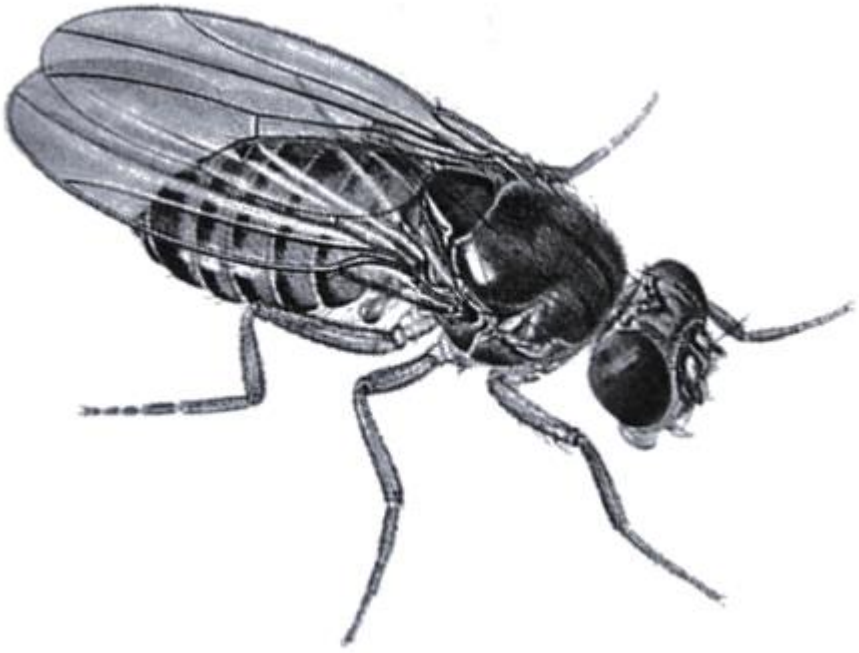


Molecular Basis of Cold Resistance: the role of *Frost* and *Muc68E*

Ana Catarina da Silva
Master's degree in Cell and Molecular Biology
Biology Department
2014

Supervisor
Jorge Vieira, Lead Researcher at IBMC
Institute for Molecular and Cell Biology
and Invited Assistant Professor at FCUP
Faculty of Sciences University of Porto

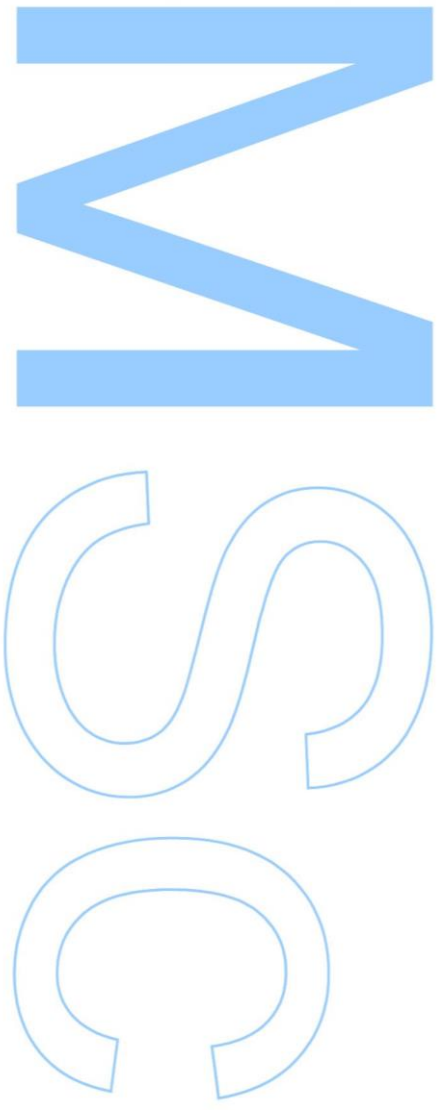
Co–supervisor
Cristina Vieira, Lead Researcher at IBMC
Institute for Molecular and Cell Biology
and Invited Assistant Professor at FCUP
Faculty of Sciences University of Porto

U.PORTO

FACULDADE DE CIÊNCIAS
UNIVERSIDADE DO PORTO

Todas as correções determinadas pelo júri, e só essas, foram efetuadas.
O Presidente do Júri,

Porto, _____/_____/_____



Resumo

Os animais ectotérmicos são especialmente afectados pela temperatura uma vez que esta acarreta consequências na sua fisiologia. As pequenas moscas da fruta do género *Drosophila* não são capazes de regular a sua temperatura corporal e por isso evoluíram uma vasta gama de comportamentos adaptativos de forma a evitar o stress provocado pela temperatura. Em particular, os limites da distribuição desta espécie e de vários outros insectos têm sido relacionados com a resposta fisiológica a baixas temperaturas.

Em *Drosophila*, o tempo de recuperação das moscas após coma induzido pelo frio tem sido usado em várias análises genéticas como uma medida de resistência ao frio. Com este trabalho vamos relacionar a expressão de *Frost* e *Muc68E,* dois genes que traduzem proteínas com características semelhantes, após coma induzido pelo frio.

O gene *Frost* é sobreexpresso cerca de cinquenta vezes após choque térmico por baixas temperaturas, embora também se apresente sobreexpresso após outros tipos de stress, apesar de aqui a resposta ser de menor magnitude. Com este trabalho, sugerimos que este gene poderá estar a responder à dissecação e não especificamente ao stress induzido pelo frio. O *Muc68E* é um gene pouco estudado, por isso fez-se inicialmente uma análise da sua estrutura e evolução molecular. Assim concluiu-se que este gene, a estar envolvido na resposta ao frio, só pode explicar variação no subgénero *Sophophora*, uma vez que não está presente no subgénero *Drosophila*. Para além disso, também realizamos comparações fenotípicas entre uma estirpe com uma mutação neste gene, e uma estirpe controlo, de forma a melhor perceber a resposta do *Muc68E* ao stress induzido pelo frio, assim como a outros tipos de stress, de forma a melhor perceber as funções deste gene

Abstract

Ectotherms are affected by environmental temperature, as it impacts their physiology. Small cosmopolitan flies from the *Drosophila* genus are not capable of regulating their body temperature, so they present different kinds of behaviour in order to avoid thermal stress. In particular, geographical distribution limits of *Drosophila* and many other insects, have been linked to their physiological and fitness responses to low temperatures.

Chill coma recovery has been used in genetic analysis as a measure of cold resistance in *Drosophila*. Here, we relate the expression after chill coma of two genes that encode proteins with similar features, Frost and Muc68E, with the cold response.

Frost is overexpressed around fifty times after cold exposure, and it has been shown to be involved in the responses to other types of stress although to a lesser extent. However, we suggest that *Frost* is actually responding to desiccation. Since *Muc68E* is a poorly studied gene, we started by analysing its structure and evolution and discovered that it is only present in the *Sophophora* subgenus – meaning that even if it is involved in cold response it is not associated with a genus-wide mechanism. We also performed phenotypical comparisons between a *Muc68E* mutant strain and its control in chill coma and a variety of other stresses in order to grasp other possible functions of this gene.

Keywords: Cold adaptation; *Frost*; *Muc68E*; stress response; gene expression

Acknowledgements

To Jorge Vieira and Cristina Vieira, I want to thank for these three years at the molecular evolution lab. You have shown me that this area is not only about knowing how to do agarose gels, but also about exploring the implications of small changes that happen over a long period of time. I am really grateful for all the conversations about the philosophy of science, your invaluable biological insight, and overall support in these years which for sure enriched my academic career as well as my personal life.

Thanks to my EvoMol comrades, Ramiro Morales-Hojas, Hélder Rocha, Inês Páscoa, João Rodrigues, Joana Santos and Lisete Moutinho for all the moments at the lab – both learning and teaching, I have had valuable experiences with all of you! Special thanks to Micael Reis for the end-of-day discussions and out-of-the-box thinking. Thanks also to my group neighbours Inês Pratas and Bruno Aguiar for their opinions and company, and to Tânia Meireles and Paula Magalhães for hope and support in those tricky RNA days.

In addition, a word of gratitude towards those who have taught me these last five years, especially Fernando Tavares, Jorge Teixeira, and Susana Pereira, for nourishing my curiosity and pushing me to develop my critical thinking. Furthermore, a word of appreciation to my master's colleagues and to my friends who supported me in many ways, even when I was talking for the n^{th} time about my "flysitter" occupation.

Turning to the backstage, thanks to my aunt Margarida for all the care and to my brother for all the laugher. To my mother, thanks is an empty word considering how very grateful I am for your love and support – the distance in nothing diminishes your presence at my side! And to complete my support system, my sincerest thanks to Pedro, a partner and core pillar in every aspect of my life.

Index

Figure Index

Table Index

List of symbols and abbreviations

Atf3 – Activating transcription factor 3

BLASTp – protein Basic Local Alignment Search Tool

CBT2 domain – Chitin-binding type-2 domain

CCRT – Chill Coma Recovery Time

cDNA – complementary Deoxyribonucleic Acid

CO – Control

C_T – Threshold cycle

Fst – Frost

GO – Gene ontology

GTR – Generalised Time-Reversible

HS – Heat Shock

Hsps – Heat shock proteins

LS – Lifespan

mRNA – messenger Ribonucleic Acid

ND – Nutrient Deprivation

Ore-R – Oregon-R

P – Significance of Pearson product-moment correlation coefficient

PCR – Polymerase Chain Reaction

EST – motif composed of proline, two glutamic acid residues, serine and threonine

PerA – Peritrophin-A domain

PM – Peritrophic Matrix

prc – pericardin

PTS – motif with prolines, threonines, and serines

qPCR – quatitative real-time Polymerase Chain Reaction

QTL – Quantitative Trait Loci

R – Pearson product-moment correlation coefficient

RCH – Rapid Cold Hardening

RNAi – Ribonucleic Acid interference

RQI – RNA Quality Indicator

RT-qPCR – Reverse Transcription quantitative real-time Polymerase Chain Reaction

SCP – Supercooling Point

SD – Standard Deviation

ST – Starvation

tBLASTn – protein-nucleotide translation Basic Local Alignment Search Tool

Chapter I – General Introduction

1.1. How is temperature important for ectothermic animals?

Adaptation to fluctuations in temperature is important to animals, especially to those that, being ectotherms, need to gather heat from the environment mainly by behavioral means since they have only limited abilities to thermo regulate via physiological adjustments [63, 134]. In insects, temperature is a major abiotic factor, resulting in potential cold or heat stress [71] with extreme temperatures being injurious and potentially lethal. Nonetheless, thermal environment does not completely constrain ectotherms. If exposed to extreme temperatures, insects may respond in different ways: they can behaviorally avoid extremes by escaping the stress source, for example alternating between shade and sun, and they can also respond by changes in morphology, life history or physiology within their lifetime (reviewed in detail by [8]).

1.2. Surviving winter – the insect cold response

It is not surprising that selection for cold resistance is stronger than for heat resistance given that summer does not pose a range of harsh conditions as variable as winter [27]. Particularly, distribution limits of ectotherms, including many insects, have been linked to their physiological and fitness responses to low temperatures [4, 146], and strong correlations exist between cold tolerance and environmental distribution [25, 76, 81]. For instance in flies, certain threshold temperatures generally limit reproduction [126] and development in most tropical and temperate species [40]. Therefore, had insects not evolved a range of physiological and molecular adaptations to tolerate low temperatures, they would be likely to suffer from cold injury, with severe consequences as increased mortality and reduced reproduction ([83, 84, 98, 109, 128, 150].

Temperature resistance in *Drosophila* can also be influenced by prior exposure to altered temperature conditions or to different photoperiods – because light conditions act as important environmental cues for many insects. Decreasing day length, for instance, typically signals that winter is coming, which frequently induces diapause [8, 16]. This process represents a different developmental pathway, initiated and regulated by unique patterns of gene expression, that results in the ability to survive seasonally environmental stress, especially by adapting the rates of growth, development and

reproduction [44]. Insects in diapause are commonly cold resistant, allowing them to overwinter [43], although the mechanisms behind this are not fully understood yet (review in [44]).

1.2.1. How do insects react to cold

The physiological behaviour of insects near the freezing point, and in particular their ability to survive ice formation in their extracellular fluid, provides a natural way of dividing this class. Insects have thus been called freeze tolerant – when they tolerate the formation of internal ice - or freeze avoidant organisms – when they avoid freezing by maintaining the body fluids liquid below the normal freezing point through a process called supercooling [90, 116, 131, 153]. Recently, however, it was realised that many insects have such a low supercooling point (SCP - the point when body water does turn to ice) that they die from cold injury before their body fluids can freeze [83, 98, 106, 129]. A good example are *Drosophila melanogaster* flies, whose SCP is between -17 and -20ºC [38] and yet die between -6 and -9ºC [79]. These have since then been labelled chill-susceptible, completing this classification [12], which is underlined in Fig. I-1 (squares with red lining).

It is still unclear why insects adopt a certain strategy for dealing with subzero temperatures, but studies have shown that in the arthropod lineages freeze avoidance is the basal strategy and that freeze tolerance evolved several times among insects, as for instance in Diptera [130]. These authors suggested that freeze tolerance is a strategy that evolved recurrently because of different reasons according with an insect's habitat. In the Northern Hemisphere, due to severe winters, insects must be able to survive low temperatures for an entire season, and the most efficient way to do that is to pursue a freeze tolerance strategy, but only for the overwinter period to avoid desiccation for instance. By contrast, weather in the Southern Hemisphere tends to be unpredictable but usually mild so, in order to survive the occasional cold storms during the summer, insects that have food in their gut can use that as ice nucleators and survive the cold inducing freeze tolerance, without spending much energy avoiding cold that is not there during warm winters.

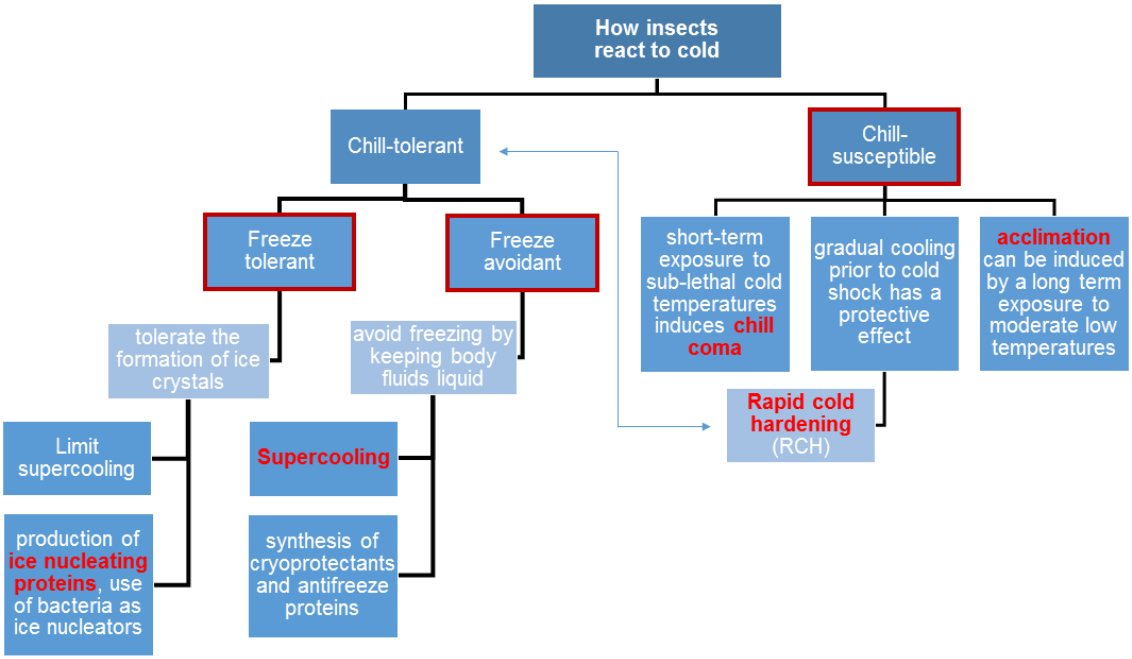


Figure I-1: Diagram summarizing the insects' reaction to cold. Squares surrounded by red lines state different classes of insects' physiological behaviour towards cold, and words highlighted in red refer to some of the processes/agents occurring in a particular class. The blue arrow indicates that RCH is more common in chill-susceptible species, but is also present in chill-tolerant organisms

In freeze avoidant insects supercooling is not the only process to guarantee overwinter survival, since synthesis of cryoprotectants, polyols, sugar and glycogen, as well as production of antifreeze proteins that adsorb ice crystals inhibiting their growth, work together as protective mechanisms that assure a response to the expected low temperatures when seasons change (thoroughly revised in [29]). Freeze tolerant insects limit supercooling by voluntarily initiating the freezing of their bodies at higher temperatures, and for that they recur to the production of ice nucleating proteins, or use particles of food and even bacteria present in the gut as ice nucleators [90, 151].

The damage caused in chill-susceptible insects varies drastically with the amount and intensity of cold exposure. A short-term exposure (minutes or hours) to sub lethal cold temperatures causes cold hardening [15] and/or a reversible state of paralysis known as chill coma [102] which results in reversible physiological changes. Long-term exposure (days or weeks) to temperatures between the normal and viable temperature range of an organism gives rise to both reversible and irreversible changes in physiology, and is named acclimation (review by [8]).

The reversible status of chill coma is only maintained when cold exposure is neither prolonged nor severe, otherwise insects are likely to die from accumulation of cold injuries [4, 39, 54, 83, 84, 98]. The mechanisms behind chill coma physiology are yet to be fully grasped, but previous studies have shown that the loss of ability to maintain

posture involves a disruption of normal neuro-muscular performance [5], and also a loss of muscular excitability in bees and flies [55, 70], which is related to a more global loss of muscular function. In both cases, it looks like these processes are strongly related to an inability to maintain trans-membrane ion homeostasis at low temperatures [78, 84, 99, 154].

When *Drosophila* are acclimated, they show physiological changes such as alteration of membrane lipid composition [61], most commonly by increasing the proportion of unsaturated fatty acids to saturated fatty acids [75, 103]. They also reveal alterations in sugar or polyol concentrations [64], and metabolic rate [13], which can affect temperature resistance. Moreover, the amino acid proline was found to act as an energy reserve and was associated with cold resistance [104]. Glycogen and proteins (especially rich in prolines) also act as energy reserves and may enhance survival at extremely cold temperatures [23].

When insects need to respond quickly to changes in environmental temperatures, there is a rapid protective mechanism against cold injury named rapid cold hardening (RCH), which was first identified as the capacity for rapid accumulation of glycerol in response to low temperature exposure [89]. We can mimic this hardening mechanism by gradually cooling flies before cold shock, and it has been studied that this RCH acts as a protective effect of acclimation [45, 131], and can increase cold tolerance in some insects [79, 152] in a matter of days or even hours. Presently, as with chill coma, the mechanisms behind RCH are still to be perfectly understood, but this is one of the fastest responses to low temperatures [89], and has been found to improve traits such as survival, activity and reproduction during cold exposure [38, 79, 109, 120, 128, 150]. Some studies suggest that insects might enhance cold tolerance in response to predictable or unexpected decreases in environmental temperature via the RCH response [45, 77, 79, 85, 89, 110], independently of being freeze tolerant or avoidant (which is represented in Fig. I-1 with the blue arrow).

1.2.2. The molecular basis of short-term cold resistance

Resistance to cold in *Drosophila* has been quantified by survival rate after cold shock [23] but it is now commonly estimated by the recovery time of flies after a non-lethal but chill coma inducing exposure to cold [11, 17, 54, 59, 71, 108, 115]. This latter approach can be particularly useful in distinguishing among the geographical distribution of *Drosophila* species [4, 54, 76, 81, 97, 98].

Many examples in *Drosophila* literature show that cold tolerance phenotypes embody an important adaptation in this genus, although a comprehensive understanding of this adaptation involves a thorough description of the genetic architecture influencing cold resistance variation, as well as the identification of the functional allelic variants underlying such phenotypes [30]. This was accomplished in a more general fashion through quantifying genetic variation along cold tolerance clines [11, 34, 53, 65, 81], via studies focused on artificial selection [4, 24] or for instance identification of mutants with altered temperature preference [137]. With analysis of gene expression it was also possible to identify genetic changes in response to selection towards cold tolerance [140] or various cold stressors [56, 57, 114, 132] and associations of variation with polymorphisms in candidate genes [3, 35]. These studies have clearly demonstrated the existence of transmissible genetic variation for cold tolerance and suggest that this variation is probably influenced by a large number of genes.

To isolate genes with large effects on traits such as thermal resistance, another method that involves the identification of small sections of a chromosome with significant impact on the studied trait of interest – the quantitative trait loci (QTL) approach – can be used [145]. One of these large studies [105] used a population of recombinant inbred lines to map QTL that affect variation in both heat- and cold-stress resistance in order to understand and identify genes controlling thermo tolerance phenotypes. In one mapped region associated with cold response, three genes that have previously been implicated in various responses to cold, *Fst*, *Sas* and *desat2,* were underlined. Those were also identified in another QTL for chill coma recovery [108]. *Fst* will be described in a more detailed fashion in Chapter II, *Sas* has been implicated in ice binding and response to freezing [80], and *desat2* is involved in cuticular hydrocarbon biosynthesis [36] and has alleles that vary in their cold tolerance [58].

The QTL in the Norry et al. study [108] also includes *Dca* (also known as *smp-30*) and *hsr-omega,* which, along with *Fst*, are named candidate genes that causally affect thermal tolerance in the right arm of chromosome *3* in another molecular analysis study [3]. More related to heat response*, hsr-omega* is thought to play a role in the dynamic coordination of nuclear and cytoplasmic transcript processing under various stress and growth conditions [74, 88], and its allelic variation underlies a significant fraction of the heat sensitivity variation in *D. melanogaster* [100, 101]. Meanwhile, *Dca* was first identified as a candidate gene for cold tolerance in *Drosophila* [56], but microarrays and qPCR techniques showed that this gene is down-regulated after a cold shock at 0ºC for 1 to 3 hours [114, 132], so *Dca* is more likely to be involved in the cold adaptation but not in the response after a cold shock. A phylogenetic analysis revealed that this gene is only present in the *Sophophora* subgenus, and a more detailed study

supported the idea of a duplication from the ancestral *regucalcin* gene, after the split of *Drosophila* subgenus and before the *Sophophora* radiation, as the origin for the *Dca* [7], so this gene cannot explain cold adaptation across *Drosophila* genus.

One of the microarray analyses mentioned before [114] aimed to examine the changes in transcript abundance associated with the cold hardening treatment and, along with *Dca* and *Fst*, the authors identified three heat shock proteins (Hsps), namely *Hsp23*, *Hsp26*, and *Hsp83*. Because Hsps are chaperone proteins that facilitate the refolding of damaged proteins, this up regulation after cold hardening (confirmed with RT-qPCR) is probably a useful function when the cell is dealing with heat or cold-denatured proteins, thus protecting insects from cold exposure. In this study other three membrane proteins, *CG13510*, *CG10912*, and *CG8778*, were also involved in cold hardening. These eight proteins make up the class of biological processes defined in Flybase (http://flybase.org/) as any process that increases freezing tolerance of an organism in response to low, non-freezing temperatures, which is associated with the GO for cold acclimation (GO:0009631). Also worthy of mention is the fact that the GO for response to cold (GO:0009409) only encompasses three more *D. melanogaster* genes: *brv1* and *brv2* involved in the cellular response to cold, and *brv3*, all of them inferred from mutant phenotype [50]. Nonetheless bear in mind that these genes are also important in other pathways, as is the case of the *brivido* proteins which are involved in the activity of calcium channels [94].

Note that overall GO (gene ontology) terms are a useful way to describe gene products in terms of their associated biological processes, cellular components and molecular functions in a species-independent manner [9]. We sum up the information about the genes with biological processes related to the response to cold in Table I-1, and also add a summary of the molecular functions of those proteins with both experimental evidence and predictions (data from Flybase).

Table I-1: Summary of the genes involved with the GO term: response to cold

Symbol	Name	Annotation ID	Cytology	Molecular function
brv3	brivido-3	*CG13762*	3A8 -3A8	calcium ion binding; calcium channel activity
CG8778	-	*CG8778*	49B11 -49B11	AU-rich element binding; enoyl-CoA hydratase activity
CG10912	-	*CG10912*	55B2 -55B2	unknown
CG13510	-	*CG13510*	58F4 -58F4	unknown
Hsp83	Heat shock protein 83	*CG1242*	63B11 -63B11	unfolded protein binding; ATP binding; ATPase activity, coupled
Hsp26	Heat shock protein 26	*CG4183*	67B2 -67B2	myosin binding
Hsp23	Heat shock protein 23	*CG4463*	67B3 -67B3	protein and actin binding
brv2	brivido-2	*CG16793*	74A5 -74A5	calcium ion binding; calcium channel activity
brv1	brivido-1	*CG9472*	76B3 -76B3	calcium ion binding; calcium channel activity
Fst	Frost	*CG9434*	85E2 -85E2	unknown
smp-30 (Dca)	Senescence marker protein-30 (Drosophila cold acclimation)	*CG7390*	88D2 -88D2	calcium ion binding

1.3. *Drosophila melanogaster* as a model organism for cold response

Drosophila species have different thermal niches and habitat requirements ranging from species with narrow and restricted distributions to cosmopolitan species [54, 133, 135]. Each tends to have its own temperature niche with a distinct optimum and a range of permissible temperatures [40].

Linking thermal variance in populations to the molecular level is facilitated by the vast genetic information available for *Drosophila* [66], especially *Drosophila*

melanogaster since its genome was sequenced 14 years ago [1] and today there is a wide range of always up to date data compiled in Flybase, a repository of genetic and molecular information, with several useful query tools that further facilitate the integration of knowledge about this species and *Drosophila* genus in general.

Particularly, *D. melanogaster* is well understood genetically and developmentally when compared to other insects, it has a well characterized life history variation in natural populations, and since they are small insects their maintenance is neither costly nor demanding. Therefore, many can be reared in the lab, with the benefit of also having a short generation time, which permits for instance evolutionary studies. Nowadays, technological advances allow the production of transgenic strains that result in well characterised breeding lines, mutants and cell lines, which enable us to test specific hypotheses relevant in thermo resistance, as for instance in the study of Hsps as an important group of candidate genes for stress resistance [47].

D. melanogaster was originally from Africa and then expanded to more temperate regions of the world, to its current worldwide species distribution. With its geographical expansion, this species was exposed to cool temperate conditions, so in order to be relatively more resistant to temperature extremes and successfully adapt to novel environments they needed to cope with many novel thermal stresses.

Although both high- and low-temperature extremes are ecologically important, as previously described, resistance to low temperatures appears to be a major climatic adaptation not only among *Drosophila* species [39, 53, 54] but also across insect populations in nature [11, 65, 125].

Despite some limitations as a model organism in global cold tolerance studies, as it is not possible to test all of the mechanisms of cold adaptation in chill-susceptible species, in the analysis of short-term cold resistance a valuable insight is provided by the existence of both tropical and temperate strains of *D. melanogaster*. This fact together with the information presented above support the use of this species in the identification of candidate genes for cold resistance – that if thoroughly characterised can be recognised and functionally evaluated in other insect species supporting the study of this important thermal limit.

Chapter II – Is *Frost* specifically involved in cold response?

2.1. Introduction

2.1.1. *Frost* – a candidate gene in short-term cold resistance

With all the investigation around the molecular basis of short-term cold resistance, one can assume that a gene which is identified in multiple studies by different authors [4, 57, 105, 108, 114] as *Frost (Fst)* is one of the most credible candidates for this type of cold resistance [118].

At the C-terminus region, Fst contains multiple tandem repeats rich in serine (S), threonine (T) and proline (P) [31] which form PEEST motifs with a structure that resembles a mucin (boxes with diagonal stripes in Fig. II-1). Mucins are exported into the extracellular space and, at the N-terminal region, Fst shows an 18 amino acid signal peptide, suggesting that this protein may be secreted into the extracellular space [57] (blue box in Fig. II-1).

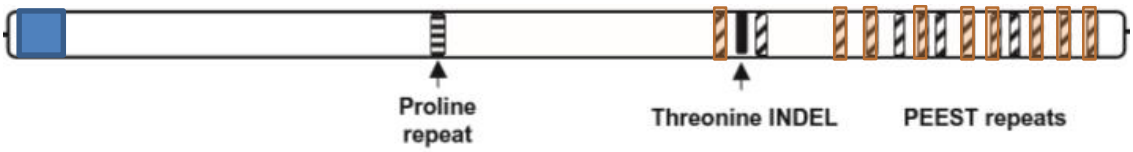


Figure II-1: Sequence features of the *Frost* gene. The region encoding the signal peptide is indicated by a blue box; the region encoding the proline repeat is indicated by a box with horizontal stripes; the regions encoding for the PEEST repeat are represented by boxes with diagonal stripes (perfect PEEST motifs are indicated with an orange shadow); the region corresponding to the threonine repeat polymorphism is indicated by a solid black box – adapted from Hoffmann et al. [67]

In *D. melanogaster*, under normal physiological conditions, *Fst* shows high to moderate levels of significant expression only in the larval and adult midgut, hindgut, and Malpighian tubules (FlyAtlas Anatomical Expression Data [121]) being higher in the latter structure. Indeed, *Fst* is 40.6 times more expressed in Malpighian tubules than in the whole fly [147]. It should be noted that, when genes involved in the classical roles of the renal system are assayed, male and female tubules show similar transcriptional levels. However, when other genes are assayed, clear differences are found between males and females. Such is the case for *Fst,* which is 31.4 times more expressed in females

than males [26]. Such a difference must be taken into account when comparing results for males and females.

This gene is included in a QTL for thermo tolerance in *D. melanogaster* [105, 108], and length variation in the *Fst* promoter region seems to explain about 1% of the variation in cold-resistance [115]. Moreover *Fst* is greatly overexpressed after cold shock in *D. melanogaster* [32, 57, 114, 118]. *Fst* is up-regulated in response to cold in eggs, third instar larvae, and 2- and 5-day-old male and female adults, with high constitutive expression being observed in those life stages in which cold does not up-regulate *Fst* [14]. The RNAi experiments performed by Colinet *et al.* [33] show that in *D. melanogaster* this gene is essential for cold tolerance. These observations led to the hypothesis that high *Fst* transcription levels are associated with cold tolerance.

There are, however, several unexpected observations under the hypothesis that *Fst* is specifically involved in the response to cold. Zhang *et al*. [155] report that the *D. melanogaster Fst* gene is up-regulated after prolonged (10h) cold exposure but not after repeated (five daily 2h exposures) or a single short (2h) cold exposure(s), and thus, *Fst* does not respond to all kinds of cold challenges. Moreover, overexpression of this gene is maximum (about 50 times) at two hours after recovering from a single sudden exposure to cold (i.e, chill-coma; [31]), and there are no changes in *Fst* gene expression during the cold treatment [31]. In addition, in *D. melanogaster* adult flies, *Fst* expression does not drop with age (http://flybase.org/) while cold tolerance rapidly declines with age [33]. There is also clinal variation for a proline repeat polymorphism at *Fst* that is not associated with cold resistance [67]. Lastly, in sharp contrast with the results of Colinet *et al.* [31], Udaka *et al.* [143] found an association between low *Fst* levels and cold resistance in *D. melanogaster*. There are also unexpected observations in other *Drosophila* species: in *D. subobscura, Fst* is overexpressed in warm-adapted (22 ºC) populations compared to cold-adapted (13 ºC) populations [87], while in *D. americana* (*Drosophila* subgenus) an association was found between *Fst* gene size variation and chill coma recovery time, but this could be the result of a body size effect [118]. *D. americana* is a temperate species (and thus more cold resistant than *D. melanogaster* that is of tropical origin) but in this species *Fst* is much less overexpressed two hours after a single cold shock [118].

Fst is also up-regulated when flies are exposed to other types of stress. Indeed, *Fst* has been found to be up-regulated when flies are exposed to virus, bacteria and fungi [6, 18, 22, 42, 92]. Surprisingly, *Fst* is up-regulated in response to infection only in mated females [127]. They have lower immune defences than virgin females, but the authors show that this finding cannot be explained by an increased positive stimulation of their immune system through higher pathogen load. However, ingestion of some pathogens,

such as the Gram-negative bacteria *Erwinia carotovora*, has a dramatic impact on the physiology of the gut, namely leading to modulation of stress response and increased stem cell proliferation and epithelial renewal [18].

Fst is also up-regulated when flies are starved or exposed to desiccation conditions [132], and when they are transferred to arid and food-free conditions [156]. Addition of hydrogen sulphide, that increases desiccation tolerance in *D. melanogaster*, further increases *Fst* mRNA levels [156]. *Fst* is also overexpressed when flies are subjected to dietary shifts [21]. Moreover, *Fst* is overexpressed when flies are exposed to hypoxia [95] as well as to Methotrexate, a synthetic folate analog that interferes with DNA synthesis, and results in the arrest of rapidly proliferating cells [2]. This gene is also up-regulated in response to starvation generated from a mitochondria signal using a tko^{25t} mutant [48], in dSAGA-specific H3 lysine 9 and 14 acetylation mutants (*dAda2b* mutants [111, 157])**,** and in $blanks^{KG00804}$ mutant testes relative to $blanks^{KG00804}$/*TM3* heterozygotes [52].

The above observations regarding the overexpression of *Fst* under a variety of conditions could suggest that *Fst* is a general stress response gene rather than a gene specifically involved in cold response. Nevertheless, the reported *Fst* overexpression under a variety of stresses is never as high as that observed 2h after chill coma recovery (about 50 times more), not to mention that most of these experiments were not performed to specifically address the possible response of *Fst* to a given stress. *Fst* expression has been assayed at different life stages (larvae and adults, for instance) and in whole individuals or in specific tissues, making any detailed comparison difficult.

In this work, we first explore the hypothesis that *Fst* is a general stress response gene, by addressing its expression changes at extreme heat conditions, nutrient deprivation, and starvation in both males and females. Most importantly, we first show that the conditions under which flies are tested are high stress near death conditions. As a control we also looked at the expression of *Fst* 2 hours after cold shock recovery, in order to compare the differences in the magnitude of this gene's response between stresses.

2.2. Methodologies

2.2.1. Phenotypical characterization

We phenotyped flies from a commonly used wild type *D. melanogaster* strain, Oregon-R (Ore-R), for lifespan, cold shock (as chill coma recovery time), heat shock,

nutrient deprivation, and starvation. Fly stocks were maintained in 250 ml bottles in uncrowded conditions. Bottles were kept at 25ºC, 50% relative humidity and 12h:12h light: dark cycle conditions on standard food (10% [mass/volume] yeast, 4% [mass/volume] wheat flour, 8% [mass/volume] sugar, 0.4% [mass/volume] salt diet, 1% agar [mass/volume], and 0.5% propionic acid [volume/volume]).

To guarantee that we had a statistically significant data set, 100 male and 100 female newly emerged adult flies were collected within 8h of eclosion (to guarantee that they were virgins), sexed under light CO_2 anaesthesia and transferred in groups of 5 to 10 ml vials with standard food and reared for 4 days at the same conditions as the stocks. We only used 4-day old flies in order to reduce the age influence in each phenotype, thus avoiding a possible ageing effect, as seen previously for both cold [71] and heat shock [69]. Note also that sorting time was less than 15 min, and flies were not anesthetised subsequently to the initial sorting because long exposures to carbon dioxide anaesthesia are known to affect cold tolerance [107].

We assess each phenotype, in the following fashion:

- For lifespan: flies resulting from the cross of 20 males with 20 females were individually collected and maintained at 25° C under 12h light and dark cycle conditions until they died. Single individuals were kept in a vial containing standard food and their condition was checked every other day and vials were changed every week;
- For cold shock: in a 4ºC cold chamber, individual flies were transferred without anaesthesia to empty vials which were sealed with parafilm, and then buried in ice. After 4 hours of cold exposure at 0ºC, we measured individual chill coma recovery time at 25ºC. Flies were considered completely recovered when able to stand up on all their legs;
- For heat shock: at room temperature, groups of 5 flies were changed into empty vials which were then placed in an incubator maintained at 37° C. Their status was checked every 30 minutes until they all died;
- For nutrient deprivation: groups of 5 flies were changed into vials with cotton imbibed in water and maintained at 25° C under 12h light and dark cycle conditions until they died. Their status was checked every 12 hours;
- For starvation: groups of 5 flies were changed into empty vials and maintained at 25° C under 12h light and dark cycle conditions until they died. Their status was checked every hour.

Also worth mentioning is the fact that time points (intervals) were chosen using information from the literature and from our own preliminary data such that we would see

flies with complete, intermediate and no survival for heat shock, nutrient deprivation, and starvation phenotypes.

2.2.2. Gene expression analyses

2.2.2.1. Sample selection

Based on the results obtained for the Ore-R strain under heat shock, nutrient deprivation, and starvation (see Results), we set the near-death stress point for each phenotype and decided on the following conditions for the reverse transcription quantitative real-time PCR (RT-qPCR) quantification:

- → For cold shock: 4-day old flies without cold shock (control – CC CO), immediately after chill coma recovery – 782.5 +- 140.3 s for Ore-R ♂ and 783.4 +- 120.4 s for Ore-R ♀ (CC I), and cold shock followed by a 2 hours recovery period at 25ºC (CC II);
- → For heat shock: 4-day old flies without heat shock (control – HS CO), 1 hour and a half of exposure to 37ºC (HS I), and 1 hour and a half of exposure to 37ºC followed by a 2 hours recovery period at 25ºC (HS II);
- → For nutrient deprivation and starvation: after 36 hours (ND) and 18 hours (ST), respectively, of exposure to the stress the living flies were collected. . 4-day old flies kept in standard vials were used as controls (CO).

For each condition we had three sets (biological replicates) of 3 females and 3 males. The flies subjected to these conditions were kept at -80ºC immediately after the exposure to the stress.

2.2.2.2. RNA extraction and cDNA synthesis

Total RNA was isolated from each set of 3 individuals using TRIzol Reagent (Invitrogen, Spain) and treated with Turbo DNA-free™ kit (Life technologies, Carlsbad, California, EUA) according to the manufacturer's instructions. The quality of the RNA samples was checked using Nanodrop (only samples with RNA with concentrations above 100 ng/uL were used) and the RNA integrity checked using Experion platform (Bio-Rad, Portugal; only samples with RQI above 7 were used).

Prior to cDNA synthesis, RNA samples were diluted to concentrations of 106,25 ng/µl (males) or 125 ng /µl (females) and 8 µl of these dilutions was used in the cDNA reactions (in order to end up with 850 ng and 1000 ng of RNA for males and females

respectively). cDNA was then synthesized by reverse transcription with SuperScript III First-Strand Synthesis SuperMix for RT-qPCR (Invitrogen, Spain), using random primers. No-template controls and reactions with RNA that was not reverse transcribed were performed in order to confirm the absence of DNA contamination.

2.2.2.3. RT-qPCR

We performed RT-qPCR experiments using the isolated cDNA. Expression levels were determined for sets of three individuals from each sex (3 biological replicates in total) for each condition tested. RT-qPCR reactions were carried out in a 20 µL final volume solution containing 10 µL of the iQ SYBR Green Supermix (Bio-Rad, Portugal), 8.5 µL of bidestilled water, 0.25 µL of forward and reverse primers (10 µM), and 1 µL of cDNA from each condition. The specific primers used to amplify *Fst* and the endogenous ribosomal protein L32 (*RpL32*; the reference gene) have an efficiency between 90% and 100% (see Table II-1). The reactions were performed on a Bio-Rad iCycler with the following program: 3 min at 95º C; 40 cycles of 30 s at 94º C, 30 s at 56º C and 30 s at 72º C followed by a standard melt curve. The threshold cycle (C_T) values of technical replicates of both control and each treatment samples did not differ by more than 0.7 cycles in any case. Fold change in expression was calculated using the $2^{-\Delta\Delta CT}$ method [96].

Table II-1: Primer sequences and efficiencies (%) for genes validated with RT-qPCR and used in the analysis. *RpL32* primer efficiency was determined with the standard curve method for serial dilutions of 1:5

Gene	Primer Sequence F/R	E %	R^2
Frost	5' TCAGGGTCAGTGGGATGG 3' 5' CCGTTGGTGGTGGTGGAG 3'	90<E<100*	<0.95*
RpL32	5' GTTTACTGCGGCGAGAT 3' 5' CGTTGGGGTTGGTGAGGC 3'	97.4	0.994

*data obtained from Reis et al. [118]

2.3. Results and Discussion

The observation that, in *D. melanogaster, Fst* is overexpressed about 50 times two hours after chill coma recovery, and that under a variety of other stresses this gene is never so highly expressed, is one of the main arguments in favour of *Fst* being specifically involved in cold response (see introduction). Nevertheless, most of these experiments were not specifically optimized to consistently address the possible

response of *Fst* to different types of stress. In fact, in different experiments, different methodologies were used, *Fst* expression was assayed at different life stages (larvae and adults, for instance), in whole individuals or in specific tissues, and it is unclear whether the conditions assayed impose similarly high stresses, making any detailed comparisons unreliable.

Firstly, then, we wanted to determine how Oregon-R (Ore-R), a wild type strain of *D. melanogaster,* performed under different types of stress in order to identify the maximum amount of each kind of stress that 4-day old flies could endure without dying. With that data, we performed an expression analyses of *Fst* mRNA through RT-qPCR in flies subjected to near-death stress (near-death was set as the time before 1% of the flies start to die), and related these results to the phenotypical data.

2.3.1. Cold shock response

Regarding the cold shock stress, as we can see in Fig. II-2, Ore-R males take on average 782.5 seconds (standard deviation SD = 140.3; sample size N=100) to recover from cold shock, while females take on average 783.4 seconds (SD=120.4; N=100).

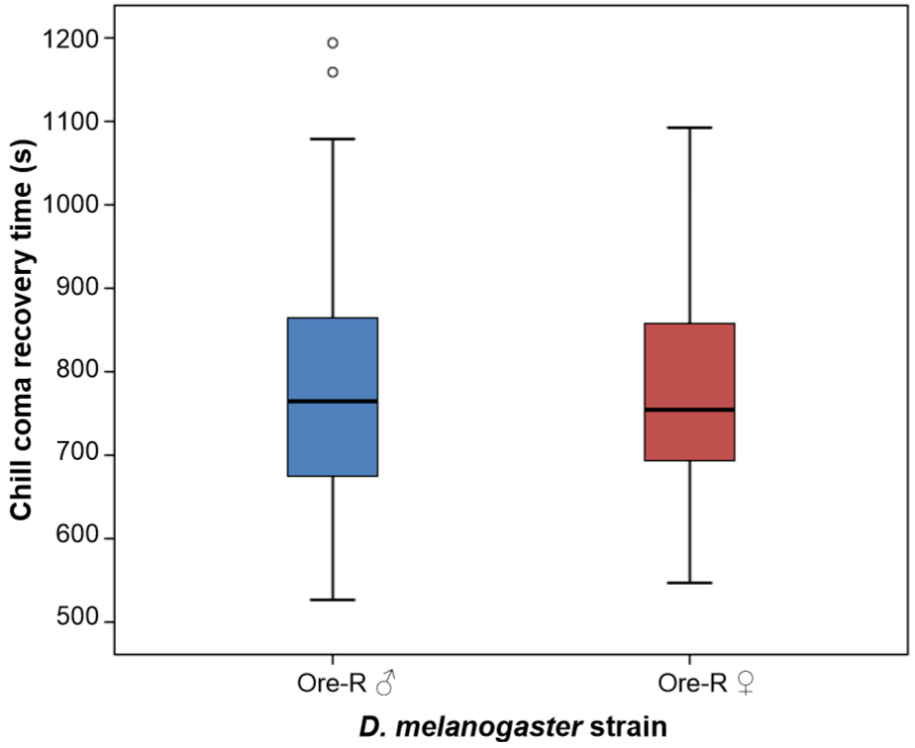


Figure II-2: Box plot representing chill coma recovery times for *D. melanogaster* Ore-R; males (blue) and females (red). The top of the box represents the 75th percentile, the bottom of the box represents the 25th percentile, and the line in the middle represents the 50th percentile that corresponds to the median. The whiskers represent the highest and lowest values that are not outliers or extreme values. Circles beyond the whiskers represent outliers

These average chill coma recovery times are in accordance with previous published results that used different *D. melanogaster* strains [4, 54, 71]. Also note that under normal conditions *Fst* has been reported to be 31.4 times more expressed in females than in males [26], but there are no statistically significant differences between Ore-R males and females (Non-parametric Mann-Whitney test; P>0.05) regarding chill coma recovery time.

2.3.2. *D. melanogaster* near-death conditions

2.3.3. Heat shock response

In order to determine for how long Ore-R flies are able to stand prolonged heat (37ºC) and the associated desiccation when placed in empty food vials and in a dry incubator, the percentage of living flies was recorded every half hour until all flies were dead. We can observe that the death rate increases linearly with exposure time from the moment there is mortality (for males Pearson product-moment correlation coefficient R=0.96; significance of Pearson product-moment correlation coefficient P < 0.001; N=8; for females R=0.89; P < 0.001; N=10), and there are no statistically significant differences between males and females regarding death rates (Non-parametric sign test; P > 0.05). There is almost no mortality (less than 5%) in the first 90 minutes of exposure to heat, and no fly was able to stand the treatment for more than five and a half hours (Fig. II-3). Given these results, we decided to assay *Fst* for heat shock response after one and a half hours of exposure to these conditions. Also, to have some sort of comparison between these results and the ones from the cold shock, we also assay *Fst* response after 2 hours of recovery from the stress.

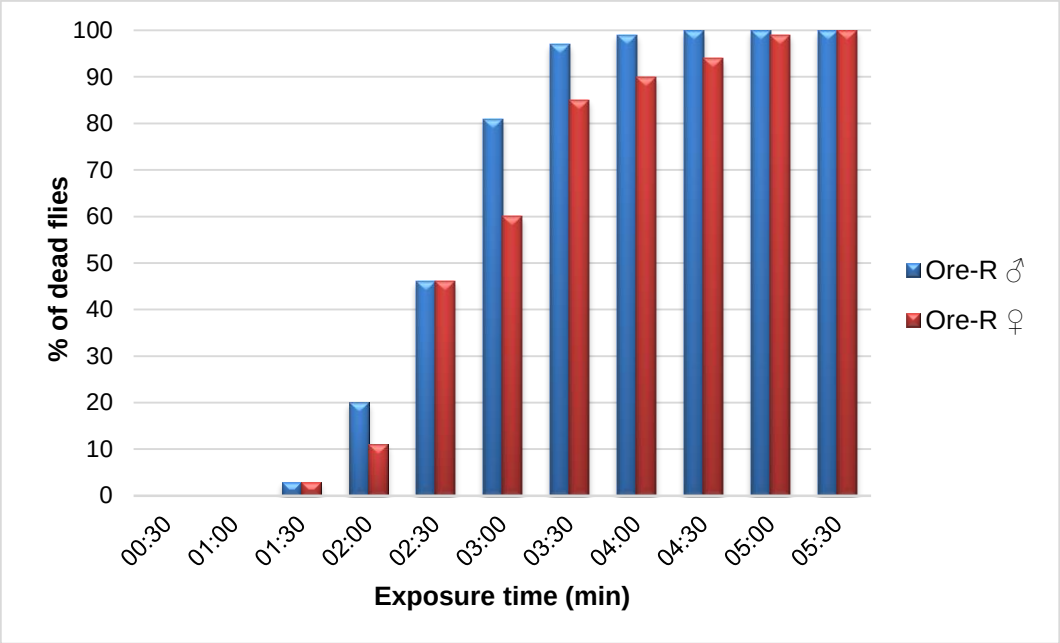


Figure II-3: Percentage of dead *D. melanogaster* Ore-R flies after exposure to heat shock; males (blue) and females (red)

2.3.4. Nutrient deprivation response

In order to determine how Ore-R is able to stand prolonged nutrient deprivation, these flies were kept in vials with cotton balls soaked in water, and the percentage of living flies was recorded every 12 hours until all flies were dead. With the results from these experiments we can tell that the death rate seems to increase linearly with exposure time since the moment there is mortality (for males $R=0.95$; $P < 0.005$; $N=6$; for females $R=0.98$; $P < 0.005$; $N=5$), and there are no statistically significant differences between males and females regarding death rates (Non-parametric sign test; $P > 0.05$). There is almost no mortality (less than 5%) in the first 24 hours, and no fly was able to stand the treatment for 72 hours (Fig. II-4). Given that there is almost no mortality after 24 hours, we decided to assay *Fst* for nutrient deprivation response at 36 hours when the average mortality is almost 25 %.

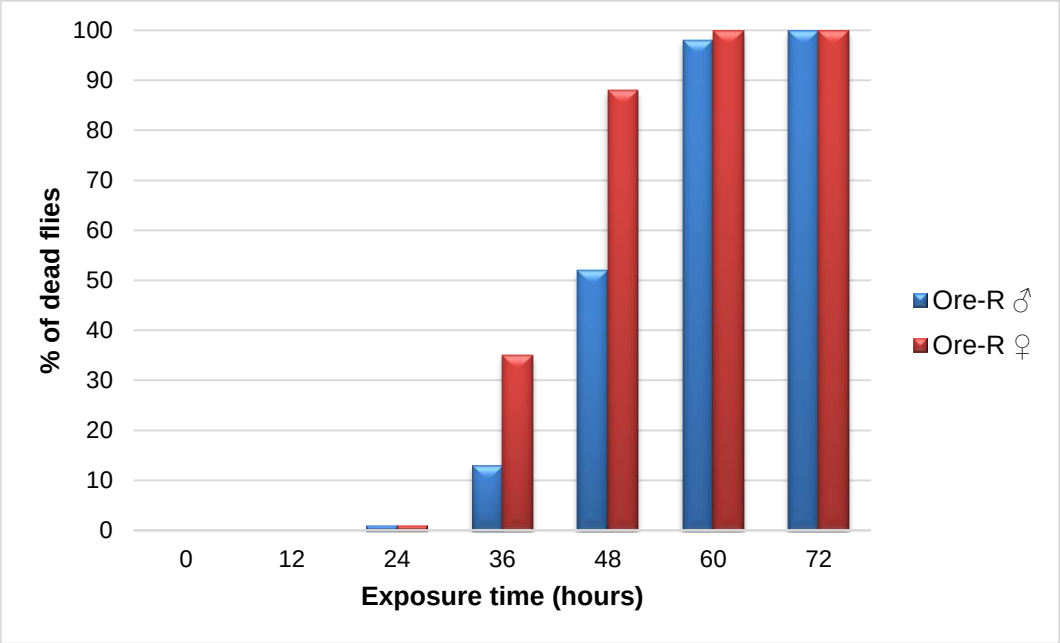


Figure II-4: Percentage of dead *D. melanogaster* Ore-R flies after exposure to nutrient deprivation; males (blue) and females (red)

2.3.5. Starvation response

In order to determine how well the Ore-R strain is able to stand prolonged starvation (nutrient and water deprivation), the percentage of living flies was recorded every hour until all flies were dead. Here, as for the previous phenotypes, the death rate seems to increase linearly with exposure time from the moment there is mortality (for males $R=0.88$; $P < 0.001$; $N=12$; for females $R=0.89$; $P < 0.001$; $N=11$), but it should be noted that there are statistically significant differences between males and females regarding death rates – males have a more pronounced death rate (Non-parametric sign test; $P < 0.005$), unlike what happens for heat shock and nutrient deprivation. There is no mortality in the first 17 hours, and no fly was able to stand the treatment for more than 28 hours (Fig. II-5). Given the above results for nutrient deprivation, where more than half of the flies are still alive after 36 hours, desiccation is likely to be a major cause of death here, since starved flies (which are without food and water) die sooner than flies with water but no food. Bearing these results in mind, we decided to assay *Fst* for starvation response at 18 hours when the average mortality is about 7%.

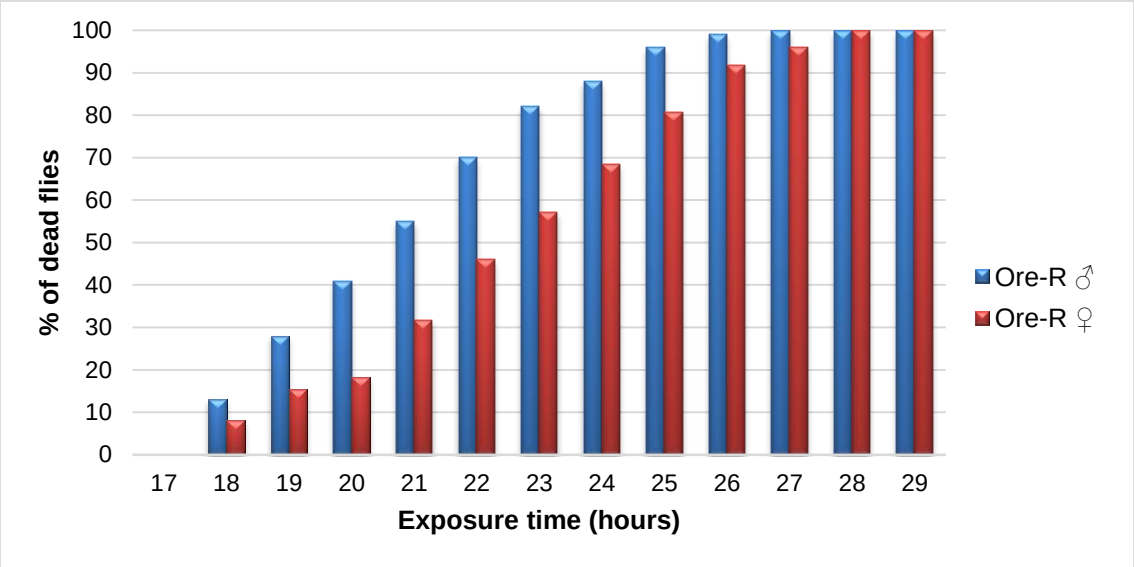


Figure II-5: Percentage of dead *D. melanogaster* Ore-R flies after exposure to starvation; males (blue) and females (red)

2.3.6. RT-qPCR expression analyses

In order to test the prediction that *Fst* could be up-regulated during recovery from cold exposure, we measured expression in Ore-R flies not exposed to cold (control flies), immediately after recovery from chill coma and two hours after recovery from chill coma. The results are presented in Fig. II-6, and as previously reported [31, 118], two hours after recovery *Fst* is overexpressed about 40 times in both males and females. *Fst* is not significantly overexpressed immediately after chill coma recovery, although the average *Fst* overexpression, around 8 times, is similar to that reported by Collinet *et al.*[31] and Reis *et al.* [118] under the same conditions.

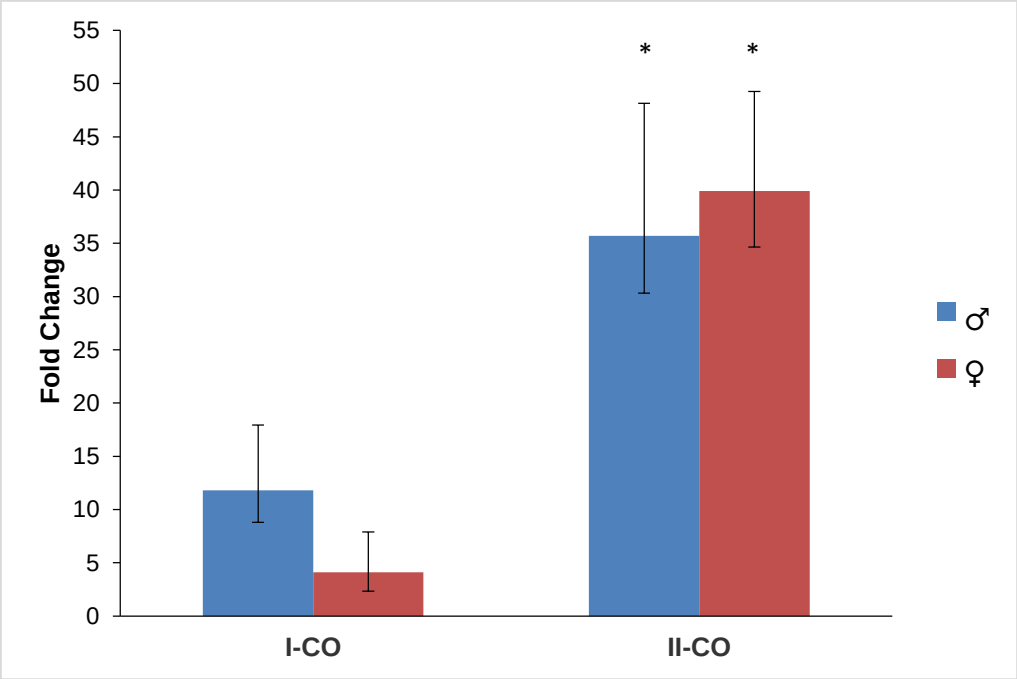


Figure II-6: Fold change in *Frost* expression after chill coma obtained by RT-qPCR using the $2^{-\Delta\Delta CT}$ method. The reference gene *RpL32* was used to normalize the expression values. Expression fold changes were addressed immediately after chill coma recovery (I-CO) and after 2 hours of recovery (II-CO) for both males (blue) and females (red) of *D. melanogaster* Ore-R strain; Error bars present a measure of the variation in biological replicates. Bars with an asterisk represent significant ($P<0.05$) values according to T-test statistics.

We also measured *Fst* expression in Ore-R flies not exposed to heat (control flies), immediately after recovery from heat shock and two hours after recovery from heat shock. Here, *Fst* displayed an overexpression two hours after recovery from heat stress (and the associated desiccation stress) for both males and females, as we can see in Fig. II-7. Under these experimental conditions, males show a *Fst* overexpression as pronounced as 2 hours after cold shock recovery, around 40 times.

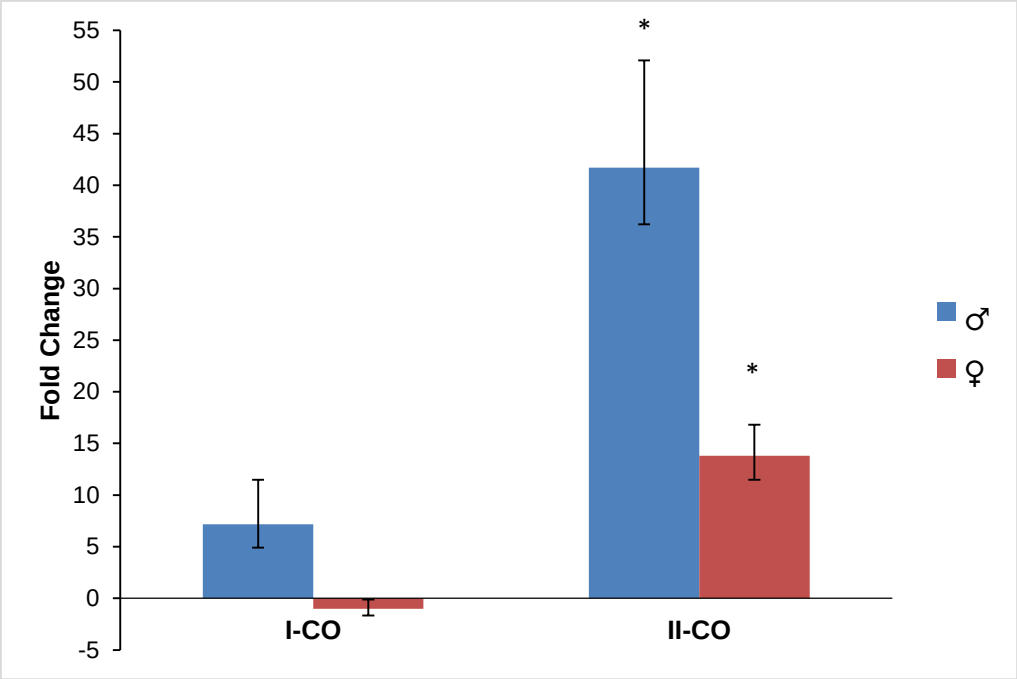


Figure II-7: Fold change in *Frost* expression after heat shock obtained by RT-qPCR using the $2^{-\Delta\Delta CT}$ method. The reference gene *RpL32* was used to normalize the expression values. Expression fold changes were addressed immediately after heat shock (I-CO) and after 2 hours of recovery (II-CO) for both males (blue) and females (red) of *D. melanogaster* Ore-R strain; Error bars present a measure of the variation in biological replicates. Bars with an asterisk represent significant ($P<0.05$) values according to T-test statistics.

When we analyse the expression of flies subjected to nutrient deprivation (36h) and starvation (18h) we note that *Fst* is only significantly overexpressed after starvation, for both males and females as we can see in Fig. II-8. *Fst* overexpression is around 40 times in females, the same result that we obtained for the expression 2 hours after chill coma recovery.

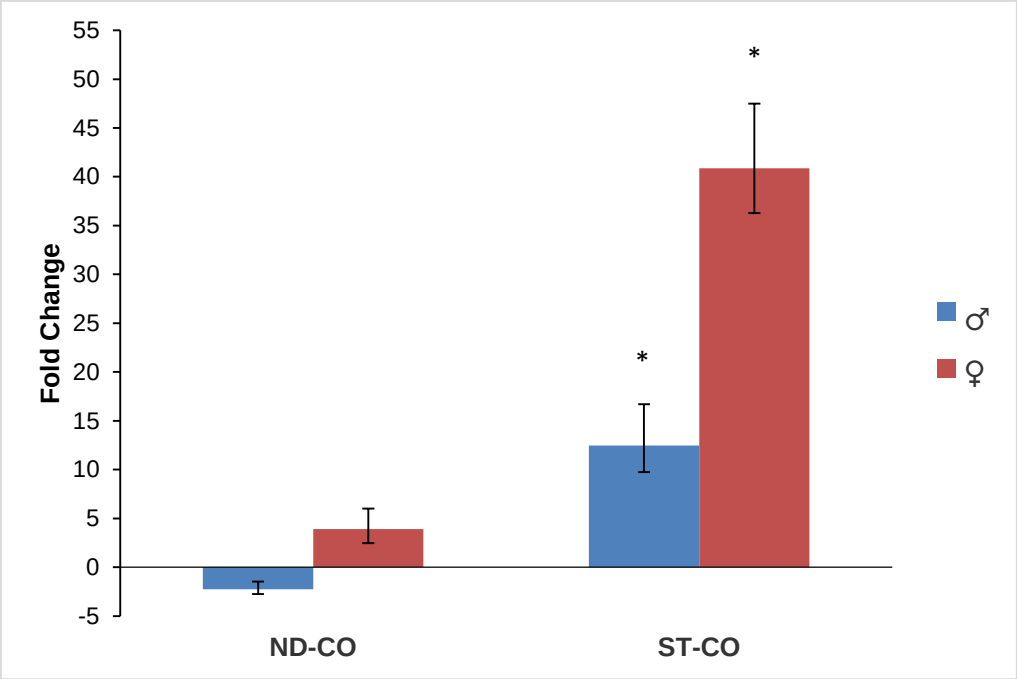


Figure II-8: Fold change in *Frost* expression after nutrient deprivation and after starvation obtained by RT-qPCR using the $2^{-\Delta\Delta CT}$ method. The reference gene *RpL32* was used to normalize the expression values. Expression fold changes were addressed after 36 hours of nutrient deprivation (ND-CO) and after 18 hours of starvation (ST-CO) for both males (blue) and females (red) of *D. melanogaster* Ore-R strain; Error bars present a measure of the variation in biological replicates. Bars with an asterisk represent significant (P<0.05) values according to T-test statistics.

Here desiccation is likely a major cause of death, and we propose that *Fst* is responding directly to that. As we obtained a 40-fold response of *Fst* not only after 2 hours of recovery from chill coma, but also after 2 hours of recovery from heath shock and after 18 hours of starvation, we can argue that *Fst* is not specifically involved in cold response, but it could be responding to desiccation instead. When flies are subjected to starvation, they die from loss of water, as we confirmed with the nutrient deprivation experiment (these flies live much longer than the ones subjected to starvation). In the way that we performed the cold/heat shock experiments, as the vials were empty, there was no food to keep the humidity in and prevent the flies from being affected by water loss as temperature changes. We previously argued (see introduction) that temperature is one of the main factors restraining the life of insects, and for *Drosophila* the ability to endure water loss to a certain degree is a major benefit when responding to different kinds of stress. With these results we can support the idea that *Fst* is involved in that role to some extent.

Chapter III – Is *Muc68E* involved in the cold response?

3.1. Introduction

3.1.1. Overall characterization, structure and function of mucins

Mucins are a group of high molecular weight, heavily glycosylated proteins produced by various types of epithelial cells in the majority of animals. These cells are present in the respiratory, digestive, and urogenital tracts, and even the skin (in some amphibians) [112], and mucins are the principal component of the mucous that lines those surfaces. They can be membrane-bound or both secreted and gel-forming [112], providing lubrication of the luminal surface of several organs, especially aiding the passage of food through digestive tracts. They also have an important role in the protection of the underlying epithelium against physical and chemical damage, such as protease degradation, pathogen invasion, and dehydration [68, 112, 142, 144].

The main characteristics of these proteins are their extended regions of tandemly repeated sequences which vary in number and length from one mucin to another [51] but contain specific amino acids: prolines (P) together with serines (S) and/or threonines (T), to which large sugar side chains attach via an O-glycosidic bond [51, 82]. These carbohydrates, which represent 50 to 90% of the protein weight [112, 149], make the mucins highly protease resistant [20], and the remaining parts of the sequence often contain conserved protein domains that mediate protein-protein interactions. Thus, mucins are capable of forming enormous networks, as seen in Fig. III-1 for a human mucin (MCU2), to which the glycosylated PTS repeats confer high water-binding capacity, a selective barrier function, and the ability to trap microorganisms [20].

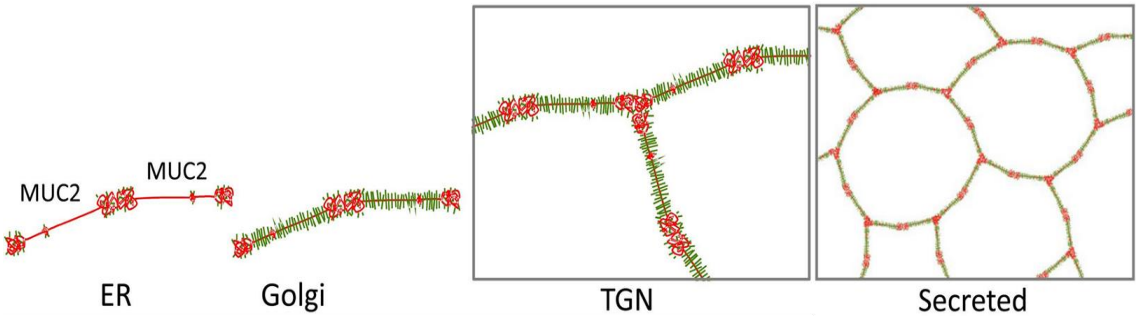


Figure III-1: Assembly of the MUC2 mucin (protein core red) into dimeric forms in the endoplasmic reticulum (ER), O-glycosylation (green) in the Golgi apparatus, formation of trimeric forms in the trans Golgi network (TGN) and a schematic picture of the secreted MUC2 polymer – adapted from Hansson [60]

3.1.2.Mucins in insects

Detailed studies in rodents and humans [10, 72] reveals that the gastrointestinal tract is covered by mucus, and most of its proprieties are related to mucins and the network formed upon its polymerization [73]. There is a great deal of research on human mucins because changes in these genes' expression and in the structures of the polysaccharides attached to them occurs in cancers of the intestine [68], and are more globally related to development and progression of cancer [72].

The use of highly glycosylated molecules as the main building block to protect the intestines is common in multicellular organisms [60]. In invertebrates, especially insects, there is a chitin and glycoprotein layer called the peritrophic matrix (PM), which is functionally similar to the mucous secretions of the vertebrate digestive tract [62]. The PM is a physical barrier, lubricating the passage of food through the midgut and protecting its epithelium from pathogen invasion [124, 139, 148]. It also regulates nutrient uptake by compartmentalizing digestive processes and prevents excretion of digestive enzymes by providing a means for enzyme recycling [62, 91, 141]. Furthermore it serves as a biochemical barrier, sequestering and, in some cases, inactivating ingested toxins [62, 91].

The existence of a mucous layer, in addition to the PM, is suggested by the fact that the apical surface of midgut epithelial cells is positive for a staining method used to detect mucous substances [19, 119]. In fact, in association with the peritrophic matrix, insect intestinal mucins [149] that have chitin-binding domains, as well as one or more mucin domains [142] (which are regions rich in threonine and serine residues with a high potential for O-linked glycosylation [46]) were identified. A representation of the cross section of the adult *Drosophila* midgut can be seen in Fig. III-2.

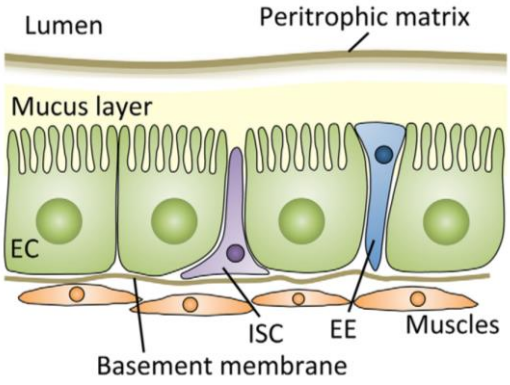


Figure III-2: A cross section of the adult *Drosophila* midgut, with several types of cells: absorptive enterocytes (ECs), secretory enteroendocrine cells (EEs), and pluripotent intestinal stem cells (ISCs). Muscle cells are present under the basement membrane of epithelial cells, and between the lumen and epithelia there is a semipermeable non-cellular structure, the peritrophic matrix. In addition, a mucus layer lies between the peritrophic matrix and ECs along the midgut – adapted from Kuraishi el al. [86]

More than 30 *Drosophila* genes have been annotated as mucin-like proteins, and the current characterization of *Drosophila* mucins should make it possible to address different functional aspects of these proteins, and to investigate the importance of mucins and mucin-type glycosylation for various physiological and developmental processes [136]. It will also allow the formulation of basic questions about human mucin-related diseases in this model system, although the functional relevance of these genes or, more generally, of mucus in the gut has not been investigated thoroughly [93].

3.1.3. *Muc68E* – is it related to *Frost?*

Analyzing the *Drosophila melanogaster* proteome, was noticed that the PEEST repeat found in *Frost* [16], thought to be a mucin-like gene, could only be found repeated more than twice in a classical mucin, *Muc68E* (Vieira J, personal communication). The amino acid composition of these two proteins is similar (in percentage, as seen in Table III-1) and the repetitive domain, which corresponds to a region without a fixed tertiary structure, occupies more than 70% of the length of both proteins. However, *Frost* was not identified as a mucin by Syed et al. because their cutoff for ST % was 25% [136].

Table III-1: Comparison of the amino acid composition in the middle repetitive region of Muc68E and Frost

Name	Gene ID	Localization	Size (aa)	P (%)	E (%)	S (%)	T (%)	ST (%)	Repetitive domain (%)
Muc68E	*CG33265*	3L: 11,960,680… 11,966,074 [-]	1799	7.6	14.9	7.5	25.3	32.9	77.4
Frost	*CG9424*	3R: 5,470,700… 5,471,876 [-]	286	8.7	17.1	11.9	12.2	24.1	73.4

Both *Muc68E* and *Frost* show high levels of expression in adult midgut, and moderate to high levels in larval midgut, as it is described in FlyAtlas Anatomical Expression Data [121]. In addition, Frost has a signal peptide which indicates that it goes towards the secretory pathway. This protein is guided into the endoplasmatic reticulum and released into the extracellular space [57]. Muc68E does not have a predicted signal peptide but, as it is part of the mucin family [136], it is expected to also be secreted.

There are not many studies on *Muc68E*, but it has been shown that this gene is enriched 2.75 times in the larval gut epithelium of *Activating transcription factor 3* (*Atf3*)

mutants relative to controls [123]. *Atf3* is important in the safeguarding of metabolic and immune system homeostasis, and its loss results in chronic inflammation and starvation responses mounted primarily by the larval gut epithelium. Therefore, *Muc68E* seems to respond to some stress conditions. As *Frost* is probably related to cold response because it is highly overexpressed after cold shock (see Chapter II), we wanted to characterize *Muc68E* and determined its possible involvement in cold response.

3.2. Methodologies

3.2.1. Evolutionary analyses

We downloaded genome sequences from Flybase (http://flybase.org/) or from NCBI (http://www.ncbi.nlm.nih.gov/) and manually annotated a set of 16 highly conserved, intronless in *D. melanogaster*, single copy genes (*Ppox*, *CG32281*, *CG33230*, *CG12170*, *Rpn7*, *CG3570*, *eIF6*, *Prpk*, *CG14270*, *CG14512*, *Arpc4*, *Rpp20*, *CG33932*, *mRpL42*, *Bet1* and *CG34117*), that in *D. melanogaster* represent 11988 bp of sequence, in 24 *Drosophila* species (*D. simulans*, *D. sechellia*, *D. melanogaster*, *D. erecta*, *D. yakuba*, *D. suzukii*, *D. biarmipes*, *D. takahashii*, *D. eugracilis*, *D. elegans*, *D. rhopaloa*, *D. ficusphila*, *D. kikkawai*, *D. bipectinata*, *D. ananassae*, *D. persimilis*, *D. pseudoobscura*, *D. miranda*, *D. willistoni*, *D. virilis*, *D. americana*, *D. mojavensis*, *D. grimshawi* and *D. albomicans*). The sequences from the different genes were concatenated and aligned using the ClustalW2 alignment algorithm as implemented in ADOPS [117].

When this software is used, nucleotide sequences are first translated and then aligned using the amino acid alignment as a guide. Only codons with a support value above 2 are then used for phylogenetic reconstruction. Phylogenetic trees were obtained using MrBayes 3.1.2 [122], using the Generalised Time-Reversible (GTR) model of sequence evolution, allowing for among-site rate variation and a proportion of invariable sites. Third codon positions are allowed to have a gamma distribution shape parameter different from that of first and second codon positions, and two independent runs of 2,000,000 generations with four chains each (one cold and three heated) are carried out. Convergence is assessed by looking at the average standard deviation of split frequencies (that is ~0.001) and at the potential scale reduction factor for every parameter (that was ~1.00). Trees were sampled every 100th generation and the first 5000 samples were discarded (burn-in). The remaining trees were used to compute the Bayesian posterior probabilities for each clade of the consensus tree.

Muc68E sequences were obtained from Flybase or from the manual annotation of *Drosophila* genomes, but since many of the species used here do not have a genome annotation, and we used species from both the *Drosophila* and *Sophophora* subgenus, all of the *Muc68E* sequences were confirmed and changed manually in all species.

Amino acid logos were obtained using WebLogo [37], the chitin binding domains of the genes in the micro-synteny analysis were recognized using ScanProsite [41], and a BLASTp of the first Muc68E chitin binding domain against those was made to set a group for the phylogenetic analysis – note that here we also included *Cht8*, a gene from the 2R chromosome, as a root for the tree. The phylogenetic tree showing the relationship between these chitin binding domains was built using MEGA6 [138], the amino acid sequences were aligned using ClustalW, and a neighbour joining tree was built using p-distance, pairwise deletion, and assuming uniform rates. The interior branch test was used (1000 bootstrap replications) to test the support of the different nodes. Synteny data was obtained using both the data available at Flybase and by performing tBLASTn analyses of the region of interest against the *D. melanogaster* proteome.

3.2.2. Gene expression analyses

Gene expression analyses was performed as previously described (see section 2.2.2). Briefly, sample selection involved proven near-death conditions (studied with the wild type *D. melanogaster* strain Ore-R), and RNA extraction and cDNA synthesis followed the same protocols and cares. RT-qPCR reactions were performed with the isolated cDNA in the same way, and expression levels were determined for sets of three individuals from each sex (3 biological replicates in total) for each condition tested. We used the same reference gene (*RpL32*), and design highly efficient primers to amplify the *Muc68E* region (see Table III-2). As previously, fold change in expression was calculated using the $2^{-\Delta\Delta CT}$ method [96].

Table III-2: Primer sequences and efficiencies (%) for genes validated with RT-qPCR and used in the analysis. Primer efficiencies were determined with the standard curve method for serial dilutions of 1:5

Gene	Primer Sequence F/R	E %	R^2
Muc68E	5' GCACAAAGCACAGGTATCAT 3' 5' ACTACTGTCACCGCAAC 3'	90.6	0.996
RpL32	5' GTTTACTGCGGCGAGAT 3' 5' CGTTGGGGTTGGTGAGGC 3'	97.4	0.994

3.2.3. Phenotypical characterization

.

We phenotyped flies from two *D. melanogaster* strains (w^{1118} 5905, and w^{1118} 27851) for lifespan, cold shock (as chill coma recovery time), heat shock, nutrient deprivation, and starvation as described above (see section 2.2.1) for the wild type *D. melanogaster* Ore-R strain. Note that all flies were kept in the same conditions as Ore-R before the stresses.

3.3. Results and Discussion

3.3.1. *Muc68E* structure and evolutionary history

Muc68E can only be found in species of the subgenus *Sophophora*, so, the first conclusion to be taken is that if *Muc68E* is somewhat involved in cold resistance, it cannot explain cold resistance variations genus-wide since this gene is missing in species of the subgenus *Drosophila*. This is in contrast with *Fst* gene that is found in all *Drosophila* species [118].

This protein can be divided into three main regions: the N-terminal region, the central highly repetitive region and the chitin binding region (Fig. III-3).

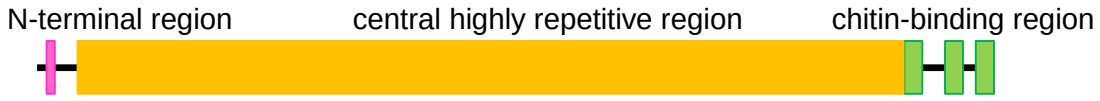


Figure III-3: Schematic of *Muc68E* functional regions

Muc68E is a mucin [136] and thus it is expected to be exported to the extracellular space. Therefore, it should harbour a clear peptide signal. Nevertheless, no signal peptide is predicted when the *D. melanogaster* Muc68E protein and the Signal IP server is used [113]. At amino acid position 24 there is, however, another methionine, and when this methionine is assumed to be the translation start, a peptide signal is, however, predicted in every *Drosophila* species analysed with the exception of *D. pseudoobscura*.

Methionine 24 is conserved in all *Drosophila* species analysed, and in some species, particularly *D. elegans*, *D. ananassae*, *D. bipectinata*, *D. biarmipes*, *D. miranda*. *D. kikkawai*, *D. willistoni,* there is no other methionine before methionine 24, so this suggests that methionine 24 may be the true start site of this protein.

The size of the *Muc68E* highly repetitive middle region varies greatly between species. This is true for both the number of repeats (from 5 up to over 80) and the size of the repeat (from 16 to 48; Fig. III-4). It should be noted that the number of repeats may be underestimated because highly degenerated repeats are not easy to recognize. The amino acid logos are very homogeneous for most species, and there are fixed differences between species, even between closely related species such as *D. melanogaster* and *D. simulans* (see, for instance, the fixed difference at repeat positions 28 and 35 in Fig. III-5). This implies either a significant amount of within gene conversion in the central region of the gene or frequent contractions and expansions of the repeats. The only amino acid pattern common to all species is P[ED][ED][ST][ST][ST] (motif 1 in Fig. III-4), that may be generalized to [Not hydrophobic][Negatively charged][Negatively charged][Small][Small][Small] (motif 2 in Fig. III-4).

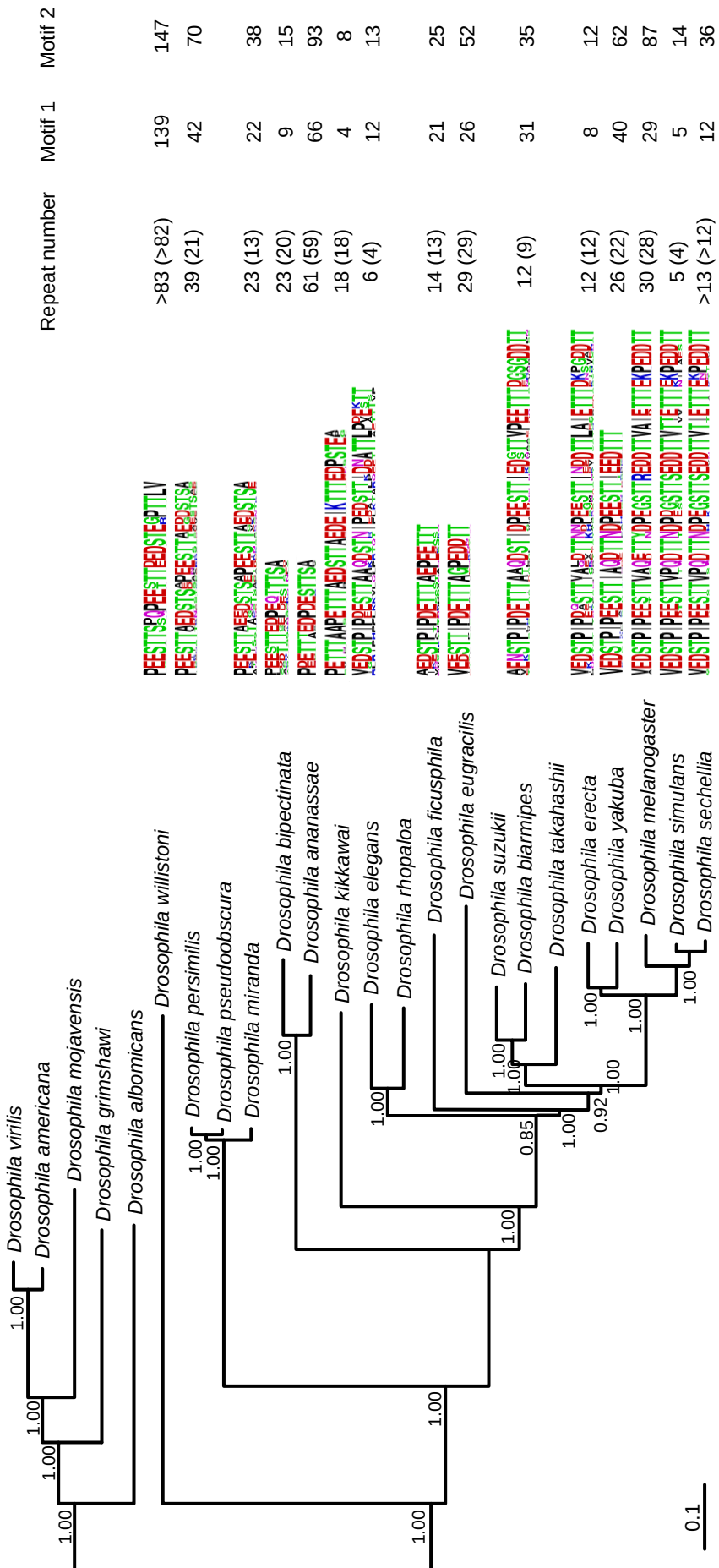


Figure III-4: Web logos analysis of Muc68E highly repetitive region across *Drosophila* genus

Figure III-5: zoom of *D. melanogaster* and *D. simulans* logos with the aminoacidic differences marked with a brown rectangle

Muc68E also shows three chitin binding domains in the C-terminal region, with a typical $CX_2GX_9CX_5CX_9CX_5WX_6CX_6C$ motif where X is any amino acid other than cysteine (green rectangles in Fig. III-3), that can be identified as a Peritrophin-A (PerA) domain, which is more commonly called chitin binding type-2 domain (CBT2 domain) [49].

The micro-synteny analysis of the *Muc68E* region (the region between genes *CG7252* and *CG43896;* Fig. III-6) reveals the insertion of the *prc* (*pericardin* gene) after the separation of *D. willistoni* lineage (a *prc* gene can be found in all species in a different region of the genome). This evolutionary event is not, however, correlated with the appearance of the *Muc68E* gene, since in *D. willistoni* a *Muc68E* gene is detected in this region, but the *prc* gene is located elsewhere in the genome. The *Muc96D* gene is present in this region in *D. americana*, *D. virilis*, *D. mojavensis* and *D. albomicans* (species from the *Drosophila* subgenus) but is elsewhere in the genomes of the *Sophophora* subgenus species. Also, this gene was apparently lost in *D. grimshawi*.

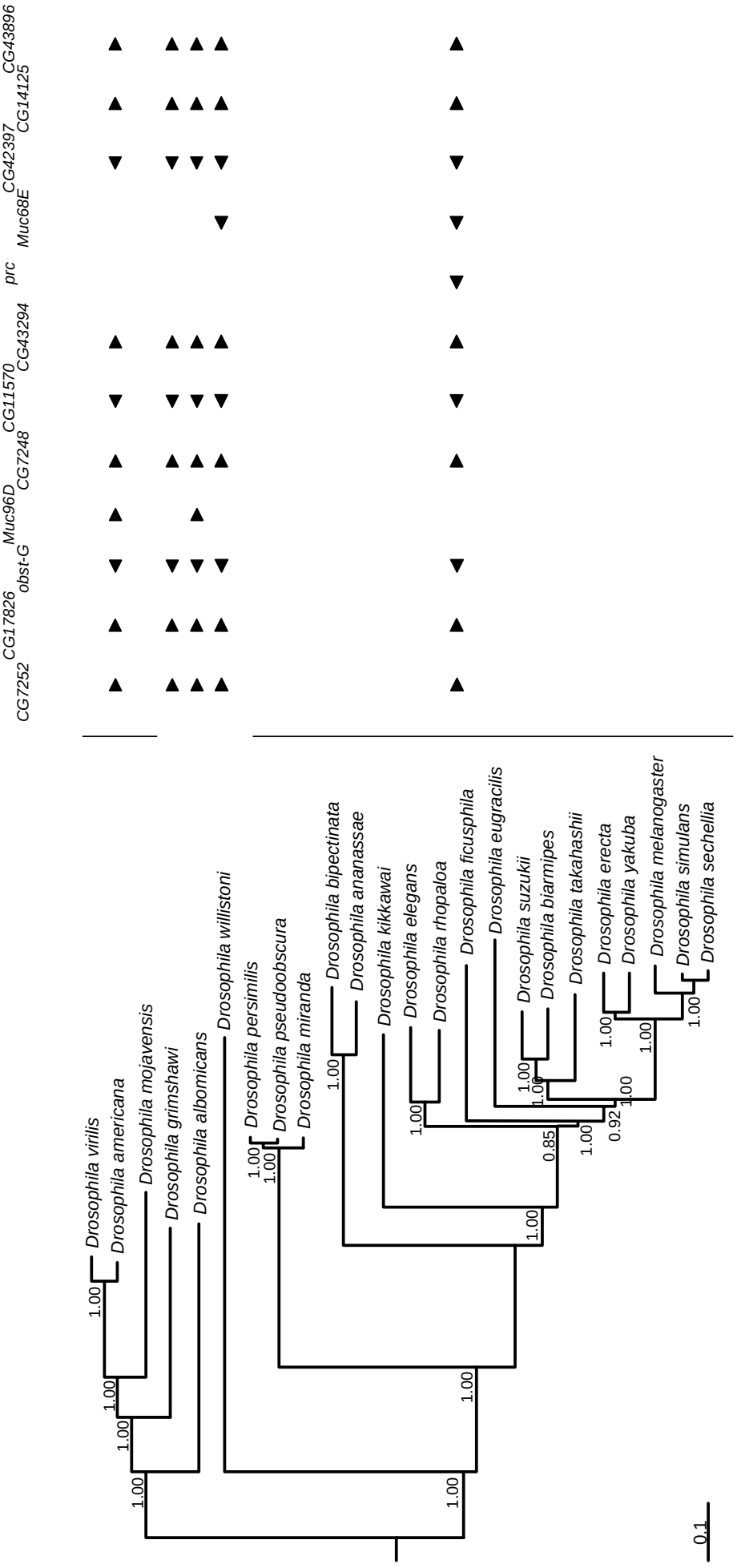


Figure III-6: Phylogenetic analysis of *Muc68E* distribution across *Drosophila* genus, and synteny map of the region surrounding this gene.

All of the genes above (used in the micro-synteny analysis) except *prc* have CBT2 domains similar to the ones in Muc68E, which follow the PerA motif ($CX_{13–20}CX_{5–6}CX_{9–19}CX_{10–14}CX_{4–14}C$). When a phylogenetic analysis of the CBT2 domains showing similarity to the first Muc68E CBT2 domain is made, it becomes apparent that the CBT2 domains of the *Muc68E* neighbours – *CG42397* and *CG14125* – are the most closely related to those of *Muc68E* (blue rectangle in Fig. III-7). It is also evident that, as noted for the *Muc68E* middle repetitive region, a significant amount of within gene conversion or frequent contractions and expansions of the CBT2 domains must be argued in order to explain the observation that CBT2 domains from the same gene usually cluster together (see *CG7252* (purple) and *CG43896* (yellow) genes in Fig. III-7).

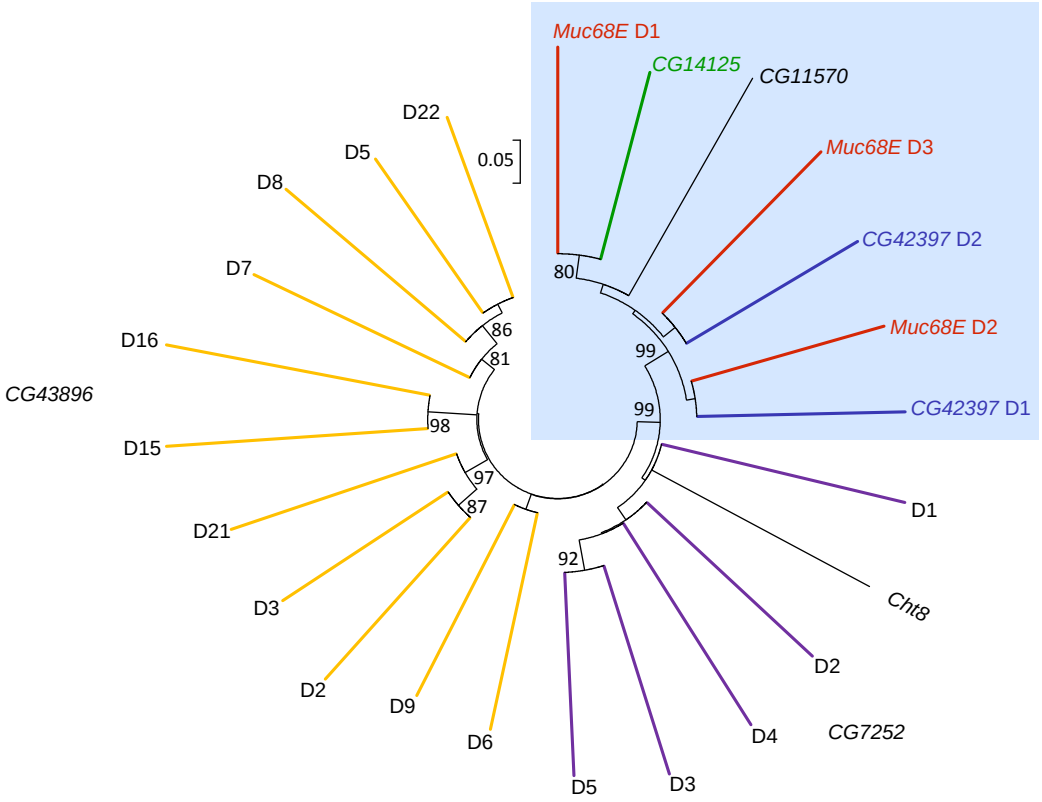


Figure III-7: Phylogenetic analysis of the CBT2 domains showing similarity to the first Muc68E CBT2 domain. This is displayed in a circular and unrooted Neighbour-joining tree in which numbers on each node indicate bootstrap percentages after 1,000 replicates. Only bootstrap values higher than 80 are shown. CBT2 domains from the same gene have branches with the same colour, specifically: yellow (CG43896), purple (CG7252), grey (Cht8), blue (CG42397), red (Muc68E), black (CG11570), and green (CG14125). Also shown is the divergence scale bar.

The *CG42397* gene is transcribed in the same orientation as the latter gene. The CG42397 protein is 178 amino acids long and shows two CBT2 domains in the C-terminal region that represent 69% of the protein. There is also a strong peptide signal predicted in the first 19 amino acids, and in contrast with Muc68E there is no highly repeated middle region, and thus this gene is not a mucin.

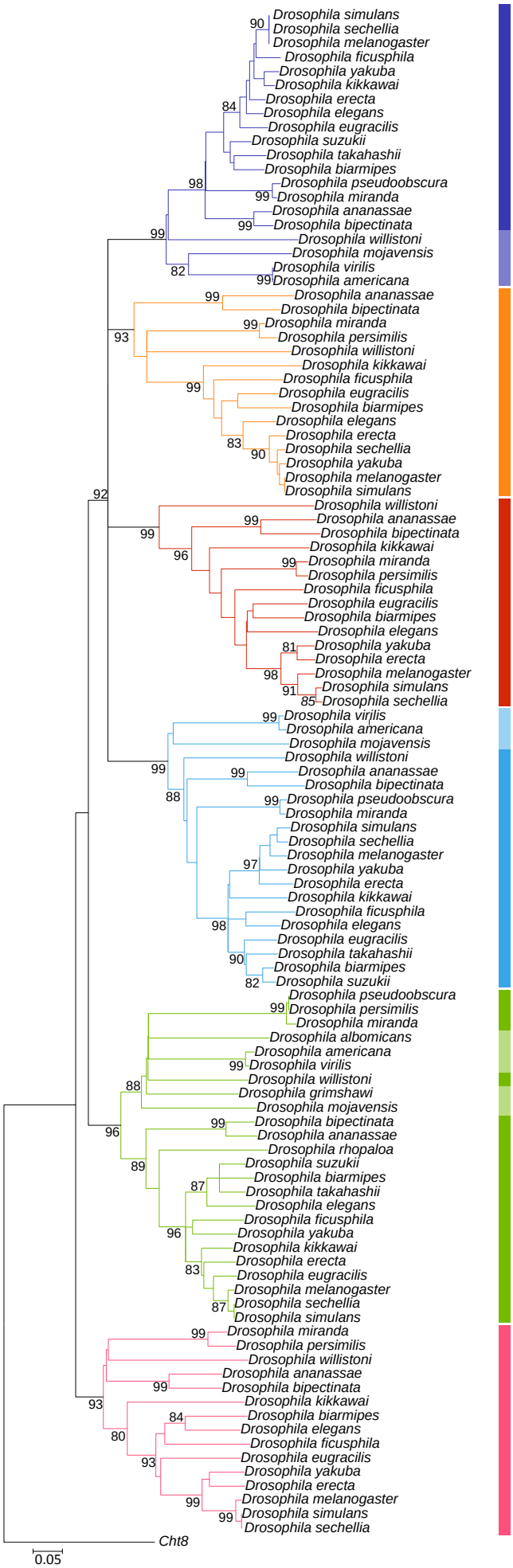


Figure III-8: Phylogenetic analysis of the CBT2 domains of Muc68E, CG42397 and CG14125 using as root the CBT2 domain of Cht8. A rooted Neighbour-joining tree in which numbers on each node indicate bootstrap percentages after 1,000 replicates is displayed. Only bootstrap values higher than 80 are shown. In each gene (represented in a colour) bold colours represent species from the *Sophophora* subgenus, while transparency represents species from the *Drosophila* subgenus (when present). Also shown is the divergence scale bar.

Nevertheless, this observation raises the possibility that *Muc68E* may have arisen as a tandem duplication of the *CG42397* gene that later on acquired the middle highly repetitive region. In this scenario, two predictions can be made: 1) phylogenetic analyses of the CBT2 domain should show the close relationship between *Muc68E* and *CG42397*; and 2) phylogenetic analyses should show that *Muc68E* is more closely related to the *CG42397* genes from the *Sophophora* lineage than the *CG42397* genes of the *Sophophora* and *Drosophila* lineages.

The first prediction is observed (Fig. III-8), although there is a polytomy that prevents us from resolving the relations between the CBT2 domains 2 and 3 of Muc68E and the CBT2 domains 1 and 2 of CG42397, but the second prediction is disfavoured by the fact that both CG42397 CBT2 domains have clusters that include *Drosophila* and *Sophophora* lineages which are closer to each other than to Muc68E *Sophophora* lineages. To sum up, the phylogenetic signal is not enough to resolve the relations between these genes due to gene conversion, for instance, as recent events can prevent the visualization of the expected molecular signature.

As we can see in Fig. III-4, the PTS repeats have different sizes and amino acid compositions in different species, although they are in some part maintained in subgroups, as for instance in the five analysed species of the *Drosophila melanogaster* species subgroup (*D. erecta, D. yakuba, D. melanogaster, D. simulans*, and *D.sechellia*), only *D. yakuba* has a smaller motif, but it is highly homogeneous with the other motifs from this subgroup.

Because the alignment of the highly repetitive middle region is intricate due to the presence of PTS repeats, we could have missed information when analysing *Muc68E* in the *Drosophila* subgenus, and that could lead to us not recognising this gene. However, with the synteny analysis we have a powerful tool that confirms our results and gives us a clear answer: *Muc68E* is not located where it should be. Further, the chitin-binding domains are really characteristic, and they are not found together with PTS repeats anywhere else across the *Drosophila* subgenus, so *Muc68E* clearly does not exist in species of that subgenus.

3.3.2. RT-qPCR expression analyses

Fst and Muc68E are the only proteins in the *D. melanogaster* proteome showing more than two PEEST motifs (Vieira J, personal communication), and this could be related to their function. In line with what was discussed before (see introduction), *Fst*

may be a mucin-like gene so, as *Muc68E* has some structural resemblance to *Frost*, it could be involved in cold response as well.

We used the same experimental settings used on Chapter II, and realised that *Muc68E* is overexpressed 2 hours after recovery from chill coma, as illustrated in Fig. III-9. Indeed, *Muc68E* shows a significant overexpression for females, around 2.5 times. For males it shows a similar average overexpression that is non-significant due to variation between biological replicates. Also, immediately after chill coma recovery, *Muc68E* is under expressed around 1.5 times but not significantly.

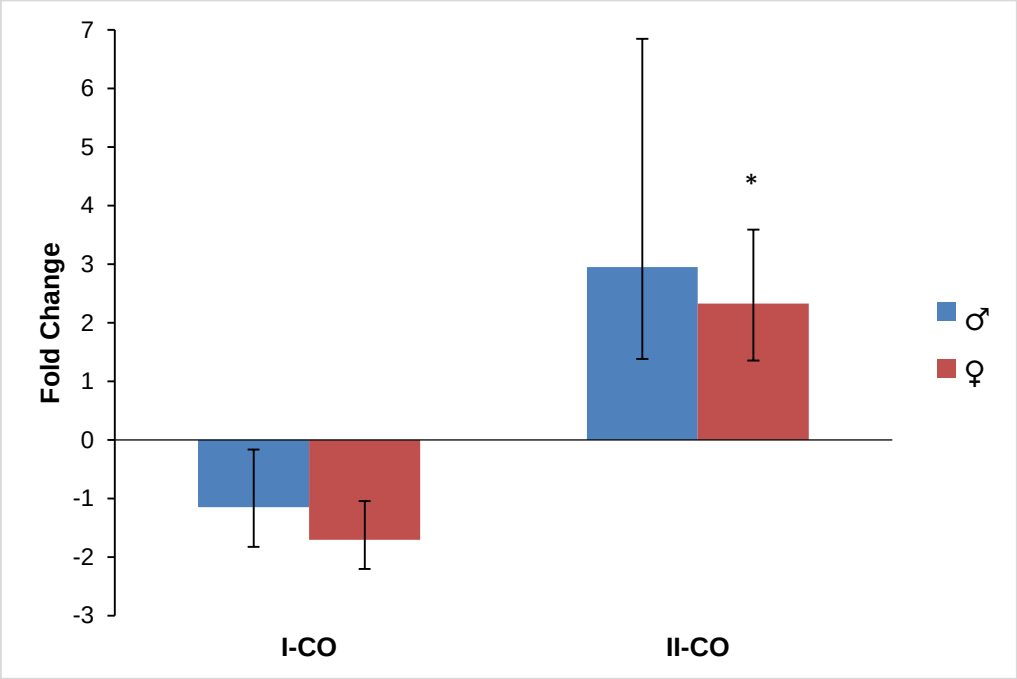


Figure III-9: Fold change in *Muc68E* expression after chill coma obtained by RT-qPCR using the $2^{-\Delta\Delta CT}$ method. The reference gene *RpL32* was used to normalize the expression values. Expression fold changes were addressed immediately after chill coma recovery (I-CO) and after 2 hours of recovery (II-CO) for both males (blue) and females (red) of *D. melanogaster* Ore-R strain; Error bars present a measure of the variation in biological replicates. Bars with an asterisk represent significant (P<0.05) values according to T-test statistics.

In males *Muc68E* is overexpressed two hours after recovery from heat stress but in females it reveals a non-significant sub expression (when comparing to the I-CO), although the numerical value is roughly the same as the one obtained for chill coma (for the same situation) (see Fig. III-10). It should be noted that *Muc68E*, in both males and females, is already significantly overexpressed immediately after exposure to heat stress, and this could to some extend reveal an important role of this gene regarding the response to variations in temperature.

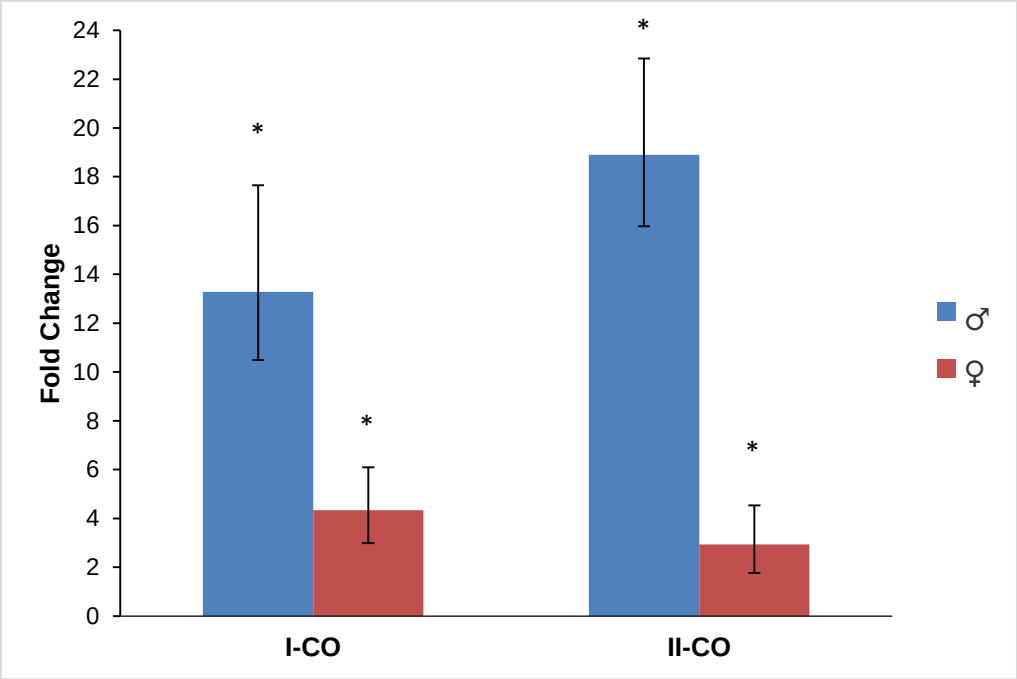


Figure III-10: Fold change in *Muc68E* expression after heat shock obtained by RT-qPCR using the $2^{-\Delta\Delta CT}$ method. The reference gene *RpL32* was used to normalize the expression values. Expression fold changes were addressed immediately after heat shock (I-CO) and after 2 hours of recovery (II-CO) for both males (blue) and females (red) of *D. melanogaster* Ore-R strain; Error bars present a measure of the variation in biological replicates. Bars with an asterisk represent significant ($P<0.05$) values according to T-test statistics.

Concerning flies subjected to nutrient deprivation (36h) and starvation (18h), we note that *Muc68E* is under expressed in both situations as can be seen in Fig. III-11, albeit due to differences in biological replicates we cannot obtain statistically significant values.

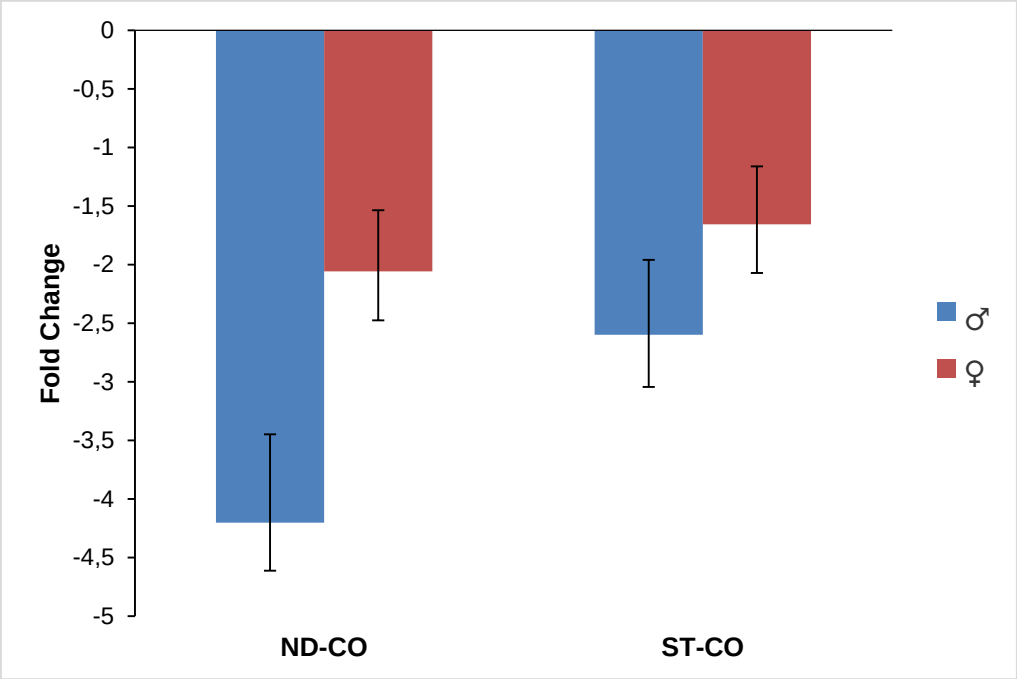


Figure III-11: Fold change in *Muc68E* expression after nutrient deprivation and after starvation obtained by RT-qPCR using the $2^{-\Delta\Delta CT}$ method. The reference gene *RpL32* was used to normalize the expression values. Expression fold changes were addressed after 36 hours of nutrient deprivation (ND-CO) and after 18 hours of starvation (ST-CO) for both males (blue) and females (red) of *D. melanogaster* Ore-R strain; Error bars present a measure of the variation in biological replicates. None of the values is significant (P<0.05) according to T-test statistics.

We also tested the involvement of *Muc68E* in cold response by analysing the phenotypical response of a *D. melanogaster* w^{1118} strain which has an insertion in the coding region of *Muc68E (*27851), and thus is a *Muc68E* mutant, against the *D. melanogaster* w^{1118} strain used as background for the mutation (5905).

3.3.3. 27851 vs 5905 – the impact of an insertion on *Muc68E* gene

It should be noted that strains 5905 and 27851 are genetically identical with the exception of the 7.5 kb *P*-element insertion at the beginning of *Muc68E* coding region. Although there are significant differences in the lifespan (LS) of males and females from the same strain (Non-parametric Mann-Whitney test; P<0.005 for all comparisons), there are no statistically significant differences (Non-parametric Mann-Whitney test; P>0.05 for all comparisons) in the LS of strain 5905 and 27851 when comparing either males or females (Fig. III-12). Therefore, the knockdown of *Muc68E* gene does not seem to compromise any essential aspect of the fly physiology. The LS differences between males and females is also observed in the commonly used strain Ore-R (Non-parametric Mann-Whitney test; P<0.001). The Ore-R strain lives, however, significantly longer than

the control strain 5905 (Non-parametric Mann-Whitney test; P>0.05 for all comparisons; Fig. III-13).

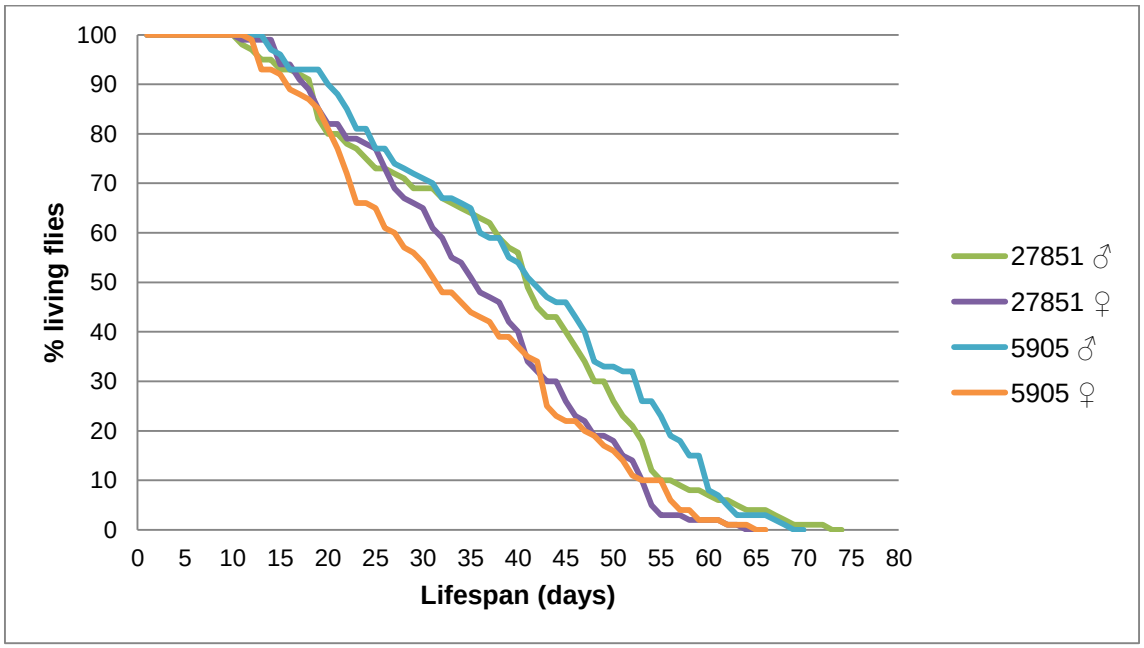


Figure III-12: Percentage of dead *D. melanogaster* 27851 and 5905 flies. 27851 ♂ Average (AVG) = 39.58 days, Standard deviation (SD) = 15.31; 27851 ♀ AVG = 35.85 days, SD = 12.92; 5905 ♂ AVG = 41.19 days, SD = 15.23; 5905 ♀ AVG = 33.89 days, SD = 14.01; sample size (N) = 100 for all the strains used.

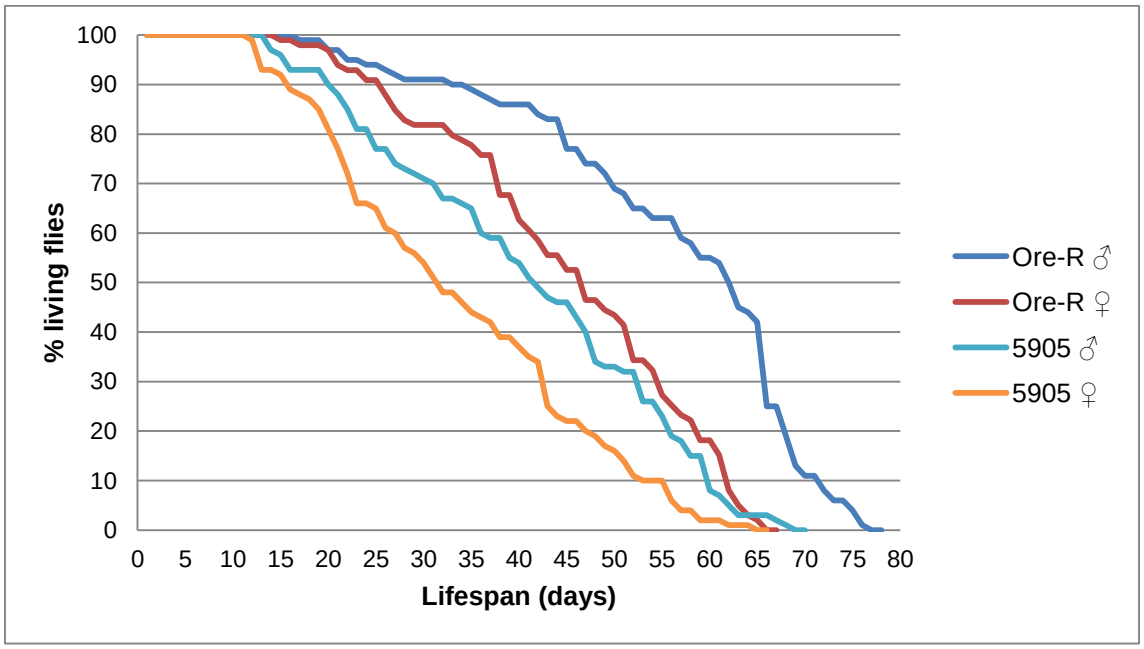


Figure III-13: Percentage of dead *D. melanogaster* Ore-R and 5905 flies. Ore-R ♂ AVG = 56.68 days, SD = 14.96, N = 100; Ore-R ♀ AVG = 45.45 days, SD = 13.54, N = 99; 5905 ♂ AVG = 41.19 days, SD = 15.23, N = 100; 5905 ♀ AVG = 33.89 days, SD = 14.01, N = 100.

3.3.3.1. *Muc68E* mutant flies recover faster from cold shock

In order to understand whether *Muc68E* could be involved in cold resistance, we compared the chill coma recovery time (CCRT) of flies from strain 5905 with the *Muc68E* 27851 mutant strain. For both the 5905 and the 27851 strains there is a highly significant statistical difference between males and females regarding CCRT (Non-parametric Mann-Whitney test; P<0.001 see Fig. III-14). For both males and females there is also a highly significant statistical difference between strain 5905 and strain 27851 regarding CCRT (Non-parametric Mann-Whitney test; P<0.001). Flies without *Muc68E* expression recover on average between 12.1% and 17.8% faster than control flies. This is at odds with the observation that *Muc68E* is overexpressed 2 hours after recovery, although there is a non-significant under expression immediately after recovery. It should be noted that both males and females from the control strain 5905 recover significantly faster (in between 7.3% and 26.2%) than Ore-R males and females, respectively (Non-parametric Mann-Whitney test; P<0.001).

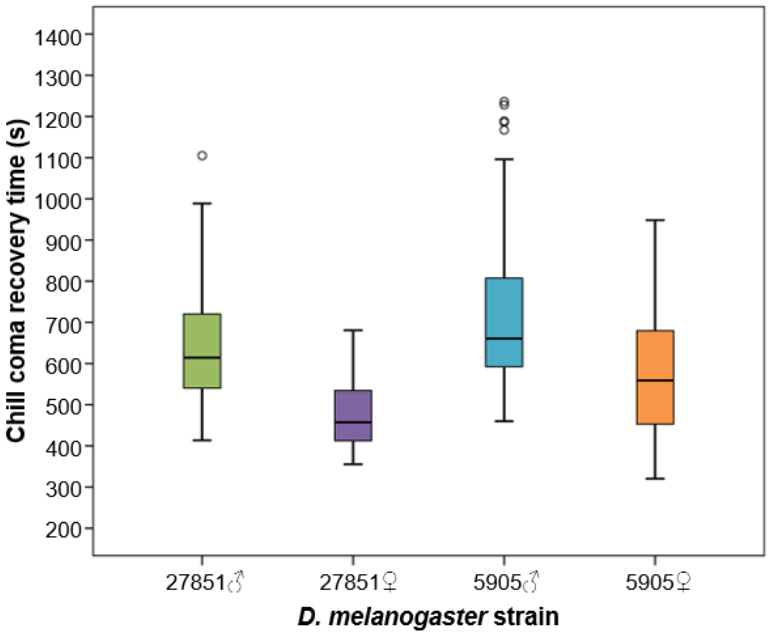


Figure III-14: Box plot representing chill coma recovery times for *D. melanogaster* 27851 males (green) and females (purple), and for 5905 males (cyan) and females (orange). The top of the box represents the 75th percentile, the bottom of the box represents the 25th percentile, and the line in the middle represents the 50th percentile that corresponds to the median. The whiskers represent the highest and lowest values that are not outliers or extreme values. Circles beyond the whiskers represent outliers. For 27851 ♂ AVG = 637.6 s, SD = 133.6; for 27851 ♀ AVG = 474.9 s, SD = 75.8; for 5905 ♂ AVG = 725.3 s, SD = 185.1; for 5905 ♀ AVG = 577.8 s, SD = 144.9; N = 100 for all the strains used.

3.3.3.2. *Muc68E* mutant flies are not sensitive to heat/desiccation stress

Since a significant *Muc68E* overexpression was observed after heat/desiccation stress in Ore-R strain, we reasoned that the viability of *Muc68E* mutants could be compromised under these stress conditions. Therefore the percentage of living flies was recorded every half hour until all flies were dead. As in the case of strain Ore-R, there is almost no mortality (less than 5%) in the first 90 minutes of exposure to heat, and no fly was able to stand the treatment for more than five and a half hours (Fig. III-15). For both strain 5905 and strain 27851, the death rate seems to increase linearly with exposure time (Table III-3), as observed for the Ore-R strain (see above). For both strain 5905 and strain 27851, there are no statistically significant differences between males and females regarding death rates (Non-parametric sign test; $P > 0.05$ for all strains; see Fig. III-15).

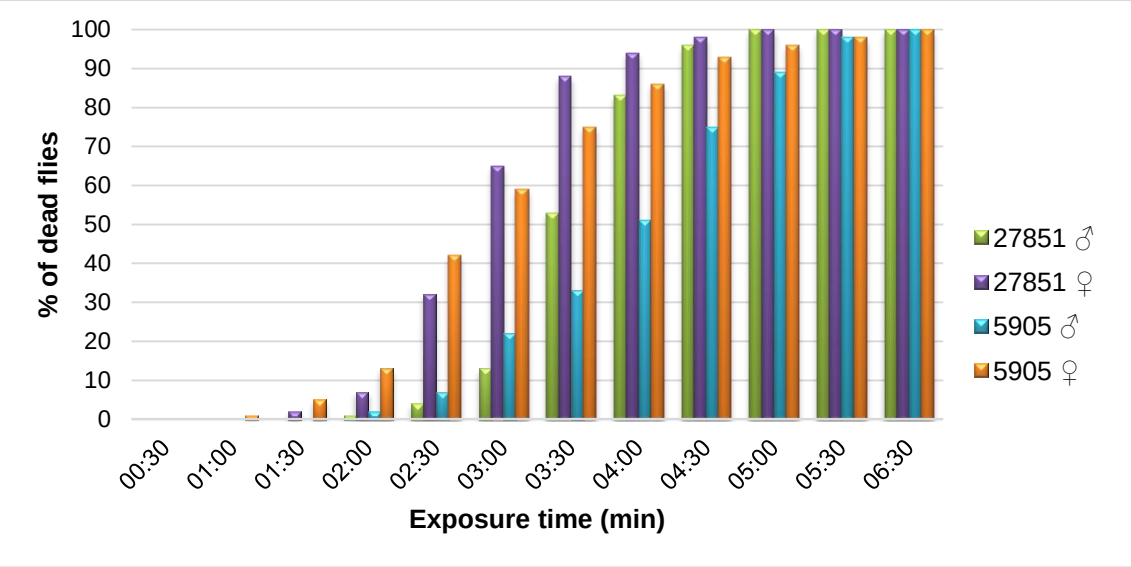


Figure III-15: Percentage of dead *D. melanogaster* 27851 and 5905 flies after exposure to heat shock

Table III-3: Correlation between death rates and exposure time (to heat shock) from the moment significant death occurs; R stands for Pearson product-moment correlation coefficient; Significance was calculated through a non-parametric sign test

Strain	Sex	N	R	Significance
5905	Males	11	+ 0.94	P<0.001
	Females	11	+ 0.90	P<0.001
	All	11	+ 0.95	P<0.001
27851	Males	9	+0.93	P<0.001
	Females	9	+0.95	P<0.001
	All	9	+0.97	P<0.001
Ore-R	Males	8	+0.96	P<0.001
	Females	10	+0.89	P<0.001
	All	10	+0.91	P<0.001

After 3 hours, there is about 50% mortality in the control strain 5905, and thus we use this time point to compare the different strains. Here, there is no statistically significant difference between strain 5905 and strain 27851 (Fisher's exact test; P>0.05). Therefore, despite the significant *Muc68E* overexpression that is observed after heat/desiccation stress in the Ore-R strain, it seems that *Muc68E* is not involved in the response to heat and associated dissection stress. It should be noted that strain 5905 is much more resistant to heat than the commonly used strain Ore-R (Fisher's exact test; P<0,001).

3.3.3.3. *Muc68E* mutant flies respond to the lack of food – nutrient deprivation

In order to determine how well the control 5905 and the 27851 *Muc68E* mutant strains are able to stand prolonged nutrient deprivation, the percentage of living flies was recorded every 12 hours until all flies were dead. For these strains, there is almost no mortality (less than 5%) in the first 14 hours, and no fly was able to stand the treatment for 72 hours (Fig. III-16). For both strain 5905 and strain 27851, the death rate seems to increase linearly with exposure time (Table III-4), and such a feature is also present in the commonly used Ore-R strain. It should be noted that there are no statistically significant differences between males and females regarding death rates (Non-parametric sign test; P > 0.05 for all strains).

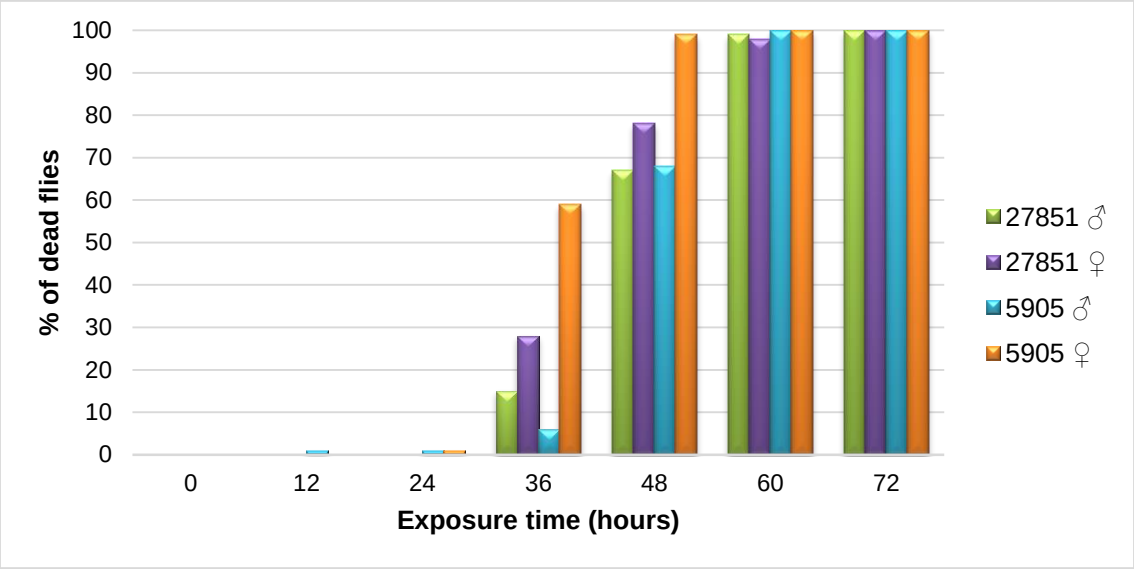


Figure III-16: Percentage of dead *D. melanogaster* 27851 and 5905 flies after exposure to nutrient deprivation

Table III-4: Correlation between death rates and exposure time (to nutrient deprivation) from the moment significant death occurs; R stands for Pearson product-moment correlation coefficient; Significance was calculated through a non-parametric sign test

Strain	Sex	N	R	Significance
5905	Males	5	+ 0.91	P<0.05
	Females	5	+ 0.98	P<0.005
	All	5	+ 0.98	P<0.005
27851	Males	6	+0.96	P<0.005
	Females	6	+0.99	P<0.001
	All	6	+0.98	P<0.001
Ore-R	Males	6	+0.95	P<0.005
	Females	5	+0.98	P<0.005
	All	6	+0.98	P<0.001

When we compare males and females together, half of the flies die in between 36 and 48 hours, and thus we use the 36 hours data point to compare the different strains. There is a statistically significant difference between strain 5905 and strain 27851 (Fisher's exact test; P<0.05). Therefore, it seems that lack of *Muc68E* could lead to increased resistance to nutrient deprivation (a 16% increase in survival by 36 hours). It should also be noted that strain 5905 is as resistant to nutrient deprivation as the commonly used strain Ore-R (Fisher's exact test; P>0.05).

3.3.3.4. *Muc68E* mutant flies do not respond to starvation

In order to determine how well the control (5905) and the *Muc68E* mutant (27851) strains are able to stand prolonged starvation, the percentage of living flies was recorded every hour until all flies were dead. For these strains, there is almost no mortality (less than 5%) in the first 18 hours, and no fly was able to stand the treatment for more than 28 hours (Fig. III-17). Given the above results for nutrient deprivation, in which more than half of the flies are still alive by 36 hours, desiccation is probably a major cause of death here, as it was shown before for Ore-R. For both strain 5905 and strain 27851, the death rate seems to increase linearly with exposure time (Table III-5). Such a feature is also present in the commonly used Ore-R strain. It should be noted that there are statistically significant differences between males and females regarding death rates (Non-parametric sign test; for 5905 $P < 0.01$; for 27851 $P < 0.05$; for Ore-R $P < 0.005$).

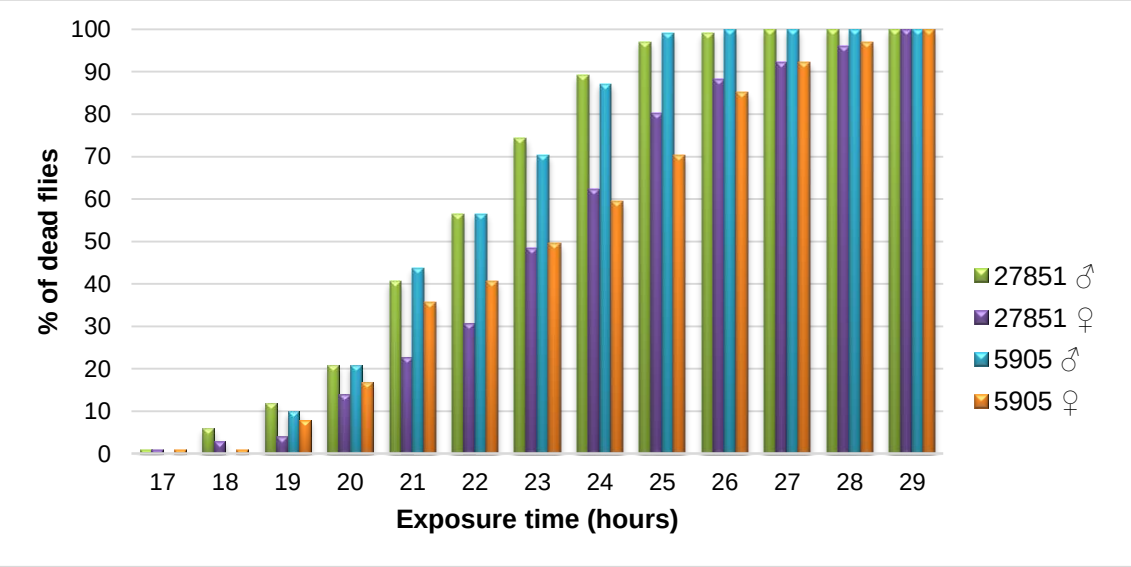


Figure III-17: Percentage of dead *D. melanogaster* 27851 and 5905 flies after exposure to starvation

Table III-5: Correlation between death rates and exposure time (to starvation) from the moment significant death occurs; R stands for Pearson product-moment correlation coefficient; Significance was calculated through a non-parametric sign test

Strain	Sex	N	R	Significance
5905	Males	9	+ 0.94	P<0.001
	Females	10	+ 0.97	P<0.001
27851	Males	10	+0.93	P<0.001
	Females	12	+0.89	P<0.001
Ore-R	Males	12	+0.88	P<0.001
	Females	11	+0.89	P<0.001

After 22 hours, there is about 50% mortality in the control strain 5905, and thus we use this time point to compare the different strains. For both males and females, there is no statistically significant difference between strain 5905 and strain 27851 (Fisher's exact test; P>0.05). Therefore, it seems that *Muc68E* is not involved in the starvation stress response. It should be noted that strain 5905 is as resistant to starvation as the commonly used strain Ore-R (Fisher's exact test; P>0.05).

To sum up, the expression analysis suggests that *Muc68E* could somehow be involved in the recovery from cold response, but this evidence is not strong as we could only identify a significant overexpression in females 2 hours of recovery after chill coma. There is also a non-significant under expression (+-1.5 times) immediately after chill coma recovery that could explain why the mutants – without a functional Muc68E protein – recover faster from chill coma, when compared to the controls.

Chapter IV – Conclusions

4.1. Global discussion and further work

One of the major findings of this work is the demonstration that *Frost,* which was thought to be specifically involved in cold response, is probably actually responding to desiccation. The way our experiments were optimized guaranteed consistency in the study of the response of *Fst* to the different types of stress analysed here – and in particular enabled us to consistently compare reliable data from different experiments. Furthermore, we showed that the conditions assayed impose high levels of stress and we did several biological replicates to ensure that we could grasp what was truly happening, making our conclusions trustworthy.

When temperature changes, flies suffer, for instance, metabolic alterations of which one of the consequences is water loss. As temperature is an important abiotic factor, *Drosophila* need to be able to adapt in order to survive when subjected to extreme temperatures (see general introduction). Our results (see Chapter II) suggest that the ability to resist water loss is somehow connected with the expression of *Fst*.

This can explain the apparently contradictory results obtained with RNAi experiments [31, 143], where an induced *Fst* mutant recovers faster than the control from chill coma. As *Fst* is highly expressed in Malpighian tubules, it could be related to the retention of water in these structures (that are somewhat analogous to the kidneys of mammals). We hypothesised that Frost acts in the tubule walls somehow regulating their permeability so that when this protein is removed the water intake is diminished and, for instance, the formation of ice crystals inside the body is reduced, causing mutants to recover faster. Another possibility is that, as chill coma is probably related to an inability to maintain trans-membrane ion homeostasis at low temperatures (see general introduction), a lack of control in water intake could lead to a break in ion homeostasis, altering the osmolality of several cell compartments which could provoke leaking and major cellular damage.

The only really critical aspects that limit the habitats where *Drosophila* can live in is temperature and availability of water [28]. The availability of water varies through seasons and also across a species distribution, therefore being able to endure some degree of water loss is beneficial not only when dealing with thermo induced stresses, but also when trying to conquer a new habitat or a more restricted ecological niche.

Several studies have analysed the expression of *Frost* across *Drosophila* genus and supposing that this gene is responding to dissection, as we propose, some of this data can be easily explained. In a particular case, after chill coma recovery low levels of *Fst* expression were observed for temperate species such as *D. americana* [118], but high levels of *Fst* were found in tropical species such as *D. melanogaster* [14, 31]. This could be accounted for by the fact that desiccation is a major source of damage in temperate areas which have harsh weather, especially during winters. Since temperate species are seasonally exposed to temperatures which can induce cold damage, for them it should be more advantageous to have basal levels of *Frost* higher than the ones of tropical species in order to cope with that stress. Thus, they do not need to overexpress Frost as this protein should be abundant since it is possibly already expressed at high levels in the basal metabolism. On the contrary, as desiccation is not a major source of damage in tropical areas which have mild weather and a characteristic high humidity level, high levels of *Frost* basal expression would only be energy consuming for tropical species. So in these species there is an overexpression of *Frost* when flies are subjected to stress induced by desiccation, which as we showed with our work can for instance be due to cold or heat shock and starvation.

To test this hypothesis we could rear *D. melanogaster* flies in a lower temperature in order to induce cold acclimation and then analyse if the basal levels of *Frost* changed as time progressed.

Regarding to the major question in Chapter III, whether *Muc68E* is involved in the cold response, even though there is an overexpression 2 hours after chill coma recovery, the magnitude of *Muc68E* fold change is very different from the one given by *Fst*, so the involvement of this gene in cold response is somewhat dubious. Nevertheless, since we showed that *Fst* is not actually directly involved in cold response but is instead responding to desiccation, we hypothesized that the same could happen with *Muc68E*.

As we previously discussed in Chapter II, and we can see in the summary below (Table IV-1), although *Muc68E* is significantly overexpressed after heat shock, this answer is somewhat different from the one displayed by *Frost*. This gene shows a significant overexpression if we compare immediately (I-CO) versus 2 hours after recovery (II-CO), while *Muc68E* shows a small down-regulation.

Table IV-1: Summary of fold change in *Frost* and *Muc68E* expression after different types of stress obtained by RT-qPCR using the $2^{-\Delta\Delta CT}$ method. The reference gene *RpL32* was used to normalize the expression values. Expression fold changes were addressed after each stress condition in *D. melanogaster* Ore-R strain. Values with blue shading represent significant (P<0.05) values according to T-test statistics.

Chill Coma ♂	I-CO	II-CO
Frost	11.81	35.71
Muc68E	-1.15	2.95

Chill Coma ♀	I-CO	II-CO
Frost	4.11	39.90
Muc68E	-1.71	2.33

Heat Shock ♂	I-CO	II-CO
Frost	7.17	41.69
Muc68E	13.28	18.90

Heat Shock ♀	I-CO	II-CO
Frost	-1.01	13.82
Muc68E	4.33	2.94

Starvation ST-CO	♂	♀
Frost	12.45	40.88
Muc68E	-2.60	-1.65

Nutrient Deprivation ND-CO	♂	♀
Frost	-2.25	3.91
Muc68E	-4.20	-2.06

Note the importance of having determined the expression through RT-qPCR on a commonly used *D. melanogaster* strain. Since Ore-R is a wild type strain, using it allows us to normalize our results across treatments and genes, which ultimately enables these comparisons. Thus we can conclude that *Muc68E* is not responding to desiccation as *Fst*, but it could be responding to the damage caused by differences in temperature, and possibly be involved in the response to heat shock.

However, when we compared the response of a *Muc68E* mutant strain (27851) to heat shock (see Fig. III-15) we could not identify statistically significant differences from the control strain 5905. As a consequence, one should be really careful when assuming a certain function for a gene based solely on its expression pattern - despite its importance, this information alone is insufficient to evaluate the importance of a given gene. That being said, even though *Muc68E* is not particularly involved in chill coma recovery, perhaps other mucins could be, due to their particular 3D structure (namely the glycosylated PTS repeats, that build into large chains of carbohydrates and sugars, which favours water retention). Since the phenotypic analysis clearly states that there is some effect in response to this stress (see Fig. III-14) and the expression analyses point in the same direction (see Fig. III-9 and Table IV-1), this evidence cannot be overlooked.

We showed that 5905 is as robust as Ore-R in regard to lifespan and resistance to nutrient deprivation, but they behaved differently in response to other stresses. So in future experiments we should analyse the expression levels of 5905 and compare them to the ones obtained for Ore-R, in order to determine whether a difference in basal levels of *Muc68E* could explain the apparently contradictory results that we found in cold responses.

Globally, this work is a contribution towards to a better understanding of the molecular basis that confines species distribution, especially the response to the lower temperature limit. It sheds light on a possible function of the *Frost* gene, which still manages to keep its secrets in spite of having been investigated by a reasonable number of different groups since it was first described in 2001. It also focuses on mucins, which despite being important in several key aspects, are mainly studied in humans due to the fact that their overexpression is related to cancer. They have been poorly studied in other classes, and we think that this work is a step towards the molecular characterization of this important group of proteins in other animals.

References

1. Adams, M.D., et al., *The genome sequence of Drosophila melanogaster.* Science, 2000. **287**(5461): p. 2185-95.
2. Affleck, J.G. and V.K. Walker, *A role for Drosophila in understanding drug-induced cytotoxicity and teratogenesis.* Cytotechnology, 2008. **57**(1): p. 1-9.
3. Anderson, A.R., et al., *Thermal tolerance trade-offs associated with the right arm of chromosome 3 and marked by the hsr-omega gene in Drosophila melanogaster.* Heredity (Edinb), 2003. **90**(2): p. 195-202.
4. Anderson, A.R., A.A. Hoffmann, and S.W. McKechnie, *Response to selection for rapid chill-coma recovery in Drosophila melanogaster: physiology and life-history traits.* Genet Res, 2005. **85**(1): p. 15-22.
5. Anderson, R.L. and J.A. Mutchmor, *Temperature acclimation and its influence on the electrical activity of the nervous system in three species of cockroaches.* Journal of Insect Physiology, 1968. **14**(2): p. 243-251.
6. Apidianakis, Y., et al., *Profiling early infection responses: Pseudomonas aeruginosa eludes host defenses by suppressing antimicrobial peptide gene expression.* Proceedings of the National Academy of Sciences of the United States of America, 2005. **102**(7): p. 2573-2578.
7. Arboleda-Bustos, C.E. and C. Segarra, *The Dca gene involved in cold adaptation in Drosophila melanogaster arose by duplication of the ancestral regucalcin gene.* Mol Biol Evol, 2011. **28**(8): p. 2185-95.
8. Ary, A.H., G.S. Jesper, and L. Volker, *Adaptation of Drosophila to temperature extremes: bringing together quantitative and molecular approaches.* Journal of Thermal Biology, 2003. **28**(3).
9. Ashburner, M., et al., *Gene ontology: tool for the unification of biology. The Gene Ontology Consortium.* Nat Genet, 2000. **25**(1): p. 25-9.
10. Atuma, C., et al., *The adherent gastrointestinal mucus gel layer: thickness and physical state in vivo.* Am J Physiol Gastrointest Liver Physiol, 2001. **280**(5): p. G922-9.
11. Ayrinhac, A., et al., *Cold adaptation in geographical populations of Drosophila melanogaster: phenotypic plasticity is more important than genetic variability.* Functional Ecology, 2004. **18**(5): p. 700-706.
12. Bale, J.S., *Insect cold hardiness: a matter of life and death.* European Journal of Entomology, 1996. **93**: p. 369-382.
13. Berrigan, D., et al., *Comparing Different Measures of Heat Resistance in Selected Lines of Drosophila melanogaster.* J Insect Physiol, 1997. **43**(4): p. 393-405.
14. Bing, X., J. Zhang, and B.J. Sinclair, *A comparison of Frost expression among species and life stages of Drosophila.* Insect Molecular Biology, 2012. **21**(1): p. 31-39.
15. Bowler, A.R.C.a.K., *Temperature Biology of Animals*. 1987, New York: Chapman and Hall.
16. Bradshaw, W.E. and C.M. Holzapfel, *Evolution of Animal Photoperiodism.* Annual Review of Ecology, Evolution, and Systematics, 2007. **38**(1): p. 1-25.
17. Bubliy, O.A., et al., *Variation in resistance and acclimation to low-temperature stress among three geographical strains of Drosophila melanogaster.* Journal of Thermal Biology, 2002. **27**(5): p. 337-344.
18. Buchon, N., et al., *Drosophila intestinal response to bacterial infection: activation of host defense and stem cell proliferation.* Cell Host & Microbe, 2009. **5**(2): p. 200-211.

19. Buchon, N., et al., *Drosophila Intestinal Response to Bacterial Infection: Activation of Host Defense and Stem Cell Proliferation.* Cell Host and Microbe, 2009. **5**(2): p. 200-211.
20. Carlstedt, I., et al., *Characterization of two different glycosylated domains from the insoluble mucin complex of rat small intestine.* J Biol Chem, 1993. **268**(25): p. 18771-81.
21. Carsten, L.D., T. Watts, and T.A. Markow, *Gene expression patterns accompanying a dietary shift in Drosophila melanogaster.* Mol Ecol, 2005. **14**(10): p. 3203-8.
22. Chamilos, G., et al., *Drosophila melanogaster as a model host to dissect the immunopathogenesis of zygomycosis.* Proceedings of the National Academy of Sciences of the United States of America, 2008. **105**(27): p. 9367-9372.
23. Chen, C.-P. and V.K. Walker, *Cold-shock and chilling tolerance in Drosophila.* Journal of Insect Physiology, 1994. **40**(8): p. 661-669.
24. Chen, C.P. and V.K. Walker, *Increase in cold-shock tolerance by selection of cold resistant lines in Drosophila melanogaster.* Ecological Entomology, 1993. **18**(3): p. 184-190.
25. Chen, I.C., et al., *Rapid range shifts of species associated with high levels of climate warming.* Science, 2011. **333**(6045): p. 1024-6.
26. Chintapalli, V.R., et al., *Functional correlates of positional and gender-specific renal asymmetry in Drosophila.* PLoS ONE, 2012. **7**(3): p. e32577.
27. Chown, S.L., *Physiological variation in insects: hierarchical levels and implications.* J Insect Physiol, 2001. **47**(7): p. 649-660.
28. Chown, S.L., J.G. Sorensen, and J.S. Terblanche, *Water loss in insects: an environmental change perspective.* J Insect Physiol, 2011. **57**(8): p. 1070-84.
29. Clark, M.S. and M.R. Worland, *How insects survive the cold: molecular mechanisms-a review*. 2008. p. 917-933.
30. Clowers, K.J., et al., *Genetic variation in senescence marker protein-30 is associated with natural variation in cold tolerance in Drosophila.* Genetics research, 2010. **92**(2): p. 103-113.
31. Colinet, H., S.F. Lee, and A. Hoffmann, *Functional characterization of the Frost gene in Drosophila melanogaster: importance for recovery from chill coma.* PLoS One, 2010. **5**(6): p. e10925.
32. Colinet, H., S.F. Lee, and A. Hoffmann, *Temporal expression of heat shock genes during cold stress and recovery from chill coma in adult Drosophila melanogaster.* FEBS Journal, 2010. **277**(1): p. 174-185.
33. Colinet, H., et al., *Rapid decline of cold tolerance at young age is associated with expression of stress genes in Drosophila melanogaster.* Journal of Experimental Biology, 2013. **216**(2): p. 253-259.
34. Collinge, J.E., A.A. Hoffmann, and S.W. McKechnie, *Altitudinal patterns for latitudinally varying traits and polymorphic markers in Drosophila melanogaster from eastern Australia.* J Evol Biol, 2006. **19**(2): p. 473-82.
35. Collinge, J.E., et al., *Latitudinal and cold-tolerance variation associate with DNA repeat-number variation in the hsr-omega RNA gene of Drosophila melanogaster.* Heredity, 2008. **101**(3): p. 260-270.
36. Coyne, J.A., C. Wicker-Thomas, and J.M. Jallon, *A gene responsible for a cuticular hydrocarbon polymorphism in Drosophila melanogaster.* Genetics research, 1999. **73**(3): p. 189-203.
37. Crooks, G.E., et al., *WebLogo: a sequence logo generator.* Genome Res, 2004. **14**(6): p. 1188-90.
38. Czajka, M.C. and R.E. Lee, Jr., *A rapid cold-hardening response protecting against cold shock injury in Drosophila melanogaster.* J Exp Biol, 1990. **148**: p. 245-54.

39. David, J.R., et al., *The fly that came in from the cold: geographic variation of recovery time from low-temperature exposure in Drosophila subobscura.* Functional Ecology, 2003. **17**(4): p. 425-430.
40. David, J.R., Allemand, R., Van Herrewege, J. and Cohet, Y., *Ecophysiology: abiotic factors*. In The Genetics and Biology of Drosophila. 1983, London and New-York: Academic Press.
41. de Castro, E., et al., *ScanProsite: detection of PROSITE signature matches and ProRule-associated functional and structural residues in proteins.* Nucleic Acids Res, 2006. **34**(Web Server issue): p. W362-5.
42. De Gregorio, E., et al., *Genome-wide analysis of the Drosophila immune response by using oligonucleotide microarrays.* Proceedings of the National Academy of Sciences of the United States of America, 2001. **98**(22): p. 12590-12595.
43. Denlinger, D., *Relationship between Cold Hardiness and Diapause*, in *Insects at Low Temperature*, R. Lee, Jr. and D. Denlinger, Editors. 1991, Springer US. p. 174-198.
44. Denlinger, D.L., *Regulation of Diapause.* Annual Review of Entomology, 2002. **47**(1): p. 93-122.
45. Denlinger, D.L.a.R.E.L., *Low Temperature Biology of Insects*. 2010, Cambridge: Cambridge University Press.
46. Devine, P.L. and I.F. McKenzie, *Mucins: structure, function, and associations with malignancy.* Bioessays, 1992. **14**(9): p. 619-25.
47. Feder, M.E. and G.E. Hofmann, *Heat-shock proteins, molecular chaperones, and the stress response: evolutionary and ecological physiology.* Annu Rev Physiol, 1999. **61**: p. 243-82.
48. Fernández-Ayala, D.J., et al., *Gene expression in a Drosophila model of mitochondrial disease.* PLoS ONE, 2010. **5**(1): p. e8549.
49. Gaines, P.J., S.J. Walmsley, and N. Wisnewski, *Cloning and characterization of five cDNAs encoding peritrophin-A domains from the cat flea, Ctenocephalides felis.* Insect Biochem Mol Biol, 2003. **33**(11): p. 1061-73.
50. Gallio, M., et al., *The coding of temperature in the Drosophila brain.* Cell, 2011. **144**(4): p. 614-624.
51. Gendler, S.J. and A.P. Spicer, *Epithelial mucin genes.* Annu Rev Physiol, 1995. **57**: p. 607-34.
52. Gerbasi, V.R., et al., *Blanks, a nuclear siRNA/dsRNA-binding complex component, is required for Drosophila spermiogenesis.* Proceedings of the National Academy of Sciences of the United States of America, 2011. **108**(8): p. 3204-3209.
53. Gibert, P. and R.B. Huey, *Chill-coma temperature in Drosophila: effects of developmental temperature, latitude, and phylogeny.* Physiol Biochem Zool, 2001. **74**(3): p. 429-34.
54. Gibert, P., et al., *Chill-coma tolerance, a major climatic adaptation among Drosophila species.* Evolution, 2001. **55**(5): p. 1063-1068.
55. GOLLER, F. and H. ESCH, *Comparative Study of Chill-Coma Temperatures and Muscle Potentials in Insect Flight Muscles.* Journal of Experimental Biology, 1990. **150**(1): p. 221-231.
56. Goto, S.G., *Expression of Drosophila homologue of senescence marker protein-30 during cold acclimation.* J Insect Physiol, 2000. **46**(7): p. 1111-1120.
57. Goto, S.G., *A novel gene that is up-regulated during recovery from cold shock in Drosophila melanogaster.* Gene, 2001. **270**(1–2): p. 259-264.
58. Greenberg, A.J., et al., *Ecological adaptation during incipient speciation revealed by precise gene replacement.* Science, 2003. **302**(5651): p. 1754-1757.
59. Hallas, R., M. Schiffer, and A.A. Hoffmann, *Clinal variation in Drosophila serrata for stress resistance and body size.* Genet Res, 2002. **79**(2): p. 141-8.

60. Hansson, G.C., *Role of mucus layers in gut infection and inflammation.* Curr Opin Microbiol, 2012. **15**(1): p. 57-62.
61. Hazel, J.R., *Thermal Adaptation in Biological Membranes: Is Homeoviscous Adaptation the Explanation?* Annual Review of Physiology, 1995. **57**(1): p. 19-42.
62. Hegedus, D., et al., *New insights into peritrophic matrix synthesis, architecture, and function.* Annu Rev Entomol, 2009. **54**: p. 285-302.
63. Heinrich, B., *The Hot-Blooded Insects. Strategies and Mechanisms of Thermoregulation*. 1993: Harvard University Press.
64. Hendrix, D.L. and M.E. Salvucci, *Polyol metabolism in homopterans at high temperatures: accumulation of mannitol in aphids (Aphididae: Homoptera) and sorbitol in whiteflies (Aleyrodidae: Homoptera).* Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology, 1998. **120**(3): p. 487-494.
65. Hoffmann, A.A., A. Anderson, and R. Hallas, *Opposing clines for high and low temperature resistance in Drosophila melanogaster.* Ecology Letters, 2002. **5**(5): p. 614-618.
66. Hoffmann, A.A., J.G. Sørensen, and V. Loeschcke, *Adaptation of Drosophila to temperature extremes: bringing together quantitative and molecular approaches.* Journal of Thermal Biology, 2003. **28**(3): p. 175-216.
67. Hoffmann, A.A., et al., *A proline repeat polymorphism of the Frost gene of Drosophila melanogaster showing clinal variation but not associated with cold resistance.* Insect Molecular Biology, 2012. **21**(4): p. 437-445.
68. Hollingsworth, M.A. and B.J. Swanson, *Mucins in cancer: protection and control of the cell surface.* Nat Rev Cancer, 2004. **4**(1): p. 45-60.
69. Hollingsworth, M.J. and K. Bowler, *The decline in ability to withstand high temperature with increase in age in Drosophila subobscura.* Experimental Gerontology, 1966. **1**(4): p. 251-257.
70. Hosler, J.S., J.E. Burns, and H.E. Esch, *Flight muscle resting potential and species-specific differences in chill-coma.* Journal of Insect Physiology, 2000. **46**(5): p. 621-627.
71. Jean David, R., et al., *Cold stress tolerance in Drosophila: analysis of chill coma recovery in D. Melanogaster.* Journal of Thermal Biology, 1998. **23**(5): p. 291-299.
72. Johansson, M.E., et al., *The inner of the two Muc2 mucin-dependent mucus layers in colon is devoid of bacteria.* Proc Natl Acad Sci U S A, 2008. **105**(39): p. 15064-9.
73. Johansson, M.E., J.M. Larsson, and G.C. Hansson, *The two mucus layers of colon are organized by the MUC2 mucin, whereas the outer layer is a legislator of host-microbial interactions.* Proc Natl Acad Sci U S A, 2011. **108 Suppl 1**: p. 4659-65.
74. Johnson, T.K., F.E. Cockerell, and S.W. McKechnie, *Transcripts from the Drosophila heat-shock gene hsr-omega influence rates of protein synthesis but hardly affect resistance to heat knockdown.* Molecular Genetics and Genomics, 2011. **285**(4): p. 313-323.
75. Kayukawa, T., et al., *Upregulation of a desaturase is associated with the enhancement of cold hardiness in the onion maggot, Delia antiqua.* Insect Biochem Mol Biol, 2007. **37**(11): p. 1160-7.
76. Kellermann, V., et al., *Phylogenetic constraints in key functional traits behind species' climate niches: patterns of desiccation and cold resistance across 95 Drosophila species.* Evolution, 2012. **66**(11): p. 3377-3389.
77. Kelty, J., *Rapid cold-hardening of Drosophila melanogaster in a field setting.* Physiological Entomology, 2007. **32**(4): p. 343-350.
78. Kelty, J.D., K.A. Killian, and R.E. Lee, *Cold shock and rapid cold-hardening of pharate adult flesh flies (Sarcophaga crassipalpis): effects on behaviour and*

neuromuscular function following eclosion. Physiological Entomology, 1996. **21**(4): p. 283-288.
79. Kelty, J.D. and R.E. Lee, Jr., *Induction of rapid cold hardening by cooling at ecologically relevant rates in Drosophila melanogaster.* J Insect Physiol, 1999. **45**(8): p. 719-726.
80. Kim, K., et al., *Expression of a functional Drosophila melanogaster N-acetylneuraminic acid (Neu5Ac) phosphate synthase gene: evidence for endogenous sialic acid biosynthetic ability in insects.* Glycobiology, 2002. **12**(2): p. 73-83.
81. Kimura, M.T., *Cold and heat tolerance of drosophilid flies with reference to their latitudinal distributions.* Oecologia, 2004. **140**(3): p. 442-9.
82. Korayem, A.M., et al., *A Drosophila salivary gland mucin is also expressed in immune tissues: evidence for a function in coagulation and the entrapment of bacteria.* Insect Biochemistry and Molecular Biology, 2004. **34**(12): p. 1297-1304.
83. Kostal, V., J. Vambera, and J. Bastl, *On the nature of pre-freeze mortality in insects: water balance, ion homeostasis and energy charge in the adults of Pyrrhocoris apterus.* J Exp Biol, 2004. **207**(Pt 9): p. 1509-21.
84. Kostal, V., M. Yanagimoto, and J. Bastl, *Chilling-injury and disturbance of ion homeostasis in the coxal muscle of the tropical cockroach (Nauphoeta cinerea).* Comp Biochem Physiol B Biochem Mol Biol, 2006. **143**(2): p. 171-9.
85. Koveos, D.S., *Rapid cold hardening in the olive fruit fly Bactrocera oleae under laboratory and field conditions.* Entomologia Experimentalis et Applicata, 2001. **101**(3): p. 257-263.
86. Kuraishi, T., A. Hori, and S. Kurata, *Host-microbe interactions in the gut of Drosophila melanogaster.* Front Physiol, 2013. **4**: p. 375.
87. Laayouni, H., et al., *Thermal evolution of gene expression profiles in Drosophila subobscura.* BMC Evolutionary Biology, 2007. **7**: p. 42.
88. Lakhotia, S.C., T.K. Rajendra, and K.V. Prasanth, *Developmental regulation and complex organization of the promoter of the non-coding hsr gene of Drosophila melanogaster.* Journal of Biosciences, 2001. **26**(1): p. 25-38.
89. Lee, D.C. and D. Chapman, *The effects of temperature on biological membranes and their models.* Symp Soc Exp Biol, 1987. **41**: p. 35-52.
90. Lee, R.E.a.D.L.D., *Insects at Low Temperature*. 1991, New York: Chapman and Hall.
91. Lehane, M.J., *Peritrophic matrix structure and function.* Annu Rev Entomol, 1997. **42**: p. 525-50.
92. Lemaitre, B. and J. Hoffmann, *The host defense of Drosophila melanogaster.* Annu Rev Immunol, 2007. **25**: p. 697-743.
93. Lemaitre, B. and I. Miguel-Aliaga, *The digestive tract of Drosophila melanogaster.* Annu Rev Genet, 2013. **47**: p. 377-404.
94. Littleton, J.T. and B. Ganetzky, *Ion channels and synaptic organization: analysis of the Drosophila genome.* Neuron, 2000. **26**(1): p. 35-43.
95. Liu, G., J. Roy, and E.A. Johnson, *Identification and function of hypoxia-response genes in Drosophila melanogaster.* Physiol Genomics, 2006. **25**(1): p. 134-41.
96. Livak, K.J. and T.D. Schmittgen, *Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method.* Methods, 2001. **25**(4): p. 402-8.
97. Macdonald, S.S., et al., *Dissecting chill coma recovery as a measure of cold resistance: evidence for a biphasic response in Drosophila melanogaster.* Journal of Insect Physiology, 2004. **50**(8): p. 695-700.
98. MacMillan, H.A. and B.J. Sinclair, *The role of the gut in insect chilling injury: cold-induced disruption of osmoregulation in the fall field cricket, Gryllus pennsylvanicus.* J Exp Biol, 2011. **214**(Pt 5): p. 726-34.

99. MacMillan, H.A., et al., *Reestablishment of ion homeostasis during chill-coma recovery in the cricket Gryllus pennsylvanicus.* Proceedings of the National Academy of Sciences, 2012. **109**(50): p. 20750-20755.
100. McColl, G., A.A. Hoffmann, and S.W. McKechnie, *Response to two heat shock genes to selection for knockdown heat resistance in Drosophila melanogaster.* Genetics, 1996. **143**(4): p. 1615-1627.
101. McKechnie, S.W., et al., *Both allelic variation and expression of nuclear and cytoplasmic transcripts of Hsr-omega are closely associated with thermal phenotype in Drosophila.* Proceedings of the National Academy of Sciences of the United States of America, 1998. **95**(5): p. 2423-2428.
102. Mellanby, K., *Low Temperature and Insect Activity.* Proceedings of the Royal Society of London. Series B - Biological Sciences, 1939. **127**(849): p. 473-487.
103. Michaud, M.R. and D.L. Denlinger, *Oleic acid is elevated in cell membranes during rapid cold-hardening and pupal diapause in the flesh fly, Sarcophaga crassipalpis.* J Insect Physiol, 2006. **52**(10): p. 1073-82.
104. Misener, S.R., C.-P. Chen, and V.K. Walker, *Cold tolerance and proline metabolic gene expression in Drosophila melanogaster.* Journal of Insect Physiology, 2001. **47**(4–5): p. 393-400.
105. Morgan, T.J. and T.F.C. Mackay, *Quantitative trait loci for thermotolerance phenotypes in Drosophila melanogaster.* Heredity, 2006. **96**(3): p. 232-242.
106. Nedved, O., *Snow white and the seven dwarfs: a multivariate approach to classification of cold tolerance.* Cryo Letters, 2000. **21**(6): p. 339-348.
107. Nilson, T.L., B.J. Sinclair, and S.P. Roberts, *The effects of carbon dioxide anesthesia and anoxia on rapid cold-hardening and chill coma recovery in Drosophila melanogaster.* J Insect Physiol, 2006. **52**(10): p. 1027-33.
108. Norry, F.M., et al., *QTL for the thermotolerance effect of heat hardening, knockdown resistance to heat and chill-coma recovery in an intercontinental set of recombinant inbred lines of Drosophila melanogaster.* Molecular Ecology, 2008. **17**(20): p. 4570-4581.
109. Overgaard, J., et al., *Metabolomic profiling of rapid cold hardening and cold shock in Drosophila melanogaster.* Journal of Insect Physiology, 2007. **53**(12): p. 1218-1232.
110. Overgaard, J. and J.G. Sørensen, *Rapid thermal adaptation during field temperature variations in Drosophila melanogaster.* Cryobiology, 2008. **56**(2): p. 159-162.
111. Pankotai, T., et al., *Functional characterization and gene expression profiling of Drosophila melanogaster short dADA2b isoform-containing dSAGA complexes.* BMC Genomics, 2013. **14**: p. 44.
112. Perez-Vilar, J. and R.L. Hill, *The structure and assembly of secreted mucins.* J Biol Chem, 1999. **274**(45): p. 31751-4.
113. Petersen, T.N., et al., *SignalP 4.0: discriminating signal peptides from transmembrane regions.* Nat Methods, 2011. **8**(10): p. 785-6.
114. Qin, W., et al., *Cold hardening and transcriptional change in Drosophila melanogaster.* Insect Molecular Biology, 2005. **14**(6): p. 607-613.
115. Rako, L., et al., *Candidate genes and thermal phenotypes: identifying ecologically important genetic variation for thermotolerance in the Australian Drosophila melanogaster cline.* Molecular Ecology, 2007. **16**(14): p. 2948-2957.
116. Ramlov, H., *Aspects of natural cold tolerance in ectothermic animals.* Hum Reprod, 2000. **15 Suppl 5**: p. 26-46.
117. Reboiro-Jato, D., et al., *ADOPS--Automatic Detection Of Positively Selected Sites.* J Integr Bioinform, 2012. **9**(3): p. 200.
118. Reis, M., et al., *A Comparative Study of the Short Term Cold Resistance Response in Distantly Related Drosophila Species: The Role of regucalcin and Frost.* PLoS ONE, 2011. **6**(10): p. e25520.

119. Ridley, E.V., et al., *Impact of the resident microbiota on the nutritional phenotype of Drosophila melanogaster.* PLoS One, 2012. **7**(5): p. e36765.
120. Rinehart, J.P., G.D. Yocum, and D.L. Denlinger, *Thermotolerance and rapid cold hardening ameliorate the negative effects of brief exposures to high or low temperatures on fecundity in the flesh fly, Sarcophaga crassipalpis.* Physiological Entomology, 2000. **25**(4): p. 330-336.
121. Robinson, S.W., et al., *FlyAtlas: database of gene expression in the tissues of Drosophila melanogaster.* Nucleic Acids Res, 2013. **41**(Database issue): p. D744-50.
122. Ronquist, F., et al., *MrBayes 3.2: efficient Bayesian phylogenetic inference and model choice across a large model space.* Syst Biol, 2012. **61**(3): p. 539-42.
123. Rynes, J., et al., *Activating transcription factor 3 regulates immune and metabolic homeostasis.* Mol Cell Biol, 2012. **32**(19): p. 3949-62.
124. Sarauer, B.L., C. Gillott, and D. Hegedus, *Characterization of an intestinal mucin from the peritrophic matrix of the diamondback moth, Plutella xylostella.* Insect Mol Biol, 2003. **12**(4): p. 333-43.
125. Schmidt, P.S. and A.B. Paaby, *Reproductive diapause and life-history clines in North American populations of Drosophila melanogaster.* Evolution, 2008. **62**(5): p. 1204-15.
126. Schnebel, E. and J. Grossfield, *Mating-Temperature Range in Drosophila.* Evolution, 1984. **38**(6): p. 1296-1307.
127. Short, S.M. and B.P. Lazzaro, *Reproductive Status Alters Transcriptomic Response to Infection in Female Drosophila melanogaster.* G3 : genes - genomes - genetics, 2013. **3**(5): p. 827-840.
128. Shreve, S.M., J.D. Kelty, and R.E. Lee, Jr., *Preservation of reproductive behaviors during modest cooling: rapid cold-hardening fine-tunes organismal response.* J Exp Biol, 2004. **207**(Pt 11): p. 1797-802.
129. Sinclair, B.J., *Insect cold tolerance: how many kinds of frozen?* European Journal of Entomology, 1999. **96**: p. 157-164.
130. Sinclair, B.J., A. Addo-Bediako, and S.L. Chown, *Climatic variability and the evolution of insect freeze tolerance.* Biol Rev Camb Philos Soc, 2003. **78**(2): p. 181-95.
131. Sinclair, B.J., et al., *Insects at low temperatures: an ecological perspective.* Trends in Ecology & Evolution, 2003. **18**(5): p. 257-262.
132. Sinclair, B.J., A.G. Gibbs, and S.P. Roberts, *Gene transcription during exposure to, and recovery from, cold and desiccation stress in Drosophila melanogaster.* Insect Molecular Biology, 2007. **16**(4): p. 435-443.
133. Stanley, S., et al., *Resistance of Species of the Drosophila Melanogaster Subgroup to Environmental Extremes.* Australian Journal of Zoology, 1980. **28**(3): p. 413-421.
134. STEVENSON and R. D., *The relative importance of behavioral and physiological adjustments controlling body temperature in terrestrial ectotherms*. Vol. 126. 1985, Chicago, IL, ETATS-UNIS: University of Chicago Press.
135. Stratman, R. and T.A. Markow, *Resistance to thermal stress in desert Drosophila.* Functional Ecology, 1998. **12**(6): p. 965-970.
136. Syed, Z.A., et al., *A potential role for Drosophila mucins in development and physiology.* PLoS ONE, 2008. **3**(8).
137. Takeuchi, K., et al., *Changes in temperature preferences and energy homeostasis in dystroglycan mutants.* Science, 2009. **323**(5922): p. 1740-3.
138. Tamura, K., et al., *MEGA6: Molecular Evolutionary Genetics Analysis version 6.0.* Mol Biol Evol, 2013. **30**(12): p. 2725-9.
139. Tellam, R.L., G. Wijffels, and P. Willadsen, *Peritrophic matrix proteins.* Insect Biochem Mol Biol, 1999. **29**(2): p. 87-101.

140. Telonis-Scott, M., et al., *Selection for cold resistance alters gene transcript levels in Drosophila melanogaster.* Journal of Insect Physiology, 2009. **55**(6): p. 549-555.
141. Terra, W.R., *Evolution of Digestive Systems of Insects.* Annual Review of Entomology, 1990. **35**(1): p. 181-200.
142. Toprak, U., et al., *Insect intestinal mucins and serine proteases associated with the peritrophic matrix from feeding, starved and moulting Mamestra configurata larvae.* Insect Mol Biol, 2010. **19**(2): p. 163-75.
143. Udaka, H., A. Percival-Smith, and B.J. Sinclair, *Increased abundance of Frost mRNA during recovery from cold stress is not essential for cold tolerance in adult Drosophila melanogaster.* Insect Molecular Biology, 2013. **22**(5): p. 541-550.
144. Van Klinken, B.J., et al., *Mucin gene structure and expression: protection vs. adhesion.* Am J Physiol, 1995. **269**(5 Pt 1): p. G613-27.
145. Vieira, C., et al., *Genotype-environment interaction for quantitative trait loci affecting life span in Drosophila melanogaster.* Genetics, 2000. **154**(1): p. 213-27.
146. Voituron, Y., et al., *To freeze or not to freeze? An evolutionary perspective on the cold-hardiness strategies of overwintering ectotherms.* Am Nat, 2002. **160**(2): p. 255-70.
147. Wang, J., et al., *Function-informed transcriptome analysis of Drosophila renal tubule.* Genome Biology, 2004. **5**(9): p. 1-21.
148. Wang, P. and R.R. Granados, *An intestinal mucin is the target substrate for a baculovirus enhancin.* Proc Natl Acad Sci U S A, 1997. **94**(13): p. 6977-82.
149. Wang, P. and R.R. Granados, *Molecular cloning and sequencing of a novel invertebrate intestinal mucin cDNA.* J Biol Chem, 1997. **272**(26): p. 16663-9.
150. Wang, X.H. and L. Kang, *Rapid cold hardening in young hoppers of the migratory locust Locusta migratoria L. (Orthoptera: Acridiidae).* Cryo Letters, 2003. **24**(5): p. 331-40.
151. Worland, M.R., P. Convey, and A. Luke ov, *Rapid cold hardening: a gut feeling.* Cryo Letters, 2000. **21**(5): p. 315-324.
152. Worland, M.R. and P. Convey, *Rapid cold hardening in Antarctic microarthropods.* Functional Ecology, 2001. **15**(4): p. 515-524.
153. Zachariassen, K.E., *Physiology of cold tolerance in insects.* Physiol Rev, 1985. **65**(4): p. 799-832.
154. Zachariassen, K.E., E. Kristiansen, and S.A. Pedersen, *Inorganic ions in cold-hardiness.* Cryobiology, 2004. **48**(2): p. 126-33.
155. Zhang, J., et al., *Divergent transcriptomic responses to repeated and single cold exposures in Drosophila melanogaster.* Journal of Experimental Biology, 2011. **214**(23): p. 4021-4029.
156. Zhong, J.F., et al., *Hydrogen sulfide exposure increases desiccation tolerance in Drosophila melanogaster.* Journal of Insect Physiology, 2010. **56**(12): p. 1777-1782.
157. Zsindely, N., et al., *The loss of histone H3 lysine 9 acetylation due to dSAGA-specific dAda2b mutation influences the expression of only a small subset of genes.* Nucleic Acids Research, 2009. **37**(20): p. 6665-6680.

Appendices

Table 2 – Phenotypical data for Chill Coma Recovery Time in the three analysed *D. melanogaster* strains

	Chill Coma Recovery time (seconds)					
Strain / # individual	Ore-R ♂	Ore-R ♀	5905 ♂	5905 ♀	27851 ♂	27851 ♀
1	786	838	936	464	493	565
2	696	718	893	454	561	482
3	556	730	977	395	538	399
4	737	695	775	475	580	457
5	672	750	1167	921	585	463
6	956	777	719	575	558	378
7	726	795	709	809	480	465
8	762	733	579	782	755	604
9	689	676	461	726	590	551
10	627	587	479	390	571	399
11	545	855	589	585	598	427
12	682	791	819	589	640	384
13	1007	608	1186	531	594	490
14	722	998	1228	659	646	615
15	798	704	1018	503	540	445
16	732	774	892	568	608	414
17	665	746	638	643	651	438
18	771	830	646	685	643	384
19	775	688	709	619	588	391
20	745	724	561	415	701	407
21	749	722	552	857	459	414
22	660	740	537	599	558	400
23	657	688	622	823	511	604
24	956	663	575	677	483	574
25	726	710	560	713	509	440
26	740	748	1189	682	415	546
27	619	682	686	609	472	571
28	673	654	646	646	444	408
29	603	737	593	817	515	562
30	717	731	680	550	695	379
31	1003	743	759	364	536	534
32	613	811	753	371	652	458

33	767	763	780	583	644	480
34	625	921	590	654	679	456
35	664	787	514	455	757	369
36	526	781	655	558	723	603
37	907	1037	535	432	541	501
38	621	912	600	466	872	512
39	677	673	610	770	741	355
40	565	998	565	326	733	449
41	742	679	626	550	1105	525
42	686	692	518	389	915	462
43	716	654	653	321	775	537
44	671	624	659	839	636	413
45	854	633	559	374	596	445
46	864	664	513	370	958	428
47	917	620	602	499	896	681
48	921	709	524	410	988	556
49	959	656	555	447	554	421
50	876	752	587	607	588	434
51	923	669	656	601	588	522
52	751	762	621	633	616	465
53	856	784	611	442	647	444
54	799	757	736	583	515	412
55	595	710	693	771	731	519
56	959	877	484	560	568	456
57	768	792	702	498	676	480
58	860	777	706	671	668	449
59	806	1014	1063	843	643	403
60	645	630	1066	409	734	386
61	746	720	1001	691	919	550
62	866	1079	675	748	510	537
63	770	930	705	520	583	514
64	710	895	699	450	618	476
65	1159	923	659	740	720	651
66	763	873	760	499	611	410
67	969	702	782	654	623	371
68	1004	695	767	511	621	381

69	835	869	664	431	698	571
70	641	832	571	416	681	392
71	1017	837	817	694	743	522
72	809	829	621	454	634	386
73	858	884	833	512	882	403
74	825	929	803	552	514	469
75	1194	1045	880	506	951	638
76	998	1039	521	560	517	445
77	813	631	714	566	729	491
78	846	1028	723	556	738	455
79	983	782	647	615	792	500
80	786	974	770	483	692	660
81	778	960	502	810	602	608
82	788	742	625	673	720	534
83	1004	745	635	458	745	480
84	1079	735	798	540	597	567
85	903	812	1236	438	624	451
86	841	897	810	445	683	423
87	930	805	642	814	873	374
88	577	920	968	525	491	537
89	941	678	593	564	724	416
90	635	671	1002	451	516	506
91	698	616	545	579	482	550
92	669	547	625	502	554	463
93	755	663	879	454	448	390
94	742	862	649	746	499	547
95	732	748	977	948	525	418
96	558	819	602	692	585	375
97	554	838	1072	846	493	444
98	823	871	1095	444	503	502
99	954	1093	656	688	498	423
100	811	846	891	449	562	453

Table 3 – Phenotypical data for Heat Shock in the three analysed *D. melanogaster* strains

Exposure time /min		30	60	90	120	150	180	210	240	270	300	330	360
Sex	Strain												
Males	Ore-R	100	100	97	80	54	19	3	1	0	0	0	0
	5905	100	100	100	98	93	78	67	49	25	11	2	0
	27851	100	100	100	99	96	87	47	17	4	0	0	0
Females	Ore-R	100	100	97	89	54	40	15	10	6	1	0	0
	5905	100	99	95	87	58	41	25	14	7	4	2	0
	27851	100	100	98	93	68	35	12	6	2	0	0	0

Table 4 – Phenotypical data for Starvation in the three analysed *D. melanogaster* strains

Exposure time /hours		17	18	19	20	21	22	23	24	25	26	27	28	29
Sex	Strain													
Males	Ore-R	100	87	72	59	45	30	18	12	4	1	0	0	0
	5905	101	101	91	80	57	44	30	13	1	0	0	0	0
	27851	100	95	89	80	60	44	26	11	3	1	0	0	0
Females	Ore-R	98	90	83	80	67	53	42	31	19	8	4	0	0
	5905	100	100	93	84	65	60	51	41	30	15	8	3	0
	27851	100	98	97	87	78	70	52	38	20	12	8	4	0

Table 5 – Phenotypical data for Nutrient Deprivation in the three analysed *D. melanogaster* strains

Exposure time /hours		0	12	24	36	48	60	72
Sex	Strain							
Males	Ore-R	100	100	99	87	48	2	0
	5905	100	99	99	94	32	0	0
	27851	100	100	100	85	33	1	0
Females	Ore-R	100	100	99	65	12	0	0
	5905	100	100	99	41	1	0	0
	27851	100	100	100	72	22	2	0

Table 6 – Characterization and identification of chitin binding type 2 (CBT2) domains of the eleven genes in the analysed syntenic region of *Muc68E*

Gene symbol	# introns in CDS	Size (aa)	Cr	Sequence location	# CBT2 domains
CG7252	1	474	3L	11,940,775.. 11,942,348 [+]	5
CG17826	1	751	3L	11,949,839.. 11,952,225 [+]	13
Obst-G	1	279	3L	11,952,301.. 11,953,317 [-]	3
Muc96D	1	881	3R	25,397,788.. 25,400,645 [-]	3
CG7248	1	796	3L	11,954,083.. 11,956,780 [+]	9
CG11570	0	PA – 214 PB – 214	3L	11,949,808.. 11,950,865 [-]	2
CG43294	0	PA – 124 PB – 124	3L	11,958,291.. 11,958,909 [+]	2
Muc68E	0	1799	3L	11,960,443.. 11,966,079 [-]	3
CG42397	1	178	3L	11,966,185.. 11,966,890 [-]	2
CG14125	1	PA – 256 PB – 263	3L	11,968,005.. 11,968,910 [+]	PA – 1 PB – 1
CG43896	PB – 9 PC – 10 PD – 10	PB – 1324 PC – 2113 PD – 2113	3L	11,969,211.. 11,976,864 [+]	PB – 19 PC – 31 PD – 31

Table 7 – Values of the threshold cycle (C_T) for *Frost*, *Muc68E*, and *RpL32* obtained by RT-qPCR in the Chill Coma experiment; CO – control; I – immediately after chill coma recovery; II – 2 hours after chill coma recovery; A, B, C stand for the three biological replicates

Chill Coma males							Chill Coma females						
	Frost			*RpL32*				*Frost*			*RpL32*		
	CO	**I**	**II**	**CO**	**I**	**II**		**CO**	**I**	**II**	**CO**	**I**	**II**
A	31.03	26.09	23.89	22.56	22.14	20.15	**A**	27.78	25.92	23.97	19.47	19.58	19.35
	30.51	26.01	23.94	22.49	22.08	19.77		27.70	25.97	23.99	19.23	19.15	19.70
B	31.67	26.79	23.98	21.90	22.12	22.61	**B**	30.40	27.28	23.59	19.97	19.66	19.70
	31.49		24.02	22.12	22.01	22.75		29.98	26.86	23.48	19.98	19.56	19.57
C	26.84	26.09	24.31	21.21	22.42	21.66	**C**	31.35	29.69	25.05	19.92	20.09	19.56
	27.05	26.40	24.58	21.48	21.94	21.90		31.41	29.99	25.37	20.32	20.18	19.75
	Muc68E			*RpL32*				*Muc68E*			*RpL32*		
	CO	**I**	**II**	**CO**	**I**	**II**		**CO**	**I**	**II**	**CO**	**I**	**II**
A	33.66	33.99	33.17	22.56	22.14	20.15	**A**	33.43	34.1	32.46	19.47	19.81	20.15
	33.85	33.3	33.14	22.49	22.08	19.77		33.06	34.07	32.66	19.23	19.85	19.97
B	34.51	35.07	31.10	21.9	22.12	22.61	**B**	32.92	35.03	33.21	19.67	19.66	19.70
	34.90		31.15	22.12	22.01	22.75		33.45	35.12	32.94	19.53	19.56	19.57
C	32.59	33.82	31.10	21.21	22.42	21.66	**C**	34.70	34.78	32.84	19.67	20.09	20.21
	33.69	34.10	31.25	21.48	21.94	21.90		33.92	34.80	32.06	19.78	20.18	19.75

Table 8 – Values of the threshold cycle (C_T) for *Frost*, *Muc68E*, and *RpL32* obtained by RT-qPCR in the Heat Shock experiment; CO – control; I – immediately after heat shock; II – 2 hours after heat shock; A, B, C stand for the three biological replicates

Heat Shock males							Heat Shock females						
	Frost			*RpL32*				*Frost*			*RpL32*		
	CO	**I**	**II**	**CO**	**I**	**II**		**CO**	**I**	**II**	**CO**	**I**	**II**
A	30.19	25.97	23.83	21.74	21.44	21.45	**A**	28.01	28.14	24.56	19.67	19.51	19.90
	30.90	26.44	24.02	21.51	21.31	21.65		27.26	27.86	24.34	19.53	19.20	19.14
B	30.57	26.11	23.78	21.92	21.23	21.71	**B**	27.78	29.19	23.8	19.47	19.93	19.15
	30.37	25.72	23.64	22.04	21.27	21.55		27.70	28.82	24.04	19.23	19.91	19.42
C	26.32	26.52	23.56	20.80	21.46	21.44	**C**	28.34	27.17	23.87	18.82	19.14	19.18
	26.40	26.08	24.01	20.84	21.28	21.43		28.59	27.74	24.27	19.33	19.54	19.19
	Muc68E			*RpL32*				*Muc68E*			*RpL32*		
	CO	**I**	**II**	**CO**	**I**	**II**		**CO**	**I**	**II**	**CO**	**I**	**II**
A	33.65	27.91	29.48	21.74	21.44	21.45	**A**	32.92	31.75	31.48	19.67	19.51	19.90
	33.06	28.69	29.11	21.51	21.31	21.65		33.45	31.58	31.07	19.53	19.20	19.14
B	34.35	30.36	28.68	21.92	21.23	21.71	**B**	33.43	31.74	33.21	19.47	19.93	19.15
	34.87	30.08	28.73	22.04	21.27	21.55		33.06	32.22	31.98	19.23	19.91	19.42
C	32.21	29.47	29.56	20.80	21.46	21.44	**C**	33.39	30.29	30.91	18.82	19.14	19.18
	31.88	30.26	29.40	20.84	21.28	21.43		33.15	30.31	31.35	19.33	19.54	19.19

Table 9 – Values of the threshold cycle (C_T) for *Frost*, *Muc68E*, and *RpL32* obtained by RT-qPCR in the Nutrient Deprivation experiment; CO – control; ND – 36 hours of nutrient deprivation; A, B, C stand for the three biological replicates

Nutrient Deprivation males

	Frost		*RpL32*	
	CO	**ND**	**CO**	**ND**
A	29.49	27.93	23.12	22.39
	28.78	28.18	22.94	22.75
B	28.92	30.56	23.17	22.76
	28.76	29.62	23.26	22.84
C	27.10	30.19	21.79	22.94
	26.70	29.97	22.10	22.39

	Muc68E		*RpL32*	
	CO	**ND**	**CO**	**ND**
A	34.26	34.45	23.12	22.39
	33.51	34.27	22.94	22.75
B	32.64	34.16	23.17	22.76
	32.66	34.23	23.26	22.84
C	32.89	36.87	21.79	22.94
	32.86	36.96	22.10	22.39

Nutrient Deprivation females

	Frost		*RpL32*	
	CO	**ND**	**CO**	**ND**
A	31.35	31.06	19.92	19.99
	31.41	30.70	20.32	20.18
B	32.13	29.29	20.23	19.91
	31.81	28.78	20.17	20.08
C	31.68	28.02	19.77	19.31
	31.40	28.24	20.06	19.12

	Muc68E		*RpL32*	
	CO	**ND**	**CO**	**ND**
A	33.37	34.08	21.21	19.99
	33.85	34.38	21.16	20.18
B	32.67	33.27	20.23	19.91
	32.25	32.98	20.17	20.08
C	33.11	32.82	19.77	19.31
	32.74	32.70	20.06	19.12

Table 10 – Values of the threshold cycle (C_T) for *Frost*, *Muc68E*, and *RpL32* obtained by RT-qPCR in Starvation experiment; CO – control; ST – 38 hours of starvation; A, B, C stand for the three biological replicates

Starvation males

	Frost		*RpL32*	
	CO	**ST**	**CO**	**ST**
A	29.49	26.63	23.12	22.64
	28.78	25.36	22.94	22.99
B	28.92	22.81	23.17	21.91
	28.76	22.91	23.26	21.97
C	27.10	23.45	21.79	21.53
	26.70	23.18	22.10	21.76

	Muc68E		*RpL32*	
	CO	**ST**	**CO**	**ST**
A	34.26	35.02	23.12	22.64
	33.51	34.95	22.94	22.99
B	32.64	32.80	23.17	21.91
	32.66	33.21	23.26	21.97
C	32.89	33.61	21.79	21.53
	32.86	33.92	22.10	21.76

Starvation females

	Frost		*RpL32*	
	CO	**ST**	**CO**	**ST**
A	31.35	25.23	19.92	19.56
	31.41	24.90	20.32	19.71
B	32.13	25.68	20.23	19.54
	31.81	25.25	20.17	19.76
C	31.68	26.93	19.77	19.31
	31.40	26.55	20.06	19.47

	Muc68E		*RpL32*	
	CO	**ST**	**CO**	**ST**
A	33.37	32.98	21.21	19.56
	33.85	32.92	21.16	19.71
B	32.67	32.66	20.23	19.54
	32.25	32.63	20.17	19.76
C	33.11	32.97	19.77	19.31
	32.74	32.94	20.06	19.47