of Warmest Quarter	-,216**	0,005	168	-0,086	0,267	168
Precipitation of Coldest Quarter	,201**	0,009	168	0,075	0,331	168

**. Correlation is significant at the 0,01 level (2-tailed).

*. Correlation is significant at the 0,05 level (2-tailed).

This underlines that some aspects of temperature and rainfall can affect both males and females, while there are others that exert more pressure on one of the sexes.

However, the correlations go in the opposite direction of what was expected. For instance, since males are less heat resistant than females, males should be less tolerant to the temperatures observed during the warmest quarter than females, meaning that selection should be stronger for males during that period. If that was the case, a positively significant correlation between mean temperature of the warmest quarter and heat resistance for males should have been observed and not for females.

1.4 Population differences

Although trends were found with climatic variables, as explained in the previous section, when results are separated by populations, i.e., when looking at the population level, there are no significant correlations between the population average heat resistance and latitude (N=9; Pearson's R=0,128 for females and R=0,497 for males; $P > 0,05$),

longitude (N=9; Pearson's R=-0,034 for females and R=0,309 for males; $P > 0,05$) or any of the analysed bioclimatic variables (Table 7).

Table 7 - Pearson Correlation between the 19 bioclimatic variables analysed and the population average heat resistance. All significant correlations were lost when considering just the average of the nine considered populations

	Females			Males		
	Pearson Correlation	**Sig. (2-tailed)**	**N**	**Pearson Correlation**	**Sig. (2-tailed)**	**N**
Annual Mean Temperature	-0,046	0,906	9	-0,465	0,207	9
Mean Diurnal Range	-0,355	0,348	9	-0,481	0,190	9
Isothermality	0,052	0,895	9	-0,298	0,436	9
Temperature Seasonality	-0,256	0,506	9	0,055	0,888	9
Max. Temperature of Warmest Month	-0,302	0,430	9	-0,577	0,103	9
Min. Temperature of Coldest Month	0,079	0,840	9	-0,336	0,377	9
Temperature Annual Range	-0,358	0,344	9	-0,069	0,860	9
Mean Temperature of Wettest Quarter	-0,394	0,294	9	-0,579	0,102	9
Mean Temperature of Driest Quarter	-0,234	0,545	9	-0,594	0,092	9
Mean Temperature of Warmest Quarter	0,213	0,583	9	0,000	0,999	9

Mean Temperature of Coldest Quarter	0,066	0,866	9	-0,341	0,370	9
Annual Precipitation	0,197	0,612	9	0,122	0,754	9
Precipitation of Wettest Month	0,179	0,644	9	0,082	0,833	9
Precipitation of Driest Month	-0,337	0,375	9	-0,126	0,747	9
Precipitation Seasonality x Coefficient of Variation	0,266	0,489	9	0,122	0,754	9
Precipitation of Wettest Quarter	0,207	0,594	9	0,104	0,791	9
Precipitation of Driest Quarter	-0,289	0,450	9	-0,194	0,618	9
Precipitation of Warmest Quarter	-0,289	0,451	9	-0,087	0,824	9
Precipitation of Coldest Quarter	0,400	0,286	9	0,230	0,551	9

Thus, when analysing heat resistance at the population level a sample much larger than the nine populations here analysed is needed in order to find such trends. Nevertheless, the previous sections show that significant differences in the heat resistance of males and females were found, as well, that the two sexes are likely to respond differently to different bioclimatic variable (mainly rainfall and temperature).

Population-dependent changes in relative heat resistance of both females and males was also analysed (

***Table** 8*).

Table 8 - Average heat resistance per population and relative heat resistance of each population. Relative Heat Resistance (RHR) is obtained by dividing the male score by the female's, this entails that the closest the RHR is to one, the more similar males and females are on that population.

Population	**Average Heat Resistance**		**Relative Heat Resistance**
	Females	Males	
Portugal	393.08	304.10	0.77
Austria	387.04	291.28	0.75
Denmark	386.32	311.87	0.81
Finland	376.10	329.63	0.88
Ukraine	372.28	288.77	0.78
Turkey	366.89	307.55	0.84
Russia	350.32	293.65	0.84
Spain	328.64	248.37	0.76
Germany	321.63	275.50	0.86

Indeed, at the extremes of the distribution (Portugal – 0,77 and Spain – 0,76) vs Finland – 0,88 and Russia – 0,84), the relative heat resistance of males and females is quite different.

However, the difference goes in the opposite direction of what is expected. In Portugal and Spain, where selection for heat resistance in males was expected to be strongest (note that temperatures are higher), in comparison with females from the same population, males are less resistant than those from Finland and Russia.

2. Selecting putative candidate genes for Heat Resistance

From a vcf file made available by the European *Drosophila* Population Genomics Consortium[59], which compiled the information of the sequencing of 48 European *D. melanogaster* populations, from 13 different countries, genes with high correlation coefficient between latitude or longitude and their SNP's in the 5'- non-coding region of genes were identified.

Genes not previously implicated in the heat resistance phenotype, with high correlation coefficients, were selected in order to add to the list of genes setting this trait. From the top scoring modules of correlation coefficients (Figure 4), four genes were selected as novel candidate genes for heat resistance, all of them varying along a longitudinal cline: *CG2224* (R= 0,853), *GluRIA* (R= -0,853), *CG6553* (R= -0,835) and *CG1146* (R= 0,832).

No suppression of expression of the four selected candidate genes yielded a lethal phenotype when crosses with Actin5c-GAL4 driver were performed.

From the previous experiment, it has been established that males and females are expected to perform very differently, therefore, their analysis must be done separately. Indeed, here the same pattern was noticed.

Since it was not possible to submit all replicates of the same cross to heat treatment in the same day, all scores were corrected to account for any possible variation that may induce variability in the final reading. This was done by standardizing the value of the heat knockdown time of each individual replicate by the mean value of the control group of the day the flies were submitted to treatment.

2.1 CG1146

In Flybase the only readily available information on the gene *CG1146* is its position on the left arm of the third chromosome together with expression information both by tissue and during developmental stages of the fly[65].

Curiously, *CG1146* is expressed throughout all different tissues, from the nervous system to the digestive tract and in reproductive organs. Moreover, it is highly expressed in the salivary gland prepupae stage (Figure 8, A). In adults, it is important to note that its expression is greater in the testis than the ovary, but the sequenced flies were not of the same age as the flies assessed in this study; therefore, the expression pattern

could be slightly different. Moreover, conditions on how this data was obtained are not available, so it could be that the setting of this experimental protocol leads to a slightly different profile.

Expression in adult flies is moderate, with a slightly higher expression on males, as expected from the tissue data, than females (Figure 8, B). Once more, there is no information for flies with precisely 7 days of age, so the number of transcripts may be slightly different. The real transcript number should fall between the presented data for 5-day old flies and 30-day old ones.

Figure 8 - RNA sequencing profile of the gene *CG1146* by (A) anatomy (B) development. Only flies with 7 days were used for the heat treatment experiment, which means that the expression profile of *CG1146* will fall between that of a 5 day old fly and 30 day old fly.
Image taken from http://flybase.org

Next, possible interactions with other proteins were inferred. For *CG1146* no known interactions were found[66]. There is also no information available about conserved putative domains on the NCBI database.

Although the function of *CG1146* is currently not understood, it is known that it encodes for a protein integrated in the cellular membrane.

The suppression of the expression of *CG1146* caused an increase in the fly's tolerance to heat exposure (Table 9). For males, this increase was not statistically different (Mann-Whitney test; N=18; $P > 0,05$) for crosses in both direction with the test group (SW flies) surviving only 2-4% less than the control group (CW flies). Whereas for females, the suppression of the expression of *CG1146* lead to an increase in heat resistance of 12-13% (Mann-Whitney test; N=18; $P < 0,001$) (Figure 9).

Table 9 - Mean, standard error, minimum and maximum, considering the corrected values for straight winged (SW) and curly winged (CW) flies. Females with suppression of gene expression showed a better heat resistance that the control group

	N	Mean ± Standard Deviation	Minimum	Maximum	N	Mean ± Standard Deviation	Minimum	Maximum
		***CG1146* ♂ x Actin5c-GAL4 ♀**				***CG1146* ♀ x Actin5c-GAL4 ♂**		
Female CW	9	1,00 ± 0,05	0,93	1,09	9	1,00 ± 0,4	0,94	1,07
Female SW	9	1,13 ± 0,06	1,05	1,22	9	1,12 ± 0,6	1,03	1,23
Male CW	9	1,00 ± 0,11	0,82	1,18	9	1,00 ± 0,9	0,85	1,15
Male SW	9	0,96 ± 0,15	0,75	1,16	9	1,02 ± 0,11	0,87	1,14

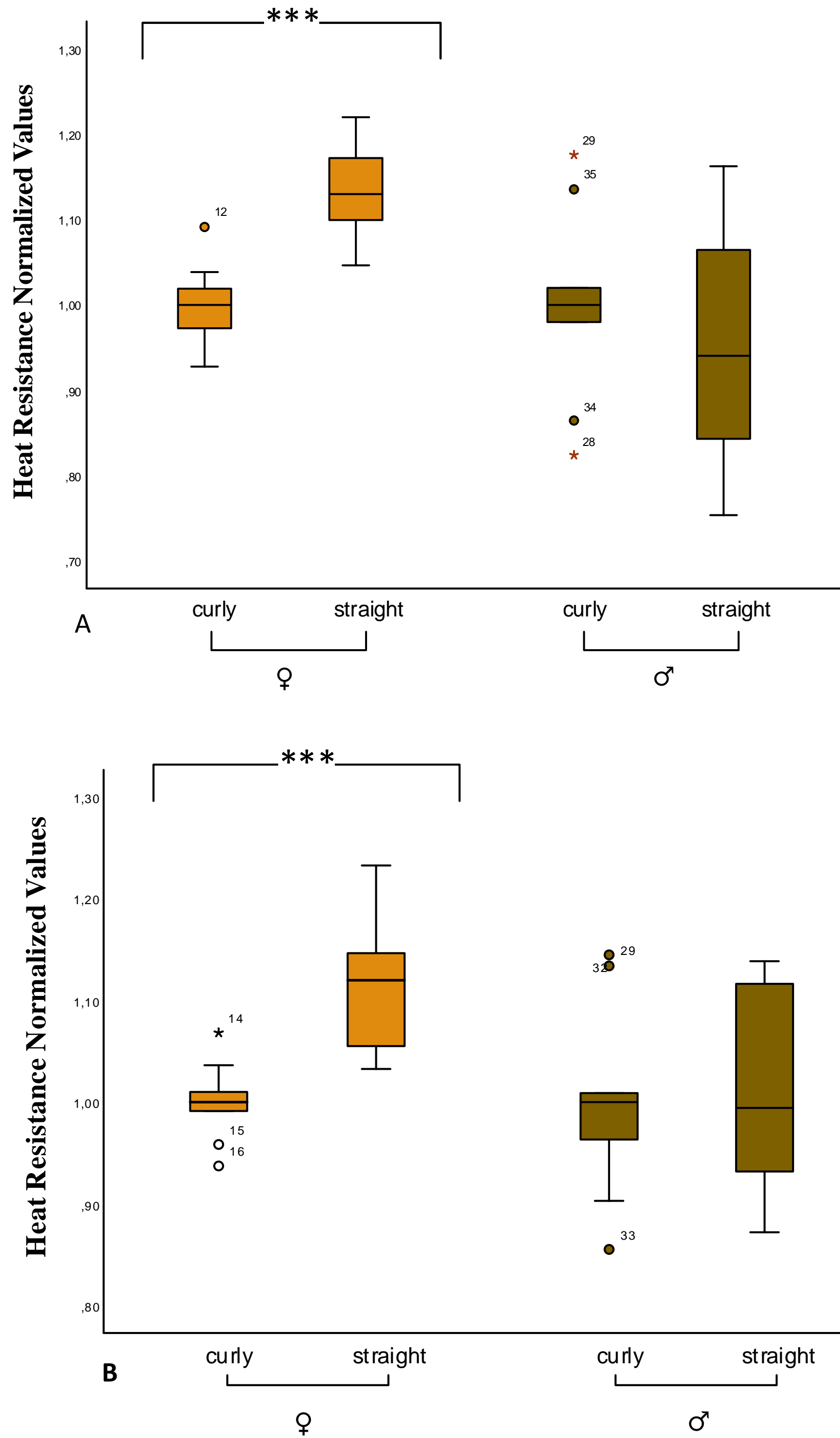


Figure 9 - Heat resistance results for gene *CG1146*. (A) *CG1146* ♂ χ Actin5c-GAL4 Driver ♀ (B) *CG1146* ♀ χ Actin5c-GAL4 Driver ♂. Curly refers to flies with wild type phenotype, straight refers to the flies with the gene expression suppression. Values are normalized relative to the control (curly flies).
*** $P < 0{,}001$

Real-Time PCR was performed, using curly winged flies as a control for normal expression under the tested conditions.

For reliable results in a real-time PCR reaction, primer efficiency must first be evaluated. Good primers should have an efficiency between 90-110%[67]. By using subsequent dilutions of the samples with a dilution factor of 5 (1:5, 1:25, 1:125, 1:625) it was possible to determine the efficiency of the designed primers (Figure 10).

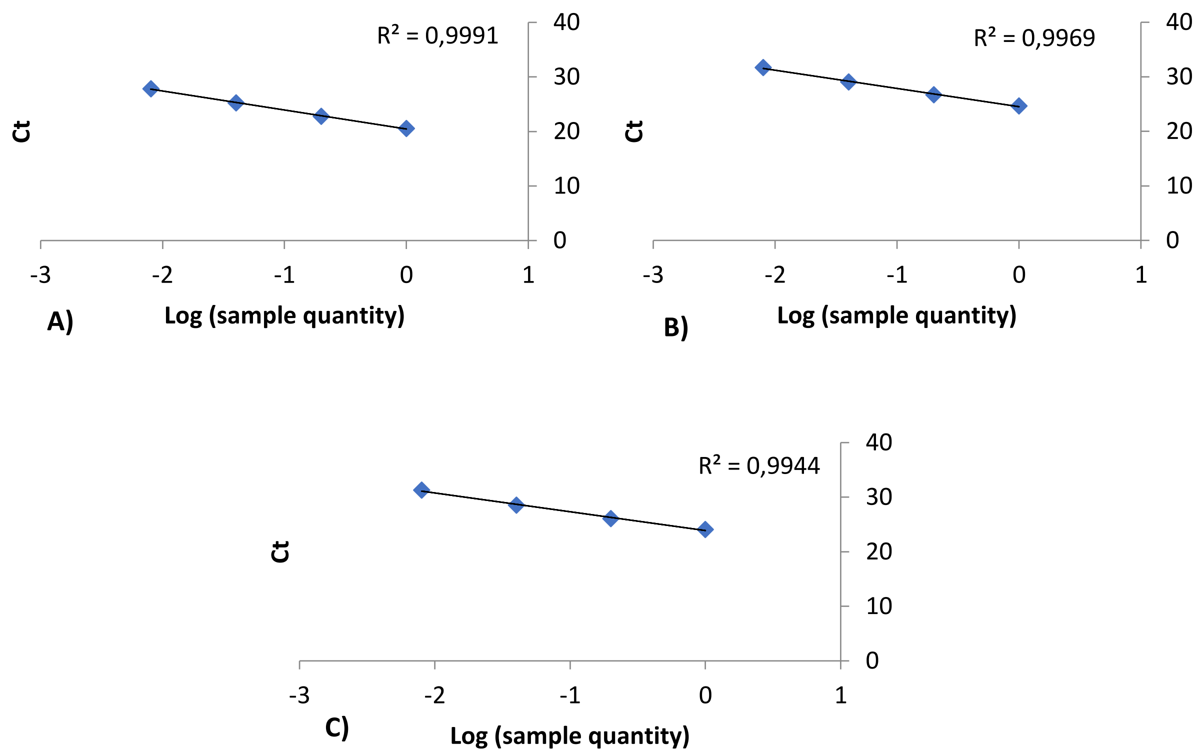


Figure 10 – Primer efficiency for real-time PCR with logarithmized results. (A) *RPL32* primer efficiency of 93,56% (B) *Actin 42A* primers had an efficiency of 99,23% which is optimal for real-time PCR. (C) The efficiency for *CG1146* primers is 95,21%, which is within the permissive values.

RPL32 and *Actin 42A* were used as reference genes, to allow the standardization of the control and test group values. Both were previously implied as good reference genes, namely for situations of heat stress[62, 63] and demonstrated good efficiency in the tested conditions: 93,56% for *RPL32* and 99,23% for *Actin 42A*. Primers for *CG1146* displayed an efficiency of 95,21% which is well within the permitted values.

For Real Time PCR experiments, four replicates were extracted. Two from each cross (Actin5c-GAL4 ♂ x RNAi ♀ and Actin5c-GAL4 ♀ x RNAi ♂) for both the control and the test group.

The RNAi crosses for *CG1146* yielded a suppression of the gene expression on the tested flies of 65,73-77,09% less than the control ($P < 0,05$ when using RPL32 as a reference gene and $P < 0,01$ when normalizing expression with *Actin 42A*; Figure 11).

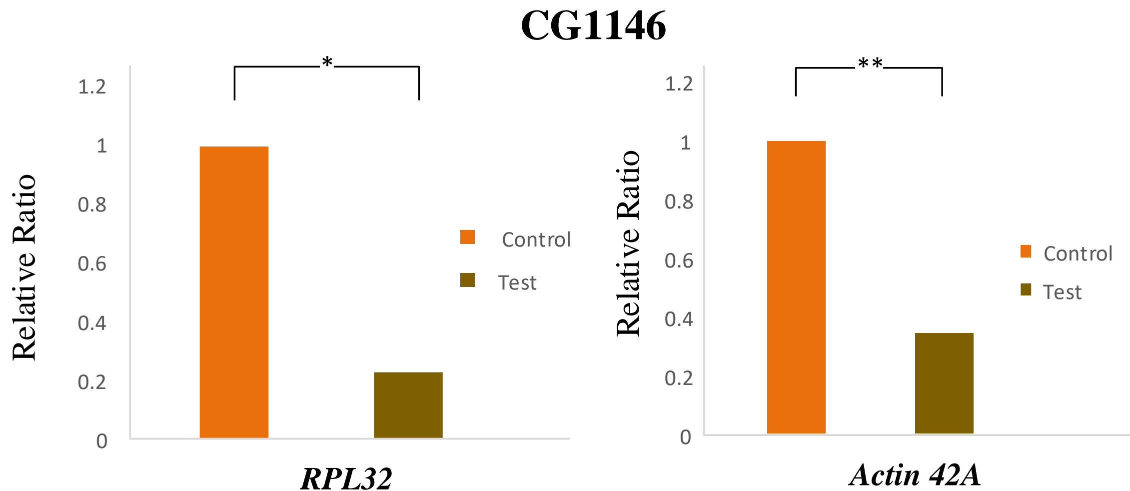


Figure 11 - Relative quantification of the suppression of *CG1146* expression. In (A) results were normalized according to reference gene *RPL32* while in (B) values were normalized according to reference gene *Actin 42A*. Two replicates of each group were performed as biological replicates, each was duplicated to create technical replicates.
** $P < 0,01$

2.2 CG6553

This genes codes for an integral component of the membrane[68] but not more is known about the molecular function of *CG6553*.

When looking at the conserved domains of the gene (Figure 12) one super family is identified, namely the Low-density lipoprotein receptor domain class A (LDLa) which is a cysteine-rich repeat the low-density lipoprotein (LDL) receptor that has been implicated in the cholesterol metabolism[69].

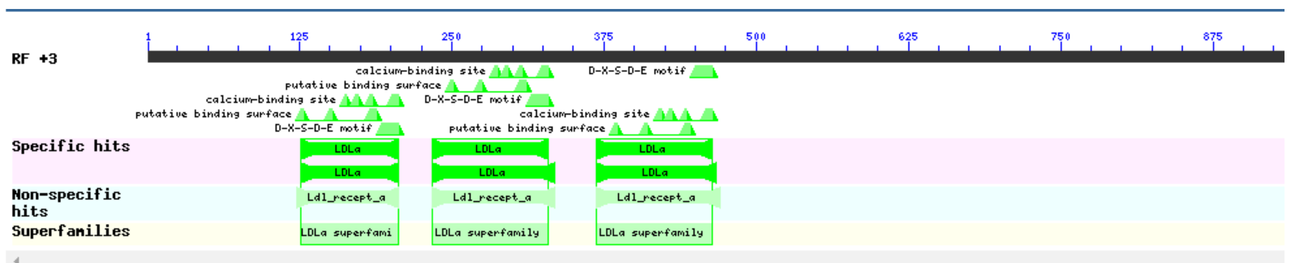


Figure 12 - Schematic representation of conserved domains in the sequence of *CG6553*. Information taken from NCBI database.

Moreover, it is important to note the presence of calcium binding, as well as the multiple hits of binding domains, which is unsurprising as the encoded protein is a protein integrated in the membrane.

No protein interactions have been described for this gene[66]. So, next it was important to infer the expression profiles both by tissue and during development. *CG6553* appears to be very minimally expressed in the fly (Figure 13**),** with very low expression in a narrow range of tissues such as the fat body of pupae and the testis of males, the head of virgin and mated females, as well as males. It also appears briefly on the digestive tract of adults and the carcass up until the 20th day.

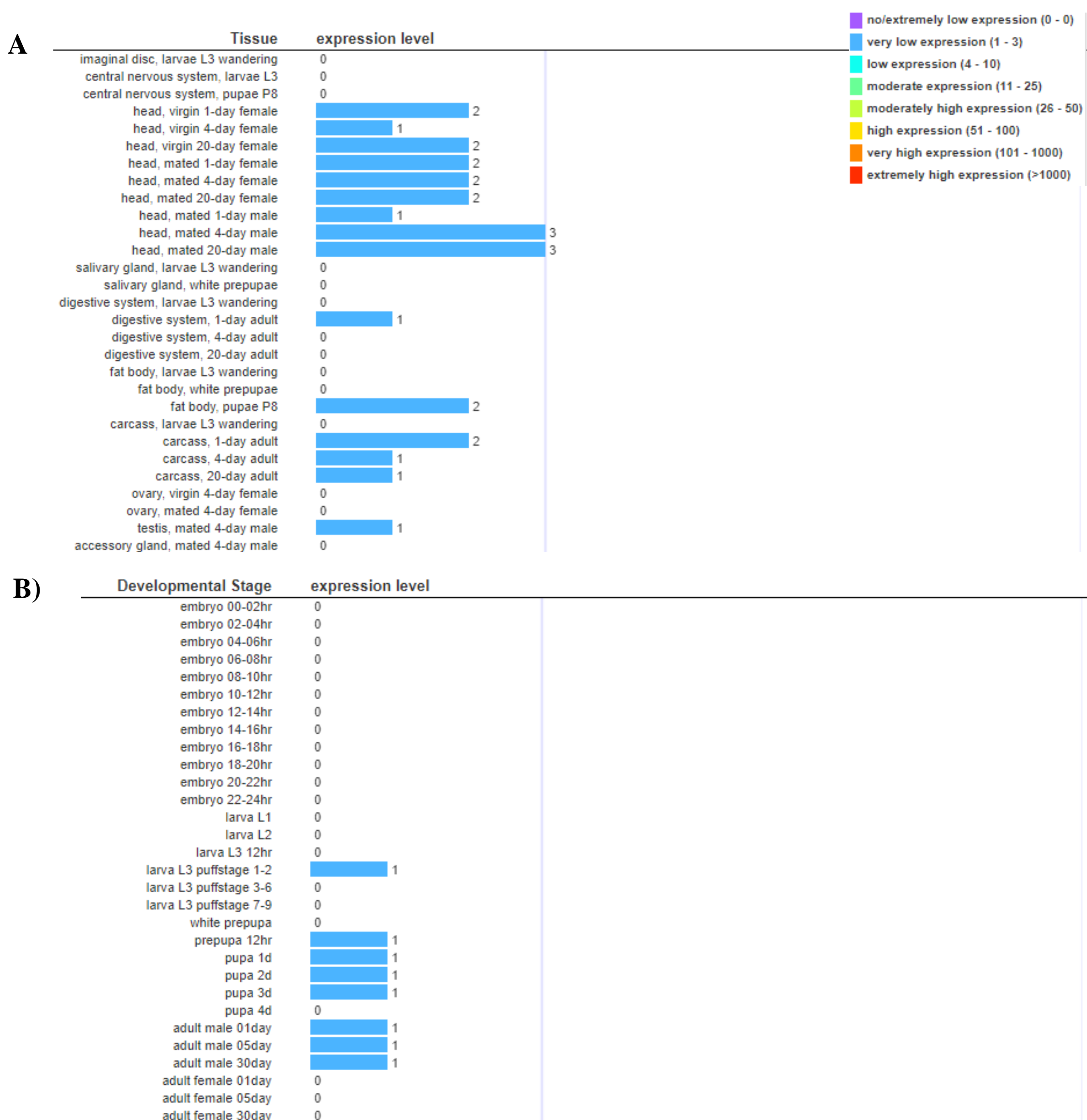


Figure 13 - RNA sequencing profile of the gene *CG6553* by (A) anatomy (B) development. Only flies with 7 days were used for the heat treatment experiment, which means that the expression profile of *CG6553* will fall between that of a 5 day old fly and 30 day old fly.
Image taken from http://flybase.org

As seen previously, throughout development *CG6553* is sparse, appearing on a very specific state of larva (Larva L3 puffstage 1-2), on the pupa stage for the first three days and then it appears to only be expressed, in a small amount, on adult male flies. Females do not appear to express *CG6553* in their adulthood (Figure 13, B).

Table 10 - Mean, standard error, minimum and maximum, considering the corrected values for straight winged (SW) and curly winged (CW) flies. Females' resistance to heat increased in the test group.

	N	Mean ± Standard Deviation	Minimum	Maximum	N	Mean ± Standard Deviation	Minimum	Maximum
		CG6553 **♂ x Actin5c-GAL4 ♀**				***CG6553*** **♀ x Actin5c-GAL4 ♂**		
Female CW	9	1,00 ± 0,07	0,85	1,10	9	1,00 ± 0,11	0,80	1,20
Female SW	9	1,14 ± 0,07	1,06	1,27	9	1,07 ± 0,05	0,99	1,12
Male CW	9	1,00 ± 0,13	0,76	1,16	9	1,00 ± 0,09	0,85	1,11
Male SW	9	1,01 ± 0,14	0,84	1,30	9	0,89 ± 0,07	0,80	1,03

For *CG6553* ♂ x Actin5c-GAL4 ♀ females display an increase of 14% in heat resistance (Mann-Whitney test; N=18; $P < 0{,}001$) while the ability of males to survive heat stress remained virtually the same (Mann-Whitney test; N=18; $P > 0{,}05$) (Table 10). Notwithstanding, in CG6553 ♀ x Actin5c-GAL4 ♂ the heat resistance of the females increased but not significantly (Mann-Whitney test; N=18; $P > 0{,}05$) and males' endurance to heat stress decreases by 11% (Mann-Whitney test; N=18; $P < 0{,}01$) (Figure 14).

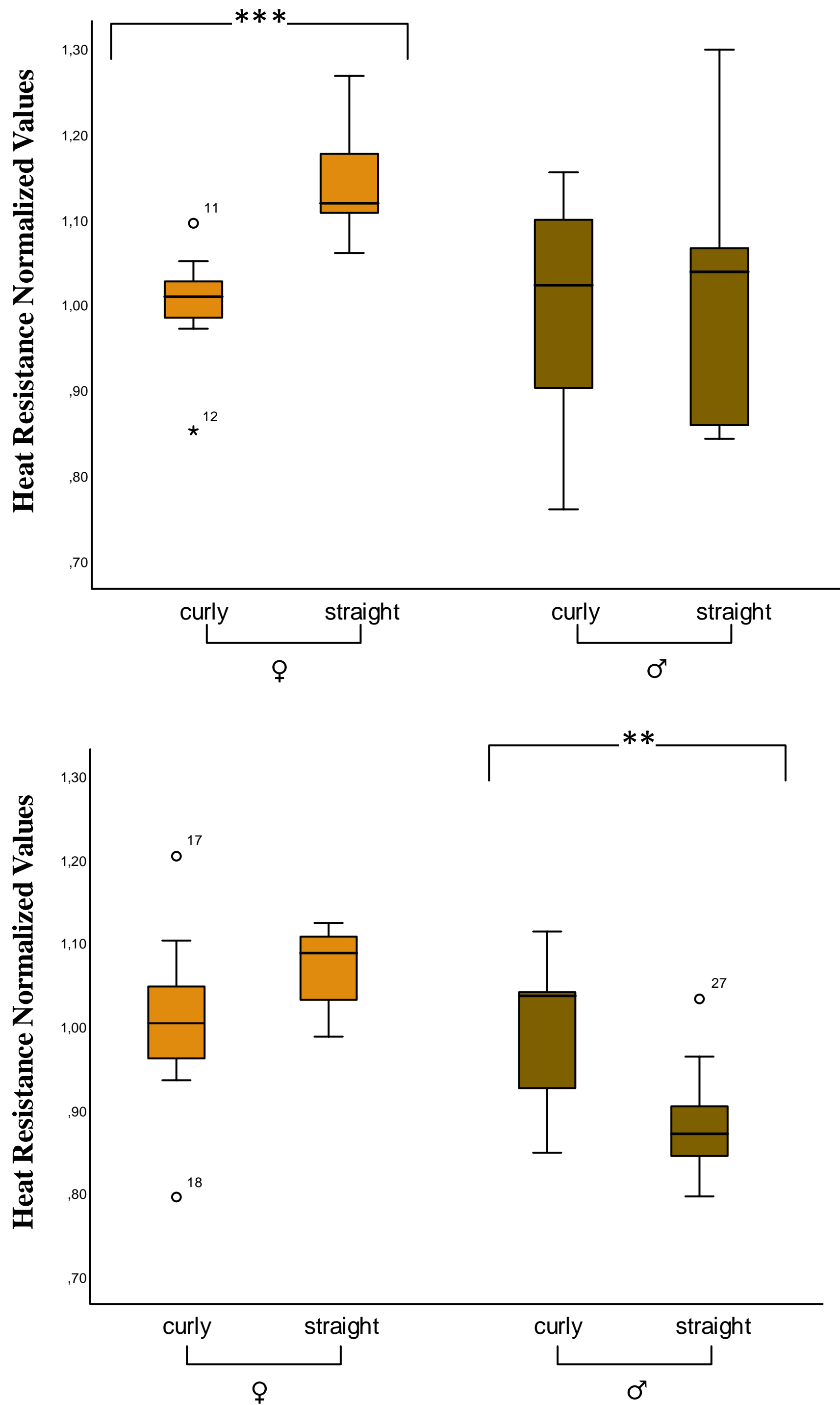


Figure 14 - Heat resistance results for gene *CG6553*. (A) *CG6553* ♂ χ Actin5c-GAL4 Driver ♀ (B) *CG6553* ♀ χ Actin5c-GAL4 ♂. Curly refers to flies with wild type phenotype, straight refers to the flies with the gene expression suppression. Values are normalized relative to the control (curly flies).
*** $P < 0{,}001$
** $P < 0{,}01$

CG6553 expression is extremely low (Figure 13) and the designed primers did not possess good efficiency, 158,04% which is above the 110% optimal value (Figure 15). As such, it was not possible to establish a percentage of suppression for *CG6553*.

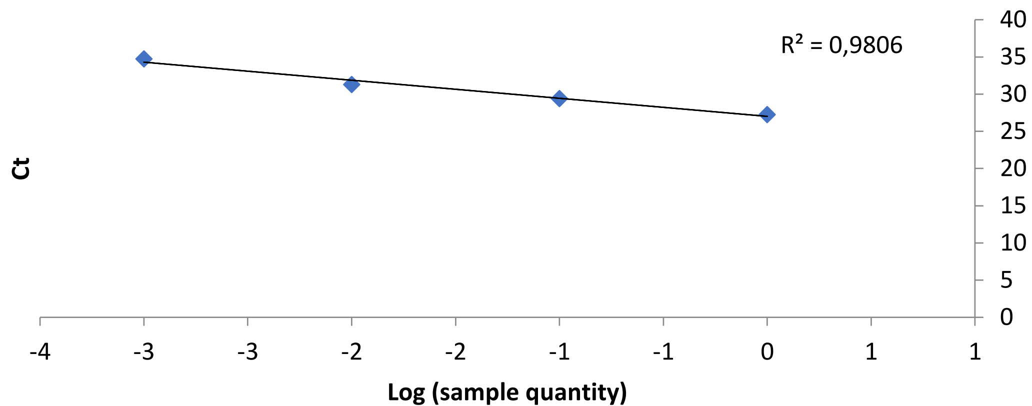


Figure 15 - *CG6553* displayed an efficiency of 158,04%, which is much higher than the optimal range of 90-110% that should be used for the Real Time PCR protocol

2.3 CG2224

This gene, which is present in the right arm of the third chromosome of *D. melanogaster* has been annotated as a Ubiquitinyl hydrolase, which enables Lys-63-specific deubiquitinase activity and thiol-dependent deubiquitinase. Thus, *CG224* encodes for an enzyme.

Currently, it is known that *CG2224* interacts with several other proteins[66] (Figure 16) none of which have been previously implicated in the survival to heat stress or the heat stress response[45].

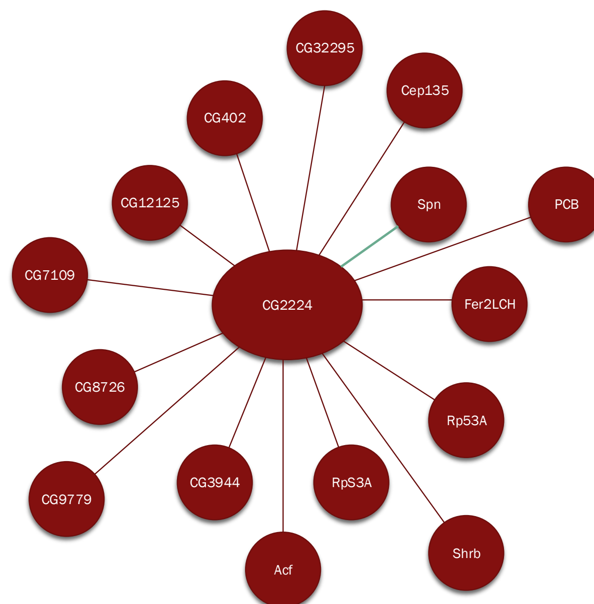


Figure 16 – All currently known interactions of the gene *CG224* in *D. melanogaster*. Information obtained from http://evoppi.i3s.up.pt/ .
In green are interactions found only in one interactome and in red interactions found in multiples but not all.

CG2224 belongs to the JAMM deubiquitinase family and has 2 transcripts and 412 orthologues, of which 23 belong to primates. In *Homo sapiens* there's two orthologues, STAMBP and STAMBPL1, both with very similar roles to that reported for the *D. melanogaster* gene[70].

Looking at the conserved domains of the sequence (Figure 17), it confirms the presence of the JAMN family motif as well as the ubiquitin interface. Furthermore, a *USP8* dimerization domain is found, which is likely due to the interaction of *CG224* with the protein *USP8* for the disassembly of the ubiquitin chain[71,72].

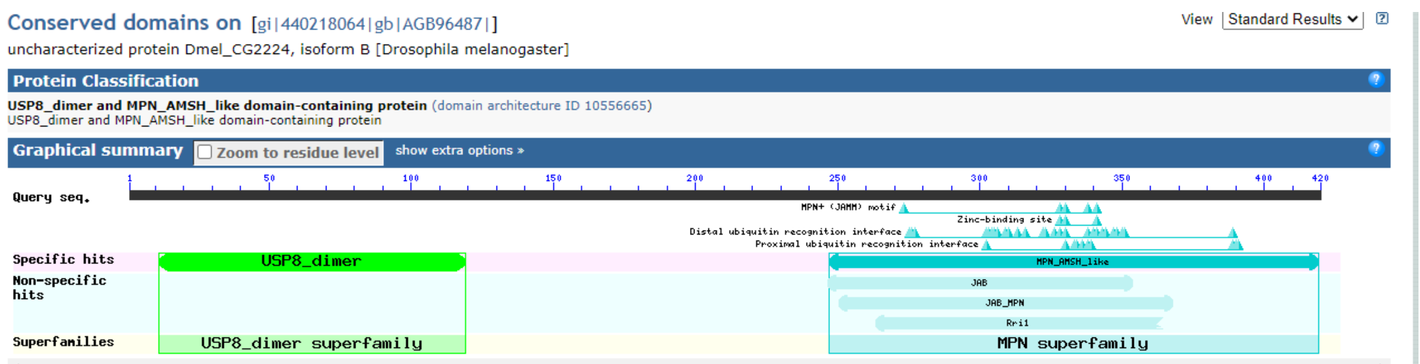


Figure 17 - Conserved domains of the *CG2224* protein. Information taken from NCBI database

Taking into account the known function of *CG2224* it is not unexpected to find a wide expression spectrum occupying with more or less expression all tissues (of those considered) and developmental phases (Figure 18). There is, however, a more pronounced expression in the reproductive system.

It is worth noting that females appear to always have a slightly lower expression than males.

Figure 18 - RNA sequencing profile of the gene *CG2224* by (A) anatomy (B) development. Only flies with 7 days were used for the heat treatment experiment, which means that the expression profile of *CG2224* will fall between that of a 5 day old fly and 30 day old fly.
Image taken from http://flybase.org

Flies where *CG2224* expression was suppressed, did not show a compromise their ability to survive heat stress (Figure 19). Indeed, variation was minimal, a decrease of 1-4% in females and 0-3% in males, and not statistically different (Mann-Whitney test; N=18; $P > 0,05$) (Table 11)

Table 11 - Mean, standard error, minimum and maximum, considering the corrected values for straight winged (SW) and curly winged (CW) flies. There was no significant change in the ability of the fly to cope with high temperatures.

	N	Mean ± Standard Deviation	Minimum	Maximum	N	Mean ± Standard Deviation	Minimum	Maximum
		***CG2224* ♂ x Actin5c-GAL4 ♀**				***CG2224* ♀ x Actin5c-GAL4 ♂**		
Female CW	9	1,00 ± 0,09	0,80	1,10	9	1,00 ± 0,03	0,95	1,05
Female SW	9	0,96 ± 0,09	0,79	1,06	9	0,99 ± 0,03	0,94	1,04
Male CW	9	1,00 ± 0,14	0,79	1,18	9	1,00 ± 0,09	0,88	1,15
Male SW	9	0,97 ± 0,10	0,86	1,14	9	1,00 ± 0,08	0,88	1,12

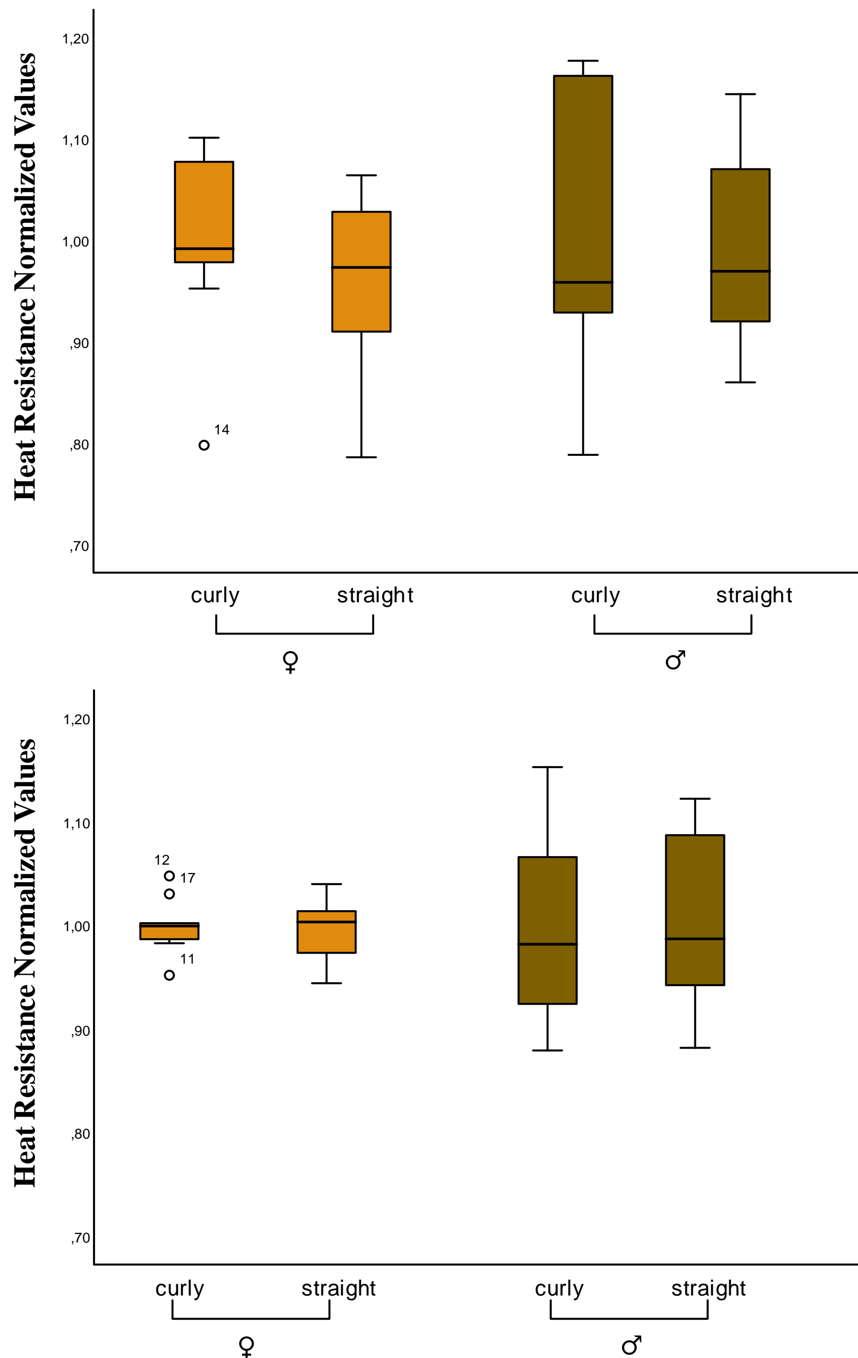


Figure 19 - Heat resistance results for gene *CG2224*. (A) *CG2224* ♂ χ Actin5c-GAL4 ♀ (B) *CG2224* ♀ χ Actin5c-GAL4 ♂. Curly refers to flies with wild type phenotype, straight refers to the flies with the gene expression suppression. Values are normalized relative to the control (curly flies).

Primers designed for *CG2224* had an efficiency of 118,10% (Figure 20), slightly above the recommended value for optimal results.

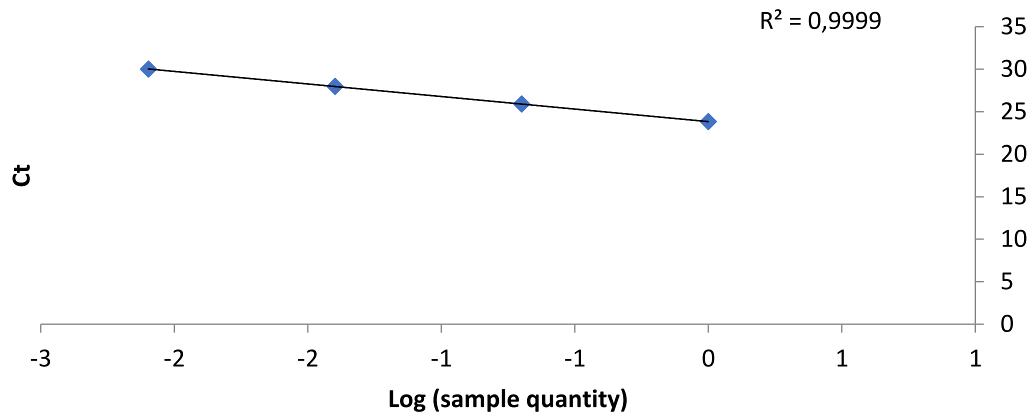


Figure 20 - Primer efficiency for *CG2224* was above the upper threshold of 110% (118,10%), but since it was the closest to the permitted values, it was still used

The suppression of *CG2224* expression was slightly lower than that obtained for *CG1146*, with a decrease in the expression of 36,8-57,51% ($P > 0,05$ when using *RPL32* as a reference gene and $P < 0,05$ when normalizing expression with *Actin 42A*). *Actin* 42A and *RPL32* efficiency was the same as shown above (Figure 10, A and B).

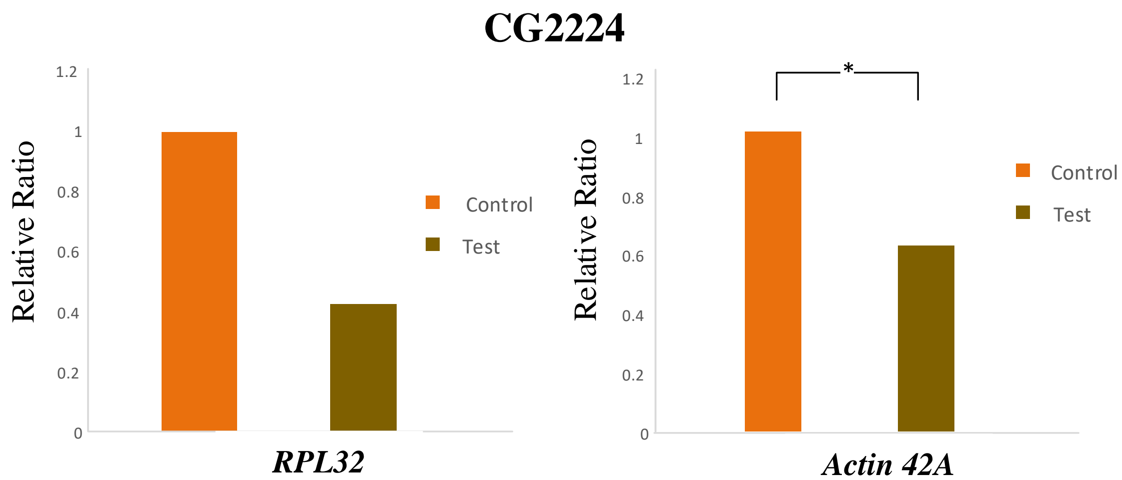


Figure 21 - Relative quantification of the suppression of *CG2224* expression. In (A) results were normalized according to reference gene *RPL32* while in (B) values were normalized according to reference gene *Actin* 42A. Two replicates of each group were performed as biological replicates, each was duplicated to create technical replicates.
* $P < 0,05$

2.4 *GluRIA*

In *Drosophila melanogaster GluRIA* encodes for the glutamate receptor 1. In the nervous system, glutamate acts as an excitatory neurotransmitter. Thus, *GluRIA* will play a role in cation transport, acting as an ion channel in the membrane.

The recognized Conserved Domains (Figure 22) confirm the annotated function since both PBP1_iGluR_AMPA and PBP2_iGluR_AMPA are domains that allow ligand-binding in glutamate receptors (AMPA receptor).

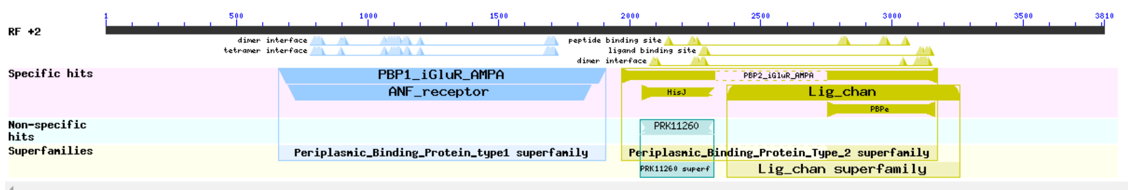


Figure 22 - Conserved domains of the *GluRIA* protein. Information obtained from the NCBI database

There are 3638 orthologues for this gene, 23 of which are in primates[73]. Furthermore, 36 paralogues have been described[74].

When looking at the interactome of *GluRIA*, one interaction was found with the gene *Ino80*[66] (Figure 23). *Ino80* encodes a component of the chromatin remodeling complex INO80 and is involved in the regulation of homeotic gene expression[75].

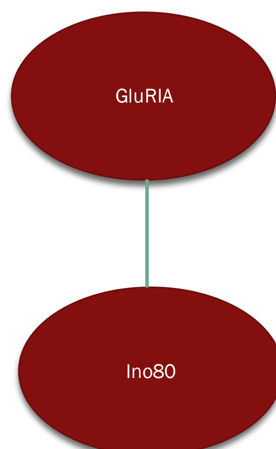


Figure 23 - Currently known interactome of the *GluRIA*. Information obtained from http://evoppi.i3s.up.pt/ .
This interaction was found in more than one interactome from the database.

The expression patterns of *GluRIA* revealed, as expected, a prominent role in the central nervous system. However, its expression, when present during development, is always quite low, before seemingly disappearing in adult females (Figure 24).

Figure 24 - RNA sequencing profile of the gene *GluRIA* by (A) anatomy (B) development. Only flies with 7 days were used for the heat treatment experiment, which means that the expression profile of *GluRIA* will fall between that of a 5 day old fly and 30 day old fly.
Image taken from http://flybase.org

Table 12 - Mean, standard error, minimum and maximum, considering the corrected values for straight winged (SW) and curly winged (CW) flies. Males' heat resistance decreased in the test group, when compared to the test group.

	N	Mean ± Standard Deviation	Minimum	Maximum	N	Mean ± Standard Deviation	Minimum	Maximum
		***GluRIA* ♂ x Actin5c-GAL4 ♀**				***GluRIA* ♀ x Actin5c-GAL4 ♂**		
Female CW	9	1,00 ± 0,07	0,89	1,08	9	1,00 ± 0,02	0,97	1,03
Female SW	9	0,96 ± 0,10	0,83	1,11	9	0,99 ± 0,03	0,95	1,03
Male CW	9	1,00 ± 0,10	0,88	1,21	9	1,00 ± 0,09	0,90	1,13
Male SW	9	0,77 ± 0,10	0,62	0,93	9	0,84 ± 0,10	0,73	0,98

The suppression of *GluRIA* expression was not lethal to the flies, and crosses were able to develop normally.

Nonetheless, males without *GluRIA* expression displayed 16-23% (Table 12) less tolerance to heat (Mann-Whitney test; N=18; $P < 0,05$), whereas females showed minimal decrease in their survival under heat stress conditions (Mann-Whitney test; N=18; $P > 0,05$) (Figure 25).

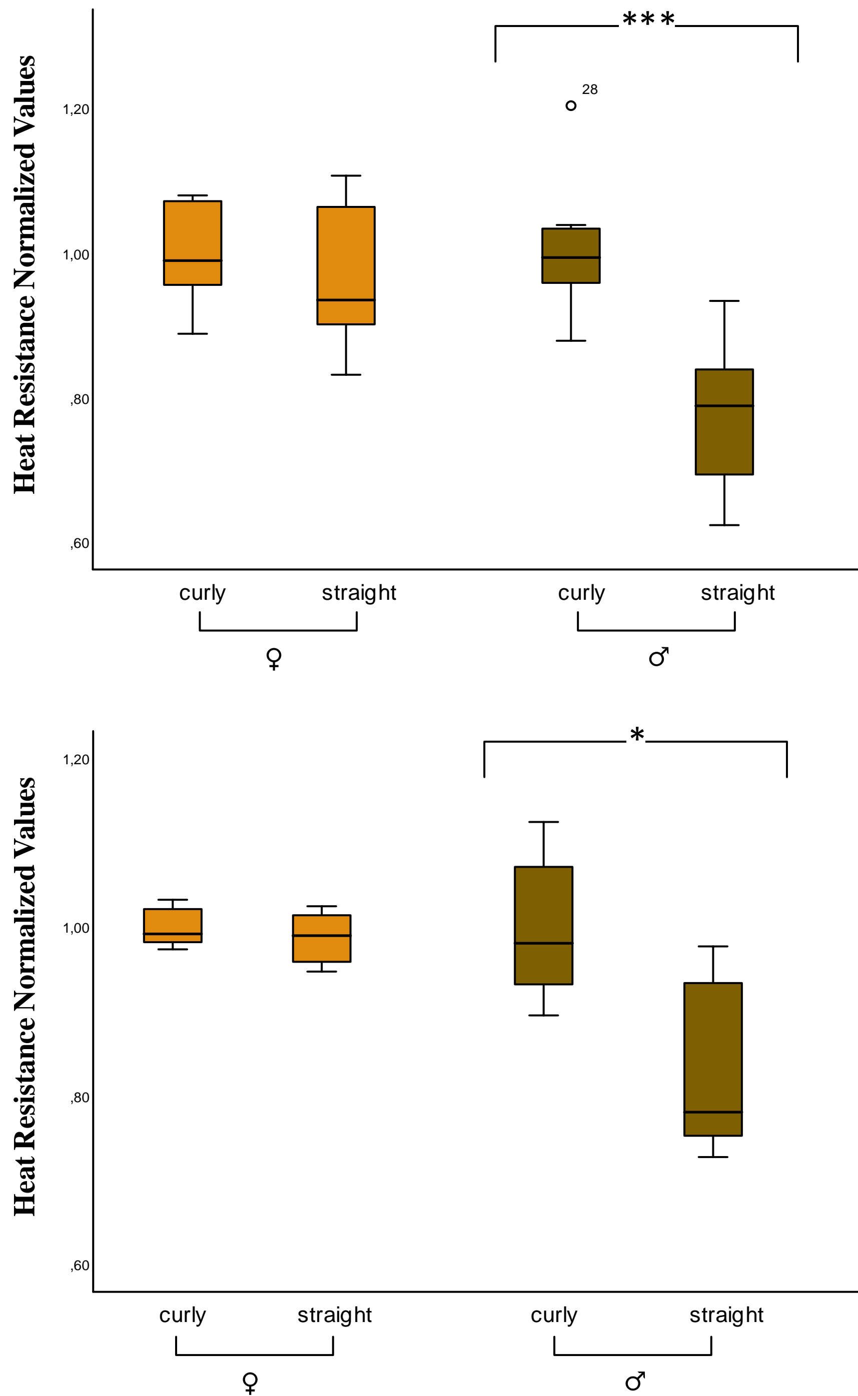


Figure 25 - Heat resistance results for gene *GluRIA* (A) *GluRIA* ♂ χ Actin5c-GAL4 ♀ (B) *GluRIA* ♀ χ Actin5c-GAL4 ♂. Curly refers to flies with wild type phenotype, straight refers to the flies with the gene expression suppression. Values are normalized relative to the control (curly flies).
*** $P < 0{,}001$
* $P < 0{,}05$

GluRIA's expression is quite low on the age group of flies used in this study. As such, and since the primers designer did not have a good efficiency, 182,83% which is well above the recommended interval of 90-110%, it was not possible to perform Real-Time PCR to evaluate this gene suppression (Figure 26).

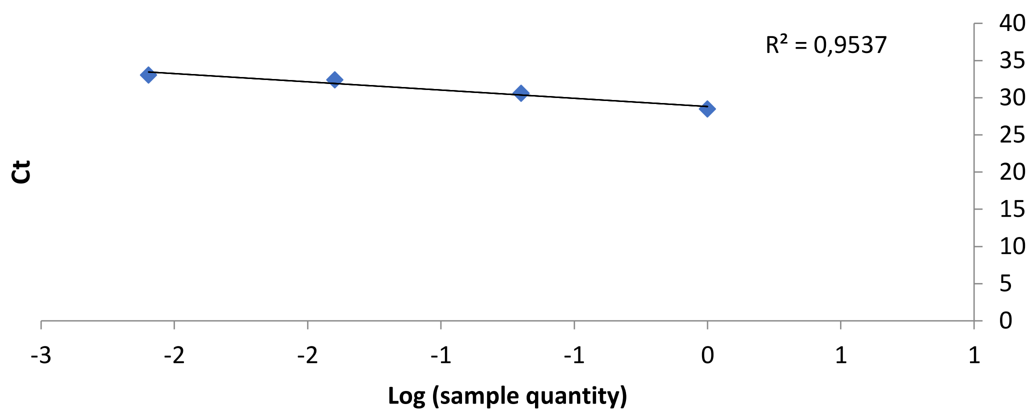


Figure 26 - *GluRIA* primers had an efficiency above the optimal interval 90%-100%, with a value of 182,83%

Chapter 5 – *Discussion*

1. *D. melanogaster* European Populations

Drosophilids are ectothermic and, therefore, highly dependable in their ability to adapt to changes in their environment in order to survive. As more studies are performed in natural populations, it has been possible to understand how local variation seems to be driven by a handful of factors, such as temperature, precipitation, wind and others[10].

For heat resistance, it was possible to show that females are much more resistant than their male counterparts across all populations, which is in accordance to what has been described in previous studies[76-79].What causes these differences is not clear; however, differences in body sizes could be one of them, as female are generally larger than males, and have, therefore, a bigger surface area with which to lose heat. This is also in concordance with other studies which have found that individuals with larger bodies are often found in populations with higher stress resistance[5, 20]

Nonetheless, these differences derive from a genetic background, as both males and females across all populations were acclimated to laboratory conditions before the beginning of the experiments to avoid possible bias by heat hardening[51, 80, 81]. However, it should be noted that strains were established from one impregnated female, i.e, each strain is an isofemale line and not an isogenic line, meaning that not all flies will have the same genotype. Although this is more representative of a natural population, it is important to keep in mind that it also poses challenges.

For instance, allelic frequencies are likely not stagnant throughout the year, since temperature will change seasonally, the selective force acting over these genes will change accordingly[82]. Despite the best efforts to collect all populations around the same time frame, doing so still does not ensure that climatic variables are the same in all countries, and thus that the same selective forces are being applied. Moreover, the heat phenotype is dependent, besides the genetic information, on the life-history of both the individual and parental flies[20, 82]. Age was not considered a factor when collecting the impregnated female used to establish a strains, this means that its genotype might have been selected for different conditions of those happening at the time of collection.

All this induces uncontrollable variables in the study that may generate some of the confounding problems found during the analysis.

The variation coefficient for males is much higher than that of females, meaning that it is harder to estimate their heat resistance than it is for females. It is important to keep this in mind when delineating experiments like this, that since males are less resistant to heat, it is possible that a successful protocol for the female flies, is not the ideal for evaluating heat resistance in male flies.

Considering how much more susceptible to heat male flies are, a shorter exposure to heat, to decrease mortality at the end of the experiment (which was over 85% for 8 out of 9 populations) and an increase in readings, to be able to better differentiate between death times, may be more appropriate for males.

Perhaps the most surprising was the lack of a geographical gradient for this phenotype, which had been previously reported many times on continents such as North America and Australia[5, 7, 51, 53, 83]. As mentioned previously, there is some debate on how exactly is the differentiation of heat resistance being done when looking at latitude. Here, no gradient was found for this phenotype, except when considering exclusively males.

In America, it is possible to sample through large regions of the continent by following one chosen latitude and without changing longitude. The same is harder to achieve in Europe, and was not accomplished in this work. Sampling more would be a way to solve this problem, but it is impossible to know how much larger the dataset would have to be to detect variation segregating along geographical gradients in Europe.

However, it was possible to detect an association between bioclimatic variables and variation in heat resistance. Since males appear to be highly susceptible to temperature, which changes predictably with latitude much more than longitude[82], this may justify the previously found correlation. While females are mostly affected by the combination of temperature and rainfall.

In the same way that female and male flies do not have the same resistance to heat stress, despite belonging to the same population, they appear to have different susceptibilities to climatic changes. And this may be because males, who are more susceptible to high temperatures, are leaving in their upper limits, while females are not.

Furthermore, in this assay it was possible to establish that for a proper analysis to be done, a sample at least as big as the one here used is needed. As when considering the 168 strains, correlations were found, but when the sample was reduced to the average reading per population, all signal was lost.

2. Candidate genes for Heat Resistance

Much of the molecular mechanisms of heat resistance remain to be understood. By knocking down the expression of candidate genes with high correlation coefficients with longitude, and phenotyping individuals with a specific protocol, it was possible to analyse the role of each gene for a complex phenotype such as heat resistance.

The Actin5c-GAL4 driver was not enough to completely suppress the expression of the tested genes, however, the results obtained end up being more biologically relevant, as in nature expression is not often 100% or 0% but a spectrum where small changes often are enough to cause phenotypical alterations.

2.1 CG1146 and *CG6553*

Both *CG1146* and *CG6553* are currently unannotated and lack information about their interactions with other genes, as well as their role within the cell.

With the data here obtained, it is not possible to infer their importance or role in the cell, besides their contribution to the studied phenotype: heat resistance.

In the case of *CG1146*, the fact that there is an increase in the heat resistance of the female flies when the expression is suppressed points towards *CG1146* being involved in another process of enough importance for the depression in heat resistance to be an acceptable loss for the organism. This may happen if the gene is directly involved in the capacity of a fly to produce descendants. For example, the *methuselah* gene (*mth)*, involved in the lifespan of the fly, undergoes something very similar, where the knockdown of this gene increased the heat and starvation resistance of the fly[84].

Sexual dimorphism has been reported before in *Drosophila melanogaster* [85-88] and thus, is the most likely explanation for the differences found between females and males when the *CG1146* expression is suppressed.

For *CG6553*, the presence of the conserved domain for calcium-binding may be a clue to its function, especially when taking into account the expression profile that is most pronounced around the head. However, further testing would be needed in order to properly infer its role in the cell, as Ca^{2+} is used as a neurotransmitter in the Central Nervous System, and is also important for the correct functioning of the mitochondria[89].

Females with reduced expression of *CG6553* are overall more heat resistant than the control group. In *CG6553* ♀ χ Actin5c-GAL4 Driver ♂ the increase is not statistically significant, nonetheless, this cross appears to have more variation than *CG6553* ♂ χ Actin5c-GAL4 Driver ♀ which might account for this difference. Sampling more replicates in this cross, may reveal no differences between crosses, with both *CG6553* ♀ χ Actin5c-GAL4 Driver ♂ and *CG6553* ♂ χ Actin5c-GAL4 Driver ♀ resulting in females with better resistance to high temperatures. The same can be seen in the males, as it appears to exist no variation on *CG6553* ♂ χ Actin5c-GAL4 Driver ♀ with males from the test group surviving roughly the same as the control group. In *CG6553* ♀ χ Actin5c-GAL4 Driver ♂ there is a decrease in the heat resistance of males with statistical significance. However, male variation is very high, and as assessed previously, the protocol might not be the best to assess their resistance to high temperatures. Sampling more replicates or diminishing the time of exposure to heat while increasing the number of readings, might give a more accurate reading.

2.2 CG2224

Deubiquitinases, also known as deubiquitinating enzymes, mediate the action of the protein modifier ubiquitin in its action as a post-translational modifier. Protein ubiquitination controls a multitude of processes in the cell, from gene transcription, protein degradation, cell division, and death, as well as multiple cellular pathways[90]. Mutations in deubiquitinases have been linked to neurological diseases[91] and thus their impairment may be important for the correct function of an individual.

CG2224 belongs to the JAMN family, which is the only class of deubiquitinase that is not a cysteine protease, but rather a Zn^{2+}-dependent metalloprotease[92].

Physiologically, the fact that the knockdown of the expression of *CG2224* did not lead to lethality could imply that it is not a necessary gene for development, which is interesting, since the knockdown of expression of almost all other members of the JAMN family led to impairment in the development of the fly[90]. Nevertheless, it must be noted that the expression of the gene was not fully suppressed, and that residual expression could be enough for the fly to develop.

A similar study to that performed here, utilized a GAL4 driver to knockdown the expression of all deubiquitinases in the fly, and much like the results here presented, achieved only a partial gene suppression, although the range of suppression was lower

here, 36,8-57,51%, compared to the 73% of that study. Moreover, the knockdown of the expression of *CG2224* resulted in flies with a mild phenotype of slower moving adults[90].

Movement can be important for flies to resist heat stress, as by moving their wings they create airflow that helps them cool[21]. Also, flies in the wild will greatly increase their chances of survival by taking refuge in a better-suited micro-environmentt[22]. Nonetheless, in this experiment, space was limited, as the flies are put into small vials in groups of 15-20, and there is no better environment for the flies to take shelter in, since the entire tube is kept at 37ºC. Thus, in these conditions, movement may not be the most important factor for surviving heat stress.

This is confirmed by the results here documented, where a slight decrease in the resistance of the flies is seen in the test group, i.e, flies with the expression knockdown of *CG2224*, yet it is not statistically significant.

It is also possible that this gene is still important for thermal resistance, but that it plays a smaller role in the survival to extremely high temperatures.

As movement as a whole, as well as muscle activity, is much more important to surviving cold stress[93, 94], investigating a possible role in cold survival, could also reveal a link to thermal resistance phenotype not tested here.

2.3 GluRIA

GluRIA, or the *glutamate receptor IA*, is expressed in the mushroom bodies (MBs) and the antennal lobe (AL) together with *GluRIB*[95]. Note that it is in the antenna that the heat neurons are present in *D. melanogaster*[21].

In *Drosophila*, glutamate is one of the major excitatory transmitters in its central nervous system. Moreover, contrary to mammals, *Drosophila* neurons do not produce both excitatory and inhibitory neurotransmitters, meaning that different parts of the brain will produce the different compounds[95].

Glutamate has been shown to be a wake-active neurotransmitter in *Drosophila,* i.e, it is a neurotransmitter that promotes the state of wakefulness in the fly. And the inactivation of glutamatergic neurons was demonstrated to decrease the total wake time in the fly as well as the duration of each bout of wakefulness[96].

Here, a decrease in the expression of *GluRIA* lead to a significant decrease in the heat resistance of males. While female flies did not appear to be significantly affected by the loss of expression.

This is not the first time that a wake promoting gene has been implied in survival to heat stress[97, 98]. Indeed, *Adar*, also known as *adenosine deaminase acting on RNA*, which encodes an RNA editing enzyme was found to be essential for flies to maintain normal waking. In this case, the knockdown of *Adar* expression led to increase sleep on the fly[99]. And a faulty expression of *Adar* also significantly decreased the ability of the fly to deal with heat stress[97-99].

It is important to consider that there are qualitative differences between daytime and nighttime sleep. Thus, an inference could be made that both correspond to distinguishable physiological states, with possible overlapping mechanisms but dissimilar biological functions[100, 101].

Although the mechanisms behind sleep are not fully understood, changes in sleep patterns, as well as lack of sleep, have been associated with performance costs[102, 103].

GluRIA seems to be expressed mainly in male flies at the age at which the flies in this study were tested. As such, it is not surprising that the main effects of the absence of *GluRIA* falls to them.

As mentioned several times throughout this study, females and males have distinct heat resistance, with males surviving on average less time under heat stress than females. This, coupled with the very little to no expression of *GluRIA* under the tested conditions on females, most likely accounts for the differences found between the sexes.

Furthermore, sexual dimorphism has been reported before in the central nervous system of *D. melanogaster*[104], as well as other traits[88, 85] this is thought to be because males and females are under very different selection pressures[86, 105]. Making it fairly common for males and females to have distinct responses under the same stimulus.

Chapter 6 – *Conclusion*

1. Heat Resistance on European Populations

This work adds to the body of knowledge aimed at looking at adaptation as a means to understand how genetic variation translates into a change in the phenotype. Especially in the European populations which are not as well characterized as those in North America and Australia.

By sampling along geographical gradients it was possible to identify a latitudinal cline for the heat resistance phenotype. Surprisingly, only in the male flies.

This opens a new line of work, which addresses how different selective forces are acting on the two sexes. Since, here, females and males appear to be differentiating under two different forces, males being mainly limited by temperature, while females are influenced by the combined effect of rainfall and temperature.

It was also possible to note that using different protocols for males and females may be more beneficial since males and females have very different resistances to heat.

Lastly, here it was established that the use of a low number of samples will likely muffle any differentiating effect acting on the populations as a whole. Thus, when looking at population averages, a large sample is needed to find correlations.

2. Identifying novel candidate genes for Heat Resistance

Unfortunately, despite the best efforts of researchers all over the world, the molecular mechanisms behind stress resistance, and in particular thermal resistance, are still not completely understood.

Here, it was possible to establish a protocol that successfully identified novel genes with a role in heat resistance (three out of the four genes used).

Despite the small sample (only four genes), these results indicate that a better approach to that of screening for phenotypic differences to later search for the genetic background causing those differences might be to simply look at genes for which there is evidence of being under selection (in this case, thermal resistance is known to differentiate along clines and so, high correlations between longitude and latitude were considered) and look at the phenotype of flies where gene expression has been suppressed.

Given the high rate of success of this approach, it is foreseen that many more heat resistance genes may be identified in the future.

Chapter 7 – *Future Perspectives*

For the effort of characterizing European populations of *D. melanogaster*, in the future, utilizing the same protocol for other populations from different countries of Europe may uncover even more correlations that were not possible to establish with the current samples.

Since the protocol here established was successful in identifying genes involved in heat resistance, in the future, looking towards the other highly correlated genes (Figure 4) that were not selected for this study, might add more information to this phenotype.

Moreover, despite now being known that these genes have a role in heat resistance, their molecular role and interactions within the cell are still not understood. Further studies could shed light not only on the function of these genes, but molecular pathways related to heat resistance.

Since *GluRIA* is not the first gene known to affect sleeping patterns in the fly that has been implicated in the resistance to heat, it would be interesting to understand in the future if the differences in the phenotype arise from a direct change in the gene expression, i.e, if the loss of the gene is what is causing the decrease in heat resistance, or if the lack of resistance to high temperatures is a side-effect of the change in the sleeping pattern of the fly.

Lastly, testing these genes, especially the *CG2224* for other thermal phenotypes, like cold resistance, could unveil more information on thermal resistance phenotypes.

In conclusion, this work supports the view that looking at local adaptation as a means to understand gene function and the impact of variants on the phenotype works very well.

Chapter 8 – *References*

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