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Redox signalling and innate immunity: a role for protein *S*-nitrosylation in the immune response of *Drosophila melanogaster*.

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Abstract

Over the past three decades, nitric oxide (NO) has been recognised as one of the most versatile and important players in many aspects of physiology, including immune responses. More recently, *S*-nitrosylation, the incorporation of a NO moiety into a protein thiol group, has emerged as a major post-translational modification (PTM) during pathophysiological responses in plants and animals. The main goal of this work was to investigate the role of *S*-nitrosylation in physiology and innate immunity of animals using the genetic reference system, *Drosophila melanogaster*.

The *S*-nitrosylated derivative of glutathione (GSH), *S*-nitrosoglutathione (GSNO), is the main non-protein *S*-nitrosothiol (SNO) in the cell and extracellular fluids. GSNO can trans-*S*-nitrosylate other thiols and is considered a reservoir of NO bioactivity. The levels of GSNO and total *S*-nitrosylation have been shown to be controlled by *S*-nitrosoglutathione reductase (GSNOR) in yeast, plants and mammals. By employing an overlapping deletion technique to knock-out *gsnor*, a role for *S*-nitrosylation in the immune response of *D. melanogaster* is proposed.

Compared to wild type flies, *gsnor* overlapping deletion flies presented lower expression of antimicrobial peptides in response to infections, and succumbed more rapidly to both Gram-positive bacterial and fungal pathogens. As the Toll pathway mediates responses against these pathogens, key components of this network were tested for their propensity to being *S*-nitrosylated.

Two CLIP-domain serine proteases of the Toll signalling pathway, Persephone (PSH) and Spätzle-Processing Enzyme (SPE), were shown to be *S*-nitrosylated both *in vitro* and *in vivo* and this process seemed to control the quaternary structure of these proteins and interfere with the immune response of *D. melanogaster*. At least for

PSH, *S*-nitrosylation at C254 has an immune significance as the expression of non-*S*-nitrosylable PSHC254S in *gsnor* knock-out flies partially recovered the resistance of these animals to infections with the entomopathogenic fungus *Beauveria bassiana*. These findings might represent a novel mechanism by which NO and *S*-nitrosylation regulate immunity.

Further results presented in this thesis reveal an interplay between reactive oxygen species (ROS) and reactive nitrogen species (RNS) in *D. melanogaster* physiology and immunity. Similarly to what has been reported in *Arabidopsis thaliana*, *gsnor* knock-out flies presented higher tolerance to the herbicide paraquat, an inducer of superoxide (O_2^-) production. Moreover, additional mutations in *Catalase* (*Cat*), a hydrogen peroxide (H_2O_2) scavenger enzyme, partially restored the immunodeficiency phenotypes of *gsnor* knock-out flies. These findings suggest an inter-relation between the levels of ROS and RNS during stress responses of plants and animals.

In addition, CRISPR/Cas9 technology was employed to generate *gsnor* knock-outs in the genome of *D. melanogaster.* These flies were shown to have no GSNOR activity, presented lower tolerance to pharmacological-induced nitrosative stress and succumbed faster to infections with *B. bassiana* compared to wild type flies. These results support the role played by GSNOR in regulating NO homeostasis and immunity in *D. melanogaster*.

Lay Summary

Nitric oxide is a reactive molecule that can bind to specific cysteine residues of proteins in a process called *S*-nitrosylation. This process can affect the function, cellular localization and interactions of proteins, and plays important roles in many aspects of physiology. Here we present a role for protein *S*-nitrosylation in the immune response of the fruit fly *Drosophila melanogaster*. Mutant flies with a deletion of the gene *gsnor* have altered levels of *S*-nitrosylation, are more susceptible to infections with fungi and Gram-positive bacteria, and fail to produce anti-microbial molecules in response to infections. A model is proposed where aberrant *S*-nitrosylation of CLIP-domain serine proteases of the Toll signalling pathway impairs the recognition of the entomopathogenic fungus *B. bassiana*, resulting in higher susceptibility. This is confirmed by the fact that transgenic *gsnor* flies expressing a mutated non-*S*-nitrosylable version of PSH (PSHC254S), but not wild type PSH, become less susceptible to infections with this pathogen. These findings shed new light on the mechanisms by which NO and *S*-nitrosylation modulate immune responses in animals.

Declaration

I hereby declare that the work presented here is my own and has not been submitted in any form for any degree at this or any other university.

Rafael Augusto Homem

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List of abbreviations

AMP	Anti-microbial peptide
BST	Biotin-switch technique
CAT	Catalase
CLIP-SPs	CLIP-domain serine proteases
DAP	Diaminopimelic acid
Def	Defensin
DEPC	Diethylpyrocarbonate
Df7305	Df(3R)Exel7305
Df7306	Df(3R)Exel7306
dNTPs	Deoxynucleotide triphosphates
Dpt	Diptericin
Drs	Drosomysin
DTT	Dithiothreitol
EDTA	Ethylenediamine tetraacetic acid
eNOS	Endothelial NOS
GSH	Reduced glutathione
GNBP	Gram-negative bacteria binding protein
GSNO	*S*-nitrosoglutathione
GSNOR	*S*-nitrosoglutathione reductase
H2O2	Hydrogen peroxide
HA	Human influenza hemagglutinin
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HEN	HEPES, EDTA, neocuproine buffer
•OH	Hydroxyl radical
IkB	Inhibitor of kappa B
IMD	Immune deficiency
iNOS	Inducible NOS
NADH	Nicotinamide adenine dinucleotide
NADPH	Reduced nicotinamide adenine dinucleotide phosphate
NOX	NADPH-oxidase

NEM	N-Ethylmaleimide
NF-κB	Nuclear factor-kappa B
NH3	Ammonia
nNOS	Neuronal NOS
NO	Nitric oxide
NOS	Nitric oxide synthase
NPR1	Nonexpressor of pathogenesis-related genes 1
O_2^-	Superoxide
PAM	Proto-spacer adjacent motif
PAMP	Pathogen-Associated Molecular Patterns
PCR	Polymerase chain reaction
PGRP	Peptidoglycan recognition protein
PPAF	Pro-phenol oxidase-activating factor
PRR	Patten Recognition Receptors
PSH	Persephone
PTM	Post-translational modification
qPCR	Quantitative real-time PCR
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
SA	Salicylic acid
SDS	Sodium dodecyl sulphate
PAGE	Polyacrylamide gel electrophoresis
SP	Serine proteases
SNO	*S*-nitrosothiol
SNP	Sodium nitroprusside
Spz	Spätzle
SPE	Spätzle-Processing Enzyme

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Chapter 1

Introduction

1.1 Innate immunity – The front line of host defence

We live in a world dominated by microorganisms, these including eukaryotes, archaea, bacteria, and viruses. Multicellular life evolved from and still shares the environment with a plethora of unicellular life. In fact, we, humans, are completely colonised by these single-cell creatures. It is estimated that bacterial cells alone outnumber human cells within an individual by an order of magnitude. The Human Microbiome Project Consortium has recently identified a total of 11,174 primary biological specimens in the microbiota of healthy human individuals (Methé et al. 2012; Huttenhower et al. 2012).

In general microorganisms are not harmful to their hosts. In many cases they can actually be beneficial by, for example, contributing to food digestion, providing vitamins and essential nutrients, and protecting against other microorganisms (Bäckhed et al. 2005; Relman 2008). However, some can cause infections that lead to debilitation and death of the host. In this way, it is crucial for the hosts to efficiently identify and eliminate potential pathogenic microorganisms.

Although the mechanism by which beneficial and pathogenic microorganism are differentiated by the host has not been completely elucidated, it is understood to involve the detection of constitutive and conserved products of microbial metabolism. The concentration and combination of different microbial products, as well as their accessibility and reactivity to specific receptors expressed by the host,

influence the mechanisms by which this recognition process occurs (Lebeer et al. 2010).

The conserved products of microbial metabolism and the host receptors mentioned above are referred as Pathogen-Associated Molecular Patterns (PAMPs) and Patten Recognition Receptors (PRRs), respectively. Once a pathogenic microorganism is detected by the host through the interaction of a PRR with a PAMP, it triggers a rapid and broad-spectrum immune response that is characterised by the production of anti-microbial peptides, pro-inflammatory mediators, phagocytosis and ultimate killing of pathogens. This immediate responsiveness to conserved microbial molecules constitutes an evolutionarily ancient immune mechanism present in all multicellular organisms, the innate immune system (Turvey & Broide 2010; Kumagai & Akira 2010; Zasloff 2002).

Often referred as the front line of the immune system, innate immunity is responsible for detecting and responding to potential harmful microorganism that succeeded in breaching through one of the host's anatomic barriers, such as epithelial and mucosal surfaces. The response involves soluble proteins and small molecules with anti-microbial activity that are either constitutively present in biological fluids (e.g. the complement proteins and defensins present in the blood of mammals), or are released from cells once they perceive the presence of a threat. These include pro-inflammatory cytokines, chemokines and reactive free radical species (Chaplin 2010; Murphy Kenneth 2011).

In contrast to adaptive immunity, where a randomly generated repertoire of antigen receptors expressed by T and B lymphocytes are employed to detect foreign and potentially dangerous molecules, innate immunity relies on a limited number of

membrane-bound or secreted PRRs that are encoded in the germline and recognize features common to many pathogens. Because it does not require genetic recombination events or a developmental phase to mediate function, like adaptive immune responses, innate immunity provides immediate action against attempted infection (Turvey & Broide 2010; Beutler 2004).

The high degree of conservation of components of the innate immune system allows researchers to employ more tractable model organisms such as *Drosophila melanogaster* to investigate the mechanisms underpinning innate immunity. Findings generated using *D. melanogaster* can be extrapolated to and tested in other animals such as mice and humans, thus providing a valuable tool to rapidly test a hypothesis.

A significant tranche of our understanding of innate immunity is credited to work carried out on the reference system, *D. melanogaster*. The discovery that the Toll signalling pathway was involved in the host defence against fungal pathogens(Lemaitre et al. 1996), and the subsequent identification of Toll-Like receptors in mammals (Poltorak et al. 1998; Medzhitov et al. 1997), are considered landmarks in the field of innate immunity.

1.2 *D. melanogaster* Immunity

Despite lacking an adaptive immune system, *D. melanogaster*, like other invertebrates, is highly efficient in mounting an effective and multifaceted immune response against microbial infections. This response involves the activation of proteolytic cascades, production of reactive oxygen species, phagocytosis and synthesis of potent antimicrobial peptides (Hoffmann & Reichhart 2002).

1.2.1 The challenge-inducible antimicrobial defence system

It is well established now that flies detect, discriminate and respond accordingly to different classes of pathogens through two distinct signalling pathways, the Toll and the immune deficiency (IMD) (Hetru & Hoffmann 2009).

The Toll signalling pathway is activated in response to the detection of microbial cell wall components of fungi and Gram-positive bacteria. Gram-positive bacteria are recognized by Peptidoglycan-recognition protein-SA (PGRP-SA) with the cooperation of Gram-negative bacteria binding protein 1 (GNBP1) and Peptidoglycan-recognition protein-SD (PGRP-SD), while fungi are recognized by Gram-negative binding protein 3 (GNBP3). This pathway can also be activated by Persephone (PSH), which detects proteases secreted by pathogens. The activation of these receptors leads to the activation of proteolytic cascades (Prs) and maturation of pro-Spätzle (pro-Spz) to Spz, the Toll ligand.

The IMD pathway predominately coordinates the response to Gram-negative bacteria. It is triggered by the activation of the transmembrane receptor Peptidoglycan recognition protein LC (PGRP-LC), as represented in Figure 1-1 and described in more details in the next section. The outcome of the activation of both Toll and IMD pathways is the NF-kB-dependent production of small peptides that are highly toxic to microorganisms, the anti-microbial peptides (AMPs) (Ferrandon et al. 2007; Hetru & Hoffmann 2009).

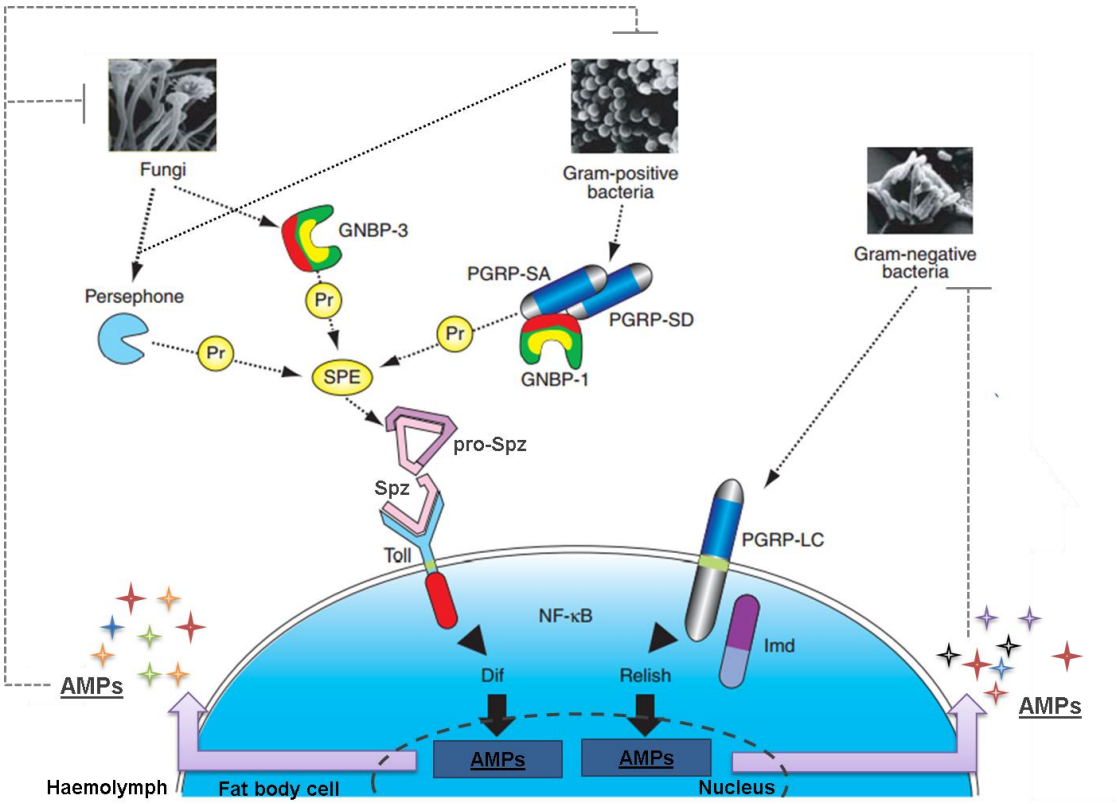


Figure 1-1: The Toll and the IMD signalling pathways

Gram-negative bacteria trigger the transmembrane receptor PGRP-LC, leading to the activation of the IMD pathway and activation of the NF-kB transcription factor Relish. In the nucleus Relish activates the production of AMPs, which are secreted to the haemolymph to combat infection. The Toll pathway can be triggered by Gram-positive bacteria recognised by haemolymph circulating receptor PGRP-SA with the cooperation of GNBP1 and PGRP-SD. It can also be triggered by fungi that are recognised by haemolymph circulating receptor GNBP-3. A third branch of this pathway is triggered by the activation of Persephone, which is activated by proteases secreted by the pathogens. Upon activation of these receptors, proteolytic (Pr) cascades lead to maturation of SPZ, the Toll ligand. Binding of SPZ to the Toll receptor leads to activation of the NF-kB transcription factor Dif. This figure was adapted from Hetru & Hoffmann (2009).

1.2.1.1 The IMD pathway

A screen for immune deficient *D. melanogaster* mutants (Corbo & Levine 1996) followed by complementation experiments identified a mutation that impaired microbial-induced expression of AMPs and reduced survival of bacteria-challenged

flies (Lemaitre et al. 1995). The recessive mutation responsible for those phenotypes was named *immune deficiency* (*imd*). The other components of this pathway were identified in a series of forward genetic screens that are reviewed in Imler (2014).

Briefly, the IMD pathway is initiated upon binding of diaminopimelic acid-containing peptidoglycan (DAP-PGN) of Gram-negative bacteria and Gram-positive Bacillus species, to membrane bound PGRP-LC and cytosolic PGRP-LE. This leads to recruitment of IMD, the adaptor protein FADD, and the caspase-8 homolog DREDD (Leulier et al. 2002; Choe et al. 2005). In the current model for downstream signalling, DREDD becomes activated by ubiquitination and cleaves IMD. This leads to ubiquitination of IMD, followed by recruitment and activation of the Tab2/Tak1 complex. Once recruited, this complex triggers activation of the IkB-Kinase (IKK) complex, which in turn phosphorylates the NF-kB protein Relish. Phosphorylated Relish is activated by proteolysis, allowing its N-terminal domain to translocate to the nucleus and activate the transcription of AMPs .In a Relish-independent fashion, PGRP-LE activation has also been shown to trigger autophagy, a critical process to protect the animal against intracellular pathogens like Listeria (Yano et al. 2008).

1.2.1.2 The Toll signalling pathway

The Toll signalling pathway was first discovered in *D. melanogaster* as a major determinant of dorsal-ventral polarity in embryos (Anderson et al. 1985) and subsequently related to *D. melanogaster* immunity (Lemaitre et al. 1996). Later this pathway was shown to be evolutionary conserved in humans (Medzhitov et al. 1997), as represented in Figure 1-2.

The Toll signalling pathway can be activated in three different ways. Lys-Type peptidoglycans (PGN) from Gram-positive bacteria are detected by the circulating recognition receptors PGRP-SA, PGRP-SD, GNBP-1, while β-(1, 3)-glucans from the fungal cell walls are detected by GNBP-3. Additionally, the Toll pathway can also be triggered by PSH, a CLIP-domain serine protease that is cleaved and activated by proteases secreted from pathogens during infection (Gottar et al. 2006; Ligoxygakis et al. 2002).

These three distinct cascades lead to the cleavage of SPE, which, in its activated form, processes pro-Spz to Spz, the Toll receptor ligand (a more detailed explanation about the activation of the Toll signalling pathway will be given in Chapter 5).

Once Spz binds to Toll, it induces dimerization of the receptor (Gangloff et al. 2008),leading to the formation of a heterotrimeric complex MyD88-Tube-Pelle (human MyD88-IRAK4-IRAK1). This induces phosphorylation and degradation of Cactus, (the Drosophila IkB), releasing the NF-kB transcription factor Dorsal-related immunity factor (Dif).Thus, this transcription factor can translocate to the nucleus and activate the transcription of immune responsive genes, including the AMPs (Valanne et al. 2011).

The main difference between the Drosophila Toll and the human TLR systems is that the former is activated upon binding of a proteolytically maturated cytokine (Spz), while the later directly interacts with PAMPs. In Figure 1-2, human TLR5 is shown interacting with flagellin, a principal component of the bacterial flagella, a portent PAMP in mammals(Hayashi et al. 2001) and plants (Zipfel et al. 2004).

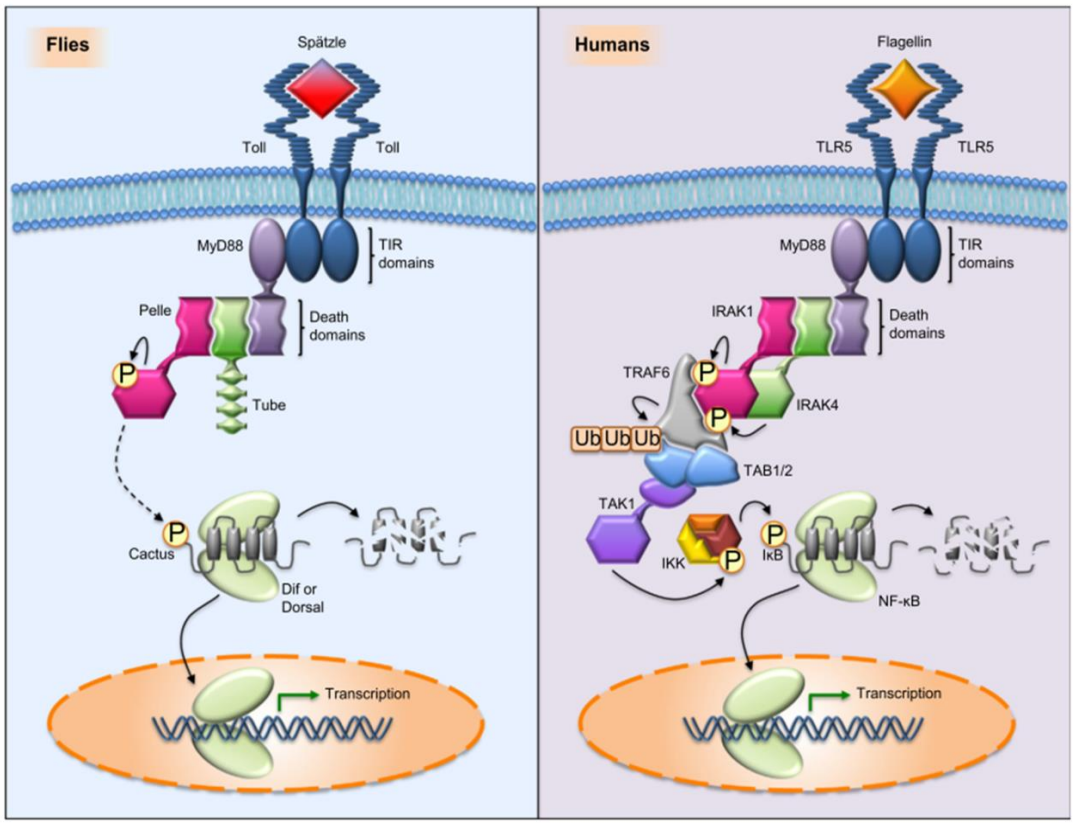


Figure 1-2: Evolutionary conservation of the Toll signalling pathway

Left - Binding of the cytokine Spätzle to the Toll triggers dimerization of the intra-cytoplasmic TIR domains, promoting binding of the adaptor protein MyD88 through its own TIR domain. MyD88 binds the adaptor protein Tube, which in turn recruits the protein kinase Pelle. Although only one signalling module is shown, there exist two modules, one on each TIR domain of the Toll dimer. Recruitment of Pelle induces its auto-phosphorylation and phosphorylation and destruction of the inhibitor Cactus. This leads to the release of the transcription factors Dif or Dorsal, which translocate to the nucleus. **Right** -In a manner analogous to flies, human TLR5 is activated by Flagellin, triggering the formation of the complex MyD88-IRAK4-IRAK1.IRAK4 phosphorylates IRAK1, triggering IRAK1 auto-phosphorylation and dissociation from the complex. IRAK1 binds TRAF6, which auto-ubiquitinates and binds the TAB/TAK1 proteins. TAK1 becomes activated and phosphorylates the IKK complex, which then phosphorylates the inhibitor IkB, leading to its degradation and the nuclear translocation of NF-KB transcription factors. In mammals, there are numerous TLR pathways involved in the recognition of different PAMPs and DAMPs. This figure was taken from Lindsay & Wasserman (2014).

1.2.2 Cellular immunity

In addition to, and working synergistically with the inducible synthesis and secretion of AMPs, immunocompetent free-circulating and sessile cells constitute another layer of *D. melanogaster* immunity. These cells, known as haemocytes, are divided in three functionally distinct groups: plasmatocytes, crystal cells and lamellocytes.

Plasmatocytes are macrophage-like cells responsible for phagocytose of microbial invaders and account for 95% of all haemocytes in the haemolymph. So far only a few genes have been implicated in phagocyte function. One of these genes is *eater*, which is predicted to encode a trans-membrane receptor with 32 EGF-like repeats in its extracellular domain. This receptor was shown to play a role in the phagocytosis of both Gram-positive and Gram-negative bacteria. In addition, loss of Eater reduces the phagocytosis of several bacterial species by up to 80% (Kocks et al. 2005).

Crystal cells and lamellocytes are both involved in the melanisation process. Lamellocytes are also responsible for encapsulation of foreign bodies that are too large to be phagocytosed. Melanin is the final product of a proteolytic cascade involving the sequential activity of the serine proteases MP1 and MP2, and pro-phenoloxidase (proPO), which is converted to phenoloxidase (PO) (Tang et al. 2006). PO catalyses the oxidation of mono and di-phenols to orthoquinones, which subsequently polymerize to form melanin. This process takes place at sites of wounding or at the surface of parasites to facilitate wound healing, encapsulation and killing of parasites (Williams 2007).

1.2.3 Serine proteases and *D. melanogaster* immunity

Serine proteases (SPs) constitute almost one-third of all known proteases. They can be distinguished by the presence of the Asp-His-Ser catalytic triad and are divided into four clans, chymotrypsin, subtilisin, carboxypeptidase Y, and Clp proteases (Hedstrom 2002). In mammals they play important roles in food digestion, immunity, reproduction, blood coagulation and fibrinolysis. Similarly, in insects they are also involved in many processes including development, coagulation and immune responses (Krem & Cera 2002).

Among the SPs present in arthropods, CLIP-domain SPs (clip-SPs) have been shown to function in cascades involved in embryonic development and immune-related processes such as melanisation and activation of the Toll signalling pathway (Jang et al. 2008). The CLIP-domain is constituted of approximately 30 to 60 amino acids, contains three disulphide bonds and is always found in the amino-terminus of the SP domain or SP homologues (SPHs) (protease-like domains lacking the catalytic serine residue). The function of these domains is still unknown; however, they are believed to be involved in mediating protein-protein interactions and in controlling the sequential activation of SP cascades (Jiang & Kanost 2000).

As mentioned before, the Toll signalling pathway is dependent on the function of CLIP-SPs to transduce the immune activation signal upon pathogen recognition. So far, six CLIP-SPs have been identified as components of the Toll signalling pathway, two being involved in embryogenesis (Easter and Snake), and four in immune signalling (Persephone, SPE, spirit and GRASS).

Clip-SPs were first described for their role in embryogenesis, where they control the initiation of the dorso-ventral polarity of embryos (LeMosy et al. 2001).

The CLIP-SP Snake was shown to be cleaved and activated by the SP Gastrulation-Defective (GD). Once activated, Snake cleaves and activates the other CLIP-SP of the Toll pathway, Easter, which in turn cleaves and actives the Toll ligand Spz.

PSH was the first CLIP-SP of the Toll pathway involved in immunity to be identified. Suppression screens of *nec* mutants, which exhibit constitutive activation of the Toll pathway (Levashina et al. 1999), led to the identification of this protein (Ligoxygakis et al. 2002). PSH was shown to be involved in triggering the immune response against fungal and Gram-positive bacteria by detecting, or, to be more precise, to be detected and cleaved by, proteases that act as pathogen virulence factors during infections (Ligoxygakis et al. 2002; Gottar et al. 2006).

The other three immuno-related CLIP-SPs (GRASS, SPE and Spirit)of the Toll pathway, plus other two CLIP-SPHs (Spheroid and Sphinx1/2), were identified in a large-scale *in vivo* RNAi screen searching for immuno-related CLIP-SPs in *D. melanogaster* (Kambris et al. 2006). SPE is the most downstream CLIP-SP in the pathway and is responsible for the cleavage and activation of Spz, the toll ligand (Jang et al. 2006).

Spirit is required for the activation of the Toll pathway in response to both fungi and Gram-positive bacteria and appears to act as an upstream component of SPE (Kambris et al. 2006). The expression of both SPE and Spirit has been shown to be induced during the immune response in a Toll pathway-dependent manner (De Gregorio et al. 2002), what provides an amplification of the immune response.

GRASS was first associated with signalling in response to Gram-positive bacterial infections (Kambris et al. 2006) but further studies demonstrated that it actually defines a common protease cascade downstream of fungal and bacterial

detection (Chamy et al. 2008). It is positioned upstream of SPE and downstream of the multi-domain serine protease ModSP (Buchon et al. 2009). To date, GRASS is the only full-length clip-SP to have its crystal structure determined (Kellenberger et al. 2011). Further studies on the structure as well on the mechanisms underpinning the regulation of these proteases will certainly contribute to a better understanding of fundamental processes as innate immune responses.

1.3 Redox signalling and immunity

Another major component of innate immunity both in animals and plants involves the inducible enzymatic production of reactive oxygen species (ROS) such as superoxide (O_2^-)/ hydrogen peroxide (H_2O_2) and reactive nitrogen species (RNS), including nitric oxide (NO) and its derivatives. These molecules not only intercept and kill pathogens, but also regulate downstream signalling pathways that lead to the full expression of the immune response.

One example of the role of ROS as immunotoxins is the NADPH-oxidase (NOX)-mediated production of O_2^- by mammalian phagocytes (Bedard & Krause 2007) and plants (Marino et al. 2012) to kill invading microorganisms. Humans deficient in NOX-mediated production of O_2^- suffer from chronic granulomatous disease, a genetic condition that is characterized by a greatly increase susceptibility to severe bacterial and fungal infections (Heyworth et al. 2003).

For the role of NO in immunity, it has been reported that polymorphisms within the promoter region of inducible NO synthase (*iNOS*), one of the enzymes responsible for NO production in humans (discussed in the next session), are related to malaria severity in children (Cramer et al. 2004). Studies have also shown that

iNOS activity and NO production are increased in severe cases of human malaria (Anstey et al. 1996; Perkins et al. 1999).

The chemical mechanisms by which RNS and ROS impose their toxicity are many, involve different targets (e.g. protein, DNA, lipid), and reactive intermediates (e.g. •OH, O_2^-, H_2O_2, NO,N_2O_3, $ONOO^-$) (Wink et al. 2011). As an example, the highly reactive hydroxyl radical (•OH), which can be formed from the reaction between $H_2O_2$and Fe^{2+}, (Fenton's reaction: $Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + \bullet OH + OH^-$), is able to hydroxylate DNA bases of bacterial invaders and to cause backbone scission via abstraction of the 2' hydrogen atoms (Imlay et al. 1988).

RNS and ROS, as mentioned before, can also regulate signalling pathways. This is due to the capacity of these molecules to post-translationally modify proteins. In this context, the amino acid cysteine (Cys) is unique. Cys residues present a nucleophilic thiol side chain group that can undergo numerous redox-based post-translational modifications such as *S*-nitrosylation (SNO), S-glutathionylation (RS-SG), disulphide bonds (RS-SR'), sulfenylation (SOH), sulfinic acid (SO2H), and sulfonic acid (SO3H) formation (Figure 1-3).

The specificity towards a given modification is spatially/temporally-dependent and is directly affected by the proximity of a target Cys to a given source of reactive species. Most of these modifications are reversible (apart from sulfonic acid, all other modification are reversible), and they can affect the structure, activity and function of proteins (Spadaro et al. 2010).

Although in principle all Cys thiols could be targets for redox modification, only a small subset of these residues can be modified in physiological conditions. Two factors appear to dictate the specificity of a given Cys: the accessibility and its

reactivity. Accessible residues are generally located at the surface of proteins (solvent exposed) and reactive residues usually present a low acid dissociation constant (pKa). A lower pKa at physiological conditions (pH~7.4) favours deprotonation and formation of thiolate anions ($-S^-$), which are far more reactive than thiols (-SH). Proximity to positively charged amino acids, hydrogen bonding, and location at the N-terminal end of an α-helix (Ncap) are features that facilitate thiol deprotonation (Paulsen & Carroll 2013).

S-nitrosylation, the covalent attachment of a NO group to the thiol side chain of cysteine, has emerged as a major mechanism for the regulation of protein signalling in health and disease (Foster et al. 2009). As will be presented in more details in the next sections of this Chapter, *S*-nitrosylation conveys a large part of the ubiquitous influence of NO on cellular signal transduction, and provides a mechanism for redox-based regulation of physiological and pathophysiological processes such as immune responses.

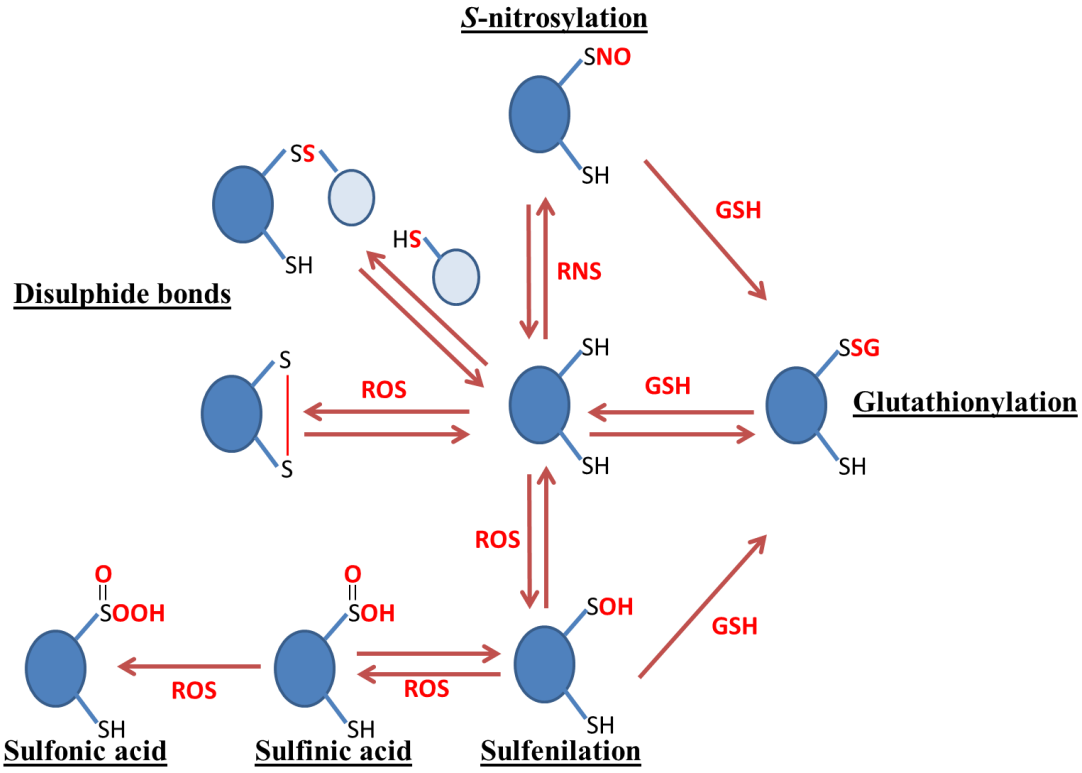


Figure 1-3: Redox-based Cys modifications

Cys thiols (SH), depending on the redox-environment, may undergo different post-translational modifications .RNS induce *S*-nitrosylation, glutathione (GSH) induces glutathionylation, ROS induce intra- and intermolecular disulphide bond formation as well as sulfenilation and formation of sulfinic and sulfonic acid. The only non-reversible reaction is the formation of sulfonic acid.

1.3.1 NO production

In animals, NO is produced during the conversion of L-Arginine to L-citrulline in a well characterised enzymatic process dependent on NADP(H) and catalysed by nitric oxide synthase (NOS) (Stuehr et al. 2004). There are three distinct NOS isoforms in mammals. Two of them, the neuronal NOS (nNOS or NOS1) and the endothelial NOS (eNOS or NOS3), are constitutively expressed and dependent on exogenous Ca^{2+}/cadmium, while the macrophage or inducible NOS (mNOS or iNOS or NOS2) is independent of exogenous Ca^{2+}/cadmium and induced in response

to infections (Alderton et al. 2001; Stuehr et al. 2004).

In *D. melanogaster* there is only one NOS, encoded by *dNOS*, which shares 43%, 39% and 40% amino acid sequence identity with the three mammalian NOS isoforms nNOS, iNOS and eNOS, respectively (Regulski & Tully 1995). In plants, although some data indicate the existence of a NOS-like enzyme, this protein has not been identified and the source of NO remains controversial (Yu et al. 2014).

As an alternative route, in conditions where NOS activity is impaired, such as in hypoxia, NO can also be produced from nitrite ($NO2^{-}$). In acid conditions, $NO2^{-}$ can be converted to NO. This reaction can also be catalysed by enzymes such as deoxy-haemoglobin, mitochondrial cytochromes and xanthine oxidase (Lundberg & Weitzberg 2005).

1.3.2 *S*-nitrosylation

PTMs of proteins play a key role in functionally amplifying the proteomic diversity of an organism without adding new genes to the genome. These modifications may modulate protein activity, interactions, compartmentalisation, and so on; consequently controlling an immense variety of biological process in all living organisms. In 1992, Stamler and co-workers described a PTM involving the coupling of a reactive cysteine thiol and a NO moiety to form a *S*-nitrosothiol (SNO) (Stamler et al. 1992). This process, known as *S*-nitrosylation, has emerged as major PTM and has been shown to modulate many different signalling systems in animals, plants and microorganisms (Hess et al. 2005; Spadaro et al. 2010).

The antioxidant tripeptide glutathione (GSH) can readily react with NO to generate *S*-nitrosoglutathione (GSNO). GSNO is the main non-protein SNO in cells

and extracellular fluids (Liu et al. 2001; Gaston et al. 1993) and acts as a bioactive reservoir of NO. As represented in Figure 1-4, in a trans-*S*-nitrosylation process, NO can be transferred from GSNO to cysteine thiols of target proteins (Protein-SH). Thus, changes in the level of GSNO directly impacts upon the level of total protein *S*-nitrosylation.

In 2001, Liu and co-workers reported the existence of a highly conserved enzyme responsible for controlling GSNO levels in yeast and mice known as GSNO-reductase (GSNOR) (Liu et al, 2001). This enzyme, which was later shown to have a conserved function in plants (Feechan et al. 2005), reduces GSNO to oxidized glutathione (GSSG) and ammonia (NH3) in a reaction dependent on the oxidation of NADH (Figure 1-4).

Loss of GSNOR function leads to accumulation of GSNO and affects SNO homeostasis in bacteria, yeast, mice and plants (Liu et al. 2001; Liu et al. 2004; Feechan et al. 2005). Interestingly, the absence of this enzyme has been associated with the higher susceptibility of plants, flies and mice to infections (Feechan et al. 2005; Kanchanawatee 2012; Tang et al. 2013), suggesting a major and evolutionary conserved role for protein *S*-nitrosylation in immunity.

The mechanisms that explain the higher susceptibility of *gsnor* mutants to infections have just started to be elucidated in plants and mice. These mechanisms will be discussed in more details in the next section of this Chapter.

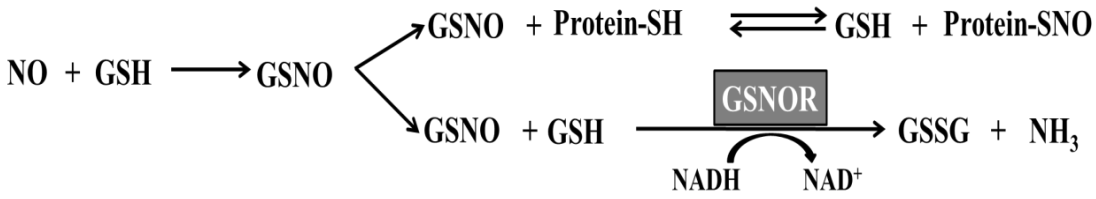


Figure 1-4: GSNOR activity indirectly controls the level of protein-SNO

GSNOR uses NADH as an electron donor to catalyse the conversion of GSNO to GSSG and NH_3, thus eliminating NO from the system. Mutations in *gsnor* change the balance towards GSNO accumulation and this leads to higher levels of global protein *S*-nitrosylation.

1.3.3 GSNOR and immunity

Aberrant levels of *S*-nitrosylation have been associated with the development of a variety of disorders such as strokes, Parkinson's, Alzheimer's and other neurodegenerative diseases (Nakamura et al. 2013). Hundreds of proteins implicated in numerous pathophysiological conditions such as asthma, diabetes, lung inflammation, innate immunity and others, have also been shown to be *S*-nitrosylated (Foster et al. 2009).

As mentioned above, an imbalance in GSNO homeostasis caused by a mutation in *gsnor* results in immunocompromised organisms. Feechan and co-workers have shown that *A. thaliana* with loss of GSNOR function presents increased cellular SNO levels and impaired plant defence responses conferred by distinct resistance (*R*) gene subclasses, thus compromising basal and non-host disease resistance (Feechan et al. 2005).

One molecular explanation for the higher susceptibility of these plants to infections regards the transcriptional cofactor Non-expresser of pathogenesis-related genes 1 (NPR1). This protein is a central regulator of plant defence signalling mediated by the plant immune activator salicylic acid (SA). It exists either as an

oligomer linked by intermolecular disulphide bonds, sequestered in the cytoplasm or a monomer which is able to translocate to the nucleus and activate the expression of immune-related genes. *S*-nitrosylation of this transcriptional co-factor induces oligomerization while infections induce monomerization. Tada and co-workers have shown that nucleation of NPR1 in response to infection is compromised in *gsnor* plants. This phenotype was shown to be related to the higher levels of NPR1 *S*-nitrosylation in *gsnor* mutants compared to wild type plants (Tada et al., 2008).

Later, Yu and co-workers have also related the higher susceptibility of these plants to infections with impairment in pathogen-triggered ROS production due to excessive *S*-nitrosylation of NOX (AtRBOHD) at Cys 890. These result also provide an interesting model relating RNS and ROS modulation during immune responses (Yun et al. 2011).

In animals, although numerous immune-related proteins have been shown to be modulated by *S*-nitrosylation (reviewed in Chapter 4), a direct mechanism relating the increased SNO levels of *gsnor*$^{-/-}$ mice (Liu et al. 2004) and the higher susceptibility of these animals to infections (Tang et al. 2013) has not been presented yet.

Aiming to investigate the role of GSNOR in animal immunity, Kanchanawatee and co-workers employed an overlapping deficiency technique (described in Chapter 3) to generate *gsnor* loss-of-function flies (*D. melanogaster*) (Kanchanawatee 2012). *gsnor* loss-of-function flies generated by this technique presented morphological and fertility-related phenotypes. Tergites, mostly the fourth and the fifth, are defective or absent in these animals. Furthermore, females retain most of the eggs in the ovaries, causing an enlargement of their abdomens. Laid eggs

are malformed, present low percentage of hatching and are unable to produce viable progeny (Kanchanawatee 2012).

In addition, these animals were shown to be highly susceptible to the entomopathogen fungus *Beauveria bassiana* but not to the Gram-negative bacteria *Erwinia carotovora* pv. *carotovora* and *E. coli* (strain MG1655). They also presented lower inducible production of the AMPs Drs and Mtk after infections, and these phenotypes were recovered when a copy of *gsnor* was re-introduced in the genome under the control of a UAS/GAL4 system. Thus, it was speculated that signalling through the Toll pathway might be impaired in $gsnor^{-/-}$ flies due to aberrant *S*-nitrosylation of target proteins integral to this signalling pathway.

Taking into account the high levels of genetic variation involved in both immune response (Tinsley et al. 2006; Bou Sleiman et al. 2015) and oxidative stress-resistance (Jordan et al. 2012; Weber et al. 2012) among wild-derived *D. melanogaster* lines, it is prudent to consider that the outcomes of experiments involving standard laboratory strains may not always be generalizable to all wild population strains. Furthermore, variability in immunocompetence has also been shown to occur among *D. melanogaster* laboratory reference strains (Okado et al. 2009; Eleftherianos et al. 2014).

Therefore, caution must be taken when comparing flies with non-identical genetic backgrounds. As described in Chapter 2, the wild type (Orengon-R) strain present in most of the experiments in this thesis was included to give a point of reference rather than as a control for genetic background. For that purpose, in most of the experiments presented here, the parental lines used to generate the overlapping deletions were included.

1.4 Aims and objectives of this study

S-nitrosylation has emerged as major PTM involved in a variety of physiologically important processes. GSNOR activity has been shown to play an important role in regulating the global levels of *S*-nitrosylation in plants, yeast and mice. GSNOR loss-of-function mice, flies and plants are known to be compromised in responding to infections, suggesting that an imbalance in SNO homeostasis affects the immune system of these organisms.

The aim of this project is to further investigate the role of GSNOR in *D. melanogaster* physiology and pathophysiology, with a main focus on immunity. In this way, *gsnor*$^{-/-}$ flies generated by the overlapping deficiency technique will be analysed for their tolerance to inducers of oxidative and nitrosative stresses, as well as to their responses to different classes of pathogens.

Furthermore, as these flies have been shown to be highly susceptible to *B. bassiana* infection and presented lower inducible expression of Toll-mediated AMPs, possible targets for *S*-nitrosylation within the Toll signalling pathway will be sought. In addition, this work aims to generate a *gsnor* knock-out mutant using CRISPR/Cas9 technology.

Chapter 2

Material and Methods

Unless otherwise stated, all chemicals and oligonucleotides used in this work were supplied by Sigma-Aldrich, UK, and all restriction enzymes were supplied by New England Biolabs (NEB), UK. Primer sequences are always written as 5' to 3'.

2.1 *D. melanogaster* stocks and maintenance

All *D. melanogaster* lines were maintained on standard yeast-cornmeal-agar medium (Live Yeast 5.7 g/L; Glucose 78.6 g/L; Agar 10.7 g/L; Maize 71.4 g/L; Propionic Acid 3.2 ml/L; Nipagin 2.7g/L), in 50 mL vials. Stocks were kept in incubators with no humidity control, at 20°C and constant darkness. Flies were transferred to new food as a matter of routine every other week. All lines used in this study are listed in Table 2-1.

Crosses and experiments were carried out at 25°C (29°C for *B. bassiana* survival assays). Carbon dioxide (CO_2) was used as the standard method of anaesthesia in the fly lab. For experiments carried out outside the fly lab (infections with *B. bassiana* and hazard group II pathogens - *E. faecalis* and *S. aureus*), flies were anesthetised prior infection by placing vials in a box with ice for at least five minutes.

In most of the experiments presented in this thesis, adult flies (a mixture of three to six day old flies) were used. After clearing flies (day one), vials were kept at 25°C for three days. Newly eclosed flies were collected, the appropriate phenotypes selected and males and females placed in separate vials. These vials were kept at 25°C for three additional days. Therefore, although most of the flies should have mated before collections at day 3, young non-mated flies were likely to be present among flies used in experiments.

Table 2-1: List of *D. melanogaster* lines used in this study

Name	Genotype and description	Origin
Oregon-R	Wild type	Finnegan lab stocks
gsnor$^{+/-}$ (*Df7305*)	*w*^{1118}*; Df(3R)Exel7305/TM6B, Tb* - *gsnor* single deficiency	Bloomington Drosophila Stock Centre (7956)
gsnor$^{+/-}$ *(Df7306)*	*w*^{1118}*; Df(3R)Exel7306/TM6B, Tb* - *gsnor* single deficiency	Bloomington Drosophila Stock Centre (7957)
gsnor$^{-/-}$ (*Df7305/Df7306*)	*w*^{1118}*;* *Df(3R)Exel7305/Df(3R)Exel7306* - *gsnor* overlapping deficiencies	Cross between *Df7305* and *Df7306*
gsnor$^{-/-}$ (*Df7306/Df7305*)	*w*^{1118}*;* *Df(3R)Exel7306/Df(3R)Exel7305* - *gsnor* overlapping deficiencies	Cross between *Df7306* and *Df7305*
UAS-gsnor	*y, w, P{y*$^{+}$*.nos-int.NLS}; attP40 UAS-gsnor* - *gsnor CDS* linked to *UAS* and integrated at attP40 (25C7)	Kanchanawatee (2012)
UAS-gsnor; Df7305	*y, w, P{y*$^{+}$*.nos-int.NLS}; attP40 UAS-gsnor* *; Df(3R)Exel7305/TM6B, Tb* -*UAS- gsnor* integrated on the second chromosome (attP40) recombined with *Df7305* on the third chromosome	Kanchanawatee (2012)
act5cGAL4	*y*^{1} *w***; P{Act5C-GAL4}17bFO1 /TM6B, Tb* - *GAL4* controlled by *Actin5C*	Bloomington Drosophila Stock

	promoter	Centre(3954)
act5cGAL4,Df7306	*y^1 w^*; P{Act5C-GAL4}17bFO1, Df(3R)Exel7306/TM6B, Tb* - *act5C-GAL4* and *Df7306* recombined in the same chromosome	Kanchanawatee (2012)
act5c:UASgsnor;Df7305 /Df7306	*UAS-gsnor/+;Df(3R)Exel7305/P{Act5CGAL4}17bFO1, Df(3R)Exel7306* - Complementation of *gsnor* under the control of *Actin5C* promoter	Cross between *act5C-GAL4,Df7306*and *UAS-gsnor;Df7305*
act5c:UASgsnor; Df7306/Df7305	*+/UAS-gsnor;Df(3R)Exel7306, P{Act5CGAL4}17bFO1/Df(3R)Exel7305* - Complementation of *gsnor* under the control of *Actin5C* promoter	Cross between *UAS-gsnor;Df7305 and act5C-GAL4,Df7306*
$Catalase^{n1}$	*Cat^{n1}/TM3, Sb, Ser* - Point mutation in *cat*	Bloomington Drosophila Stock Centre (4014)
Cat^{n1},Df7305	*Cat^{n1}, Df(3R)Exel7303/ TM6B, Tb* - *Cat^{n1}*and *Df7305* recombined on the same chromosome	This study
Cat^{n1}, Df7305/Df7306	*Cat^{n1},Df(3R)Exel7305/Df(3R)Exel7306*	Cross between *Cat^{n1},Df7305*and *Df7306*
Df7306/Cat^{n1},Df7305	*Df(3R)Exel7305/Cat^{n1},Df(3R)Exel7306*	Cross between *Df7306* and *Cat^{n1},Df7305*
UAS-psh-HA	*y, w, P{y^+.nos-int.NLS}; attP40 UAS-psh* - *psh CDS* linked to *UAS* and	This study

	integrated at attP40 (25C7)	
UAS-pshC254S-HA	*y, w, P{y$^+$.nos-int.NLS}; attP40 UAS-pshC254S-HA* - *pshC254S CDS* linked to *UAS* and integrated at attP40 (25C7)	This study
UAS-spe-HA	*y, w, P{y$^+$.nos-int.NLS}; attP40 UAS-spe-HA* - *spe CDS* linked to *UAS* and integrated at attP40 (25C7)	This study
UAS-psh-HA;Df7305	*UAS-psh-HA/UAS-psh-HA; Df7305/ TM6B, Tb, Hu* - Flies carrying *UAS- psh* on the second chromosome and *Df7305* on the third chromosome	This study
UAS-pshC254S-HA;Df7305	*UAS-pshC254S-HA/UAS-pshC254S-HA; Df7305/ TM6B, Tb, Hu* -Flies carrying *UAS- pshC254S* on the second chromosome and *Df7305* on the third chromosome	This study
Ubi-GAL4	*w*; P{Ubi-GAL4.U}2/CyO* - Expresses GAL4 in all cells under the control of the *Ubiquitin* promoter	Bloomington Drosophila Stock Centre(32551)
Ubi-GAL4; Df7306	*Ubi-GAL4; Df7306/ TM6B, Tb* - Flies carrying*Ubi-GAL4* on the second chromosome and *Df7306* on the third	This study
Ubi-GAL4:UAS-psh	*Ubi-GAL4/UAS-psh-HA* - *psh-HA* expression in wild type flies under the control of the *Ubi* promoter	Cross between *Ubi- UAS-psh-HA* and *Ubi-GAL4*

Ubi-GAL4:UAS-pshC254S	*Ubi-GAL4/UAS-pshC254S-HA* - *pshC254S-HA* expression in wild type flies under the control of the *Ubi* promoter	Cross between *Ubi- UAS-pshC254S-HA* and *Ubi-GAL4*
Ubi-GAL4:UAS-spe	*Ubi-GAL4/UAS- spe -HA* - *spe-HA* expression in wild type flies under the control of the *Ubi* promoter	Cross between *Ubi- UAS- spe-HA* and *Ubi-GAL4*
Ubi-GAL4:UAS-psh; Df7305/Df7306	*Ubi-GAL4/UAS-psh-HA; Df(3R)Exel7305/Df(3R)Exel7306* - *psh-HA* expression in *gsnor* overlapping deficiencies under the control of the *Ubi* promoter	Cross between *UAS-psh-HA;Df7305* and *Ubi-GAL4; Df7306*
Ubi-GAL4:UAS-pshC254S; Df7305/Df7306	*Ubi-GAL4/UAS-pshC254S-HA; Df(3R)Exel7305/Df(3R)Exel7306* - *pshC254S-HA* expression in *gsnor* overlapping deficiencies under the control of the *Ubi* promoter	Cross between *UAS-pshC254S-HA;Df7305* and *Ubi-GAL4; Df7306*
Spätzle	Spz^{rm7}/*TM6C* - Spätzle loss of function mutation, sensitive to Gram-positive bacteria and fungi	Bruno Lemaitre lab
gsnor null mutant (CRISPR)	w^{*}; $gsnor^{DsRed}$- *gsnor* deletion generated using CRISPR/Cas9	This study

2.2 Septic infection assay

The Gram-positive bacteria used to infect flies by septic injury were: *Enterococcus faecalis* (NCTC 775) and *Staphylococcus aureus* (NCTC 8325). Bacteria were grown on LB agar plates and single colonies were transferred to 5 mL

LB media and allowed to grow overnight at 37°C and 200 rpm. Before infections, optical densities (OD) were adjusted to 1. About 30 female flies aged 3-6 day old were anaesthetized by chilling on ice and pierced with a tungsten needle previously dipped in the bacterial cultures. As a control experiment, flies were infected with heat-inactivated *E. faecalis* (grown overnight in 5 mL of Luria-Bertani Broth and autoclaved for 15 min at 121°C). Flies were transferred to new vials containing standard fly food and kept at 29℃, an optimal temperature to pathogens growth (Neyen et al. 2014). Dead flies counted within two hours of infection were not considered for the experiment because these were likely to be killed during the infection procedure and not due to the infection. The number of surviving flies was recorded once a day.

2.3 Feeding infection assay

About 15 female flies aged 3-6 day old were fed on solutions contaminated with the Gram-negative entomopathogenic bacterium *Pseudomonas entomophila* (Vodovar et al. 2005). Bacteria were plated on LB agar plates supplemented with 1% skimmed milk to select for protease-positive colonies (which produced clear halos around the colony). A single positive colony was pre-cultured in 40 mL of LB in a 500 mL conical flask for 8 hours at 30℃. This culture was diluted 16x to a final volume of 400 mL LB in 2 L conical flask and kept overnight at 30℃. Next morning, cells were pelleted by centrifugation at 2500 g, 4℃ for 15 minutes. The Supernatant was almost completely removed and around 2 mL of it was kept and used to resuspend the pellet. Flies were fed by two different methods and these involved two different concentrations of bacteria. In the first method, concentrated bacterial

suspensions were mixed with the same volume of 10% sucrose solution, making a final of 5% sucrose. Flies were starved for two hours prior being transferred to vials containing a filter paper soaked on this highly concentrated bacterial solution. After seven hours feeding on bacteria-contaminated solution, flies were transferred to new vials with standard fly food and kept at 29℃. The number of surviving flies was recorded once a day. For the second method of infection, the O.D. of the bacterial suspension was adjusted to 4 and mixed with the same volume of 10% sucrose solution to make a final O.D. of 2 and 5% sucrose. Flies were starved for two hours and transferred to vials containing cotton rolls soaked in this bacterial solution. Vials were kept at 29℃, new bacterial solution was added every other day and the number of surviving flies was recorded once a day.

2.4 Fungal infection assay

Beauveria bassiana spores were grown on potato dextrose agar (PDA) medium supplemented with 50 μg/mL chloramphenicol at 29℃ until the fugal mycelium had fully covered the plate. To induct spore formation, the plate was protected from light and left in a fume hood until completely desiccated. The dried plate was stored in the dark at 4℃. About 20 male and/or female flies aged 3-6 day old were anaesthetized by chilling on ice and transferred to a 2 mL microcentrifuge tube containing about 1 cm^2 of a plate of *B. bassiana* spores and mycelium. Tubes were gently shaken by hand for 2 minutes. Flies were transferred to new vials containing standard fly food and kept at 29℃, an optimal temperature to pathogens growth (Neyen et al. 2014). Any dead flies counted within two hours of infection were not considered for the experiment. The number of surviving flies was recorded

once a day.

2.5 Paraquat and SNP tolerance assays

Flies were tested to their tolerance to the herbicide paraquat and to the NO donor sodium nitroprusside (SNP). About 15-30 flies aged 4-7 day old were transferred to empty vials and starved for two hours at 25°C. After that, flies were transferred to vials containing a cotton roll soaked in 5% sucrose solution supplemented with different concentrations of paraquat or SNP. Vials were kept at 25°C and the number of survivors was recorded once a day after the treatment. Fresh solution was added to the cotton rolls every other day when appropriate.

2.6 Genomic DNA extraction from *D. melanogaster*

This protocol is an adaptation of the method used by the Johnston lab, Columbia University, NY, which is available online, and was described in Kanchanawatee's PhD thesis(2012). Initially 30 flies were frozen (-70℃) for a period in excess of five minutes and then homogenised with a disposable plastic grinder in 400 µL of 100 mM Tris-HCl pH 7.5, 100 mM EDTA, 100 mM NaCl and 0.5 % SDS. The homogenate was incubated at 65℃ for 30 minutes and then 800 µL of 1.67 M potassium acetate and 4 M LiCl were added to the tubes, followed by incubation on ice for 10 minutes and a subsequent centrifugation at 14,000 rpm for 15 minutes. Equal volumes of the supernatant were transferred to two new clean tubes, and precipitation perform by adding 700 µL isopropanol followed by centrifugation at 14,000 rpm for 15 minutes. The pellet was resuspended in 100 µL sterile distilled water.

2.7 Total RNA extraction and cDNA synthesis

Five adult female flies were frozen in liquid nitrogen (N2) and subsequently ground in 500 µL of Trizol (Invitrogen) using disposable plastic grinder. The homogenate was left at room temperature for 5 minutes followed by centrifugation at 12,000 rcf for 10 minutes at 4℃. 180 µL of the supernatant was transferred to a new microcentrifuge tube and60 µL of chloroform was added to each tube. The homogenate and chloroform were mixed vigorously by hand followed by incubation at room temperature for 3 minutes and centrifugation at 10,000 rcf for 15 minutes at 4℃. The upper phase (~80 µL) was transferred to a new microcentrifuge tube. 100 µL of isopropanol was added to each tube followed by incubation at room temperature for 5 minutes and centrifugation at 12,000 rcf for 10 minutes at 4℃. The supernatant was replaced by600 µL of 75 % ethanol followed by centrifugation at 7,500 rcf for 5 minutes at 4℃. The supernatant was removed, and the RNA pellet was dried in a laminar flow cabinet. The pellet was resuspended in 55 µL of diethylpyrocarbonate (DEPC) -treated water.

Before cDNA synthesis, RNA samples were quantified using a NanoDrop spectrophotometer (Thermo Scientific) and appropriate dilutions were made to ensure all samples contained equal amounts of RNA. Reverse transcription was performed using an Omniscript RT Kit (Qiagen) according to the manufacturer's instructions.

2.8 Quantitative real-time PCR

The relative expression of the anti-microbial peptide genes *Diptericin* (*Dpt*), *Drosomycin* (*Drs*), *Defencin* (*Def*) and *Metchnikowin* (*Mtk*) was quantified by PCR

using SYBR Green I Master Mix, in a Light Cycler 480 system (Roche).An initial denaturation step of 5 minutes at 95℃ was followed by 45 cycles of 10 seconds at 95℃, 20 seconds at 60℃ and 20 seconds at 72℃. An additional melting curve step (5 seconds at 95℃, 1 minute 65℃ followed by a progressive increase to 97℃ with a ramp rate set to 1.1℃/second) was used to confirm the presence of a single amplification product in the reaction. The incorporation of SYBR green was measured at the end of every extension step and continuously melting analysis. All cDNA samples were diluted 10 fold and compared against a standard curve (0, 10, 10^2, 10^3 and 10^4 fold dilutions) generated from the same pool of cDNA. This pool was obtained from infected wild type Oregon-R flies.

The levels of expression of the housekeeping gene *Ribosomal protein 49* (*Rp49*) were used as an internal reference to calculate the relative expression of AMPs and the results presented as the ratio AMP/*Rp49*. Primers (Table 2-2) and conditions used to quantify the expression of these genes have been described before (Ryu et al. 2008; Romeo & Lemaitre 2008; Kanchanawatee 2012).

Table 2-2: Primers used for Q-RT-PCR

Gene	Primer sequences
Rp49	**R** - AGATCGTGAAGAAGCGCACCAAG **F** - CACCAGGAACTTCTTGAATCCGG
Dpt	**R** - GCTGCGCAATCGCTTCTACT **F** - TGGTGGAGTGGGCTTCATG
Drs	**R** - CGTGAGAACCTTTTCCAATATGATG **F** - TCCCAGGACCACCAGCAT
Def	**R** - TGCAGCATAGCCGCCAGAA **F** - TTGCAGTAGCCGCCTTTGAACC
Mtk	**R** - GATGCAACTTAATCTTGGAGCG **F** - TTAATAAATTGGACCCGGTCTTGGTTGG

2.9 Total protein extraction and quantification

Flies were homogenised in 20 μL/fly extraction buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 5 mM EDTA, 0.1% Triton X-100) freshly supplemented with protease inhibitors [50 μg/ml TPCK (N-tosyl-L-phenylalaninyl-chloromethylketone), 50 μg/ml TLCK (N-alpha-tosyl-L-lysinyl-chloromethylketone) and 0.5 mM PMSF (phenylmethanesulfonyl fluoride)]. Samples were centrifuged at 13,000 rpm at 4°C for 15 minutes and the supernatant was transferred to new pre-chilled tubes.

For both recombinant protein purified from *E. coli* (section 2.12) and total protein extracted from flies, the Bradford Assay (Bradford, 1976) was used to measure protein concentrations. Bovine serum albumin (BSA) (NEB, UK) was used to generate the standard curves and a standard protocol was followed. When appropriate, samples were divided in small aliquots and kept at 80°C.

2.10 SDS-PAGE and western blots

Protein samples were mixed with a 4X stock of sample buffer to a final concentration of 50 mM Tris-HCl pH 6.8, 2% SDS, 0.02%bromophenol blue and 10% glycerol with or without 50 mM dithiothreitol (DTT). Next, samples were heated at 85°C for 10 min before separating on gels of appropriate polyacrylamide percentage. Coomassie Blue staining, gels were washed in ddH_2O before incubating in staining solution (0.25% Brilliant Blue R, 40%methanol, 7% acetic acid) for 30 min to one hour. Gels were then de-stained overnight in de-staining solution (40% methanol, 10% acetic acid) and photographed or used for in gel digestion and mass spectrometry analysis.

For western blots, proteins were transferred on to nitrocellulose membranes

either overnight at a constant voltage of 20V or for 2-3 hours at 90V. Proteins were visualised on the membranes before blocking by staining with Ponceau S (0.1% Ponceau S, 5% acetic acid) for 1 min followed by rinses with H_2O to remove background staining. Photographs were taken before the stain was completely removed with PBS + 0.1% tween (PBS-T). After that, membranes were blocked for 1 hour at room temperature using 5% dried skimmed milk in PBS-T and incubated with primary antibodies (Table 2-3) either at 4°C overnight or for 1-2 hours at room temperature. After washing the membrane to remove the excess of primary antibody, appropriate secondary antibodies (Table 2-3) coupled to horseradish peroxidase (HRP) were incubated with the membrane for 1 hour at room temperature. SuperSignal West Pico/Dura Chemiluminescent Substrate (Thermo Scientific) was added to the membranes and bands were detected on X-ray films. All antibodies were diluted in 5% skimmed milk / PBS-T.

Table 2-3: List of antibodies used

Primary antibody	Host species	Manufacture
Anti-HA (clone 12CA5)	mouse	Roche
Anti-MBP HRP conjugated	mouse	NEB
Anti-Biotin HRP conjugated	goat	Cell Signalling

Secondary antibody	Host species	Manufacture
anti-mouse IgG HRP-linked	goat	Cell Signalling

2.11 Constructs for protein expression in *E. coli* and *D. melanogaster*

The coding sequence of *psh* and *spe* were amplified using Phusion high-fidelity polymerase (NEB, UK) from freshly synthetized cDNA of wild type Oregon-R flies. Primers were designed to add the nucleotides CACC at the 5' end of each

fragment (Table 2-4), thus allowing TOPO®cloning (Life Technologies). The PCR products were gel-purified and cloned into the pENTR™/D-TOPO® vector according to the manufacturers' instructions.

Constructs were transformed into *E. coli* competent cells and selected on Kanamycin plates. Single colonies were isolated, submitted to plasmid purification and validated by sequencing. Inserts from positive constructs were transferred by Gateway® cloning (LR reactions following manufacturers' instructions – Life Technologies) into the following plasmids: pETG-40A (for expression of N-terminal MBP-fusion proteins in *E. coli*), to generated pETG-40A-*psh* and pETG-40A-*spe*, and pUASt-HA (for expression of C-terminal HA-tagged proteins in *D. melanogaster*) to generate pUASt-*psh*-HA and pUASt-*spe*-HA. Recombinant clones were selected on ampicillin plates and confirmed by sequencing.

pUASt-*psh*-HA and pUASt-*spe*-HA constructs were purified using the QIAfilter plasmid midi kit (Qiagen), in accordance to the manufacturers' instructions. DNA quality and concentrations were measured using a NanoDrop spectrophotometer ND 1000, and 50 µg of each construct was sent to Genetic Services Inc. for efficient transformation into the attP40 site of the recipient *D. melanogaster* line, through a process of co-microinjection with phiC31 integrase (Markstein et al. 2008).

Table 2-4: Primers used for TOPO® cloning

Amplicon	Primer sequences
psh coding sequence	**F** - CACCATGCCATTGAAGTGGTC **R** - TTACTTCACCCGATTGTCCGG
spe coding sequence	**F** - CACCATGGCTTCTACGGAACG **R** - TCATGGCTCCAATTTCTGCTTT

2.12 Recombinant protein expression and purification

E. coli Rosetta-Gami2 cells were transformed with the constructs pETG-40A-*psh* and pETG-40A-*spe*and selected on LB agar plates supplemented with ampicillin (50 mgL-1). Single colonies were selected and grown overnight in 5mL LB medium supplemented with ampicillin at 37°C and 250 rpm. Overnight cultures were diluted in 500 mL of LB medium also supplemented with ampicillin (50 mgL-1) in 2 L conical flask and incubated at 37°C, 250 rpm until O.D. reached 0.5-0.6. IPTG was added to a final concentration of 1 mM and cultures were further incubated for four hours. Cells were harvested by centrifugation at 6000 g for 15 minutes, washed twice with phosphate buffer saline (PBS) and resuspended in lysis buffer (50 mM KHPO4 pH 8, 300 mM NaCl, 1 mg/ml lysozyme, 25 U/ml Benzonase nuclease, 0.1% Triton-X-100, 10 mM β-mercaptoethanol, 50 µg/ml TPCK, 50 µg/ml TLCK and 0.5 mM PMSF) and incubated with gentle rocking at room temperature for 30 minutes. The lysate was then centrifuged at 4°C at 15,000 rpm for 15 minutes and the supernatant collected. MBP fusion proteins were then purified by gravity-flow using amylose affinity chromatography columns (NEB) according to the manufacturers' instructions. The fusion proteins were run in a SDS-PAGE gel and analysed by mass spectrometry.

2.13 *S*-nitrosylation assays

The biotin-switch technique (Jaffrey et al. 2001) was employed to detect *S*-nitrosylation of recombinant proteins exposed to NO donors and endogenously modified proteins expressed in *D. melanogaster*. A diagram of the method is presented in Figure 2-1.

For *in vitro* assays 2 µg of recombinant protein was treated with different concentrations (100, 500 and 1000 µM) of NO donors (CysNO or GSNO) for 20 minutes in dark. As a positive control, samples were treated with 0.1% SDS before the addition of NO donors to expose other cysteine thiols that would not be accessible otherwise.

Excess of NO donor was removed using Zeba™ Spin Desalting Columns (7K MWCO, 0.5 mL, ThermoFisher Scientific). Free thiols were blocked in one volume of blocking buffer (250 mM HEPES, 1 mM EDTA, 0.1 mM Neocuproine, 5% (w/v) SDS, 50 mM NEM) for 30 minutes at 55ºC. The blocking buffer was removed by precipitating proteins with two volumes of cold acetone. After 20 minutes at -20ºC, samples were centrifuged at 15000 g for five minutes at 4ºC. Samples were washed three times with 70% acetone and resuspended in 85 µL of $HENS_{1\%}$ buffer (250 mM HEPES, 1 mM EDTA, 0.1 mM Neocuproine,1% (w/v) SDS). *S*-nitrosothiols were reduced by adding sodium ascorbate to a final concentration of 25 mM and the newly freed thiols were biotinylated by adding biotin-HPDP (*N*-[6-(biotinamido)hexyl]-3′-(2′-pyridyldithio)-propionamide) to a final concentration of 0.4 mM Samples were kept on a rocker plate for one hour. Proteins were precipitated and washed with acetone as described above. At this point, proteins were either re-suspended in 30 µL of $H_{25}ENS_{1\%}$ (25 mM HEPES, 1 mM EDTA, 0.1 mM Neocuproine, 1% SDS), run on

a non-reducing SDS-PAGE and submitted to western blots with an anti-Biotin antibody, or resuspended in 300 µL of $H_{25}ENS_{1\%}$and submitted to streptavidin pull-down.

For the second options, before the pull-down step, 20 µL was taken from each sample, mixed with DTT and loading buffer to a final concentration of 100 mM and 1x, respectively, heated for five minutes at 90ºC and kept at -20ºC. The remaining 280 µL were mixed with 1.5 mL of neutralisation buffer (25 mM HEPES, 1 mM EDTA, 0.1 mM Neocuproine, 100 mM NaCl, 0.5% Triton X-100) in 2 mL tubes. 20 µL of streptavidin beads, pre-washed and resuspended in 100 µL of neutralisation buffer, was added to each sample and incubated at 4ºC on a rocking plate overnight. Next morning, samples were washed five times with 500 µL of wash buffer (25 mM HEPES, 1 mM EDTA, 0.1 mM Neocuproine, 600 mM NaCl, 0.5% Triton X-100) and resuspended in 20 µL of elution buffer (25 mM HEPES, 1 mM EDTA, 0.1 mM Neocuproine, 1%β-mercaptoethanol v/v). After 30 minutes at room temperature, beads were spun down for one minute at room temperature and maximum speed. Samples were collected, and detected by western blot using an anti-HA antibody.

For *in vivo S*-nitrosylation assays, flies were homogenised in extraction buffer (HEN, 0.5% Triton X-100, 50 µg/ml TPCK, 50 µg/ml TLCK and 0.5 mM PMSF) and samples were centrifuged at 13,000 rpm at 4°C for 15 minutes. Supernatant was collected and submitted to the biotin switch as described above, but omitting the addition of NO donors. An anti-Biotin antibody was used to detect total protein *S*-nitrosylation and an anti-HA antibody was used to detect *S*-nitrosylation of HA-tagged proteins after streptavidin pull-down.

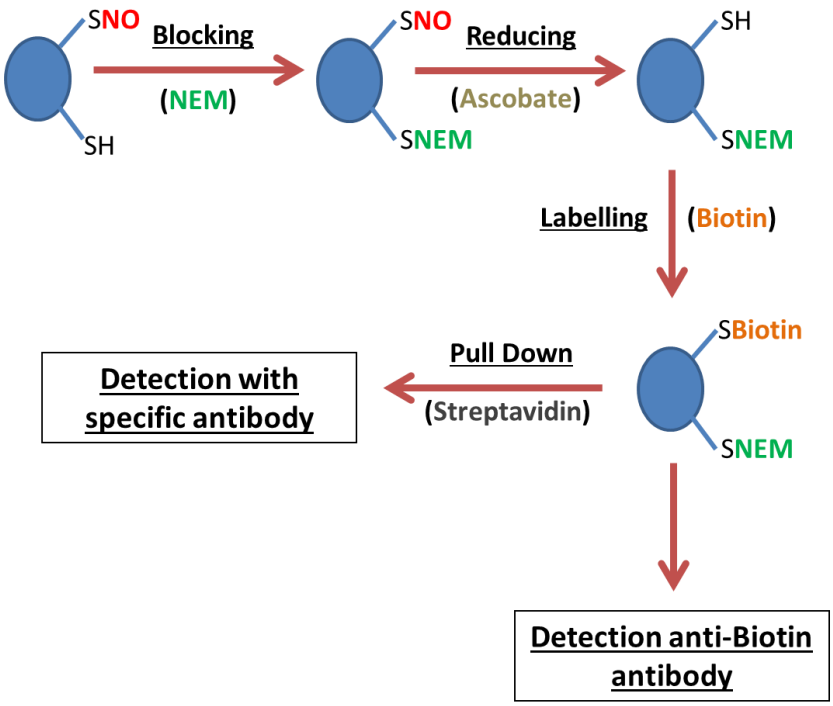


Figure 2-1: Schematic diagram of the Biotin-Switch Assay

The first step (Blocking) involves the covalent modification off free thiols (SH) with N-Ethylmaleimide (NEM). Next, in a Reducing step, Ascorbate is employed to convert S-nitrosylated thiols (SNO) to SH. In the Labelling step, nascent free thiols are biotinylated with biotin-HPDP. Biotinylated proteins can be detected using an anti-Biotin antibody or pulled down using recombinant streptavidin and detected with specific antibodies.

2.14 Identification of *S*-nitrosylation sites

2µg of purified recombinant protein was treated with 200µM GSNO for 20 minutes at room temperature to induce SNO formation. Non-treated samples were used as negative controls. N-Ethylmaleimide (NEM) was used to block non-*S*-nitrosylated free thiols and iodoacetamide was used to replace NO from SNOs after ascorbate treatment. Samples were run on a reducing SDS-PAGE, stained with bromophenol blue and excised from the gel for trypsin digestion. Tryptic digested samples were analysed by LC-MS/MS.

A cysteine residue blocked by NEM increases its peptide mass by 125.126, while a cysteine residue blocked by iodoacetamide increases its peptide mass by 57.051. In this way, by relying on the sequential blocking-reducing-blocking (NEM-Ascorbate-Iodoacetamide) steps after exposure to a NO donor, it was possible to infer whether a cysteine residue was *S*-nitrosylated or not by looking at the increase in the mass of its peptide

2.15 Site-directed mutagenesis

All site-directed mutagenesis reactions were performed using the Quick-Change II XL kit (Agilent Technologies) according to manufacturers' instructions. Two complementary primers were synthesized to each desired mutation (Table 2-5) and used in the PCR reaction following the conditions: 5 µL of 10x reaction buffer, 10 ng of plasmid DNA (psh-pENTR™/D-TOPO®), 125 ng each of each complementary primer, 1µL of dNTP mix (10 mM), 3 µL of QuikSolution reagent, 2.5 U of Pfu Ultra HF DNA polymerase and distilled water to a final volume of 50 µL. The reaction parameters were as follow: 95°C for 50 sec, 52°C for 50 sec, and 68°C for 4 min, 18 cycles. After that, parental plasmids were digested with Dpn I (10 U) at 37oC for 1 hour and the Dpn I-treated DNA was transformed into XL10-Gold ultracompetent cells. The mutated expression plasmid was purified and the mutated site was confirmed by DNA sequencing using M13 (5' – TGTAAAACGACGGCCAGT – 3') and T7 (5' – CCCTATAGTGAGTCGTATTAC – 3') primers.

Table 2-5: Primers used for site-directed mutagenesis of *psh*

Mutation	Primer sequences
C40S	**F** - GATACCATGCCCGGTATCTCCAGAACATCCTCCGATTG **R** - CAATCGGAGGATGTTCTGGAGATACCGGGCATGGTATC
C46S	**F** - CAGAACATCCTCCGATTCTGAGCCCCTAATCGATG **R** - CATCGATTAGGGGCTCAGAATCGGAGGATGTTCTG
C254S	**F** - GATAACATCCGGCCCGCTTCTCTCCATACGGATGCCACC **R** - GGTGGCATCCGTATGGAGAGAAGCGGGCCGGATGTTATC

2.16 Protein structure modelling and analysis

The web server I-TASSER (http://zhanglab.ccmb.med.umich.edu/I-TASSER/)(Zhang 2008) was used to generate a 3Dprediction model of *D. melanogaster* PSH. The same web server was used to generate a superimposition of PSH and GRASS. PyMOL software (PyMOL Molecular Graphics System, Version 1.5.0.5 Schrödinger, LLC) was used to view and analyse protein structures.

2.17 CRISPR/Cas9 constructs for generating *gsnor* knock-out mutants

To delete *gsnor* from the genome of *D. melanogaster* and replace it by a visible marker, gRNAs were designed to direct the Cas9 to specific DNA sequences to both sides of the gene and a plasmid carrying the visible marker *DsRed* was used as a template donor for homology-directed repair.

2.17.1 The gRNA construct

Efficient target recognition by the CRISPR/Cas9 system requires around 20 nucleotides of homology between the gRNA and its genomic target. It also requires the presence of a 3 base pair (bp) proto-spacer adjacent motif (PAM) sequence, NGG, at the 3' end of the genomic target sequence. To find specific target sequences

in intergenic regions flanking *gsnor*, the "CRISPR Optimal Target Finder" web tool (http://tools.flycrispr.molbio.wisc.edu/targetFinder/) (Gratz et al. 2014) was used. The chosen target sequences are presented in Table 2-6.

Table 2-6: gRNA sequences

Target sequence + PAM	Strand	Genome location
ACATAAGAGTATCTTCATTGGGG	-	3R:10870883..10870905
TAAAAGCTCACCGGGACTCAGG	+	3R:10872425..10872446

Both gRNA target sequences were cloned into the same plasmid (pCFD4)as described before (Port et al. 2014). First, primers were designed (Table 2-7) to integrate both gRNA sequences into a PCR product amplified from pCDF4. This PCR product was then cloned by homology directed cloning (Gibson Assembly® Cloning Kit - NEB) back into a BbsI digested fragment of pCFD4. In this way, one of the target sequences is cloned in frame with an upstream U6-1 promoter and a downstream gRNA core region, and the other target sequence is cloned in frame with an upstream U6-3 promoter and second downstream gRNA core region (illustrated in Figure 2-2).

Table 2-7: Primers for cloning two gRNAs into plasmid pCFD4

Target sequences flanked by regions with homology to pCFD4	
Fwd.	TATATAGGAAAGATATCCGGGTGAACTTCGACATAAGAGTATCTTCATTGGTTTTAGAGCTAGAAATAGCAAG
Rev	ATTTTAACTTGCTATTTCTAGCTCTAAAACTAAAAGCTCACCGGGACTCCGACGTTAAATTGAAAATAGGTC

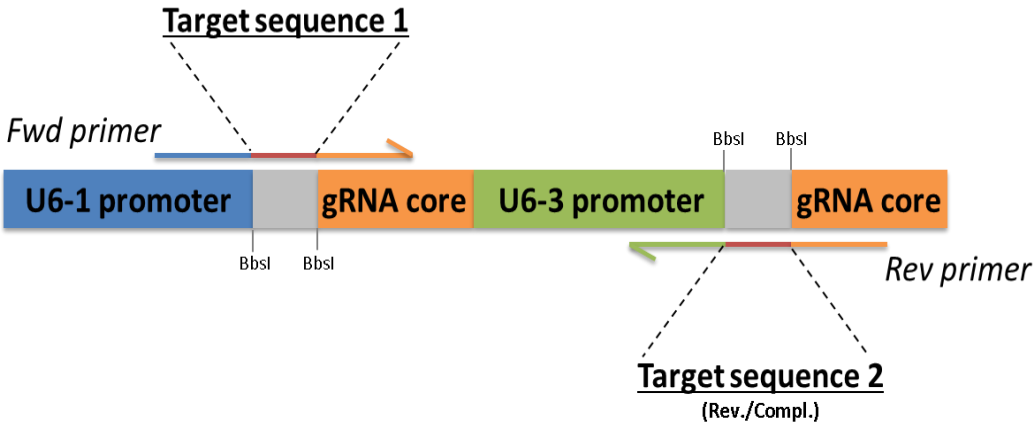


Figure 2-2: Cloning of two gRNAs into the plasmid pCFD4

gRNA target sequences are integrated into forward and reverse primers which are used to amplify a region of the plasmid. PCR products are cloned into pCFD4 by homology directed cloning. This figure is adapted from http://www.crisprflydesign.org/.

2.17.2 The donor plasmid

Homology arms adjacent to the Cas9-generated double strand breaks were amplified from the genome of *D. melanogaster* and cloned into the plasmid pHD-DsRed-attP, which presents a 3xP3-DsRed marker surrounded by two multiple cloning sites (MCS1 and 2) (Figure 2-3). Primers (Table 2-8) were designed to integrate restriction sites at the 5' and 3' ends of the homology arms, being PstI (5' end) and a BglII (3' end) integrated in the homology arm upstream of *gsnor*, and NdeI (5' end) and EcoRI (3' end) integrated in the homology arm downstream of *gsnor*. The fragments amplified by PCR from the genome of wild type flies were sequentially cloned into MCS1 and 2 of the plasmid, replicated in *E. coli* and confirmed by sequencing.

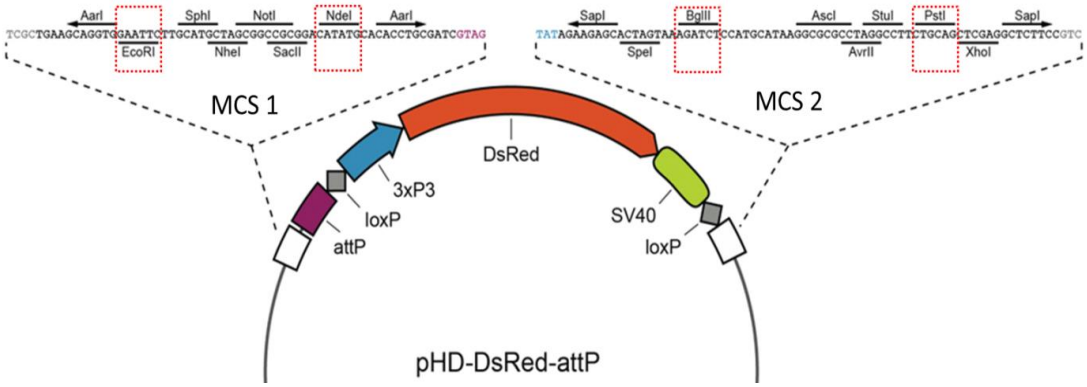


Figure 2-3: pHD-DsRed-attP donor plasmid

Fragments with homology to regions upstream and downstream of gsnor where amplified from the genome and cloned into the MCS 1 and 2 using the restriction sites highlighted by the red boxes.

Table 2-8: Primers used to amplify the homology arms cloned into pHD-DsRed-attP

Product size	Primer sequences
923 bp	**F** - CACTGCAGCGTATCTCTACGGATATCC (*PstI* site) **R** - CAAGATCTTTGGGGGTCGGATTACTGTC (BglII site)
926 bp	**F** - ATACATATGAAGCTCACCGGGACTCAG (NdeI site) **R** - GATAGAATTCACACTGACGATGTGATCCACATAG (EcoRI site)

2.17.3 Generation of transgenic fly lines

Both constructs (pCFD4-gRNAs and pHD-DsRed-attP-gsnor-homology arms) were purified and sent to Genetic services Inc., where they were co-injected into vasa-Cas9 embryos ($y^{[1]}$ *M{vas-Cas9}ZH-2A* $w^{[1118]}$*/FM7c*– BDSC stock 51323) and selected for the expression of DsRed in the eyes of adult flies. DsRed-positive flies were sent back to our laboratory, made homozygous for *DsRed* and kept as a stock. To confirm that the integration took place at the right genomic position, the primers present in Table 2-9 were used to amplify a hybrid fragment (region of integration) from the genome of transgenic flies. PCR products were confirmed by

sequencing using the same primes. Genomic DNA from wild type flies was used as a negative control.

Table 2-9: List of primers used to confirm *DsRed* integration

Primer	Product size	Sequences
P3'-F/R	**1415 bp**	**F** - GCCTCCGATTTGGTTTGTTG
		R - CTTGGAGCCGTACTGGAACTG
P5'-F/R	**1037 bp**	**F** - CACAACGAGGACTACACCATC
		R - CTCCTTTGTGTTCGTTTGCTC

2.18 GSNOR activity assay

The activity of GSNOR was assayed spectrophotometrically by measuring the rate of NADH oxidation in the presence of GSNO. Total proteins from flies were extracted in HE buffer (25 mM HEPES, 1 mM EDTA pH7.7) supplemented with protease inhibitors (50 μg/ml TPCK, 50 μg/ml TLCK and 0.5 mM PMSF) and quantified by Bradford assay. Samples were diluted accordingly to include 75 μg of total protein per reaction. For all GSNOR activity assays, proteins were incubated in 1 ml of HE buffer with the addition of 350 μM NADH and 350 μM GSNO. All samples were blanked just after the addition of GSNO and NADH oxidation was measured by following the absorbance at 340 nm for 10 minutes. As a negative control, GSNO was omitted from the reactions.

2.19 Statistical Analysis

Paired data were evaluated by Student's t-test. One-way ANOVA with the Tukey post-hoc test was used for multiple comparisons. Value of $p < 0.05$ was considered statistically significant. Results are shown as mean ± SEM. Survival

curves in Chapter 6 were estimated using the Kaplan-Meier test (GraphPad PRISM4 software) and statistical significances were tested by the Log-rank test.

Chapter 3

GSNOR and redox homeostasis in *D. melanogaster*

3.1 Introduction

Nitric oxide (NO) is a membrane diffusible, free radical gas. It is synthesised by a NO synthase (NOS) during the conversion of L-arginine to L-citrulline and can act as both an intra- and an intercellular messenger by modifying cysteine thiols, hydroxyphenyl groups of tyrosine residues and transition metal centres of proteins. For these reasons it is involved in different aspects of physiology, including neuronal transmission, vasodilatation and immunity (Schmidt & Walter 1994).

S-nitrosylation, the incorporation of a nitroso group to a reactive cysteine thiol to form an *S*-nitrosothiol (SNO), is now considered a major NO-mediated redox-based post-translational modification, and an increasing number of proteins related to different signalling pathways in animals, bacteria and plants have been shown to be modified by this process (Hess et al. 2005; Astier et al. 2012).

The *S*-nitrosylated derivative of the antioxidant tripeptide glutathione (GSH), *S*-nitrosoglutathione (GSNO), is the main non-protein SNO in cells and extracellular fluids (Liu et al. 2001; Gaston et al. 1993). This molecule, which acts as a bioactive reservoir of NO, can transfer NO to cysteine thiols of target proteins in a process called trans-*S*-nitrosylation. Thus, changes in the level of GSNO directly impacts upon the level of total protein *S*-nitrosylation. In this context, alterations in the metabolism of GSNO indirectly affect the metabolism of SNOs.

In 2001, Liu and co-workers showed that formaldehyde dehydrogenase (GS-FDH) is the enzyme responsible for metabolising GSNO with ammonia (NH3) and

glutathione disulphide (GSSG) being the end products of the reaction. This enzyme, which was renamed GSNO reductase (GSNOR), was shown to be necessary for SNO homeostasis in bacteria, yeast and mice as *gsnor* loss-of-function mutants presented higher levels of protein SNO (Liu et al. 2001; Liu et al. 2004).

Later, this mechanism was shown to be conserved in the plant model *Arabidopsis thaliana*(Feechan et al. 2005). Interestingly, *gsnor* loss-of-function plants are compromised in disease resistance, suggesting a major role for protein *S*-nitrosylation in plant immunity. More recently, *gsnor* loss-of-function has also been related to the immunodeficiency of mice against *Klebsiella pneumonia* (Tang et al. 2013), suggesting a role for protein *S*-nitrosylation in mammalian immunity.

Little is known about the role of *gsnor* in *D. melanogaster*. Only recently has a *gsnor* loss-of-function fly been generated (Kanchanawatee 2012). Here, by employing genetic and pharmacological tools, the function of GSNOR in *D. melanogaster* physiology and redox biology is further investigated.

3.1.1 Background results - The overlapping deletion technique

A very powerful approach towards studying the function(s) exerted by a given protein in a cell, tissue or organism, is to knock-out the corresponding gene. When feasible, this methodology can generate valuable phenotypic information and lead to a better understanding of the potential roles played by the given protein in the physiology of an organism.

To characterise the function(s) of GSNOR in *D. melanogaster*, the overlapping deletion technique (Figure 3-1) was used to generate *gsnor* loss-of-function flies (Kanchanawatee 2012). This method consists of crossing the two

deficiency lines *Df(3R)Exel7305/TM6B* and *Df(3R)Exel7306/TM6B*,in this thesis referred as Df7305 and Df7306,which have genomic deletions that overlap in a region covering the *gsnor* gene. Progeny from this cross, carrying both deletions, referred to here as *gsnor*$^{-/-}$ flies, can be selected by the absence of the balancers (*TM6B*).

Protein extracts from *gsnor*$^{-/-}$ flies do not show GSNOR activity. As presented in Figure 3-2, protein extracts from these flies, but not from wild type flies, are unable to metabolise GSNO. This can be detected spectrophotometrically by monitoring the conversion of NADH to NAD^+ at 340 nm. NADH, which is a cofactor, acting as an electron donor during the reaction, absorbs light at 340 nm while it's oxidised form, NAD^+, does not.

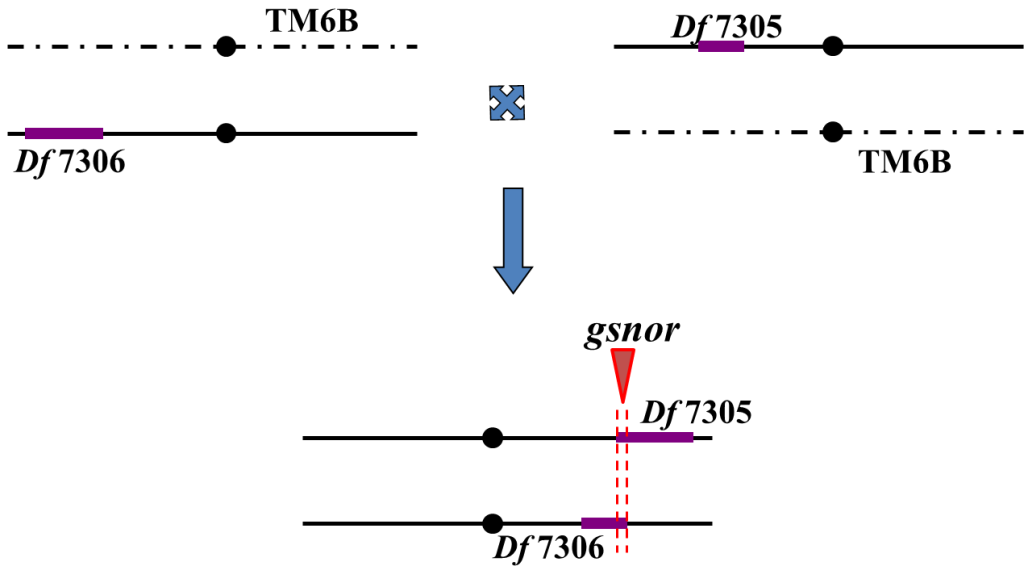


Figure 3-1: *gsnor*$^{-/-}$ flies generated by overlapping deletion technique

The deficiency lines *Df7305* and *Df7306* contain deletions on the third chromosome that precisely overlap on *gsnor* and thus can be crossed to generate *gsnor*$^{-/-}$ flies. Both deficiencies are homozygous lethal and kept as stocks over the balancer *TM6B*, which carries the mutations *Tb* and *Hu* with dominant phenotypes. *gsnor*$^{-/-}$ female flies are sterile so this line cannot be maintained as a stock.

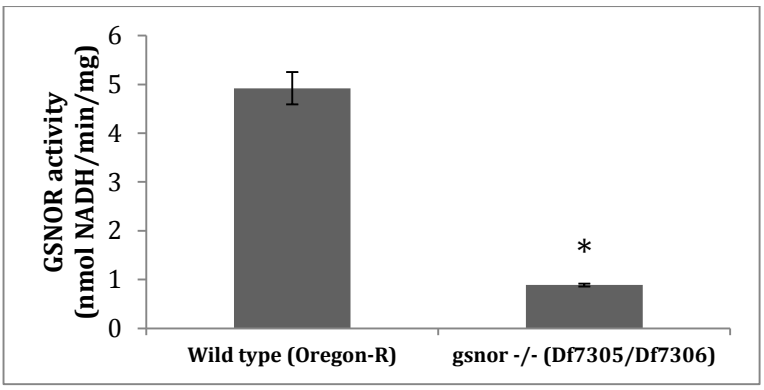


Figure 3-2: GSNOR activity of wild type and *gsnor*$^{-/-}$ overlapping deletion flies

Protein extracts from *gsnor*$^{-/-}$ overlapping deletion and wild type flies were assayed for GSNOR activity by spectrophotometrically measuring the rate of NADH oxidation in the presence of GSNO. Activity is shown per mg of protein per minute. Data points represent mean ± SEM (n=3), with asterisks indicating significant difference from the wild type samples (Student's t test, * $P < 0.01$).

3.3 *gsnor*$^{-/-}$ flies present higher levels of total *S*-nitrosylation

An increase in total *S*-nitrosylation has been associated with *gsnor* mutations in yeast (Liu et al. 2001), mice (Liu et al. 2004) and plants (Feechan et al. 2005). To investigate whether GSNOR activity is important for maintaining *S*-nitrosylation homoeostasis in *D. melanogaster*, the biotin-switch technique (BST) (Forrester et al. 2009) was employed to compare the SNO levels between *gsnor*$^{-/-}$ and wild type flies. An overview of the technique is illustrated in Chapter 2.

Briefly, this technique involves the extraction of total proteins from flies, blocking of free thiols (SH), reduction of S-nitrosothiols (SNO) to SH, and further labelling of SHs with N-[6-(Biotinamido)hexyl]-3'-(2'-pyridyldithio)propionamide (Biotin-HPDP). Ascorbate is used as the SNO reducing agent in the assay because it does not reduce other redox-based post-translational modifications of cysteine

residues such as glutathiolation, sulfenylation and disulphide bridges. After biotin labelling, or the "switch" step, proteins are precipitated and run on a non-reducing SDS-PAGE. Western blots using an anti-Biotin antibody are employed to detect biotinylated proteins, which should correspond to *S*-nitrosylated proteins (Described in details in Chapter 2).

To confirm that this method was specific and could be applied for detecting endogenously generated *S*-nitrosylation in protein extracts of *D. melanogaster*, experiments were carried out where omission of ascorbate and biotin-HPDP were used as negative controls. As presented in Figure 3-3, the anti-Biotin antibody did not detect any non-specific proteins in the proteome of wild type flies when Biotin-HPDP was omitted from the assay. The antibody, however, detected biotinylated proteins when only ascorbate was omitted from the BST, indicating that reductions of SNOs to SHs occurred even without the addition of the reducing agent. This might be explained by the labile nature of SNO bounds, which can be decomposed by exposure to light - although precautions were taken to reduce light exposure to minimum. Nevertheless, the intensity and number of proteins detected when both ascorbate and Biotin-HPDP were added to the samples were much higher, suggesting that the signal being detected was specific to biotinylation of free SHs originated from ascorbate-dependent SNO reduction.

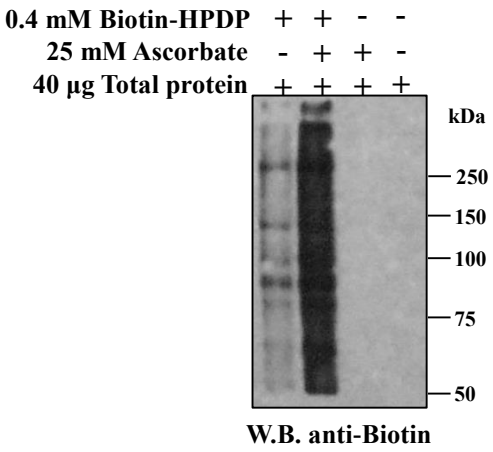


Figure 3-3: Detection of biotinylated proteins in the proteome of wild type flies after the BST

Protein extracts from wild type flies were submitted to the BST. Ascorbate and/or biotin-HPDP were omitted from reactions to test the specificity of the assay towards SNO and cross-reaction of the anti-Biotin antibody against proteins in the proteome of *D. melanogaster*. After the BST, were run in a non-reducing 8% SDS-PAGE, transferred to a nitrocellulose membrane and analysed by Western blot (W.B.) with an anti-Biotin antibody.

The BST was then used to compare the levels of *S*-nitrosylation between wild type and *gsnor*$^{-/-}$ flies. As it has been observed in other organisms (Liu et al. 2001; Liu et al. 2004; Feechan et al. 2005), absence of GSNOR activity leads to an increase in the levels of protein *S*-nitrosylation in *D. melanogaster*. As presented in Figure 3-4, the levels of protein *S*-nitrosylation (reflected by the levels of biotinylation) were higher in the protein extract of non-infected (N.I.) *gsnor*$^{-/-}$ flies compared to the protein extract of wild type flies in the same conditions. When flies were challenged by septic injury with the bacterial pathogen *Enterococcus faecalis* (*E.f.*), the levels of protein *S*-nitrosylation increased in wild type flies, suggesting that this process is responsive to infection. However, no increase in protein *S*-nitrosylation was seen in *gsnor*$^{-/-}$ flies two hours post infection (H.P.I.). Indeed, a reduction in the signal

intensity of some bands can be detected after infection.

One speculative explanation for the contrasting phenotypes between infected *gsnor*$^{-/-}$ and wild flies could be related to the regulation of dNOS activity. *S*-nitrosylation has been proposed to induce monomerization of human NOS3, which inhibits its activity (Ravi et al. 2004). Thus, it is possible that higher levels of *S*-nitrosylation in *gsnor*$^{-/-}$ flies inhibits further production of NO by dNOS in response to infection, and prevents further increases in protein *S*-nitrosylation.

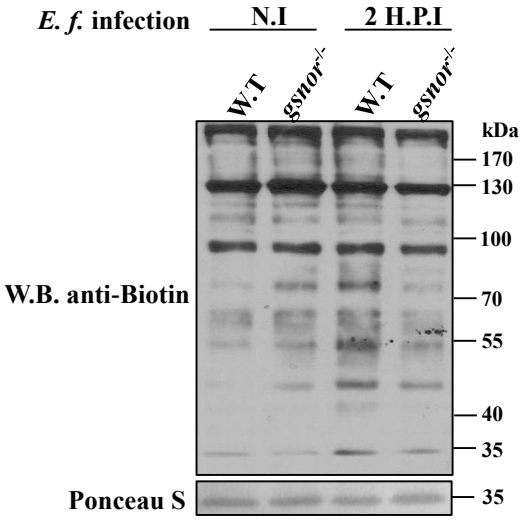


Figure 3-4: Increased SNO levels in non-infected *gsnor*$^{-/-}$ flies

Protein extracts from *gsnor*$^{-/-}$ and wild type flies at resting conditions and two hours after infection with *E. faecalis* were submitted to the BST to compare the levels of total protein S-nitrosylation. Groups of 10 female flies were used in these experiments. A small tungsten needle coated with *E. faecalis* (from an overnight culture, O.D. 1) was used to infect flies by pircing the thorax of the animals. Flies were kept at 25°C for two hours and submitted to protein extraction and quantification. The same amount of protein (~20 µg) was used in the assay. After the BST, proteins were run in an 8% non-reducing SDS-PAGE, transferred to a nitrocellulose membrane and an anti-Biotin antibody was used to detect biotinylated proteins. This experiment was repeated twice with similar results.

3.3 *gsnor*$^{-/-}$ flies are less tolerant to SNP-induced nitrosative stress

Sodium nitroprusside (SNP) is a water-soluble sodium salt consisting of a

ferrous ion surrounded by five cyanide moieties and a nitrosylated group - $Na_2[Fe(CN)_5NO]$. It is used as a NO releasing pro-drug in humans and has been shown to induce nitrosative stress in *D. melanogaster* when orally administrated (Lewinsky et al. 2012). To investigate whether GSNOR activity is important for flies to cope with the stress caused by this chemical compound, *gsnor*$^{-/-}$ and wild type flies were fed overnight on a 5% sucrose solution supplemented with 2 mM SNP, and the number of survivors was recorded next morning. To ensure synchronous feeding of all individuals, a two hours starvation period preceded treatments and, as a control, half of the flies were fed on 5% sucrose solution without SNP (Figure 3-5).

While 2 mM SNP killed less than 10% of the wild type flies after 16 hours of treatment, almost 80% of the *gsnor*$^{-/-}$ flies were dead after the same period of time. *gsnor*$^{-/-}$ and wild type flies fed only on 5% sucrose solution presented 92% and 97% survival, respectively, after 16 hours treatment. These results indicate that *gsnor*$^{-/-}$ flies are less tolerant to SNP treatment-induced nitrosative stress and this phenotype is likely to be related to the lack of GSNOR activity.

However, it should be pointed out that to make a stronger conclusion about these results, further methods should be employed to measure feeding rates between treatments. For instance, if *gsnor*$^{-/-}$ flies fed more than wild type flies, these could have a direct implication on the survival of these flies. There is a number of different fly feeding approaches (Reviewed in Marx 2015) that could be used to answer this question. One such approach is the capillary feeder assay (Ja et al. 2007), which allows precise and real-time measurement of food ingestion by flies.

Another point to be considered when analysing these results regards the fact that 24 hours fasting periods have been shown to induce oxidation of protein thiols in

D. melanogaster (Menger et al. 2015). Thus, if a two hours starvation period induces oxidative stress, this could have a stronger impact on *gsnor*$^{-/-}$ flies. Therefore, the effects of two hours starvation should on the redox status of the flies should also be assessed in the future.

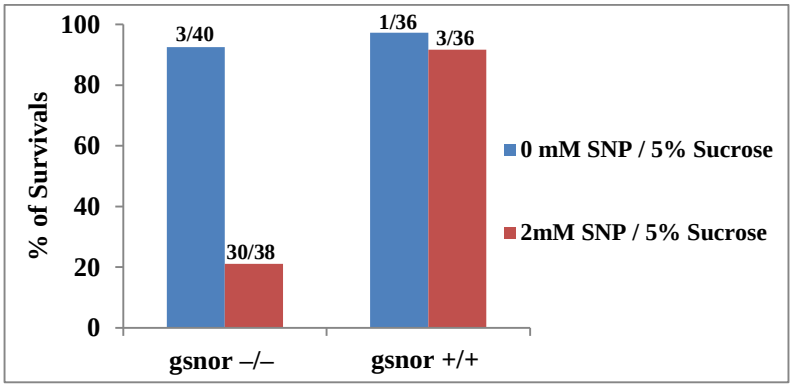


Figure 3-5: *gsnor*$^{-/-}$ flies are less tolerant to SNP treatment

Groups of 36 to 40 adult female flies were transferred to empty vials and starved for two hours at 25°C. Flies were then transferred to vials containing a cotton roll soaked in 5% sucrose solution supplemented, or not, with 2 mM SNP. Vials were kept at 25°C and the number of survivors was recorded after 16 hours. Graphs represent the percentage of survivals after given time. The number on the top of each bar chart shows the number of deaths after 16 hours over the total number of flies tested.

In addition to NO, SNP can also release cyanide anions *in vivo*, that can be highly toxic to animals (Belani et al. 2014). To further investigate whether the reduced tolerance of *gsnor*$^{-/-}$ flies to SNP was caused by the lack in GSNOR activity, and to confirm that the toxicity is caused by the release of NO and not cyanide anions, survival assays were carried out to analyse the tolerance of *gsnor* complementation flies as well as *gsnor* over expresser flies in comparison to *gsnor*$^{-/-}$ overlapping deficiencies and wild type flies.

Complementation flies (*Ubi-GAL4*/*UAS-gsnor*; *Df7305*/*Df7306*) were generated by crossing the $gsnor^{-/+}$single deficiency *Df7306* carrying an *Ubi-GAL4* construct inserted on the second chromosome (*Ubi-GAL4*; *Df7306*) and the other $gsnor^{+/-}$ single deficiency *Df7305* carrying an *UAS-gsnor* construct also on the second chromosome (*UAS-gsnor*; Df7305). The over-expresser flies - O.E. (*Ubi-GAL4*/*UAS-gsnor*; +/+) were generated by crossing an *Ubi-GAL-4*; +/+ line and an *UAS-gsnor*; +/+ line. As a $gsnor^{+/+}$ control in these experiments, *Ubi-GAL-4*; +/+ flies were used.

As presented in Figure 3-6, when compared to wild type flies, both female (Figure 3-6-A) and male (Figure 3-6-B) $gsnor^{-/-}$ flies are shown to be significantly less tolerant to SNP. However, *gsnor* seems to play a more important role in the response of females to this stress as while only 20% of the female $gsnor^{-/-}$ flies survived after one day of SNP treatment, 50% of the males were still alive. In addition, complementation of these flies with *UAS-gsnor* driven by *Ubi-GAL4*partially restored the tolerance of females but had no effect on males. Furthermore, over-expression of *gsnor* in wild type female flies (*gsnor* O.E.) made these animals more tolerant to SNP treatment, but had no impact on the tolerance of male flies. *gsnor* O.E female flies were significantly more tolerant to SNP compared to wild type flies whereas *gsnor* O.E male flies showed similar survival to the other genotypes.

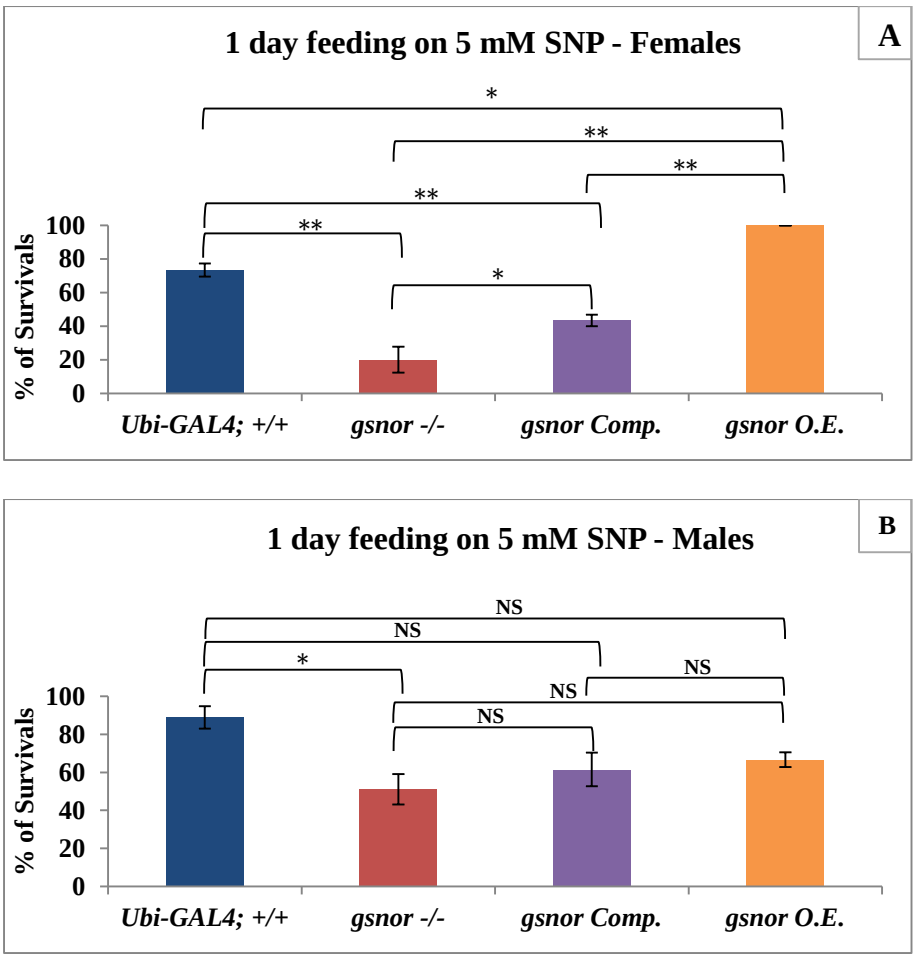


Figure 3-6: GSNOR activity confers tolerance to SNP toxicity.

Groups of 15 males (A) and female (B) flies, aged between 4 and 7 days, were transferred to empty vials and starved for two hours at 25°C. After that, flies were transferred to vials containing a cotton roll soaked in 5% sucrose solution supplemented with 5 mM SNP. Vials were kept at 25°C and the number of survivors was recorded one day after the treatment. Based on one-way ANOVA ($F_{3,8}$ = 5.27 p< 0.05 for males and $F_{3,8}$ = 56.68 p< 0.001 for females), followed by Tukey HSD test, NS indicates non-significant differences, * and ** indicate significant differences between treatments, p ≤ 0.05 and p ≤ 0.01 respectively. The data represent the mean of three vials with 15 flies each (±SE). This experiment has been repeated twice with similar results.

When these experiments were extend to analyse the effect of SNP treatment on the survival of flies over a longer period of time (Figure 3-7), the importance of GSNOR activity in conferring tolerance to SNP was even more evident, especially for female flies. As presented in Figure 3-7-A, 100% of *gsnor* O.E females survived

for three days of continuing treatment with 5 mM SNP, while all the other genotypes showed only 15-20% survival.

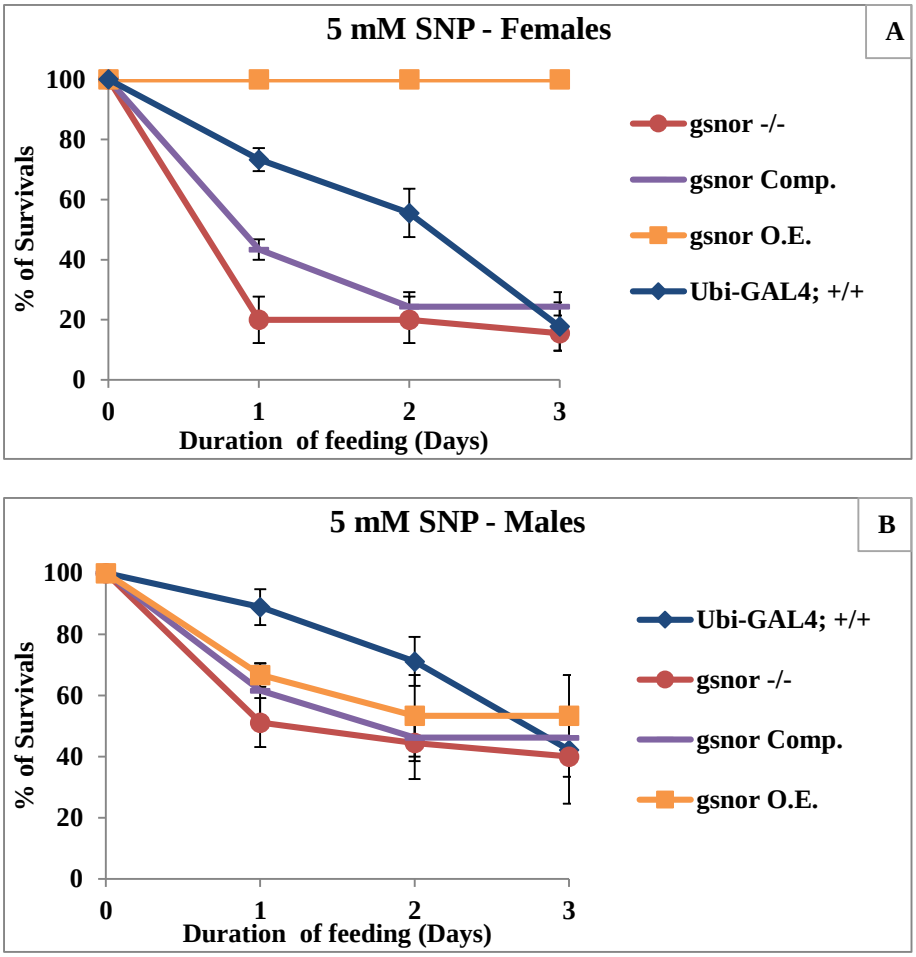


Figure 3-7: Survival of flies in response to SNP treatment

Groups of 15 female (A) and male (B) flies, aged between 4 and 7 days, were transferred to empty vials and starved for two hours at 25°C. After that, flies were transferred to vials containing a cotton roll soaked in 5% sucrose solution supplemented with 5 mM SNP. Vials were kept at 25°C and the number of survivors was recorded at one, two and three days after the first treatment. Fresh solution was added to the cotton roles every day. The data represent the mean of three independent experiments (±SE).

NO released by SNP is likely to be scavenged by GSH, forming GSNO and SNOs. Due to the lack of GSNOR activity, GSNO accumulates, increases the levels

of total SNO, and this potentiates the deleterious effects of SNP in *gsnor*$^{-/-}$ flies. The results presented here indeed suggest that GSNOR activity is required for flies to tolerate SNP-induced nitrosative stress. Interestingly, the activity of this enzyme seems to be more important for female flies to tolerate SNP.

However, if this untested trend is real, this phenotype could also be explained by differences in the amount of food ingested by males and females in the same period of time. If females consumed more SNP-treated food than males, this could certainly impact the response to the drug. Thus, further tests to compare food consumption of males and females should be employed to test this hypothesis. Another observation is the fact that *gsnor* over expression does not increase SNP tolerance in males although it does it substantially in females. The reasons for this phenotype are not clear, could be related to the differences in redox status between males and females, but will need further studies for any conclusions to be drawn.

Interestingly, it has been reported before that male and female flies also respond differently to GSNO treatment (Lewinsky et al. 2013). These authors have shown that, in a concentration-dependent manner, larvae fed on GSNO generated male flies with increased superoxide dismutase (SOD), glutathione *S*-transferase (GST) and glucose-6-phosphate dehydrogenase (G6PDH) activities and decreased catalase (CAT) activity. However, females generated from the same treatments did not show any significant alteration in G6PDH and CAT activities. Furthermore, the concentrations of GSNO necessary to alter enzyme activity in males were significantly lower when compared to female flies.

Thus, it is possible that male flies are able to respond more effectively to SNP-induced nitrosative stress by either up or down-regulating the activity of

antioxidant-related enzymes. A decrease in CAT activity, for example, might be a strategy employed by plants and animals to counteract nitrosative stresses. As will be discussed in more details in the end of this chapter, a mutation in *Cat* suppresses some of the phenotypes caused by *gsnor* loss-of-function in plants (Brzezek 2014) and flies.

3.2 *gsnor*$^{-/-}$ flies are more tolerant to paraquat-induced oxidative stress

Paraquat (1,1-dimethyl-4,4-bipyrimidyl chloride) is a nonselective herbicide widely used for broadleaf weed control. It is highly toxic to animals and its toxicity has been attributed to the excessive generation of reactive oxygen species (ROS) and oxidation and depletion of NADH from cells (Suntres 2002). It has been reported that loss of *gsnor* function in *Arabidopsis thaliana* induces higher tolerance of these plants to paraquat and reduces paraquat-induced programed cell death (PCD) (Chen et al. 2009). Although the reason for this phenotype is not completely understood, *gsnor* is believed to act downstream of superoxide generation as, according to Chen et al. (2009), both *gsnor*$^{-/-}$ and wild type plants produced similar levels of superoxide in response to paraquat treatment.

Aiming to further characterise the phenotypes associated with knocking-out *gsnor* in *D. melanogaster,* and to explore a possible mechanism conserved between plants and animals, *gsnor*$^{-/-}$ and wild type flies were compared with regards to their capacity to tolerate paraquat-induced oxidative stress. Higher concentrations of paraquat were used for females as pilot experiments showed that males died too rapidly in 20 mM paraquat while females survived much longer in the same concentration. As presented in Figure 3-8, when fed on 20 mM paraquat, more than

80% of male wild type flies were dead within 24 hours of feeding. However, for the same conditions, only 30% of female wild type flies died. Thus, males and females were next fed on 5% sucrose solutions containing 10 and 30 mM paraquat, respectively (Figure 3-9 A and B).

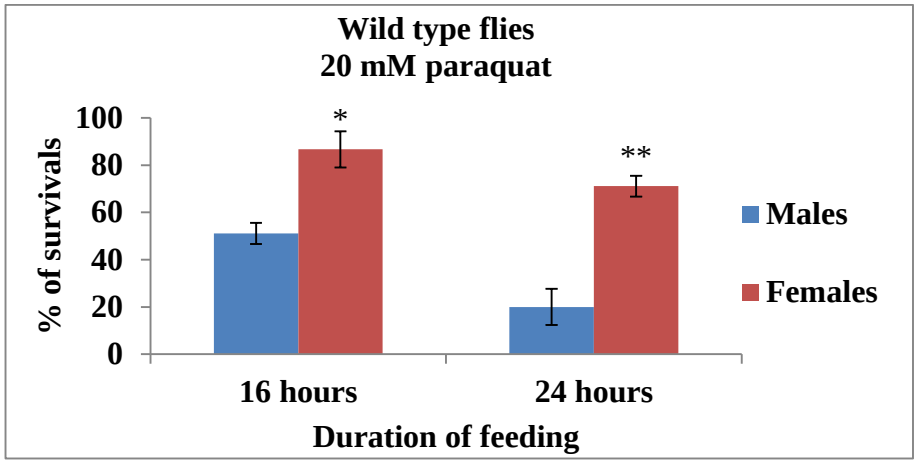


Figure 3-8: Wild type female flies are more tolerant to paraquat than males

Groups of 15 wild type Oregon-R flies, five days old, were transferred to empty vials and starved for two hours at 25°C. After that, flies were transferred to vials containing a cotton roll soaked in 5% sucrose solution supplemented with 20 mM paraquat. Vials were kept at 25°C and the number of survivals was recorded at 16 and 24 hours after the animals started feeding. Bars with asterisk indicate a statistically significant difference between the males and females (Independent Samples t-test, *P < 0.05, **P < 0.01).

As presented in Figure 3-9-A, male *gsnor*$^{-/-}$ flies are more tolerant to paraquat than either wild type or *gsnor*$^{-/+}$ single deficiency flies. These results indicate that GSNOR activity plays a role in the tolerance of *D. melanogaster* to paraquat and suggests that the mechanism behind this tolerance is conserved between plants and animals.

However, as it can be noted in Figure 3-9-B, although *gsnor*$^{-/-}$ female flies also seemed more tolerant to paraquat than wild type female flies, the percentage of survivals was similar to that presented by the single deficiency flies. Perhaps the concentration of paraquat used for the experiments with females was too high and the tolerance conferred by the deletion of *gsnor* was not sufficient to increase survival in these conditions. It is also possible that heterozygous deletions have a phenotype only in female flies, conferring some tolerance to these animals.

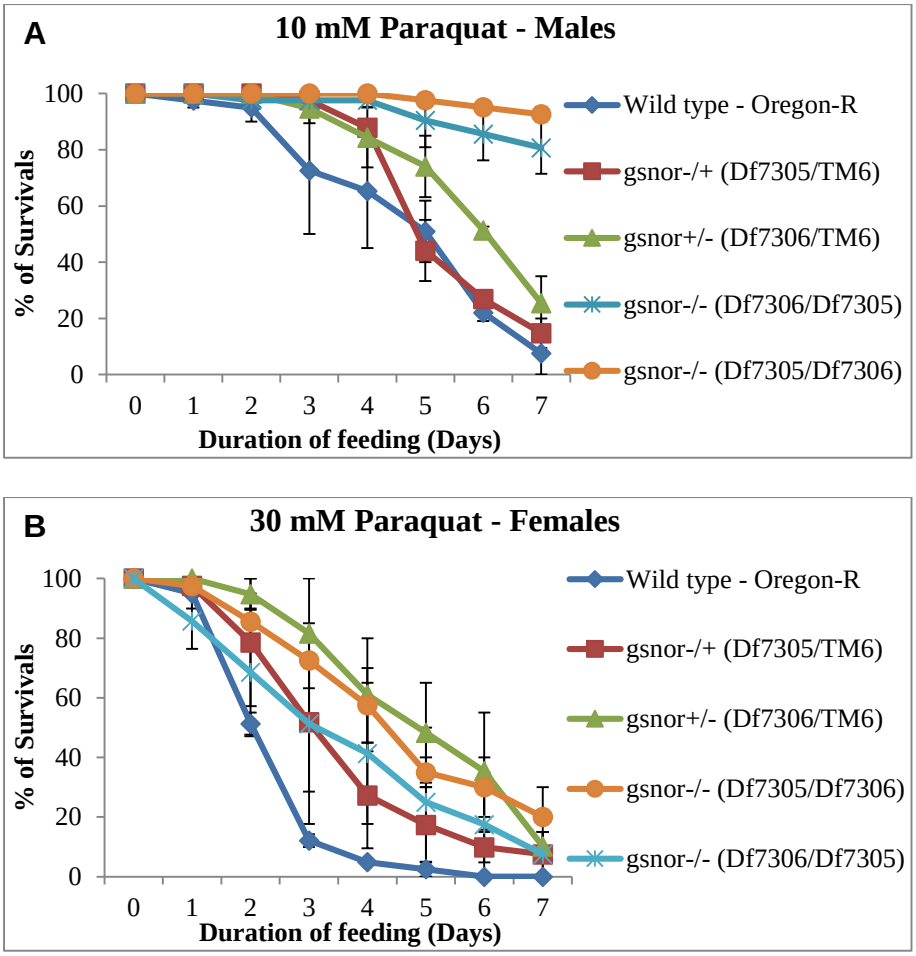


Figure 3-9: *gsnor*$^{-/-}$ flies are more tolerant to paraquat.

Groups of 20 male (A) or female (B) flies, aged between 3 and 6 days, were transferred to empty vials and starved for two hours at 25°C. After that, flies were transferred to vials containing a cotton roll soaked in 5% sucrose solution supplemented with 10 mM (A) or 30

mM (B) paraquat. Vials were kept at 25°C and the number of survivors was recorded every day for seven days. Fresh solution was added to the cotton roles every day. The data represent the mean of two independent experiments (±SE).

Sexually dimorphic genetic effects on *D. melanogaster* response to paraquat treatment have been reported before. The Keap1/Nrf2 system is conserved from mammals to flies and protects organisms against the detrimental effects of oxidative stress by activating the transcription of antioxidant-related genes. Heterozygous mutations in *D. melanogasterkeap1*, the inhibitor of the transcription factor Nrf2, increased the oxidative stress tolerance to paraquat of males but had no effect on the tolerance of female flies (Sykiotis & Bohmann 2008).

Interestingly, the NO donor S-nitroso-N-acetylpenicillamine (SNAP) has been shown to induce *S*-nitrosylation of Keap1 in rat pheochromocytoma (PC12) cells, and this process was shown to induce activation of Nrf2 (Um et al. 2011). Thus, it is plausible to speculate that the higher tolerance of $gsnor^{-/-}$ flies to paraquat is caused by higher levels of Keap1 *S*-nitrosylation. This could lead to an increase in Nrf2 nuclear translocation and transcription activation of redox-detoxification-related genes such as superoxide dismutase (SOD) and catalase (CAT). Higher expression of these genes could protect flies against deleterious effects of paraquat-induced oxidative stress.

In accordance with our results, gender-related differences in *D. melanogaster* response to paraquat have been reported before. Chaudhuri and co-workers (2007) have shown that male flies are more susceptible to paraquat than females. By using paraquat as an inducer of Parkinson-like symptoms in flies, these authors have also related paraquat-induced oxidative stress with loss of dopaminergic neurons in the

fly's brain.

D. melanogaster is a valuable model system to study human neurodegeneration (Hirth 2010). Interestingly, it has been reported that the rate of Parkinson's disease is 1.5 times greater among men compared to women (Wooten et al. 2004). Although the results presented here suggest a sex-related difference in response to paraquat in flies, as discussed before, differences in feeding behaviour between males and females could influence the results for this type of assay.

Therefore, feeding-independent strategies to induce oxidative stress should be tested. One alternative would be to compare the response of these flies to higher concentrations of oxygen (hyperoxia) or to inject the animals with different concentrations of paraquat using a microinjection system. Both methods would help uncovering not only the difference in response between genders, but also between genotypes as we still do not know whether *gsnor* knockouts affect feeding behaviour in flies.

3.5 Male and females flies respond differently to *B. bassiana* infection

There is a clear difference in the way male and female flies cope with oxidative stress. Stress induced by SNP, a NO donor, seems to have a greater effect on the survival of females, while paraquat, an inducer of ROS production, shows more toxicity to males. A deletion of *gsnor*, as expected, caused a lower tolerance of flies to SNP, however, it increased the tolerance to paraquat.

Previous results from our lab have shown that $gsnor^{-/-}$ female flies were more susceptible to infections caused by the entomopathogenic fungus *B. bassiana* (Kanchanawatee 2012). Changes in the redox state are known to play a major role in

immunity (Wink et al. 2011; Jabaut & Ckless 2012). The rapid production of both ROS and NO is one of the earliest immune responses of an organism after detecting a pathogen. Because male and female flies behave differently in response to oxidative and nitrosative stresses, it seemed sensible to investigate whether *gsnor* loss of function also compromises the disease response of male flies to *B. bassiana*.

Before analysing the effect of *gsnor* loss of function in the flies, the survival of wild type males and female flies were compared in response to *B. bassiana* infection. Interestingly, as presented in Figure 3-10, there was also a clear difference in the survival of male and female flies infected with this microorganism. At least for the conditions used to infect flies in the laboratory, where groups of 15-30 flies are transferred to 2 mL tubes containing spores of *B. bassiana* and gently shaken for two minutes, male flies survived longer than females after infections. While only 25% of female flies survived for 14 days of the experiment, around 70% of the males were still alive. These results have been reproduced at least three times and different *D. melanogaster* genotypes have shown similar responses.

Caution should be taken when interpreting these results. For example, one could argue that females are bigger than males and this difference in body size would mean that a bigger surface area is exposed to the spores during the infection procedure. This would lead to a higher load of spores being attached to a female's body and a more aggressive infection as a consequence. However, the amount of spores used to infect the flies is excessively high and this is probably an unlikely reason for the differences presented by males and females.

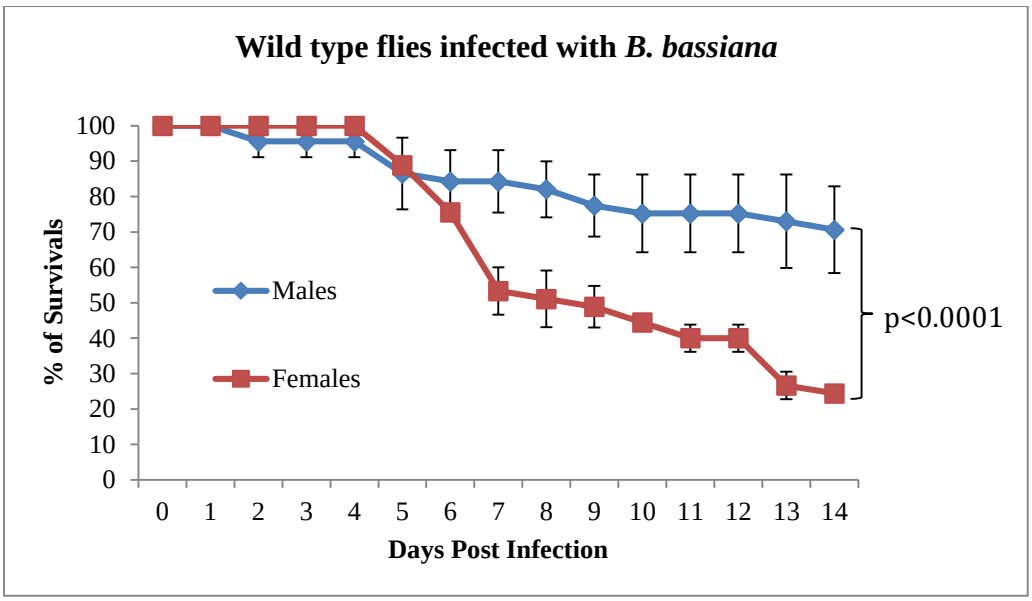


Figure 3-10: Female flies are more susceptible to *B. bassiana* than males

Groups of 15 males or females wild type Oregon-R flies, aged between three and six days, were transferred to 2 mL tubes containing similar amounts of spores of *B. bassiana* and gently shaken for two minutes. Flies were transferred to new vials containing standard cornmeal media and kept at 29°C. The number of flies surviving was recorded every 24 hours after infections and the flies that were alive were transferred to new vials every other day. The Log-rank test demonstrated a statistically significant differences in survival of wild-type males and females ($p<0.0001$). Error bars represent ±SE of three vials with 15 flies each. This experiment has been repeated three times using different *B. bassiana* spores and different *D. melanogaster* genotypes and presented similar results.

Another hypothesis for the higher susceptibility of female flies to *B. bassiana*, as mentioned before, could be related to oxidative stresses response. As presented before, *D. melanogaster* males and females respond differently to these stresses. If there is a correlation between oxidative and immune responses in flies, this could lead to differences in in the way males and females respond to infections. To investigate whether GSNOR activity is as important to *D. melanogaster* male's immunity against *B. bassiana* as it has been shown to be for female's (Kanchanawatee 2012), the survival of *gsnor*$^{-/-}$ male flies infected with this

microorganism was analysed.

As is the case with females, *gsnor*$^{-/-}$ male flies were more susceptible to *B. bassiana* infection than wild type and *gsnor*$^{-/+}$ single deficiency flies (Figure 3-11-A). Furthermore, as highlighted in Figure 3-11-B, complementation of *gsnor*$^{-/-}$ flies with *UAS-gsnor* driven by *Act-GAL4* restored the resistance of these flies to *B. bassiana* infections. These results indicate that GSNOR activity is also required for male flies to respond effectively to *B. bassiana* infections.

It is worth noting that the virulence of *B. bassiana* varies according to the age of the spores. These variations have a direct effect on the survival of infected flies, and for this reason the results from different survival experiments should not be compared. For each survival experiment, spores from the same culture were used to make sure they were similarly virulent to the flies. The high mortality presented in Figure 3-11 is likely to be due to the virulence of the particular spores. This might also explain the sudden drop in survival of *gsnor*$^{-/-}$ at day four after infection

Because male flies seem to be more resistant to *B. bassiana* infections, sometimes the differences in susceptibility between wild type and *gsnor*$^{-/-}$ flies are not as clear as with female flies. This is more evident when less virulent spores are used. For this reason, females, which present more evident and consistent disease phenotypes, were chosen for most of the survival assays presented in this work.

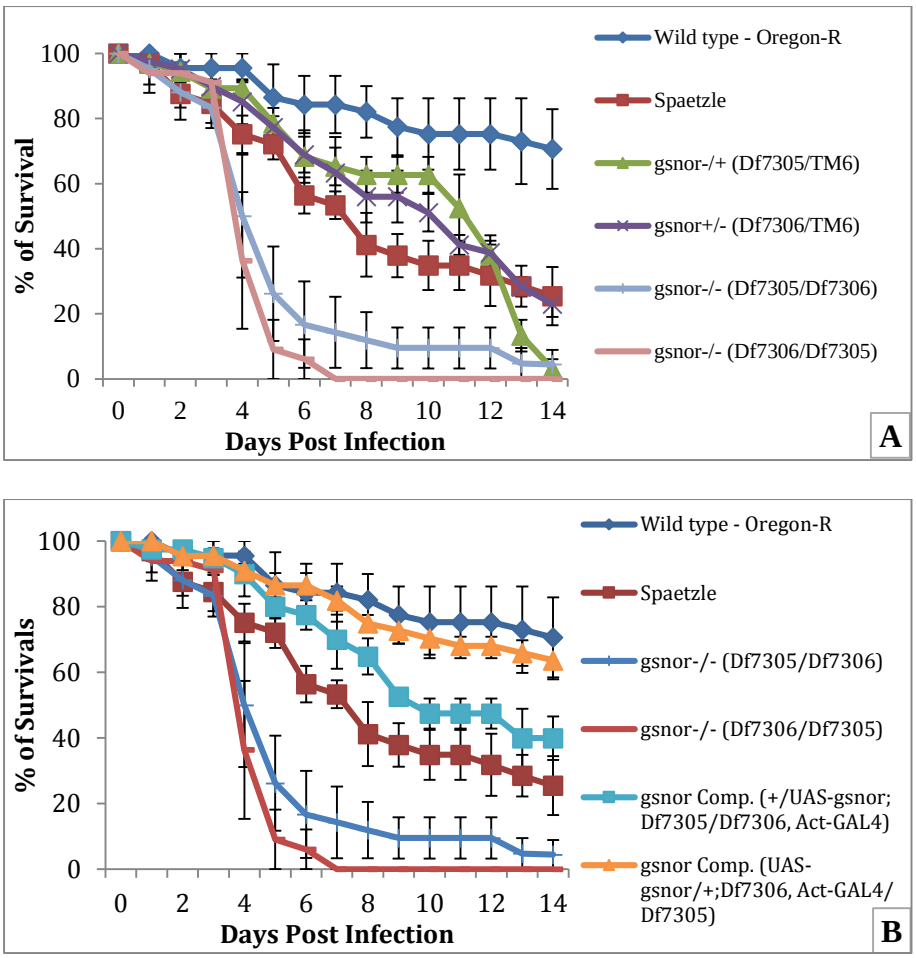


Figure 3-11: *gsnor*$^{-/-}$ males flies are highly susceptible to *B. bassiana* infection

Groups of 15 males flies, aged between three and six days, were transferred to 2 mL tubes containing similar amounts of spores of *B. bassiana* and gently shaken for two minutes. Flies were transferred to new vials containing standard cornmeal media and kept at 29°C. The number of flies surviving was recorded every 24 hours after infections and the flies that were alive were transferred to new vials every other day. Graph A highlights the differences in survival between *gsnor*$^{-/-}$ flies, *gsnor*$^{-/+}$ single deficiencies, wild type and spätzle flies. Graph B highlights the restoration in the survival of *gsnor*$^{-/-}$ flies complemented with *UAS-gsnor/Act-GAL4*. Data for both graphs were generated in the set of experiments. The data represent the mean of three vials with 15 flies each (±SE). This experiment has been repeated twice with similar results.

3.5 *Cat*$^{-/+}$ heterozygosity partially suppresses *gsnor*$^{-/-}$ immunodeficiency

gsnor$^{-/-}$ flies were shown to be more susceptible to infections caused by the

entomopathogenic fungus *B. bassiana* (Kanchanawatee 2012). In *Arabidopsis thaliana*, additional mutations in one of the catalase genes (*Cat3*) have been shown to suppress some of the phenotypes caused by a mutation in *gsnor*, including disease susceptibility to the bacterium *Pseudomonas syringae* DC300 (*avrRps4*) (Brzezek 2014).

Catalase is a key antioxidant enzyme. It protects the cells against oxidative stress-associated damage by catalysing the conversion of H_2O_2 to H_2O and O_2. In *D. melanogaster*, there are two genes coding for proteins with catalase activity. *Cat* codes for an intracellular catalase (Griswold & Matthews 1993), while an i*mmune-regulated catalase* (*IRC*) codes for a secreted enzyme with catalase activity that has recently been shown to be important in *D. melanogaster* gut immunity (Ha et al. 2005).

To explore whether a mutation in a *cat* gene, like in *A. thaliana*, could recover the phenotypes caused by the deletion of *gsnor* in *D. melanogaster*, $gsnor^{-/-}$ flies carrying a point mutation in *Cat* ($Cat^{n1/+}$) were generated and analysed for susceptibility to *B. bassiana* infection. *Cat* is located on the third chromosome (3L:18,822,604..18,828,188) and Cat^{n1} has a recessive lethal phenotype. $Cat^{n1/+}$ flies have been shown to have only 44% catalase activity compared to wild type flies (Mackay & Bewley 1989). To generate $gsnor^{-/-}$ overlapping deficiencies flies carrying Cat^{n1}, this allele was first recombined to the chromosome with the deletion *Df7305* to generate the stock Cat^{n1}, *Df7305/TMB*. These flies were then crossed with *Df7306/TMB* to generate Cat^{n1}, *Df7305/Df7306*, which were used for experiments.

As presented in Figure 3-12, Cat^{n1} had a positive effect on the survival of $gsnor^{-/-}$ flies infected with *B. bassiana*. The percentage of survivors of the $gsnor^{-/-}$

flies carrying *Cat*^{n1} was increased in comparison to the percentage of survivors of *gsnor*$^{-/-}$ flies without *Cat*^{n1}and similar to that of single deficiencies and wild type flies. Although flies carrying only *Cat*^{n1} have not been included in this experiment, *gsnor* single deficiencies carrying *Cat*^{n1} were included as positive controls and presented an improvement in survival compared to single deficiencies without *Cat*^{n1}.

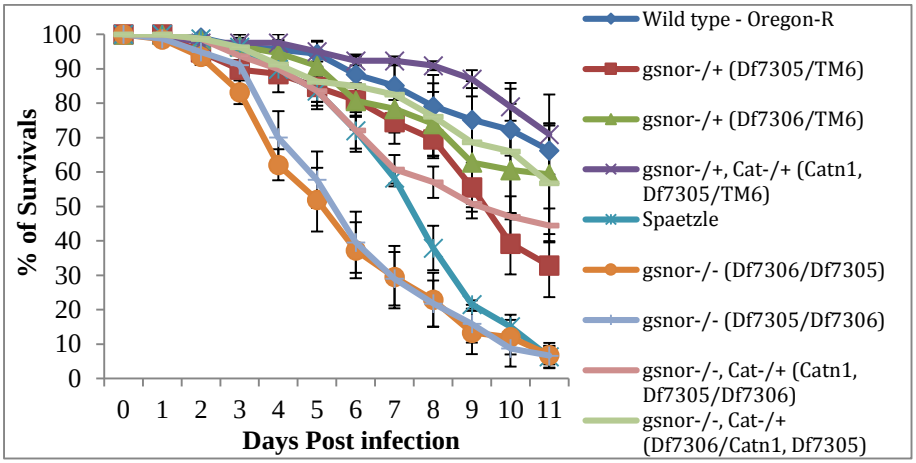


Figure 3-12: *Cat*^{n1} supresses *gsnor*$^{-/-}$ immunodeficiency phenotype

Groups of 20 females flies, aged between three and six days, were transferred to 2 mL tubes containing similar amounts of spores of *B. bassiana* and gently shaken for two minutes. Flies were transferred to new vials containing standard cornmeal media and kept at 29°C. The number of flies surviving was recorded every 24 hours after infections and the flies that were alive were transferred to new vials every other day. Oregon-R and *spätzle* flies were used as positive and Toll negative controls, respectively. All *gsnor* single and overlapping deficiencies, with and without *Cant*^{n1}, are included in the graph. The data represent the mean of four independent experiments (±SE), each experiment with three vials, and each vial with 20 flies.

These results suggest that both *Cat* and *gsnor* take part in a process involved in controlling the immune response of *D. melanogaster* to *B. bassiana*, and perhaps other pathogens. This process seems to be evolutionary conserved between plants and animals as similar results were seen in *A. thaliana* (Brzezek 2014). Although not

completely understood, the mechanisms behind this process are likely to involve a balance between the levels of SNOs and H_2O_2 during immune response.

Despite being toxic to cells, H_2O_2 also functions as an important molecule in immune responses, either directly by inducing the formation of more reactive ROS and killing invader microorganisms, or indirectly as a signalling molecule in the immune response (Torres et al. 2006). H_2O_2 originates from the dismutation of superoxide (O_2^-), which, during immune responses, is predominantly produced by a class of NADPH oxidases enzymes (NOXs) (Bedard & Krause 2007).

In *A. thaliana*, two NADPH oxidases, AtRBOHD and AtRBOHF, have been associated with pathogen-triggered ROS production (Torres et al. 2002). Interestingly, one of these oxidases, AtRBOHD, and its homologues in humans and *D. melanogaster,* were demonstrated to be *S*-nitrosylated. This modification was further shown to impair O_2^- production (Yun et al. 2011).

Thus, one possible explanation for Cat^{n1} suppression of $gsnor^{-/-}$ immunodeficiency phenotype could be related to a decrease in O_2^- generation due to excessive *S*-nitrosylation of dNOX in the $gsnor^{-/-}$ overlapping deficiencies. Lower levels of O_2^-, and H_2O_2 as a consequence, would partially compromise the immune response of these animals. However, a reduction in catalase activity, conferred by Cat^{n1}, could restore the levels of H_2O_2, partially supressing the phenotypes.

Another point to be considered when discussing the interplay between H_2O_2 and NO is the shared feature of these two molecules in signalling through post-translation modification of cysteine thiols. An example of this interplay is highlighted when we look at the bacterial transcriptional factor oxygen stress regulator (OxyR). This transcription factor, depending on the redox state of the cell,

can be regulated by different thiol modifications, such as *S*-nitrosylation, *S*-glutathiolation and disulphide bonding and, according to the modification, it activates the transcription of specific redox-responsive genes (Kim et al. 2002). Thus, perhaps an increase in H_2O_2 could activate the expression or activity of immune-related proteins that were supressed by GSNO.

Moreover, it has been known for a long time that both NO and H_2O_2modulate each other's production and turnover. One example is the interaction between NO and Cat (Deisseroth & Dounce 1970) where NO negatively regulates CAT activity *in vitro* (Clark et al. 2000; Brown 1995). Intriguingly, the pre-treatment of murine macrophage-like cells with NO donors has been shown to induce the production of CAT and protect the cells against H_2O_2-induced apoptosis (Yoshioka et al. 2006). On the other hand, in the same cell model, catalase itself appears to induce the expression of iNOS (Jang et al. 2004).

Another hypothesis relates to the fact that ROS could decompose GSNO (Manoj & Aravindakumar 2000; Manoj & Aravindakumar 2003). An increase in H_2O_2 levels could decrease GSNO levels and restore the balance caused by the absence of GSNOR activity. Moreover, although H_2O_2 can be directly decomposed by CAT, it can also be removed by GSH, generating GSSG and water (Gill & Tuteja 2010). All these speculations stress the complexity of the interactions and interplay between H_2O_2 and NO or SNOs, and also *Cat* and *gsnor*. Any changes in the level of either SNO or H_2O_2 seem to have an effect on the other. Disturbing NO bioavailability by mutating *gsnor* seems to interfere with the redox balance homeostasis and the same is true for a mutation in *Cat* and consequent increase in H_2O_2. However, for a reason that remains to be elucidated, a balance seems to be

partially restored when both enzyme activities are reduced.

3.6 Conclusions

GSNOR activity is essential for the maintenance of redox homeostasis in *D. melanogaster*. It controls total levels of protein *S*-nitrosylation and is necessary for flies to cope with nitrosative and oxidative stresses induced by chemical compounds. An intriguing difference between males and females is shown to occur not only in response to chemical inducers of nitrosative/oxidative stresses but also in response to infection with the entomopathogenic fungus *B. bassiana*.

The higher susceptibility of *gsnor*$^{-/-}$ flies to *B. bassiana* was further investigated. It was found that *gsnor*$^{-/-}$ male flies, similarly to what has been reported for females, succumb more rapidly to infections when compared to wild type flies. Furthermore, based on the fact that a mutation in *Cat* restores some of the phenotypes of *gsnor*$^{-/-}$ in plants, an interplay between *Cat* and *gsnor* during the immune response of *D. melanogaster* was explored. In what seems to be a conserved mechanism between plants and animals, an additional point mutation in *dCat* partially restored the immunodeficiency phenotype of *gsnor* loss of function flies and made these animals less susceptible to *B. bassiana*.

In the next chapter, the effects of *gsnor* loss of function in *D. melanogaster* immunity are further explored. By testing different classes of pathogens and analysing the expression of immune responsive genes, it is proposed that a dysfunction of the Toll signalling pathway is causing the immunosuppression phenotype associated with the deletion of *gsnor*.

Chapter 4

GSNOR and *D. melanogaster* immunity

4.1 Introduction

Drosophila research has played leading roles in many fields of science and all started over a hundred years ago when discoveries made by Thomas Hunt Morgan and colleagues established the chromosomal basis of inheritance (Morgan 1910; Morgan 1915), After that, the fruit fly has become one of the most studied multi-cellular model organisms and it has laid the ground for numerous discoveries in the fields of genetics and epigenetics, developmental biology, neurobiology and immunology (Wangler et al. 2015; Jennings 2011).

Given the evolutionary conservation of many signalling pathways that control development, metabolism and immunity, plus the practical advantages of working with a small and genetically simple organism, *D. melanogaster* has become one of the most powerful models with which to study biological processes in animals. Knowledge obtained from studies on fruit flies can often be extrapolated to other animals, such as humans. It is estimated that approximately 75% of disease-related genes in humans have functional orthologues in the Drosophila genome (Reiter et al. 2001).

Although Drosophila, like other insects, lacks an adaptive immune system, this does not deprive these organisms from responding rapidly and efficiently against microbial infections. These rapid and effective responses are achieved by employing a sophisticated innate immune system (Lemaitre & Hoffmann 2007), and this system

is conserved from insects to humans (Buchon et al. 2014).

The hallmark of innate immunity is a challenge-inducible production of anti-microbial peptides (AMPs), which, in flies are produced in, and secreted from, the fat body (equivalent to the mammalian liver) into the haemolymph (equivalent to mammalian blood), where they combat invader microorganisms (Hoffmann 2003). Once this system is triggered, it leads to the activation of Nuclear Factors kappa B(NF-kB transcription factor), resulting in the expression of immune-inducible genes, including AMPs (Lemaitre & Hoffmann 2007). There are two main immune responsive signalling pathways in *D. melanogaster* and they are referred as IMD and Toll. The Toll, which is presented in more details in chapter 5, is responsible for sensing and mediating the response to fungal and Gram-positive bacterial infections. The IMD pathway senses and mediates the response to Gram-negative bacterial infections mainly (Buchon et al. 2014).

S-nitrosylation, the reaction of a NO moiety with a thiol group (SH) of a cysteine to form an *S*-nitrosothiol group (SNO), has been shown to play a key roles in the immune responses of plants and animals. In mammals, *S*-nitrosylation has been shown to modulate innate immunity by modifying multiple targets downstream of TLRs activation (Marshall & Stamler 2001; Into et al. 2008; Kelleher et al. 2007).

NF-kB proteins are among these targets. Both p50 (Marshall & Stamler 2001)and p65 (Kelleher et al. 2007) subunits have been show to undergo *S*-nitrosylation at C63 and C38, respectively, impairing DNA binding and signal transduction. In addition, the activity of the inhibitory κB kinase β (IKKβ) has been shown to be blunted by *S*-nitrosylation at C179 (Reynaert et al. 2004). Furthermore, *S*-nitrosylation of the adaptor protein MyD88 has been shown to disrupt its

interaction with the cytoplasmic domain of TLR (Into et al. 2008). These results suggest that NO, through *S*-nitrosylation of key proteins, modulates NF-κB signalling at multiple points of the signalling pathway.

S-nitrosylation has also been related to regulating innate immunity within the lung of mice by modifying and inducing quaternary structural alterations of the surfactant protein-D (SP-D) (Atochina-Vasserman et al. 2009; Guo et al. 2008). These studies have shown that *S*-nitrosylation of this protein disassembles the homo-dodecamer complex into homo-trimer complexes, producing macrophage chemotaxis and triggering a pro-inflammatory response. Interestingly, the homo-dodecamer has been proposed to bind to TLR2 and 4 (Ohya et al. 2006)and to inhibit LPS-induced inflammatory response (Yamazoe et al. 2008). Thus, it appears that *S*-nirosylation modulates NF-κB signalling both down- and upstream of TLR activation.

An important player in the *S*-nitrosylation process is the low-molecular-weight SNO *S*-nitrosoglutathione (GSNO), the *S*-nitrosylated form of glutathione (GSH). Through trans-*S*-nitrosylation, GSNO can transfer NO to other proteins and the levels of protein *S*-nitrosylation are believed to be in equilibrium with the levels GSNO. As a consequence, the enzyme GSNO reductase (GSNOR), which metabolises GSNO, indirectly controls the levels of total SNO. In addition to and probably as a consequence of its essential role in SNO metabolism, GSNOR has been shown to be an important player during the immune response of plants (Feechan et al. 2005) and mammals (Tang et al. 2013).

Arabidopsis plants with a mutation in *gsnor* have increased levels of total *S*-nitrosylation and are highly susceptible to fungal and bacterial infections (Feechan et al. 2005). Similarly, *gsnor*$^{-/-}$ mice, in which SNO levels drastically increase after

intraperitoneal injection of LPS (Liu et al. 2004), have recently been shown to be more susceptible to pulmonary infections caused by *Klebsiella pneumonia*(Tang et al. 2013).

Thus, to further investigate the role played by GSNOR in immunity, *gsnor* knock-out flies generated by the overlapping deletion technique were analysed against different classes of pathogen infections. Further, the response of these animals to the infections were molecularly characterised by monitoring the expression of immune-responsive genes.

4.1.1 Background results

Previously, by employing an overlapping deficiencies technique, Kanchanawatee (2012) successfully generate *gsnor*$^{-/-}$ flies and challenged these flies against different types of microorganisms. Interestingly, *gsnor*$^{-/-}$ flies did not show any evident immune deficiency phenotype when infected with Gram-negative bacteria but were susceptible to infections caused by the entomopathogenic fungus *B. bassiana*, as discussed in the previous chapter.

4.2 Gut immunity is not affected in *gsnor*$^{-/-}$ flies

Fruit flies live on decaying matter and feed on fermenting medium. Therefore, the gut of these animals is in constant contact with a large number of microorganisms, including potential pathogens. For this reason, a rapid and efficient mechanism to identify and combat potential pathogens is very important in the gut. The IMD pathway plays a major role in Drosophila gut immunity, triggering a local and systemic production of AMPs after pathogens recognition (Vodovar et al. 2005).

Reactive oxygen species (ROS) produced by the NADPH oxidase Duox, acting as microbicides also play an important role in Drosophila gut immunity (E. Ha et al. 2005).

To investigate whether GSNOR is also required for gut immunity, *Pseudomonas entomophila*, a Gram-negative natural bacterial pathogen of Drosophila that causes high death rates when ingested by flies (Vodovar et al. 2005), was administrated to flies diet and the survival rates of *gsnor*$^{-/-}$ and WT flies were compared (Figure 4-1). To test if bacterial concentration and feeding time on bacterial solution could interfere with the final result, two methods of infection were carried out, as explained bellow. For each method of infection, experiments were repeated three times.

In the first method, flies were fed on a highly concentrated bacterial solution for a period of seven hours before being transferred to new vials with standard cornmeal agar medium, and kept at 29°C (Figure 4-1-A). This method of infection produced a rapid and high mortality of all lines tested. After 48 hours, only 10% of the WT flies were alive and *gsnor*$^{-/-}$ flies behaved similarly. In the second method, flies were allowed to feed on a more dilute bacterial solution (O.D. 2 in 5% sucrose solution), but the treatment was constant for the whole experiment with fresh bacterial solution being added every other day (Figure 4-1-B). This method of infection slowed down the death rates; however, like the first method of infection, the survival rates of all lines were similar at day four after infections, including *relish* flies.

The fact that *relish* flies did not present any difference in survival compared to wild type flies makes it difficult to draw any clear conclusion from this

experiment. The similar response between *relish* and wild type flies goes against what has been reported before (Vodovar et al. 2005). Although the reason for this is not clear, it could be related to the infection procedure. While Vodovar and co-workers (2005) starved flies for food and water for five hours prior transferring to contaminated food, only two hours were employed for these experiments.

I any case, the survival rates observed for the two *gsnor*$^{-/-}$ overlapping deficiencies (originated from reciprocal crosses), for both methods of infection, did not differ from the survival rates observed for the WT and *gsnor*$^{+/-}$ single deficiency flies. This suggests that GSNOR activity is not relevant to gut immune responses against *P. entomophila*.

Similar results, meaning no differences in survival between *gsnor*$^{-/-}$ and control flies, were seen by Kanchanawatee (2012) when flies were fed on *Erwinia carotovora* pv. *carotovora* 15 (*Ecc*15), a Gram- negative bacteria that is capable of persisting in the gut of *D. melanogaster* and triggers both local and systemic immune responses following oral infection (Basset et al. 2000).

Altogether, these results suggest that GSNOR activity is not required for a normal gut immune response against *Pe* and *Ecc*15. This support the hypothesis that the IMD signalling pathway, which is responsible for defence against Gram-negative bacterial infections and plays a major role in gut immunity, is completely functional in *gsnor*$^{-/-}$ flies.

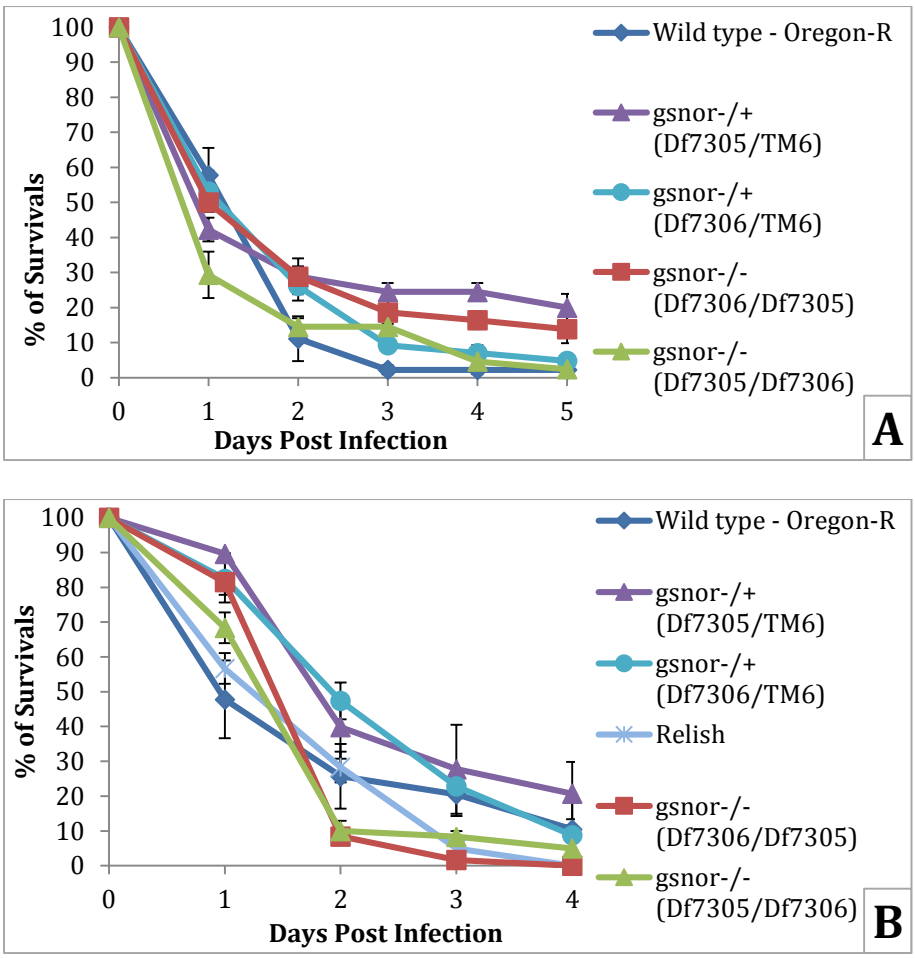


Figure 4-1: Survival of flies after of *P. entomophila* infections

A: Groups of 15 female flies, aged between three and six days, were starved for two hours in empty vials at 25°C before being transferred to vials containing filter papers soaked in a concentrated bacterial solution (this solution was made by mixing the same volumes of 10% sucrose solution and a *P. entomophila* pellet from an overnight culture). After seven hours feeding on the solution, flies were transferred to vials containing fresh standard fly food and kept at 25°C. The number of survivors was recorded every 24 hours. The data represent the mean of three independent experiments (±SE). **B**: Groups of 20 female flies, aged between three and six days, were starved for two hours in empty vials at 25°C before being transferred to vials containing cotton rolls soaked in a 5% sucrose solution and *P. entomophila* (O.D. 2). Flies were kept in the vials at 25°C for four days and fresh bacterial suspension was added to the cotton rolls once a day. The number of survivors was recorded every 24 hours. The data represent the mean of three vials with 15 female flies each (±SE).

4.3 *gsnor*$^{-/-}$ flies are highly susceptible to Gram-positive bacteria

S*taphylococcus aureus* and *Enterococcus faecalis* are Gram-positive bacteria which have been shown to induce the expression of AMPs in *D. melanogaster* after septic infection (Lemaitre et al. 1997). Both bacteria have also been shown to cause greater death rates of flies with mutations in components of the Toll signalling pathway and have been used to uncover the molecular mechanisms underpinning innate immunity (Bischoff et al. 2004; Gottar et al. 2006; Kambris et al. 2006; Marek & Kagan 2012).

gsnor$^{-/-}$ flies were more susceptible to the fungus *B. bassiana.* Because the Toll signalling pathway, which mediates the immune response against fungal infections, also mediates the immune response against and Gram-positive bacterial infections, we wanted to know whether these flies could also be more susceptible to Gram-positive bacteria. If so, this would point towards a compromise of the Toll signalling pathway in *gsnor*$^{-/-}$ flies.

To test this hypothesis, flies were infected with *S. aureus* and *E. faecalis* by septic infection. Needles coated in bacteria were used to injury the thorax of the animals and the survivals rates were assessed.

4.3.1 Heat-killed *S. aureus* inoculation

To ensure that flies did not die due to the procedure of infection or due to a bacterial sepsis-like response, heat-killed *S. aureus* were used in control experiments (Figure 4-2).

This experiment confirmed that the procedure, here referred as “septic infection”, does not kill the flies, and dead bacteria do not cause any type of hyper

immune response that culminates in death of the animals. The small increases in the number of dead flies seen for the WT and *gsnor*$^{+/-}$ single deficiency flies (around 5%) are probably due to wounding caused during the infection procedure. Intriguingly, both *gsnor*$^{-/-}$ overlapping deficiencies (originated from the reciprocal crosses) presented a similar reduction in survival rates of around 20% after septic infection with heat-killed *S. aureus*.

Although, these differences are not significant in relation to the controls, they are consistent and reproducible. Similar survival curves were seen when these flies were infected with the Gram-negative bacterium *Escherichia coli* MG1655 (*E. coli* MG1655) and also when needles were coated in sterile LB media only (Kanchanawatee, 2012).

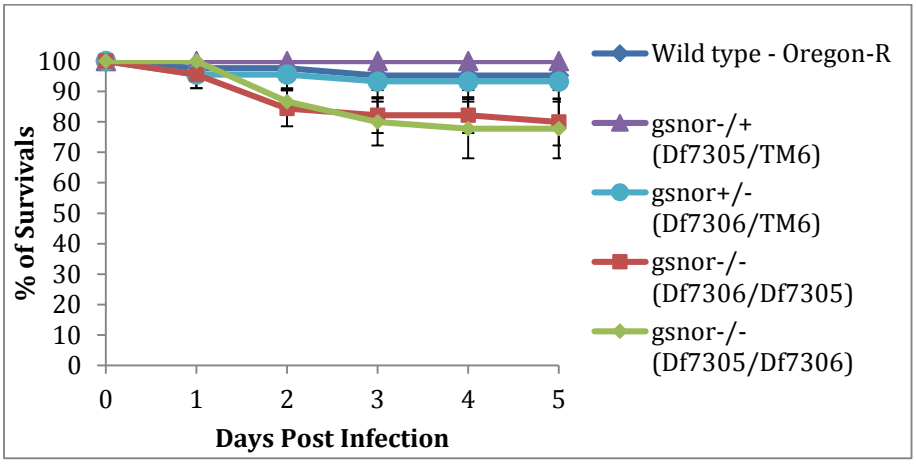


Figure 4-2: Heat-killed *S. aureus* does not kill *gsnor*$^{-/-}$ flies

Groups of 15 female flies, aged between three and six days, were pierced with a needle coated in heat-killed *S. aureus*. Flies were transferred to new vials containing standard cornmeal-agar media and kept at 29°C for five days. The number of survivors was recorded every 24 hours after infection for five days. The data represent the mean of three independent experiments (±SE)

4.3.2 *S. aureus* and *E. faecalis* infections

Knowing that neither the procedure of infection nor dead bacteria induced death of the animals, survival curves were generated after infections with *S. aureus* and *E. faecalis*. In these experiments, the survival of $gsnor^{-/-}$ flies was compared to that of wild type and $gsnor^{+/-}$ single deficiency lines. In addition, complementation lines were also included.

Figure 4-3-A and Figure 4-4-A highlight the survival of $gsnor^{-/-}$ flies in comparison with two controls, wild type (Oregon-R) and immunocompromised *spätzle* flies, after *S. aureus* and *E. faecalis* infections, respectively. Figure 4-3-B and Figure 4-4-B present the survival of $gsnor^{-/-}$, $gsnor^{+/-}$ and $gsnor^{-/-}$ complemented with *UAS-gsnor* driven by *Act-GAL4* (GSNOR Comp.), after *S. aureus* and *E. faecalis* infections, respectively.

Similarly to the infections with *B. bassiana*, $gsnor^{-/-}$ flies were more susceptible to Gam-positive bacterial infections. Both bacteria killed $gsnor^{-/-}$ flies more rapidly than all the other controls, including the immunocompromised *spätzle* mutants. As can be seen in Figure 4-3 and Figure 4-4, while approximately 90% of $gsnor^{-/-}$ were dead within 24 hours after infections with both bacteria, only about 20% of the wild type flies died after the same period of time. Furthermore, complementation of $gsnor^{-/-}$ with *UAS-gsnor/Act-GAL4* restored the survival rates of these flies. These results suggest that GSNOR plays an important role in the immune response of *D. melanogaster* to *S. aureus* and *E. faecalis*.

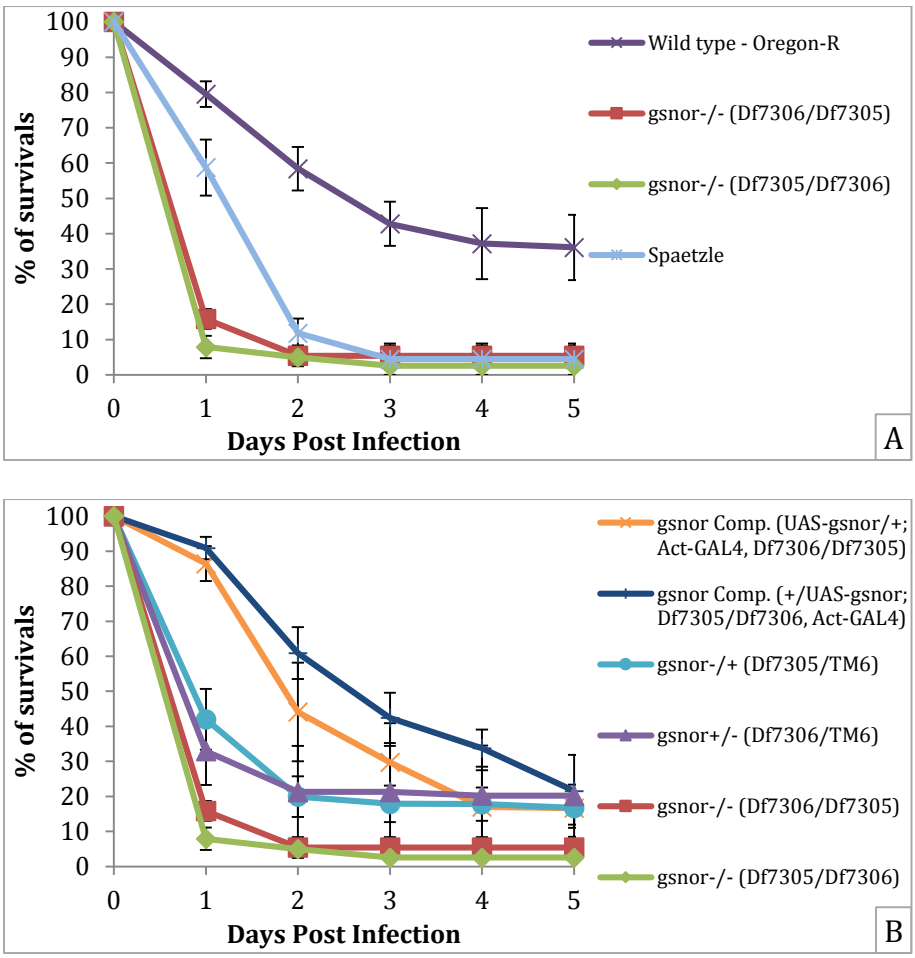


Figure 4-3: *gsnor*$^{-/-}$ flies are highly susceptible to *S. aureus*

Groups of 30 female flies, aged between three and six days, were infected with a needle coated in *S. aureus*. Flies were transferred to new vials containing standard cornmeal-agar media and kept at 29°C for five days. The number of survivors was recorded every 24 hours after infection for five days. The data represent the mean of three independent experiments (±SE). Graph A highlights the differences in survival between *gsnor*$^{-/-}$, WT and Spätzle flies. Graph B highlights the restoration in the survival of *gsnor*$^{-/-}$ flies complemented with UAS-gsnor/Act-GAL4.

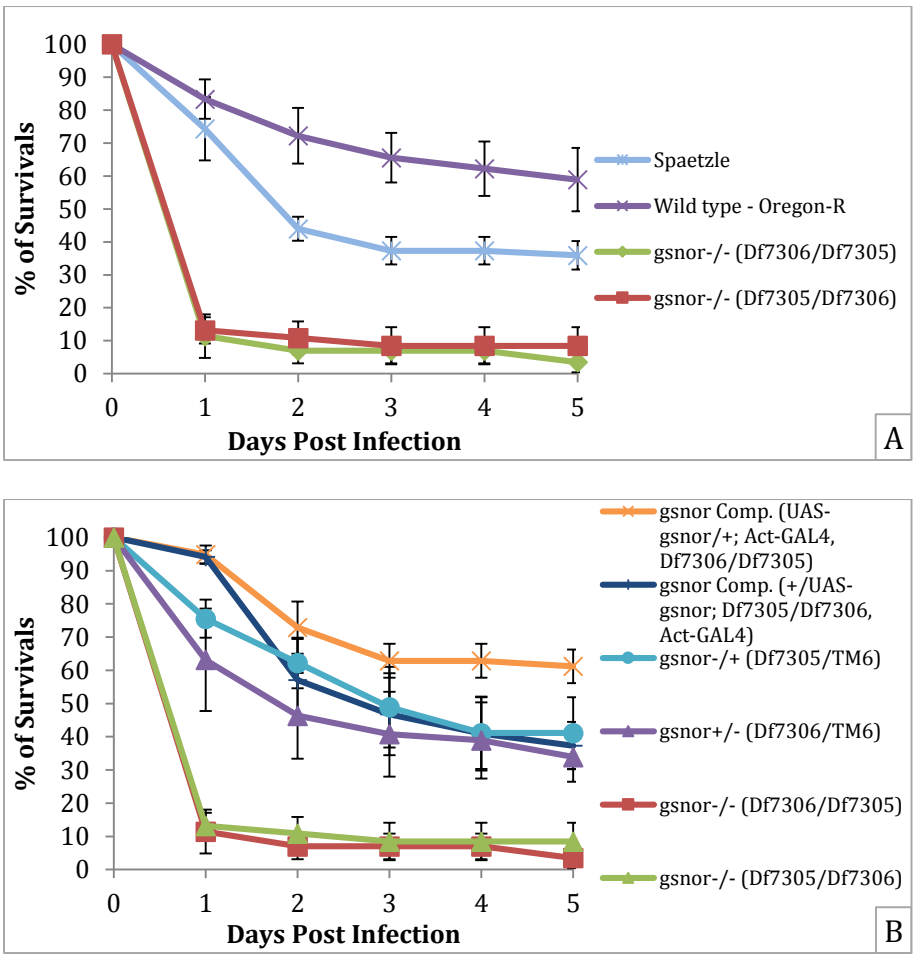


Figure 4-4: *gsnor*$^{-/-}$ flies are highly susceptible to *E. faecalis*

Groups of 30 female flies, aged between three and six days, were infected with a needle coated in *E. faecalis*. Flies were transferred to new vials containing standard cornmeal-agar media and kept at 29°C for five days. The number of survivors was recorded every 24 hours after infection for five days. The data represent the mean of three independent experiments (±SE). Graph A highlights the differences in survival between *gsnor*$^{-/-}$, WT and Spätzle flies. Graph B highlights the restoration in the survival of *gsnor*$^{-/-}$ flies complemented with UAS-gsnor/Act-GAL4.

4.4 Challenge-inducible expression of AMPs is reduced in *gsnor*$^{-/-}$ flies

One of the hallmarks of *D. melanogaster* immunity is the challenge-inducible expression of AMPs. The expression of these genes is controlled by two signalling pathways, IMD and Toll. While the IMD pathway is mainly triggered by Gram-negative bacteria, Fungal and Gram-positive bacterial infections mainly trigger the Toll signalling pathway (Lemaitre & Hoffmann 2007).

From previous results, we knew that the induction of AMP expression was reduced in *gsnor*$^{-/-}$ flies infected with entomopathogenic fungus *B. bassiana*, compared to WT flies. This reduction was associated with the higher susceptibility of these animals to infections (Kanchanawatee, 2012).

Thus, to investigate whether the susceptibility to Gram-positive bacteria could also be explained by a reduction in the challenge-inducible expression of AMPs after bacterial infections, qRT-PCR was conducted after *E. faecalis* septic infections. Batches of five flies were challenged using a needle coated in *E. faecalis* and kept at 29°C. After eight hours, the total RNA of these flies was extracted and the mRNA was reverse transcribed to cDNA. Expression of the AMP genes *Metchnikowin* (*Mtk*), *Drosomycin* (*Drs*), *Defensin* (*Def*) and *Diptericin* (*Dpt*) was normalised to the expression of the housekeeping gene *Rp49*,and the results were plotted relative to the level of expression in infected wild type flies (Figure 4-5).

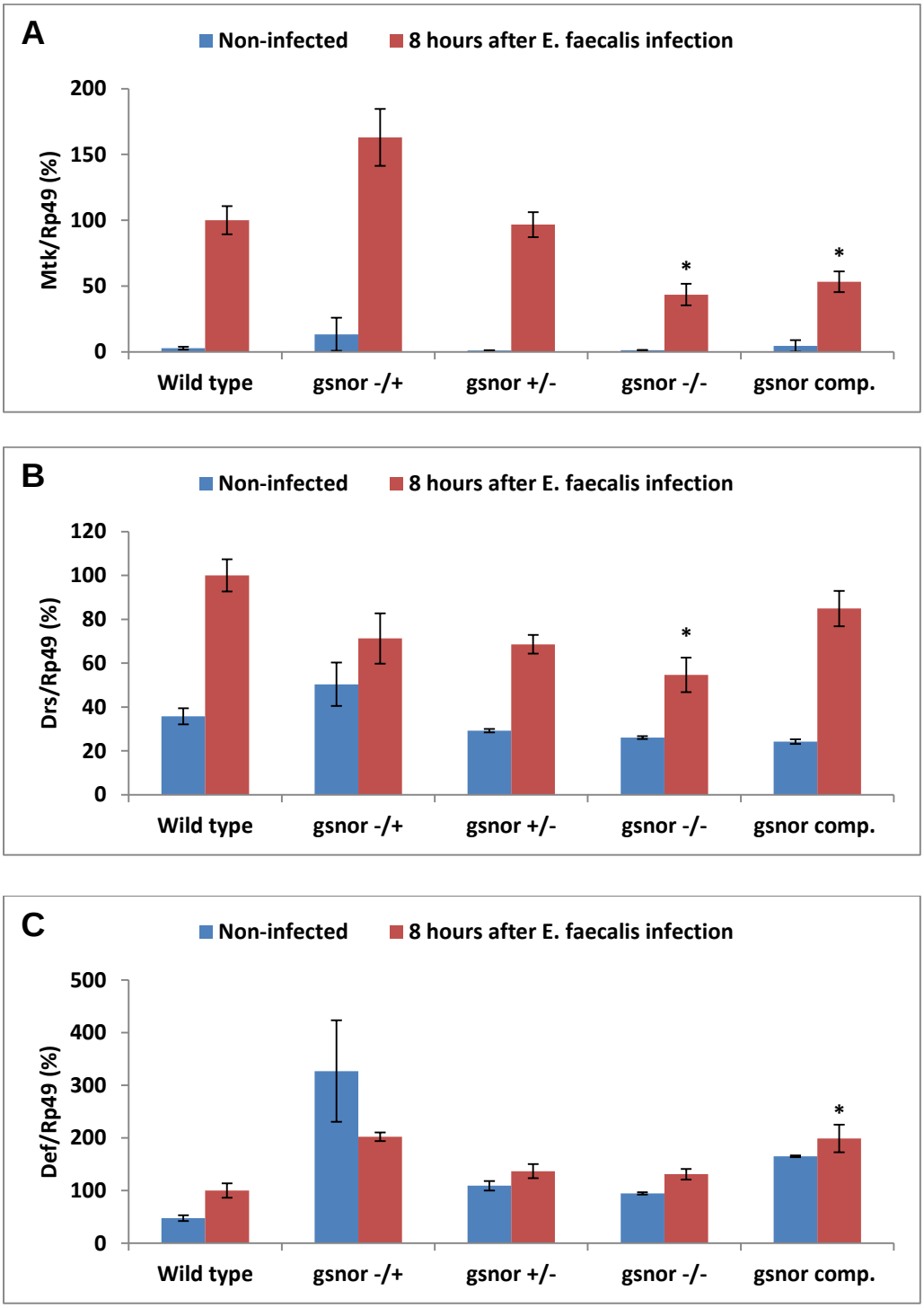
Drs and *Mtk* presented lower expression after *E. faecalis* infection in *gsnor*$^{-/-}$ flies (Figure 4-5 A and B). These AMPs are often used as read-outs of the Toll signalling pathway and have been show to present antimicrobial activity against fungi (Fehlbaum et al. 1994; Levashina & Ohresser 1995). However, *Mtk* has also been shown to present antimicrobial activity against Gram-positive bacteria

(Levashina & Ohresser 1995). The results are similar to what was seen when flies were infected with *B. bassiana* (Kanchanawatee, 2012), this implies a dysfunction of the pathway signalling the expression of these two AMPs after fungal and Gram-positive bacterial infections. Moreover, the expression of both AMPs was slightly, although not with statistical significance, increased in the UAS-*gsnor*/GAL4 complementation strains, suggesting that *gsnor* knock-out might be responsible for the reduction in the expression of these AMPs after Gram-positive bacterial infection.

Expression of *Def*, which can be regulated by both the IMD and Toll pathways (Lemaitre et al. 1996) and shows antimicrobial activity against Gram-positive bacteria only (Levashina & Ohresser 1995), was not significantly different between $gsnor^{-/-}$ and wild-type flies after septic infection with *E. faecalis*(Figure 4-5C). However, $gsnor^{-/-}$ flies complemented with UAS-*gsnor*/A*ct*-GAL4 presented a higher expression of this AMP after *E. faecalis* infection compared to wild type flies. These results can partially explain the restoration in survival rates of $gsnor^{-/-}$ complemented flies infected with Gram-positive bacteria.

The expression of *Dpt*, which is a read-out of the IMD pathway, was significantly reduced after *E. faecalis* infection in $gsnor^{-/-}$ flies in comparison with wild type flies. Moreover, unlike the other AMPs tested, the expression of *Dpt* did not change in the complementation lines (Figure 4-5D). From these results we can conclude that despite not being more susceptible to Gram-negative bacterial infections, $gsnor^{-/-}$ flies also present a reduction in the expression of an antimicrobial peptide that is mainly regulated by the IMD pathway. Thus, it is possible to speculate that the expression of this AMP is controlled by both the IMD and the Toll pathways,

probably through interactions between the transcription factor DIF and Relish.



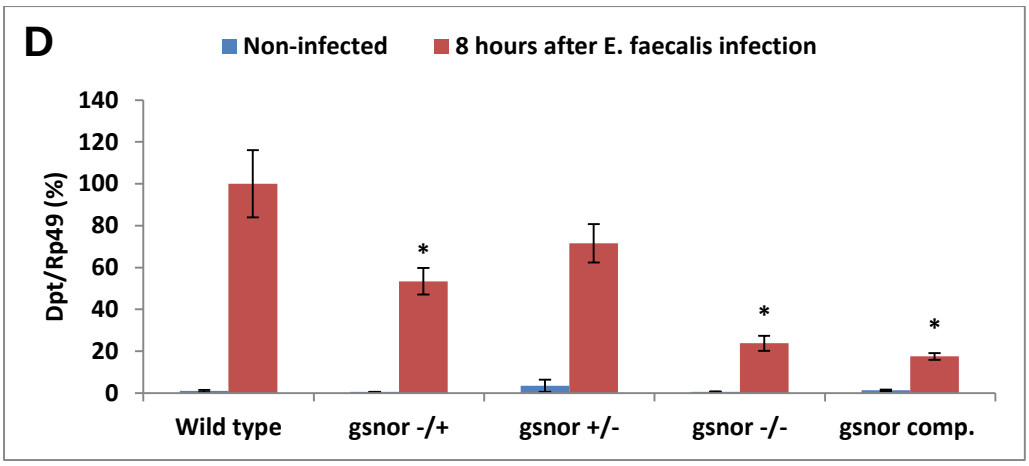


Figure 4-5: The challenge-inducible expression of AMPs is compromised in *gsnor*$^{-/-}$ flies

Relative expression of *Mtk* (A), *Drs* (B), *Def* (C) and *Dpt* (D) before and after infections with *E. faecalis*. Groups of 5 female flies, aged between three and six days, were pierced with a needle coated in a culture of *E. faecalis*. Flies were transferred to new vials containing standard cornmeal-agar media and kept at 29°. After eight hours, the total RNA of the flies was extracted and the mRNA was reverse transcribed to cDNA. The levels of expression of the target genes were normalised to the level of expression of the housekeeping gene Rp49. 100% values correspond to the level of expression of the target genes in WT flies infected with *E. faecalis*, eight hours after infection. The data represent the mean from three separate vials with 5 flies each (±SE). Based on one-way ANOVA [$F_{4,10}$ = 15.10 p< 0.001 for *Mtk* (A), $F_{4,10}$ = 4.23 p< 0.05 for *Drs* (B), $F_{4,10}$ = 8.11p< 0.005 for *Def* (C) and $F_{4,10}$ = 16.01p< 0.001for *Dpt* (D)] followed by Dunnett's test, values indicated by the symbols (*) are statically significant (p<0.05).All samples were compared to infected Wild type flies.

4.5 Conclusions

The data presented in this chapter supports a role for GSNOR in *Drosophila melanogaster* immunity. Given that *gsnor*$^{-/-}$flies succumbed very rapidly to infections caused by *B. bassiana*, *S. aureus* and *E. faecalis* but presented no clear dysfunction in the response against *P. entomophila*, GSNOR activity seems to be required for the immune response of flies against Gram-positive bacteria and fungi but not for immune response against Gram-negative bacteria. Furthermore, complementation

experiments using the UAS/GAL4 system to express *gsnor* in *gsnor*$^{-/-}$ flies support the hypothesis that the loss of *gsnor* induces higher susceptible to infections.

This higher susceptibility appears to be, at least in part, caused by a compromised production of AMPs after pathogen challenge. *gsnor*$^{-/-}$ flies presented a clear reduction in the inducible expression of the AMPs *Drs*, *Mtk* and *Dpt*, but show no changes for the expression of *Def*. However, in comparison to infected wild type flies, the expression of *Def* increased twofold in infected *gsnor*$^{-/-}$ complemented with UAS-*gsnor*/*Act*-GAL4, suggesting that both overproduction and reduction of GSNOR might have an effect on AMP expression.

Altogether, these results point towards an impairment of the Toll signalling pathway. However, it remains to be investigated whether *gsnor* loss of function and consequent compromise of the Toll signalling pathway affect the tolerance or the resistance of these flies to infections. Thus, further experiments with the aim of quantifying microbial growth in infected flies should be undertaken.

In the next chapter, a possible molecular explanation for the dysfunction of the Toll signalling pathway in *gsnor*$^{-/-}$ flies is presented. Components of this signalling pathway are shown to undergo *S*-nitrosylation *in vitro* and *in vivo* and a mechanism that links *S*-nitrosylation and innate immunity in *D. melanogaster* is proposed.

Chapter 5

S-nitrosylation and the Toll signalling pathway

5.1 Introduction

D. melanogaster can discriminate between infections caused by different classes of microorganism through two main signalling pathways, the IMD and the Toll. The first senses and drives the response against Gram-negative bacterial infection, while the second is involved in sensing and responding to fungal and Gram-positive bacterial infections. The activation of both pathways lead to the production of small peptides with anti-microbial activity (Ferrandon et al. 2007).

The Toll signalling pathway (Figure 5-1), which shares many features with the mammalian Toll-like receptor (TLR) / NF-κB signalling pathway, was first discovered in *D. melanogaster* as a major determinant of dorso-ventral polarity in embryos (Anderson et al. 1985). The identification of a role for this pathway in *D. melanogaster* immunity (Lemaitre et al. 1996), followed by the discovery that TLRs are involved in activation of the mammalian innate and adaptive immune responses (Poltorak et al. 1998; Iwasaki & Medzhitov 2004; Medzhitov et al. 1997) stablished an evolutionarily conserved role for Toll in immune functions.

In *D. melanogaster*, the Toll signalling pathway (Figure 5-1) can be triggered by the interaction of circulating recognition molecules, such as peptidoglycan-recognition proteins (PGRP) and glucan-binding proteins (GNBPs) with Lys-Type peptidoglycans (PGN) from Gram-positive bacteria and β-(1,3)-glucans from the fungal cell wall, respectively. The recognition of such molecules triggers a protease

cascade that converges into the activation of a modular serine protease (ModSP)(Buchon et al. 2009). Downstream of ModSP is the Gram-positive–specific serine protease (Grass) (Kambris et al. 2006), which was later shown to also be important for the activation of the pathway against fungal pathogens (El Chamy et al. 2008). Furthermore, four other serine proteases (spirit, spheroide, and sphinx1/2) were identified as being downstream of Grass but upstream of the Spätzle-Processing enzyme (SPE) (Kambris et al. 2006).

Additionally, this signalling pathway can also be activated through direct cleavage of the serine proteases Persephone (PSH) by virulence factors secreted by fungi and/or bacteria (Gottar et al. 2006; Chamy et al. 2008). Recently, PSH has also been associated with triggering the pathway in response to endogenous damage-associated molecular patterns (DAMPs) present in the haemolymph of apoptosis-deficient *D. melanogaster* larvae mutants (Ming et al. 2014a).

The activation of these three distinct cascades leads to the cleavage of SPE, which, in its activated form, processes the Toll receptor ligand Spätzle (Spz) from pro-Spz to Spz (Jang et al. 2006). Once Spz binds to the Toll receptor, it triggers conformational changes that induce the formation of the heterotrimeric complex MyD88-Tube-Pelle, leading to the phosphorylation and degradation of Cactus, the *D. melanogaster* inhibitor of kappa B (IkB). Degradation of cactus releases the nuclear factor kappa B (NF-kB) transcription factor Dif, allowing their translocation to the nucleus, where it induces the transcription of immune responsive genes, including AMPs (Valanne et al. 2011).

Overlapping deficiencies *gsnor*$^{-/-}$ knockout flies, as presented in the previous chapter, behave similarly to wild type flies when infected with Gram-negative

bacteria. However, these animals show higher susceptibility to the entomopathogenic fungus *B. bassiana* and to the Gram-positive bacteria *S. aureus* and *E. faecalis*. Taking together, these results suggest that the Toll, but not the IMD, signalling pathway is affected in *gsnor*$^{-/-}$ flies.

Indeed, the expression of the AMP genes *Drs* and *Mtk*, which are mainly controlled by the Toll pathway, were lower in *gsnor*$^{-/-}$ flies after pathogen challenge compared to wild type flies. Thus, it has been hypothesised that higher levels of *S*-nitrosylation in *gsnor*$^{-/-}$ overlapping deficiency flies may cause aberrant modification of target proteins and interfere with the signalling transduction in Toll pathway.

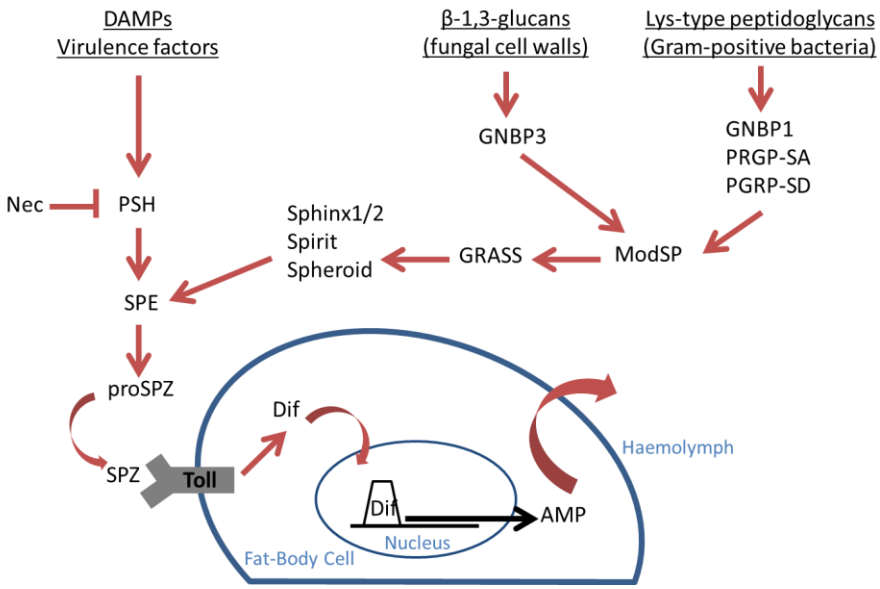


Figure 5-1: The Toll signalling pathway of *D. melanogaster*

The Toll signalling pathway can be divided in three branches with regard to the molecules it recognises in the haemolymph. The receptors GNBP1, PRGP-SA and PRGP-SD are activated by Lys-type PGN present in the cell wall of Gram-positive bacteria. GNBP3 recognises fungi through binding to β-1,3-glucans. PSH can be activated through direct cleavage by pathogen-secreted proteases. It can also be activated in response to tissue damage by still uncharacterised molecules. All three branches activate a protease cascade that culminates with the processing of pro-Spz to Spz, the Toll ligand. Once Spz binds to the Toll it internalise the signal to the cell through a phosphorylation cascade that frees the NF-

kβ Dif to translocate to the nucleus and activate the transcriptions of immune effector genes.

5.2 CLIP-domain SPs in the Toll pathway are targets for *S*-nitrosylation

A possible explanation for the immune deficiency phenotypes of *gsnor*$^{-/-}$ flies is the impairment of components of the Toll signalling pathway due to aberrant *S*-nitrosylation of key cysteine residues. Previous results generated by Kanchanawatee (2012) suggested that PSH could be a possible target for *S*-nitrosylation in the Toll signalling pathway.

Here I confirm this hypothesis and show that *S*-nitrosylation of PSH is an important process during the immune response of *D. melanogaster* against *B. bassiana*. In addition, this process is shown to be true for another CLIP-domain serine protease of the Toll signalling pathway, SPE.

5.2.1 PSH is *S*-nitrosylated *in vitro*

PSH is a 42.5 kDa, 394 amino acid long protein, composed of a signal peptide (residues 1-22), a CLIP-domain (residues 23-143) and a serine protease domain (residues 144-394). It is secreted to the haemolymph as a zymogen and requires proteolytic maturation to become active. This is achieved after the zymogen is recognised and cleaved between H143 and I144 by a protease secreted by pathogens (Ligoxygakis et al. 2002; El Chamy et al. 2008) or by damage-associated proteases (Ming et al. 2014a). Once PSH is activated it initiates a protease cascade that culminates with the cleavage and activation of Spz, the Toll ligand.

First discovered in a suppressor screen for the necrotic phenotype in *D. melanogaster* (Ligoxygakis et al. 2002), PSH was believed to function as a guard of

the immune system, being a substrate for proteases that act as pathogen virulence factors. One such virulence factor is the subtilisin protease PR1, which is secreted by entomopathogenic fungi to degrade the cuticle of insects during infection. PR1 has been shown to cleave PSH and activate the Toll signalling pathway leading to the production of the AMP Drs in *D. melanogaster* (Gottar et al. 2006; Chamy et al. 2008).

There are 17 cysteine residues in PSH and we have hypothesised that one or more of these residues could be *S*-nitrosylated and that this could regulate PSH function. In order to test if PSH is *S*-nitrosylated *in vitro*, the full coding sequence of *psh* was cloned into the gateway compatible vector pETG-40A in frame with an N-terminal Maltose Biding Protein (MBP) coding sequence and expressed in *E. coli* Rosetta-Gami-2 cells. MBP was used because it increase solubility as attempts to express this protein using 6x-His and GST tags have failed to produced soluble recombinant PSH. The Rosetta-Gami-2 strain was chosen because, in addition to supplying tRNAs for the expression of eukaryotic proteins, it has mutations in both the *thioredoxin reductase* (*trxB*) and *glutathione reductase* (*gor*) genes that enhance disulphide bond formation in these cells.

The fusion protein MBP-PSH was purified using amylose affinity chromatography (Figure 5-2) and analysed by the biotin-switch technique (BST) (detailed in Chapter 2). This method selectively reduces SNOs, replacing NO by a Biotin-HPDP, which can be detected by western blot using an anti-Biotin antibody (Figure 5-3).

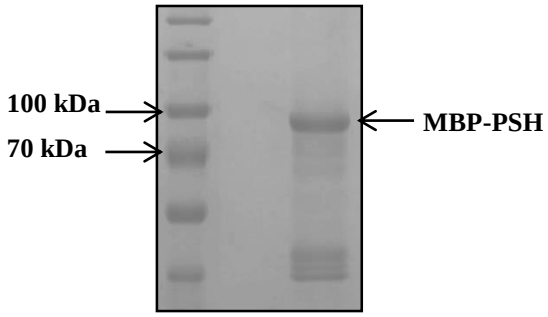


Figure 5-2: Recombinant MBP-PSH purified by amylose affinity chromatography

E. coli Rosetta-gami-2 cells carrying the construct pETG-40A-PSH were used to express the fusion protein MBP-PSH. 500µL of overnight cultures kept at 37°C were used to inoculate new 500mL cultures. These cultures were incubated at 37°C and their optical densities were monitored until they reached 0.6. IPTG was added to a final concentration of 1mM and after five hours of induction at 37°C the cells were harvest by centrifugation at 4°C. Bacterial cell walls were broken down using a lysozyme solution and lysates were passed through amylose affinity chromatography columns. After several washes, purified MBP-PSH was eluted with 10mM maltose solution and an aliquot was run on a reducing 10% SDS-PAGE. MBP and PSH have an estimated molecular mass of 42.5 kDa each.

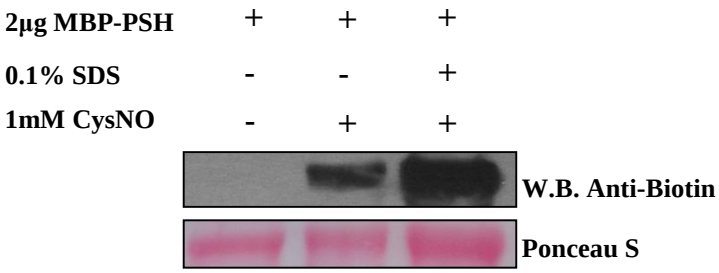


Figure 5-3: MBP-PSH is *S*-nitrosylated *in vitro*

2µg of recombinant protein was treated with 1 mM CysNO and submitted to the BST. The addition of 0.1% SDS before CysNO treatment was used as a positive control. SDS is a surfactant that breaks secondary and tertiary structures of proteins, thus exposing cysteine thiols that would be buried otherwise and would not react with NO.A CysNO non-treated sample was used as negative control. After the BST, samples were run on a 12% SDS-PAGE, transferred to a nitrocellulose membrane and submitted to western blotting (W.B.) using an anti-Biotin antibody. After transfer, membranes were stained with Ponceau S to confirm that similar amounts of proteins were being compared. This experiment was repeated at least three times with similar results.

From the results presented above, it is clear that recombinant MBP-PSH can be *S*-nitrosylated *in vitro*. As seen in Figure 5-3, the levels of biotinylation, which reflect the levels of *S*-nitrosylation before the biotin-switch, are much higher in CysNO treated samples. The addition of 0.1% SDS, used as positive control, exposes buried cysteine residues that would not be exposed on the protein when on its native conformation, and this leads to higher levels of biotinylation as a consequence.

5.2.2 PSH is *S*-nitrosylated *in vivo*

To further investigate whether *S*-nitrosylation of PSH happens *in vivo,* transgenic flies expressing PSH tagged with the human influenza hemagglutinin peptide (HA) were generated. The *psh* coding sequence was cloned into the vector pUASg_HA.attB and propagated in *E. coli*. Purified constructs were checked by sequencing and sent to Genetic Service, Inc. to be co-injected with phiC31 integrase into embryos of *D. melanogaster* carrying an attP site on the second chromosome (attP40). Homozygous lines were made and sent back to our laboratory.

Flies carrying *UAS-psh-HA* were crossed with flies carrying *Ubi-GAL4* to generate *UAS-psh-HA*/*Ubi-GAL4* flies (*psh-HA* flies), which were tested for expression of PSH-HA by western blot with anti-HA antibody (Figure 5-4). *Ubi-GAL4* drivers were chosen instead of *Act5C-GAL4* because the later produced no progeny when reared at 25°C. Although the levels of expression induced by these two drivers have not been compared, we have assumed that the lethality caused by *Act5C-GAL4* was caused by excessive expression of *UAS-psh-HA*. Thus, *UAS-psh-HA* and the other serine proteases expressed in transgenic flies under the control of

the UAS/GAL4 system were always driven by *Ubi-GAL4* at 20°C.

As presented in Figure 5-4, a protein of about 55 kDa was detected by the anti-HA antibody in protein extracts from *UAS-psh-HA/Ubi-GAL4* but not from WT (Oregon-R) flies. This suggests that the molecular mass of PSH in a reducing 12% SDS-PAGE is greater than the 42.5kDa predicted molecular mass. This is consistent with the molecular mass of MBP-PSH seen for the *in vitro* assays where the size of MBP-PSH was also greater than expected.

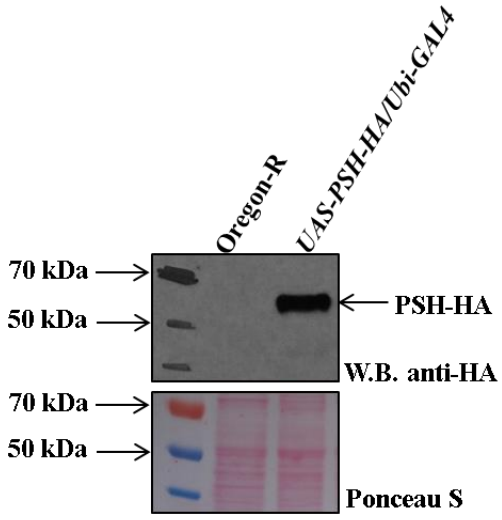


Figure 5-4: PSH-HA expression in *D. melanogaster*

Total proteins from Oregon-R and UAS-psh-HA/Ubi-GAL4 flies were extracted, run in reducing SDS-PAGE, transferred to a nitrocellulose membrane, stained with Ponceau S to confirm that similar levels of protein were being compared, and then exposed to an anti-HA antibody.

To determine if PSH-HA is *S*-nitrosylated *in vivo*, protein extracts from *UAS-psh-HA/Ubi-GAL4* flies were submitted to the BST. Streptavidin was used to pulldown biotinylated proteins and anti-HA antibody was used to detect the presence of PSH-HA among the biotinylated proteins. As input controls, 20 μL aliquots were taken from the total protein extracts before streptavidin pulldowns. As seen in Figure

5-5, PSH-HA is indeed endogenously *S*-nitrosylated. This suggests that *S*-nitrosylation of PSH may be a biologically relevant process in *D. melanogaster*.

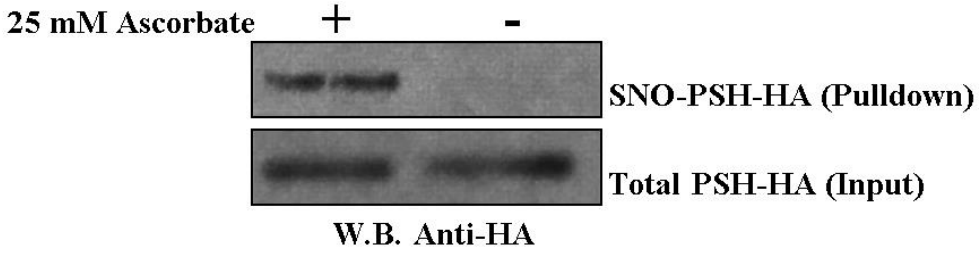


Figure 5-5: PSH-HA is *S*-nitrosylated *in vivo*

Total protein extract from 10 female UAS-psh-HA/Ubi-GAL4 flies was submitted to the BST followed by streptavidin pulldown and western blots with an anti-HA antibody. 25mM sodium ascorbate was used to reduce SNOs. Sodium ascorbate was omitted from the reaction as a negative control for the assay. To confirm that similar amounts of total protein were being compared, sample aliquots were taken before the pulldown step and used in parallel western blots. As shown on the left side of the top blot, addition of sodium ascorbate induces de-*S*-nitrosylation and allows the protein to be biotinylated. Biotinylated proteins bind to streptavidin (Pulldown step) and can be detected by Western blot (W.B.) using a specific antibody (Anti-HA). Omission of sodium ascorbate, on the contrary, does not induce de-*S*-nitrosylation of the protein, not allowing it to be biotinylated and selected in the Pulldown step. Thus, it cannot be detected on the W.B. with Anti-HA as seen of the right side of the top blot.

5.2.3 *S*-nitrosylation of PSH is immune-responsive

To test if PSH *S*-nitrosylation is a relevant biological process in *D. melanogaster* immunity, transgenic flies expressing PSH-HA in both *gsnor*$^{-/-}$ (*UAS-psh-HA*/*Ubi-Gal4*; *gsnor*$^{-/-}$) and wild type (*UAS-psh-HA*/*Ubi-Gal4*;+/+) backgrounds were challenged with the bacterial pathogen *E. faecalis* and assayed for PSH-HA *S*-nitrosylation at different time points after infection.

As presented in Figure 5-6, the levels of PSH-HA *S*-nitrosylation in wild type

flies fluctuates during infection, being reduced after 20 minutes, increased after two hours and reduced again after four hours. This reflects the dynamic nature of this PTM and suggests that de-*S*-nitrosylation of PSH could be important for the activation of the response, and *S*-nitrosylation could be necessary to keep the protein inactive and avoid over-responsiveness.

On the other hand, in *gsnor*$^{-/-}$ overlapping deficiency flies, the level of PSH-HA *S*-nitrosylation does not reduce in response to infection at any time point (Figure 5-7). Actually, the opposite seems to happen in these flies, with *S*-nitrosylation levels being lower before infection but becoming higher after infection compared to wild type flies. As shown in Figure 5-8, PSH-HA is clearly de-*S*-nitrosylated at 20 minutes after infection in wild type flies but the opposite happens in *gsnor*$^{-/-}$ flies, where the level of *S*-nitrosylation is increased.

As wound damage has recently been shown to trigger the Toll signalling pathway through activation of PSH (Ming et al. 2014b), the changes in the levels of *S*-nitrosylation presented here could also be associated with the activation of this protein induce by wound damage caused by the needle. Thus, it would be very interesting to repeat these experiments using a *B. bassiana* model, where damage to the fly is caused solely by the pathogen penetrating the cuticle.

In any event, these results show that the levels of *S*-nitrosylation of PSH change in response to the activation of the protein, being this activation triggered by wound or by the pathogen, and these variations are compromised in *gsnor*$^{-/-}$ flies.

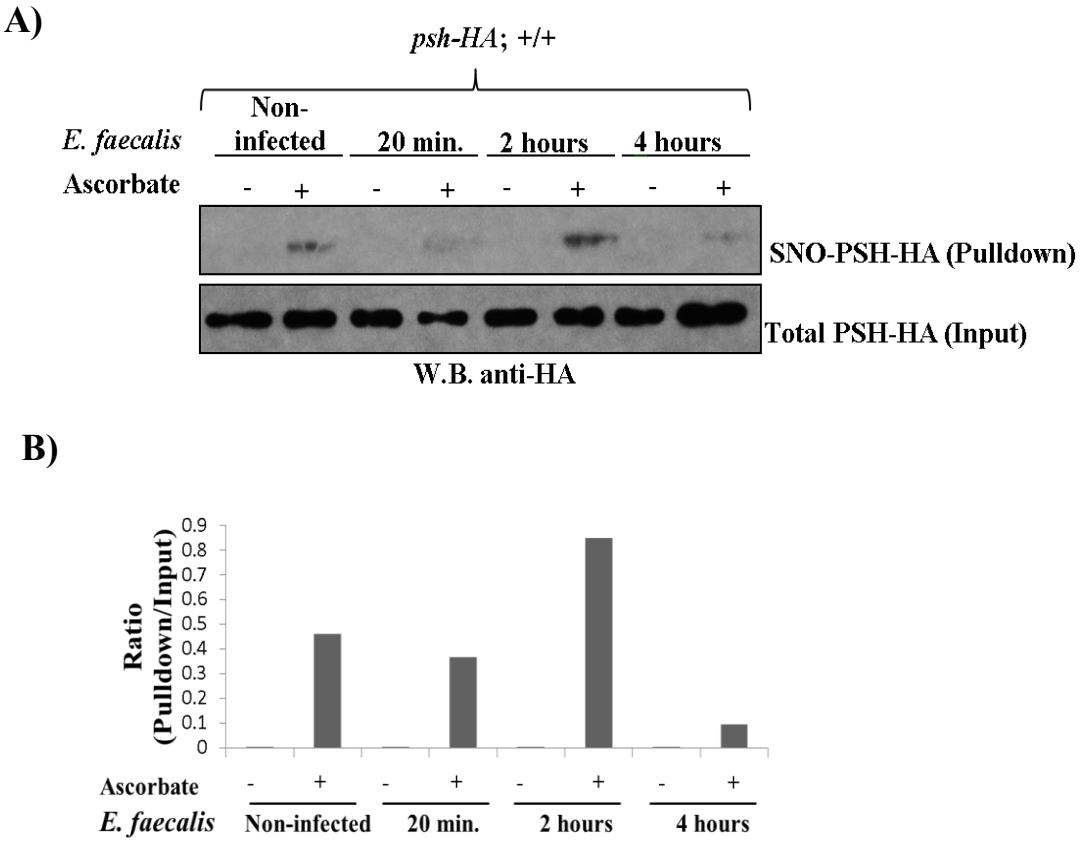


Figure 5-6: PSH-HA *S*-nitrosylation in wild type flies after pathogen infections

Batches of 10 female *UAS-psh-HA/Ubi-GAL4*; +/+ flies, aged between 3 to 6 days old, were anaesthetized by cooling on ice and septic injured with a needle previously dipped in an *E. faecalis* suspension with an O.D. of 1. Flies were transferred to standard fly food and kept at 29°C. After 20 minutes, two and four hours after infections, flies were transferred to 1.5 mL tubes and snap frozen on liquid nitrogen. Non-infected flies were kept at 29°C for four hours. Total proteins were extracted and submitted to the BST followed by streptavidin pulldown. Before the pulldowns, 20 µL aliquots were taken to be used as input controls. Pulldown and Input proteins were analysed by western blot with an anti-HA antibody (**A**) and blots were quantified by densitometry (**B**). As shown on these figures, the levels of PSH *S*-nitrosylation (Ascorbate treated non-infected, 20 min, 2 hours and 4 hours after *E. faecalis* infections) in wild type flies vary over time, being the strongest and the weakest signals detected within two and four hours after infection, respectively. These variations suggest that S-nitrosylation of this protein is responsive to infection.

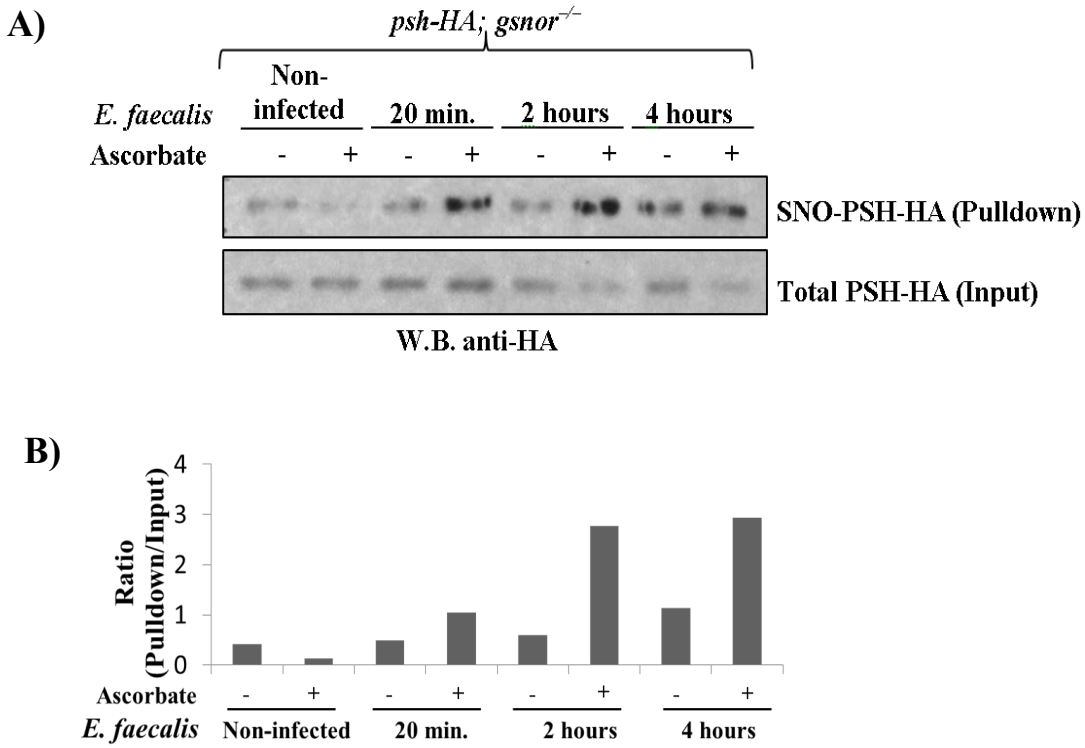


Figure 5-7: PSH-HA *S*-nitrosylation in *gsnor*$^{-/-}$ flies after pathogen infections

Batches of 10 female *UAS-psh-HA/Ubi-GAL4; Df7305/Df7306 (psh-HA; gsnor*$^{-/-}$*)* flies, aged between 3 to 6 days old, were infected with *E. faecalis* by septic injured with a needle previously dipped in a bacterial suspension with an O.D. of 1. Flies were transferred to standard fly food and kept at 29°C. After 20 minutes, two and four hours after infection, flies were transferred to 1.5 mL tubes and snap frozen on liquid nitrogen. Non-infected flies were kept at 29°C for four hours. Total proteins were extracted and submitted to the BST followed by streptavidin pulldown. Before the pulldowns, 20 µL aliquots were taken to be used as input controls. Pulldown and Input proteins were analysed by western blot with an anti-HA antibody (**A**) and blots were quantified by densitometry (**B**). As shown on these figures, the levels of PSH *S*-nitrosylation (Ascorbate treated samples) in *gsnor*$^{-/-}$flies vary over time, being increased after infection with *E. faecalis* (20 min) and remaining strong within two and four hours after infections. One interpretation for these results is the absence of GSNOR activity compromises PSH de-*S*-nitrosylation in *gsnor*$^{-/-}$ flies.

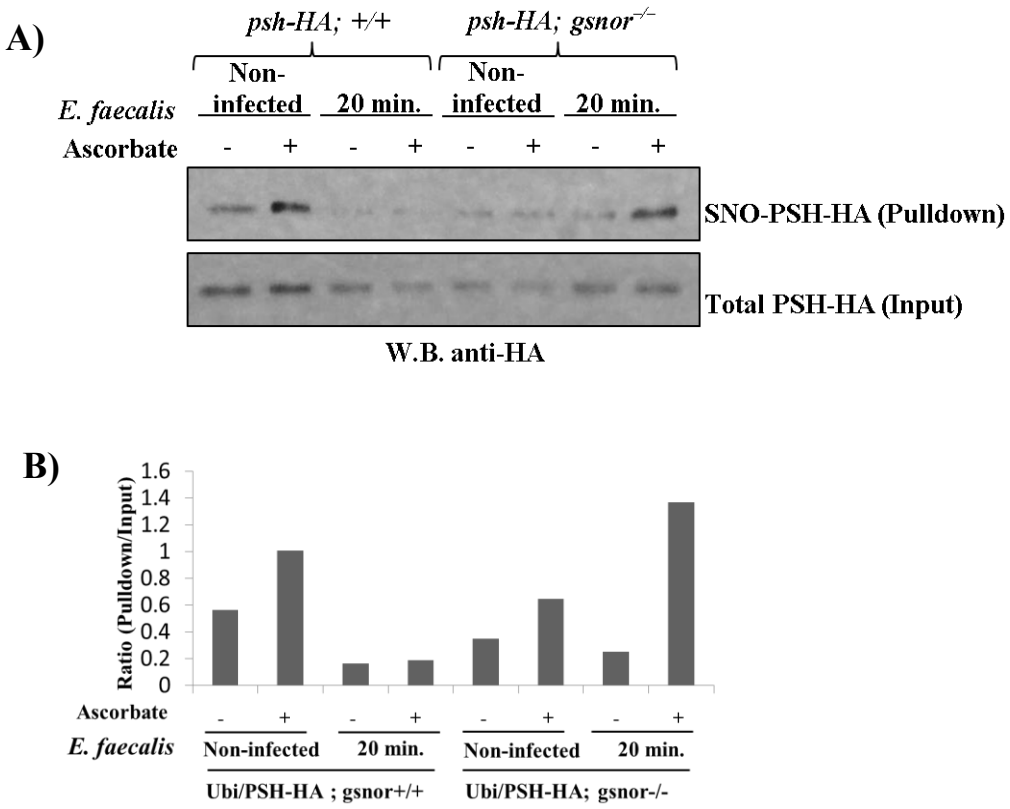


Figure 5-8: PSH-HA *S*-nitrosylation in wild type and *gsnor*$^{-/-}$ flies after pathogen infections

Batches of 10 female *UAS-psh-HA/Ubi-GAL4; +/+*(*psh-HA*; +/+) and *UAS-psh-HA/Ubi-GAL4; Df7305/Df7306* (*psh-HA*; *gsnor*$^{-/-}$) flies, aged between 3 to 6 days old, were infected with *E. faecalis* by septic injured with a needle previously dipped in a bacterial suspension with an O.D. of 1. Flies were transferred to standard fly food and kept at 29°C for 20 minutes. After that, flies were transferred to 1.5 mL tubes and snap frozen on liquid nitrogen. Non-infected flies were kept at 29°C for 20 minutes. Total proteins were extracted and submitted to the BST followed by streptavidin pulldown. Before the pulldowns, 20µL aliquots were taken to be used as input controls. Pulldown and Input proteins were analysed by western blot with an anti-HA antibody (**A**) and blots were quantified by densitometry (**B**). As shown on these figures, the levels of PSH *S*-nitrosylation (Ascorbate treated samples) 20 min after infections with *E. faecalis* decrease in wild type flies while it increases in *gsnor*$^{-/-}$ flies. These results indicate that loss of GSNOR activity in *gsnor*$^{-/-}$ flies affect PSH S-nitrosylation in response to infections.

5.2.4 C254 is a site of *S*-nitrosylation

To identify possible *S*-nitrosylation sites among PSH cysteine residues, an adaptation of the BST, here referred as the sequential cysteine blocking technique, followed by LC-MS/MS analysis was carried out. This technique, which is described in details in Chapter 2, involves treatment of the purified protein with a NO donor, blocking of free SHs with N-Ethylmaleimide(NEM), labelling of SNOs with iodoacetamide and in-gel tryptic digestions followed by LC-MS/MS. Differences in peptide masses caused by the incorporation of NEM or iodoacetamide can be discriminated by LC-MS/MS, allowing the identification of possible sites of *S*-nitrosylation within the protein.

Although 10 cysteine residues of PSH were identified by LC-MS/MS, seven residues were not present in the tryptic peptide mixture. This is probably due to the size of the fragments containing the remaining residues. Thus, further analysis using a different peptidase or a combination of peptidases to digest PSH should be employed to try to capture all cysteine residues in the peptide mixture.

Even though, out of the 10 cysteine residues identified after trypsin digestion C40, C46, C254 and C324 were found to be modified by iodoacetamide once PSH was treated with GSNO. These results suggest that these residues could be potential targets for *S*-nitrosylation. Based on protein sequence and functional information of PSH available on UniProt database, C324 is predicted to form a disulphide bridge with C300. Thus, this cysteine was not considered for further analyses.

In order to confirm that these cysteine residues are *S*-nitrosylation sites, site direct mutagenesis was employed to generate the following single amino acid replacements in MBP-PSH constructs: C40S, C46S and C254S. Protein expression

and purification followed the same methods described for the wild type protein (MBP-PSH WT). After producing recombinant proteins, the WT and mutated versions were tested for *in vitro S*-nitrosylation by the BST followed by streptavidin pulldown of biotinylated proteins and western blot using an anti-MBP antibody (Figure 5-9).

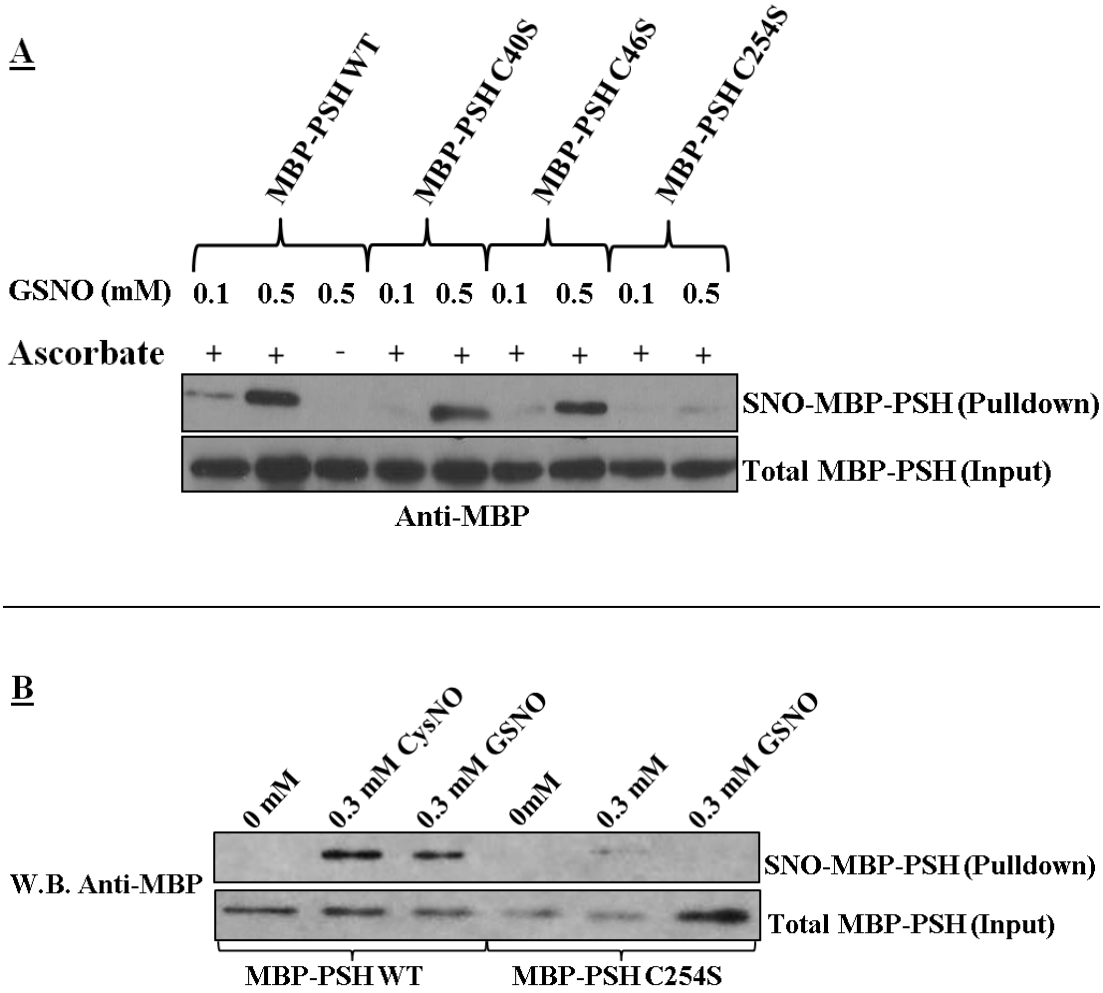


Figure 5-9: C254 is the main target for *S*-nitrosylation in PSH

A - 2µg of MBP-PSH WT, C40S, C46S and C254S were treated with different concentrations of GSNO and submitted to the BST. Streptavidin was used to pulldown biotinylated peptides, which were eluted with β-Mercaptoethanol and detected by western blot using an anti-MBP antibody. As an input control, 20µL aliquots were taken from each sample before streptavidin pulldown. As shown on the top blot, GSNO-induced *S*-nitrosylation of wild type MBP-PSH is concentration-dependent, and the detection of the signal depends on the addition of

ascorbate. Similarly, mutated versions MBP-PSHC40S and MBP-PSHC46S are S-nitrosylated when treated with 0.1 and 0.5 mM GSNO. However, mutated version MBP-PSHC254S is not *S*-nitrosylated when treated with 0.1 mM GSNO and only weakly *S*-nitrosylated when treated with 0.5 mM GSNO.

B - 2µg of MBP-PSH WT and C254S were treated with 0.3 mM of two NO donors, GSNO and CysNO. Samples were submitted to the BST followed by streptavidin pulldown and western blot with anti-MBP antibody. Non-treated samples (0 mM) were used as negative controls. As an input control (Total), 20µL aliquots were taken from each sample before streptavidin pulldowns. As shown on the top blot, 0.3 mM of both NO donors (CysNO and GSNO) induce *S*-nitrosylation of wild type MBP-PSH. However, mutated version MBP-PSHC254S is not *S*-nitrosylated when treated with 0.3 mM GSNO and only weakly *S*-nitrosylated when treated with 0.3 mM CysNO.

C254 was found to be the main target of *S*-nitrosylation as the level of MBP-C254S *S*-nitrosylation was similar to background after the BST (Figure 5-9-A). MBP-C40S and MBP-C46S showed similar intensity signals as the WT protein, suggesting that these two residues do not undergo *S*-nitrosylation. However, it is important to note that a faint signal was still present in MBP-C254S, suggesting that another cysteine, probably to a less extent, might also be *S*-nitrosylated by CysNO but not by GSNO (Figure 5-9-B).

To confirm that C254 is the main site of *S*-nitrosylation *in vivo* as well as *in vitro*, transgenic flies expressing a mutated C254S version of PSH with a HA tag attached to the C-terminal of the protein (PSHC254S-HA) were generated as described before for wild type PSH-HA. Total protein from flies expressing PSH-HA and PSHC254-HA were submitted to the BST, pulled down with streptavidin and compared by western blot with an anti-HA antibody. To induce high levels of *S*-nitrosylation, prior protein extraction, flies were starved for two hours and then fed on 5 mM SNP in 5% sucrose solution for one hour. Control flies were fed on 5%

sucrose solution only.

As presented in Figure 5-10, while PSH-HA is *S*-nitrosylated in both conditions, and shows an increase in the signal after SNP treatment, *S*-nitrosylation of PSHC254S-HA is not detectable at any of the two conditions tested. These results suggest that C254 is indeed the main target for *S*-nitrosylation in PSH and this modification is responsive to increases in NO concentrations.

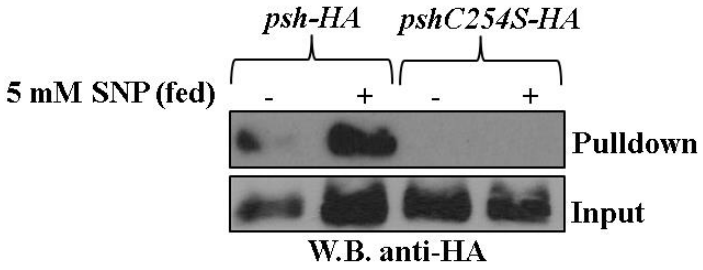


Figure 5-10: C254 is the main target of *S*-nitrosylation *in vivo*

Batches of 10 female transgenic flies expressing either PSH-HA or PSHC254-HA, aged between four and seven days, were starved for two hours in empty vials at 25°C and subsequently fed on 5% sucrose solution supplemented or not with 5 mM SNP for one hour at 25°C. Total proteins were extracted and submitted to the BST followed by streptavidin pulldown of biotinylated proteins and western blot using an anti-HA antibody. Total proteins were taken before the pulldowns (Input).

Based on protein structure prediction using the webserver I-TASSER (**http://zhanglab.ccmb.med.umich.edu/I-TASSER**), out of the 17 cysteine residues present in PSH, C254 is the most solvent exposed one, indicating that this residue is more likely to be *S*-nitrosylated than the other. According to this protein structure prediction, other cysteine residues could also be solvent exposed, however, to a less extend compared to C254. Once the crystal structure of PSH is resolved, we will be able to infer with certainty on the positions of these residues and this will certainly help clarify possible targets for S-nitrosylation. Meanwhile, further analysis of PSH *S*-nitrosylation sites, such as and mass spectrometry of peptides digested with alternative proteases and additional site-directed mutations followed by the BST

should be undertaken.

Moreover, superimposition of the predicted structure of PSH and the experimentally determined structure of GRASS (PDB 2xxl), another CLIP-domain serine protease from *D. melanogaster* Toll signalling pathway, which shares 33% amino acid sequence identity with PSH(Align Sequences Protein BLAST), suggests that C254 does not form a disulphide bridge (Figure 5-11). This is an indication that this cysteine, which is highly conserved among other serine proteases, does not have a structural role but might have a regulatory function. Altogether these results suggest that the function of this cysteine residue involves redox modulation by *S*-nitrosylation.

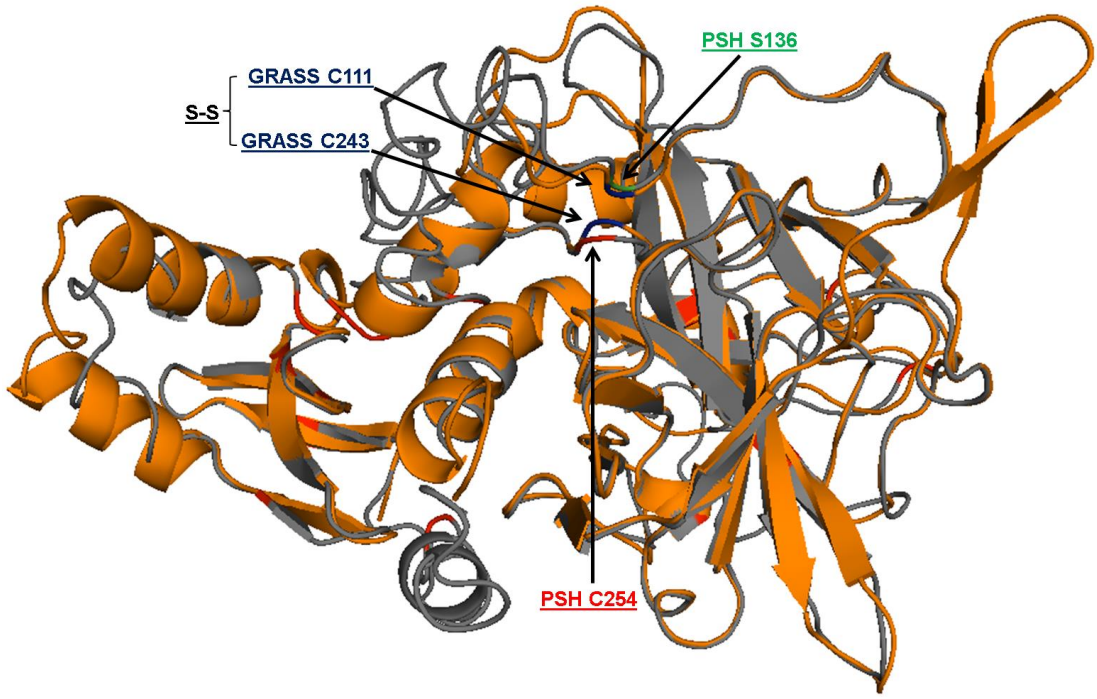


Figure 5-11: Superimposition of PSH and GRASS

The web server I-TASSER (http://zhanglab.ccmb.med.umich.edu/I-TASSER) was used to generate a model for PSH. Based on structural similarity using a TM-align structural alignment program (I-TASSER), PSH model was superimposed on the closest model available in the PDB database, which is GRASS, a closely-related CLIP-domain serine protease from *D. melanogaster* Toll signalling pathway. The PSH model is coloured grey and GRASS is coloured orange. GRASS C111 and C243, which form an inter-domain disulphide bridge, are highlighted in blue. PSH cysteine residues are highlighted in red including PSH C254, which is arrow pointed. PSH C254 is not in proximity to any other cysteine residues to form a disulphide bridge.

5.2.5 C254Spartially restores *gsnor*$^{-/-}$ immunodeficiency

C254 is a target for *S*-nitrosylation in PSH. To investigate if this cysteine residue plays a functional role in *D. melanogaster* immunity, transgenic flies carrying *UAS-pshC254S-HA* inserted at attP40 integration site (*UAS-pshC254S-HA/UAS-pshC254S-HA; +/+)* were crossed with *gsnor*$^{+/-}$ (*Df7305/TMB6)* flies to generate *UAS-pshC254S-HA/UASpshC254S-HA; Df7305/TMB6* progeny. These were crossed with *Ubi-GAL4/Ubi-GAL4; Df7306/TMB6* to generate *gsnor*$^{-/-}$ overlapping deficiencies expressing PSHC254S-HA (*Ubi-GAL4*/*UASpshC254S-HA*; *Df7305/Df7306*). The same approach was used to generate *gsnor*$^{-/-}$ overlapping deficiencies expressing wild type *PSH-HA* (*Ubi-GAL4*/*UAS-psh-HA*; *Df7305/Df7306*) and these flies were used for further experiments.

Survival curves were generated against the Gram-positive bacterium *E. faecalis* and the entomopathogenic fungus *B. bassiana,* pathogens to which *gsnor*$^{-/-}$ flies show increased susceptibility. As presented in Figure 5-12, the expression of PSHC254S-HA partially restored the resistance to *B*. *bassiana* in *gsnor*$^{-/-}$ flies [strains *UAS-pshC254s/Ubi-GAL4; gsnor-/-*(*Df7305*/*Df7306*) and *Ubi-GAL4/ UAS-pshC254s; gsnor-/-*(*Df7306*/*Df7305*)], while expression of PSH-HA [strains *UAS-psh/Ubi-GAL4; gsnor-/-*(*Df7305*/*Df7306*) and *Ubi-GAL4/ UAS-psh; gsnor-/-* (*Df7306*/*Df7305*)] had no effect on the survival of these flies against this pathogen.

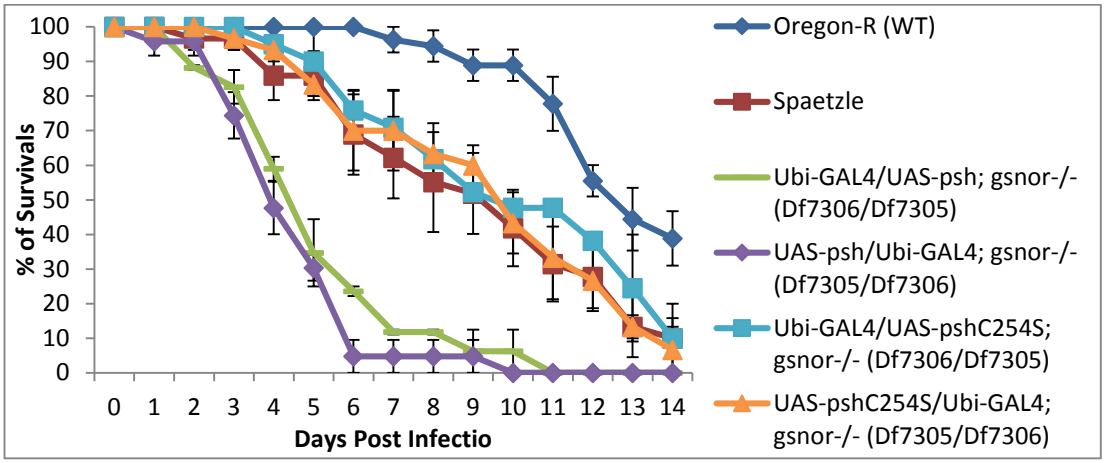


Figure 5-12: *pshC254S-HA* partially recovers *gsnor*$^{-/-}$ immunodeficiency against *B. bassiana*

Groups of 15 female flies, aged between three and six days, were transferred to 2 mL tubes containing spores of *B. bassiana* and gently shaken for two minutes. Flies were transferred to new vials containing standard cornmeal media and kept at 29°C. The number of flies surviving was recorded every 24 hours after infections and the flies that were alive were transferred to new vials every other day. The data represent the mean of three independent experiments (±SE) and show the survivals of *gsnor*$^{-/-}$ overlapping deficiency flies from both reciprocal crosses.

On the other hand, PSH-HA had a positive effect in restoring resistance of *gsnor*$^{-/-}$ flies to *E. faecalis*. As seen in Figure 5-13 both *gsnor*$^{-/-}$ strains expressing PSH-HA [*UAS-psh/Ubi-GAL4; gsnor-/-*(*Df7305*/*Df7306*) and *Ubi-GAL4/ UAS-psh; gsnor-/-*(*Df7306*/*Df7305*)] presented an improvement in survival compared to *gsnor*$^{-/-}$ strains expressing PSHC254S-Ha [*UAS-psh/Ubi-GAL4; gsnor-/-*(*Df7305*/*Df7306*) and *Ubi-GAL4/ UAS-psh; gsnor-/-*(*Df7306*/*Df7305*)] and gsnor-/- itself. [*+/+; gsnor-/-* (*Df7305*/*Df7306*) and [*+/+; gsnor-/-* (*Df7306*/*Df7305*)]. These results suggest that C254 plays a more significant role during the immune response to *B. bassiana.*

Although proteases from bacteria have been related to the cleavage and activation of PSH (El Chamy et al. 2008) , this protein is not the main mechanism by

which *D. melanogaster* detects Gram-positive bacterial infections, as *psh* mutants are only slightly more susceptible to these microorganism, while highly sensitive to *B. bassiana* (Ligoxygakis et al. 2002; El Chamy et al. 2008). Thus, *pshC254* seems to be important in the response against fungal infections, where proteases secreted by the pathogen play an important role in the establishment of disease.

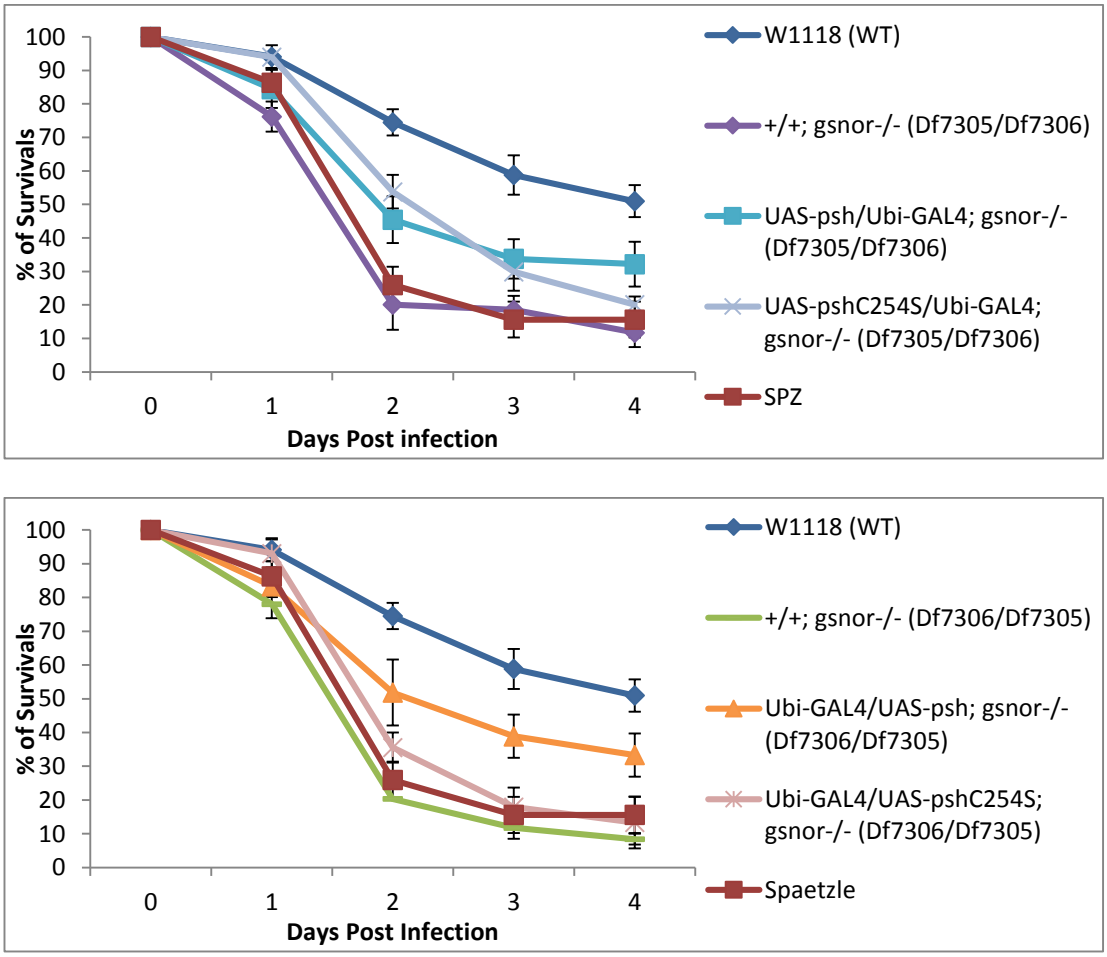


Figure 5-13: *pshC254S-HA* does not recover *gsnor*$^{-/-}$ immunodeficiency against *E. faecalis*

Groups of 20 female flies, aged between three and six days, were infected with a needle coated in *E. faecalis* (O.D. 1). Flies were transferred to new vials containing standard cornmeal-agar media and kept at 29°C for five days. The number of survivors was recorded every 24 hours after infection for four days. The data represent the mean of three independent experiments (±SE). Top and bottom graphs were generated using data from the

same experiment. Each graph shows the survival of flies generated from one of the reciprocal crosses done to generate the overlapping deficiencies.

5.2.6 SPE is a target for *S*-nitrosylation

Although PSH has been shown to be activated by proteases secreted by Gram-positive bacteria (El Chamy et al. 2008), it is not of major importance during the immune response of flies against this class of pathogen (Ligoxygakis et al. 2002; Gottar et al. 2006). Furthermore expression of *pshC254S* only partially restores the immunodeficiency phenotype of $gsnor^{-/-}$ flies against *B. bassiana* and has no effect on the response to Gram-positive bacteria. For these reasons and the fact that $gsnor^{-/-}$ flies were shown to be susceptible to Gram-positive bacteria as well as to fungi, additional potential targets for *S*-nitrosylation in the Toll signalling pathway of *D. melanogaster* were considered for furthers studies.

The CLIP-domain serine protease SPE, which cleaves and activates Spz, defines a common protease cascade downstream of fungal, bacterial, and virulence factors detection. The protease domain of SPE shares 32% amino acid sequence identity with PSH, with six conserved cysteine residues, including C254 (Figure 5-14).

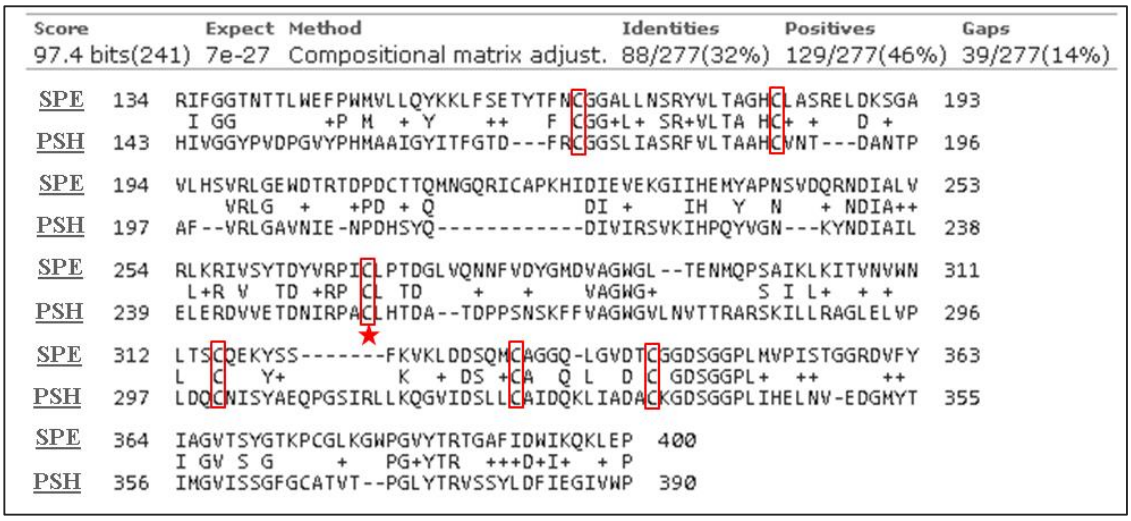


Figure 5-14: Amino acid sequence alignment of SPE and PSH

Basic Local Alignment Search Tool (BLAST) was used to align the amino acid sequences of SPE and PSH. These proteins show 32% amino acid sequence identity with six conserved cysteine residues (highlighted inside red boxes), including PSHC254 (highlighted with a red star).

In order to investigate whether this protein could also be *S*-nitrosylated, recombinant MBP-SPE and SPE-HA were expressed in *E. coli* and in *D. melanogaster*, respectively. The methodology and plasmids used to express these two constructs were the same as described for producing MBP-PSH and PSH-HA (Chapter 2). As shown in Figure 5-16, SPE is indeed *S*-nitrosylated both *in vitro* and *in vivo*.

Even though the *S*-nitrosylation site in SPE remains to be identified, these preliminary results suggest that PSH is not the only protein in the Toll signalling pathway that is *S*-nitrosylated, and suggests that NO modulates Toll-mediated immunity through modification of multiple targets in this pathway. This might explain why *gsnor*$^{-/-}$ flies are also susceptible to Gram-positive bacterial infections.

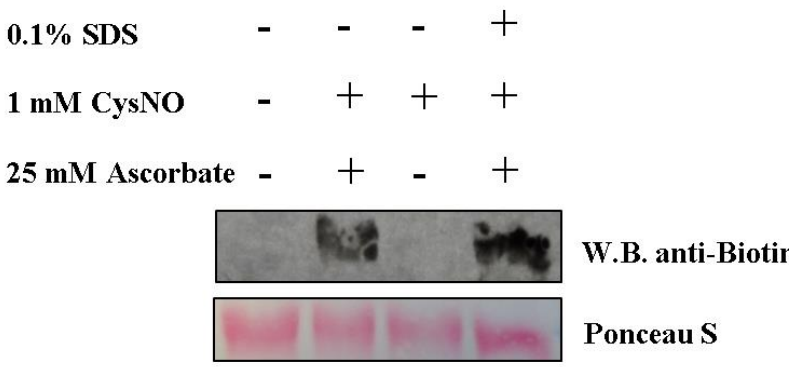


Figure 5-15: MBP-SPE is *S*-nitrosylated *in vitro*

2µg of recombinant protein was treated with 1 mM CysNO and submitted to the BST. The addition of 0.1% SDS before CysNO treatment was used as a positive control because SDS denatures the protein exposing all cysteine thiols to the NO donor resulting in a stronger signal in the BST. An untreated sample was used as negative control. As a second negative control, ascorbate treatment was omitted before the addition of the biotin donor. After the BST, samples were run on a 12% SDS-PAGE, transferred to a nitrocellulose membrane and submitted to western blotting (W.B.) using an Anti-Biotin antibody. After transfer the membrane was stained with Ponceau S to confirm that similar amounts of proteins were being compared. This experiment was repeated at least three times with similar results.

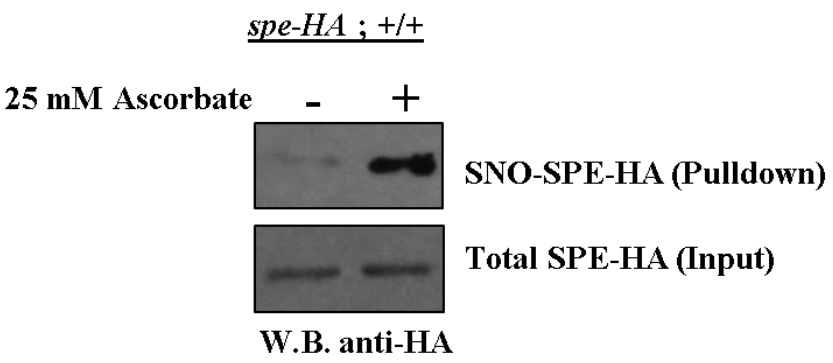


Figure 5-16: SPE-HA is *S*-nitrosylated *in vivo*

Total protein extract from 10 female *UAS-spe-HA/Ubi-GAL4* flies was submitted to the BST followed by streptavidin pulldown and western blots with an anti-HA antibody. Half of the protein extract was treated with 25mM sodium ascorbate during the BST while the other half was treated with sodium chloride as a negative control. To confirm that similar amounts of total protein were being compared, sample aliquots were taken before streptavidin pulldowns and used in parallel western blots. This experiment was repeated at least three times with similar results.

5.3 *S*-nitrosylation induces conformation changes of PSH and SPE

Treatment of MBP-PSH and MBP-SPE with the NO donor GSNO induces the formation of molecular complexes that are detected by the anti-MBP antibody in non-reducing conditions. GSH does not induce the formation of these complexes and addition of DTT converts them to monomers. These results indicate that NO from GSNO is inducing the formation of disulphide bridge-linked complexes (Figure 5-17 for MBP-PSH and Figure 5-18 for MBP-SPE). Treatment of MBP-SPE with ascorbate does not induce monomer formation, indicating that de-*S*-nitrosylation does not mediate monomerization, a process likely to be controlled by thioredoxins. Furthermore, non-*S*-nitrosylable MBP-PSHC254S is less responsive to GSNO in assembling into a high molecular weight complex (Figure 5-17), suggesting that *S*-nitrosylation of this particular cysteine might be important for this process.

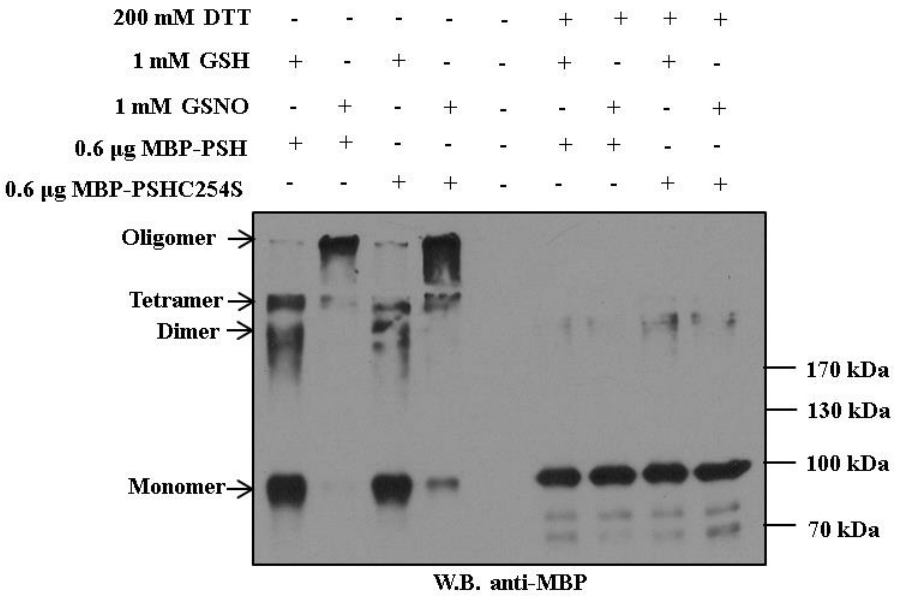


Figure 5-17: GSNO induces oligomerisation of MBP-PSH

0.6 µg of purified MBP-PSH and MBP-PSHC254S were treated with either 1 mM GSH or 1 mM GSNO at room temperature. After 20 minutes, desalting columns were used to remove GSH or GSNO from the samples and protein sample buffer with 200 mM DTT was added to half of each sample while only sample buffer was added to the other half. Samples were

incubated at 90°C for 10 minutes before being loaded on 8% SDS-PAGE. Proteins were transferred to nitrocellulose membranes and incubated with anti-MBP-HRP antibody. Addition of the NO donor GSNO induces the formation a high molecular weight complex (Oligomer) and consequent disappearance of Monomer, Dimers and Tetramers. This effect is less evident when GSNO is added to MBP-PSHC254S, as seen by a still strong Monomer band. These results indicate C254 plays a role in GSNO-induced PSH oligomerisation. The addition of DTT induces almost complete monomerization of PSH, suggesting this process is redox regulated. The smaller bands below the monomers in DTT treated samples are probably generated by self-cleavage and are likely to correspond to domains linked by disulphide bridges within PSH.

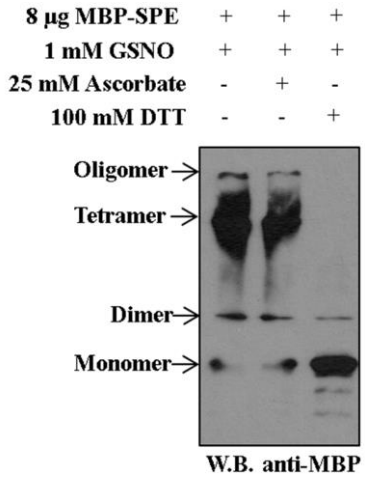


Figure 5-18: GSNO induces oligomerisation of MBP-SPE

8 µg of purified MBP-SPE samples were treated with 1 mM GSNO at room temperature. After 20 minutes, desalting columns were used to remove GSNO from the samples and protein sample buffer was added to all the samples. 100 mM DTT was added to one of the samples to reduce disulphide bridges and 25 mM sodium ascorbate was added to another sample to confirm that these complexes are not reduced by it. Samples were incubated at 90°C for 10 minutes before being loaded on 8% SDS-PAGE. After run, proteins were transferred to nitrocellulose membranes and incubated with anti-MBP-HRP antibody. Addition of the NO donor GSNO induces the formation a high molecular weight complex (Oligomer). Subsequence addition of DTT but not Ascorbate induces monomerization of MBP-SPE. These results suggest a GSNO-dependent, redox-modulated control of the quaternary structure of SPE. The smaller bands below the monomers in DTT treated samples are probably generated by self-cleavage and are likely to correspond to domains linked by disulphide bridges within SPE.

These observations led to us to investigate whether this process is

biologically relevant by looking at the formation of these complexes (or oligomers) in protein extracts from whole animals. Indeed, as presented in Figure 5-19, the addition of 0.5mM GSNO to protein extracts from wild type flies expressing PSH-HA induced the formation of oligomers in non-reducing conditions (-DTT). These oligomers could be detected by western blot with an anti-HA antibody and almost completely disappeared when 200 mM DTT was added to the samples. Interestingly, non-infected *gsnor*$^{-/-}$ flies expressing PSH-HA present higher levels of oligomers compared to non-infected wild type flies expressing the same construct.

Furthermore, PSHC254S-HA seems to present lower levels oligomerisation compared to PSH-HA when expressed in transgenic flies. In addition, similarly to the *in vitro* assays, PSHC254S-HA high molecular weight complex formation is less responsive to GSNO than PSH-HA, indicating that C254 might play a role in mediating this process (Figure 5-20). Furthermore, the disassembly of this complex seems to be triggered by *B. bassiana* infection. Altogether, these results point towards an immune-responsive SNO-dependent process that involves oligomerisation of PSH and SPE.

The involvement of *S*-nitrosylation in controlling oligomerisation/monomerization of proteins during immune responses has been shown in *A. thaliana*. *S*-nitrosylation of the transcription factor NPR1, a master regulator of salicylic acid (SA)–mediated defence genes in plants, induces the formation of a disulphide-linked oligomer in the cytoplasm preventing its translocation to the nucleus (Tada et al. 2008). These authors have also shown that NPR1 oligomers accumulate to higher levels in the cytoplasm of *gsnor* plants.

Thus, we speculate that oligomerisation of PSH are an integral part of the

function exerted by this protein during immune signalling in *D. melanogaster*, and that *S*-nitrosylation plays an important role in this process. By interfering with the quaternary structure of the protein, what probably correlates with its functions, NO finely tune the immune response to avoid over reactiveness. Deletion of *gsnor,* however, might aberrantly increase PSH *S*-nitrosylation and oligomer formation and cause a dysfunction of the system. This process seems to be conserved among CLIP-domain serine protease as SPE also undergoes oligomerisation in response to GSNO, and also accumulates more in *gsnor*$^{-/-}$ flies, as shown in Figure 5-21.

The formation of CLIP-domain serine protease oligomers has been reported before. Prophenoloxidase-activating factor (PPAF)-II, a member of the melanisation cascade, which belongs to the non-catalytic clip-domain SP family, forms a ~600 kDa homo-oligomer upon cleavage by its upstream protease, PPAF-III (Piao et al. 2005). In this case, homo-oligomers are functionally active while the monomers are inactive.

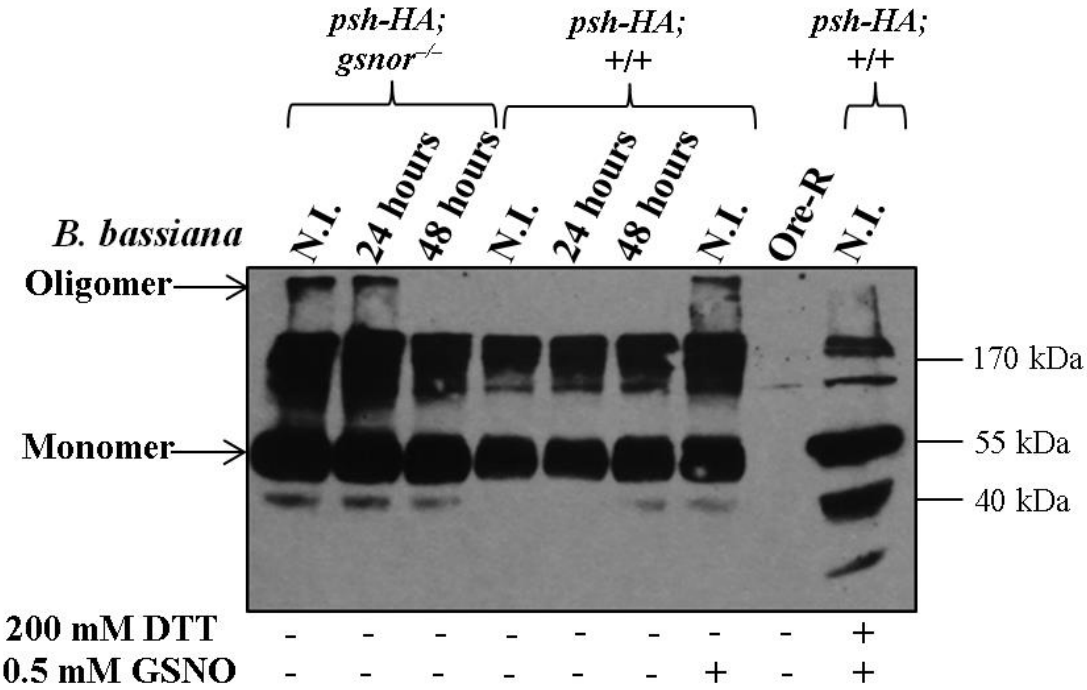


Figure 5-19: PSH-HA forms oligomers *in vivo* in response to GSNO

Groups of 10 flies expressing PSH-HA in a wild type background (*Ubi-GAL4/UAS-psh-HA*;+/+) or in a *gsnor*$^{-/-}$ background (*Ubi-GAL4/UAS-psh-HA; Df7305/Df7306*) were infected with *B. bassiana* spores and kept at 29 °C for 24 and 48 hours. Non-infected flies were kept at 29 °C for 48 hours. Proteins were extracted and quantified by the Bradford assay. 20 µg of total proteins was loaded in each well. 60 µg of proteins from psh-HA; gsnor+/+ was divided in three separate tubes with 20 µg of total proteins each. One of them was untreated; another one was treated with 0.5 mM GSNO for 20 minutes, and the third was treated with 0.5 mM GSNO for 20 minutes followed by 200 mM DTT. Sample buffer was added to all samples and they were kept at 90°C for 10 minutes before loading on a 10% SDS-PAGE. After electrophoresis, proteins were transferred to a nitrocellulose membrane and submitted to western blot with an anti-HA antibody. As seen on the top of the blot, PSH oligomers are only present in *gsnor*$^{-/-}$ flies (non-infected and 24 hours after infection), and the presence of these oligomers disappear within 48 hours after *B. bassiana* infections. In wild type flies treated in the same conditions PSH oligomers are not detected. However, when protein extracts from the same wild type flies are treated with exogenous nitric oxide donors (0.5 mM GSNO),PSH oligomers are detected. These results suggest that PSH oligomerization is GSNO dependent and increase in *gsnor*$^{-/-}$ flies. The smaller bands below the monomer (~40kDa) in DTT treated sample are probably generated by self-cleavage and are likely to correspond to domains linked by disulphide bridges within PSH.

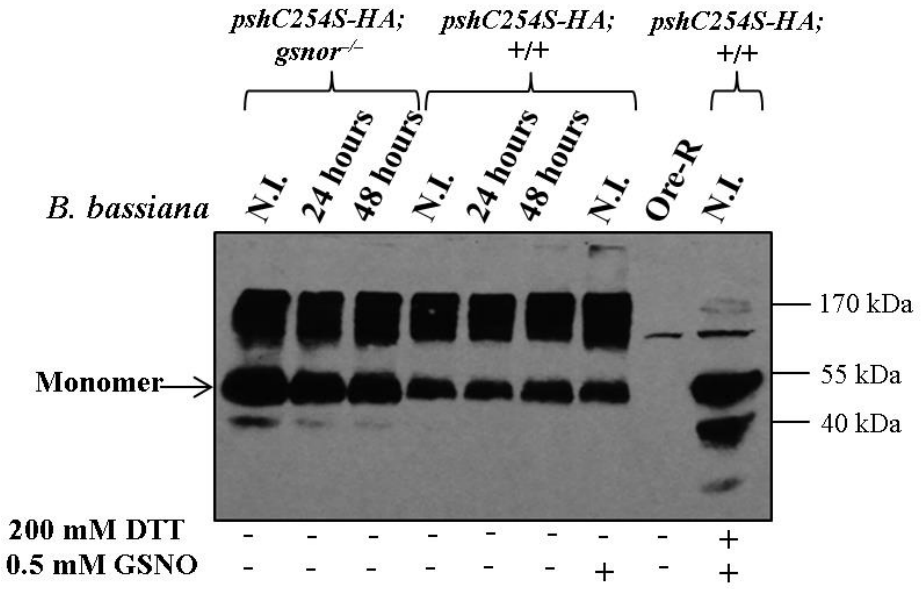


Figure 5-20: C254S mutation reduces GSNO-induced oligomer formation *in vivo*

PSHC254S-HA oligomers do not accumulate in *gsnor*$^{-/-}$ flies and are less responsive to GSNO treatment. Groups of 10 flies expressing PSHC254S-HA in a wild type background (*Ubi-GAL4/UAS-pshC254S-HA; +/+*) and in a *gsnor*$^{-/-}$ background (*Ubi-GAL4/UAS-pshC254S-HA; Df7305/Df7306*) were infected with *B. bassiana* spores and kept at 29 °C for 24 and 48 hours. Non-infected flies were kept at 29 °C for 48 hours. Proteins were extracted and quantified by the Bradford assay. 20 µg of total proteins was loaded in each well. 60 µg of proteins from *psh-HA; gsnor*$^{+/+}$flies was divided in three separate tubes with 20 µg of total proteins each. One of them was untreated; another one was treated with 0.5 mM GSNO for 20 minutes, and the third was treated with 0.5 mM GSNO for 20 minutes followed by 200 mM DTT. 1X final concentration of sample buffer was added to all samples and they were kept at 90°C for 10 minutes before loading on a 10% SDS-PAGE. After electrophoresis, proteins were transferred to a nitrocellulose membrane and submitted to western blot with an anti-HA antibody. Differently to the results present in Figure 5-19 for PSH-HA, PSHC254S-HA does not form oligomers in *gsnor*$^{-/-}$ flies. Even when protein extracts are treated with exogenous nitric oxide donors (0.5 mM GSNO) only a small fraction of PSHC254S-HAis converted to oligomers. These results suggest that C254 is involved in PSH oligomerization. The smaller bands below the monomer (~40kDa) in DTT treated sample are probably generated by self-cleavage and are likely to correspond to domains linked by disulphide bridges within PSH.

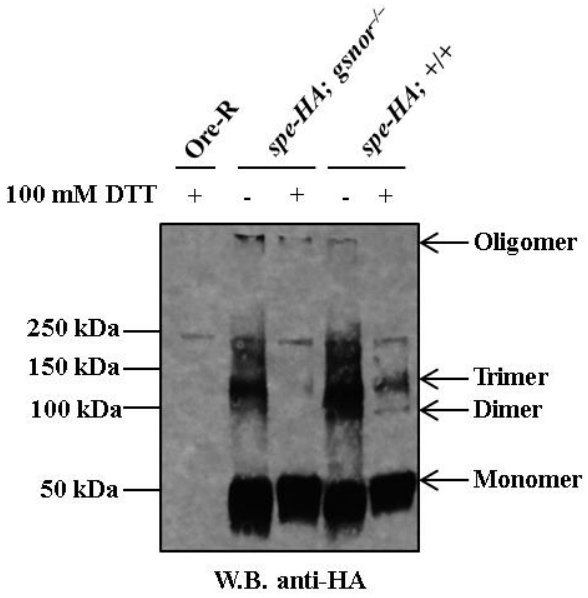


Figure 5-21: SPE-HA oligomers accumulate more in *gsnor*$^{-/-}$ flies

Groups of 10 flies expressing SPE-HA in *gsnor*$^{-/-}$ (Ubi-GAL4/UAS-spe-HA; *gsnor*$^{-/-}$) or wild type background (Ubi-GAL4/UAS-spe-HA; *gsnor*$^{+/+}$) were submitted to total protein extraction. The Bradford assay was used to quantify and adjust protein concentrations to the same level. Half of each sample was treated with 100 mM DTT and all the samples were treated with 1X sample buffer at 90°C for 10 minutes. Sample were loaded on a 10% SDS-PAGE, protein were separated by electrophoresis and transferred to a nitrocellulose membrane. A western blot with an anti-HA antibody was carried out to detect the SPE-HA. The same amount of total proteins from wild type Oregon-R flies was used as a control for non-specific binding of the antibody. As seen on the top of the blot, bands corresponding to SPE oligomers are more intense in *gsnor*$^{-/-}$ flies compared to wild type flies while bands corresponding to dimers and trimer are more intense in wild type flies compared to *gsnor*$^{-/-}$ flies. These results suggest loss of GSNOR activity might affect SPE oligomerisation in *gsnor*$^{-/-}$ flies.

5.4 Conclusion

In this chapter we sought to identify mechanisms that could explain the higher susceptibility of *gsnor*$^{-/-}$ flies to fungi and Gram-positive bacteria. As presented in Chapter 3, *gsnor*$^{-/-}$ flies present higher levels of global *S*-nitrosylation. In addition, these animals fail to induce the expression of AMPs in response to infections, and succumb more rapidly to certain classes of pathogens (Chapter 4). These observations led to the speculation that components of the Toll signalling pathway could be modulated by *S*-nitrosylation.

Either excessive *S*-nitrosylation or absence of de-*S*-nitrosylation of target proteins in the Toll pathway could perhaps compromise the signalling process and affect immune responses. In fact, previous results generated by Kanchanawatee (2012) suggested that PSH was a possible target for *S*-nitrosylation in the Toll signalling pathway. Thus, to further investigate if PSH was a target for this modification and if this process relates to the immunodeficiency phenotype of *gsnor*$^{-/-}$ flies, tagged versions of this protein were expressed both in *E. coli* for *in vitro* studies, and in transgenic flies for *in vivo* studies.

The data presented in this Chapter strongly suggest that PSH is *S*-nitrosylated *in vitro* and *in vivo*. In addition, this process was shown to be responsive to infection and augmented in *gsnor*$^{-/-}$ flies. These results indicate that *S*-nitrosylation of PSH is a functionally relevant process during *D. melanogaster* immune responses and is dysregulated in *gsnor*$^{-/-}$ overlapping deficiency flies.

The cysteine residue C254 was identified as the main target of *S*-nitrosylation in PSH and survival assays using transgenic flies expressing PSH-HA and PSHC254S-HA imply the biological relevance of this residue for *D. melanogaster*

immunity. The expression of PSHC254S-HA but not PSH-HA was enough to partially restore the immunodeficiency phenotype of *gsnor*$^{-/-}$ overlapping deficiency flies against *B. bassiana* infection. However, it had no effect on the survival against Gram-positive bacteria. These results led us to explore additional targets for *S*-nitrosylation within the Toll signalling pathway that could help understand the higher susceptibility of *gsnor*$^{-/-}$ flies to Gram-positive bacteria.

Thus, it was also found that another CLIP-SP of the Toll signalling pathway, SPE is also a target for *S*-nitrosylation both *in vitro* and *in vivo*. This enzyme is activated as a result of infections by both fungi and Gram-positive bacteria. Although these results might present further explanations for the immune-deficiency phenotype of *gsnor*$^{-/-}$ flies, further experiments will be necessary to confirm this hypothesis. The first will be the identification of the cysteine residue that is modified. Then, it will be necessary to determine whether expression of a cys-to-ser mutated version of SPE could partially or completely rescue the immunodeficiency of *gsnor*$^{-/-}$ flies against Gram-positive bacterial infections.

In addition, *S*-nitrosylation was shown to induce oligomerisation of PSH and SPE. This process, which is induced by NO donors *in vitro* and *in vivo*, seems to play a role in the immune signalling process since *B. bassiana* infection disfavours oligomer formation. Interestingly, oligomers are seen more abundantly in the protein extracts of *gsnor*$^{-/-}$ flies compared to wild type flies. These results suggest that higher SNO levels in *gsnor*$^{-/-}$ flies favour oligomerisation of the CLIP-SPs PSH and SPE, and perhaps other CLIP-SPs.

Based on these observations, a model is proposed (Figure 5-22) where *S*-nitrosylation orchestrates oligomerisation/monomerization of CLIP-SPs during

immune responses. In this simplified model, CLIP-SP zymogens are present in the haemolymph of flies as monomers, oligomers and other disulphide-linked intermediates. The state of these conformations is directly related to the redox environment and the levels of SNO. S-nitrosylation of CLIP-SPs favours oligomerisation while pathogen infections favour monomerization. Monomers can be activated during pathogen infections and this triggers immune responses. One of the outcomes of these responses is an increase in SNO levels. Higher SNO levels induce oligomerisation over monomerization and modulate the immune response.

In basal conditions this process would be beneficial for the flies to avoid over immune responsiveness, therefore inhibiting the signalling in a tightly controlled manner. However, in a scenario where de-*S*-nitrosylation is compromised, aberrant *S*-nitrosylation of CLIP-SPs could blunt or retard the activation of the pathway, leading to the immunodeficiency phenotypes that we observed for *gsnor*$^{-/-}$ overlapping deficiency flies.

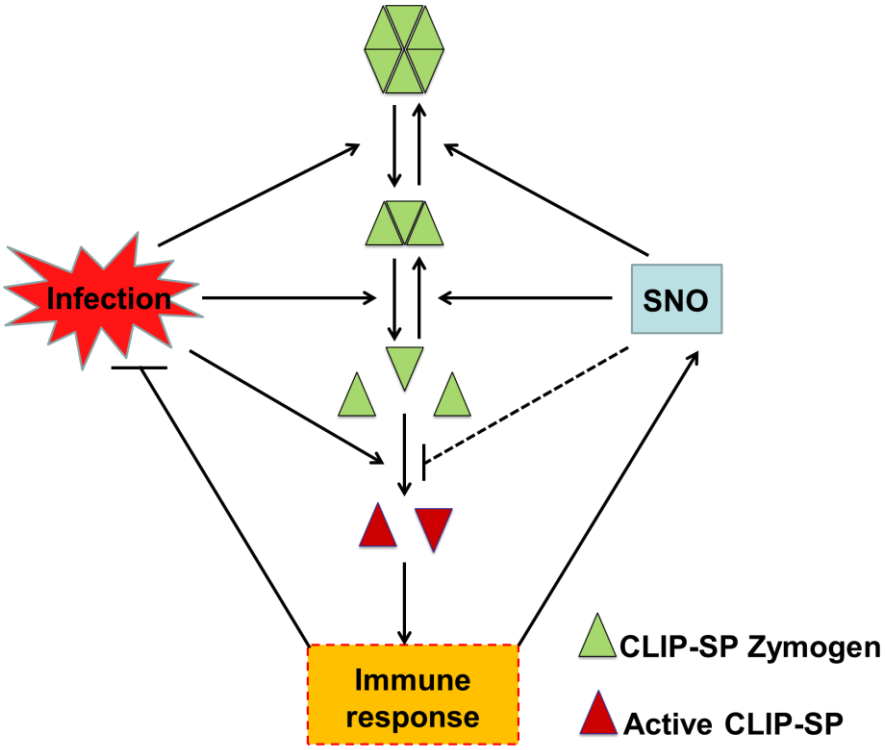


Figure 5-22: A model for redox-regulation of CLIP-SPs in *D. melanogaster* immunity

CLIP-SP zymogens form disulphide-linked intermediates. *S*-nitrosylation of CLIP-SPs favours oligomerisation while pathogen infections favour monomerization and protein activation. Activation of CLIP-SPs leads to immune responses that will combat infection and increase global *S*-nitrosylation (SNO). Higher SNO levels prevent over-responsiveness to infections by inducing the formation of oligomers.

Chapter 6

Employing CRISPR to generate *gsnor* mutants

6.1 Introduction

Over the course of my PhD project, a new genome editing technology has been developed. The type II clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated (Cas) system is a powerful and promising technology for targeting, editing and regulating the genomes of various organism (Reviewed in Sander & Joung 2014). This system is an adaptive immune mechanism of bacteria and archaea against foreign nucleic acids, such as viruses or plasmids (Sorek et al. 2008; Horvath & Barrangou 2010; Wiedenheft et al. 2012). It is based on the induction of double-strand break (DSB) in DNA by a single polypeptide nuclease, the Cas, guided to target sites by the guide RNA (gRNA). The gRNA is a chimeric sequence consisting of two small RNAs, the CRISPR RNA (crRNA), which is complementary to a target DNA sequence, and the trans-activating CRISPR RNA (tracrRNA), which is required for Cas9 nuclease activity (Figure 6-1).

DSBs are repaired by either non-homologous end joining (NHEJ) or homology-directed repair (HDR) (Lieber 2010). NHEJ is an error-prone process that frequently leads to the generation of small insertions and deletions (indels). During HDR, the repair of DSBs is achieved by precisely copying sequence from a donor template. Thus, while NHEJ can induce mutation by incorporating indels, HDR can introduce any changes pre-incorporated in the donor template. Recognising the potential of this system to genome engineering, Jinek et al. (2012) have adapted this

platform to work in the genome of other organisms. This breakthrough has since greatly contributed towards speeding-up genome editing and biological research.

The main advantage of CRISPR/Cas9 over other technologies that employ site-directed nuclease-mediated DSBs, such as Zinc- finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs), is that, unlike the latter, CRISPR/Cas9 does not require the generation of a unique protein for each genomic manipulation. In contrast, it directs a common nuclease to specific DNA sequences by short, readily generated RNA molecules. The only limitation for targeting any 20 nucleotide long specific genomic region is the presence of a 3 base pair proto-spacer adjacent motif (PAM) sequence, NGG or NAG. CRISPR/Cas-mediated editing of the *D. melanogaster* genome has been used for cell lines (Bassett et al. 2015) and whole organisms (Gratz et al. 2013; Port et al. 2014; Ren et al. 2014; Gratz et al. 2014).

Despite being an efficient way of generating *gsnor* loss of function flies, the overlapping deficiencies technique inevitably generates hemizygosity for a considerable number of genes. Even though most of the phenotypes we have observed can be partially or fully restored by complementing the flies with a copy of *gsnor*, we cannot rule out that some of the phenotypes that we have observed are affected by hemizygosity for one or more of the genes within the deletions used. Thus, by making use of CRISPR/Cas9 technology, I sought to generate a *gsnor* loss of function *D. melanogaster* mutant.

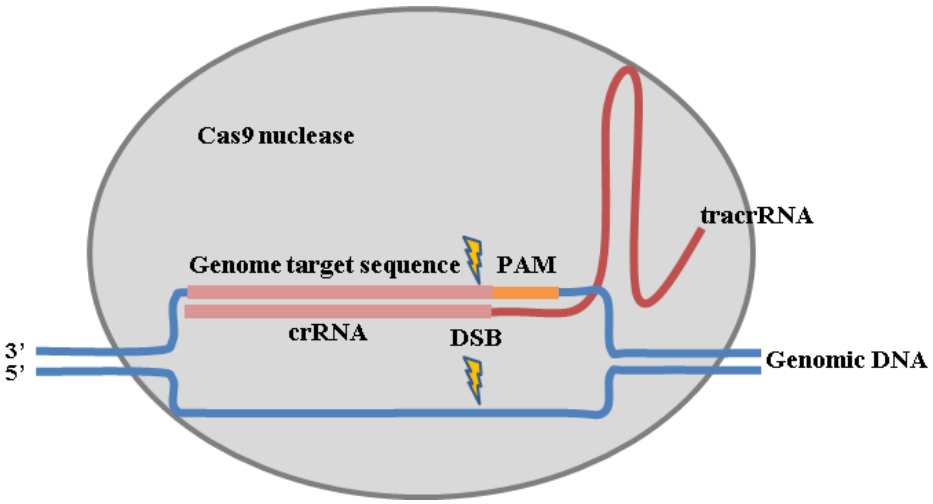


Figure 6-1: CRISPR/Cas9 targeted double-strand break.

The gRNA is a chimeric molecule constituted of a crRNA and a tracrRNA. The sequence of the crRNA is complementary to a target sequence in the genome. With the aid of the tracrRNA, Cas9 endonuclease is recruited to the sequence in the genome where the crRNA binds to the DNA, and cuts off the DNA at both strands. The target sequence must be followed by a NGG or NAG protospacer adjacent motif (PAM) which is required for Cas9 cleavage.

6.2 The strategy

CRISPR/Cas9-mediated HDR using two gRNAs, targeted to both sides of a gene, together with a donor plasmid as template, has been demonstrated to be an efficient strategy to delete genes of interest in *D. melanogaster* (Gratz et al. 2013; Port et al. 2014; Gratz et al. 2014). Aiming to completely and precisely delete *gsnor* from the genome of *D. melanogaster* and replace it by a visible marker, gRNAs were designed to direct the Cas9 to specific DNA sequences to both sides of the gene. A plasmid carrying the visible marker *DsRed* was chosen to be used as a template donor (Figure 6-2).

CRISPR targets within the 5' and 3' regions of *gsnor* were identified using a web-based tool (tools.flycrispr.molbio.wisc.edu/targetFinder) (Gratz et al. 2013), and a single plasmid (pCFD4) expressing both gRNA-*gsnor*-5' (gRNA-5') and gRNA-

gsnor-3' (gRNA-3') under the control of U6 promoters was generated as described before (Port et al. 2014) and detailed in Chapter 2. The donor template vector was generated by cloning ~900 bp homology sequences of upstream and downstream regions of *gsnor* flanking the visible marker *3xP3-DsRed* into the plasmid pHD-DsRed-attP (Gratz et al. 2014)(detailed in Chapter 2). This plasmid contains a Φ31C attP site that is transferred to the genome together with DsRed. This integration site will be used in the future to introduce a wild type copy of *gsnor* in the same genomic location, avoiding epigenetic-related interference in gene expression. The loxP sites flaking DsRed can be used to excise the marker from the region by crossing these flies to flies expressing the Cre recombinase, leaving only the attP site (Figure 6-2).

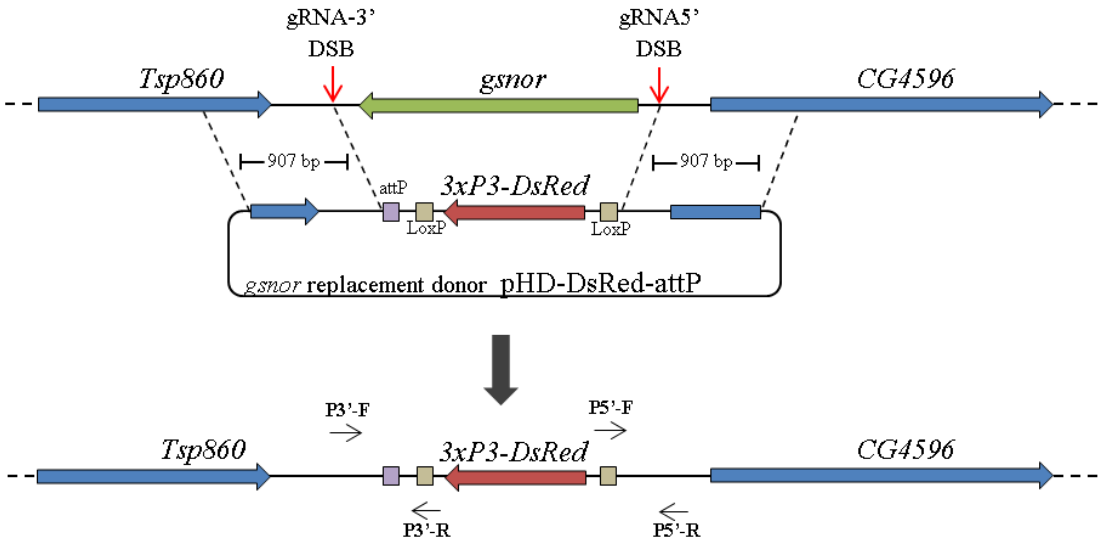


Figure 6-2: Strategy to generate *gsnor* knock-out flies using CRISPR/Cas9

gRNAs were designed to direct the Cas9 nuclease to specific sequences in intergenic regions flanking *gsnor* (gRNA-3' and gRNA-5'). Cas9-induced DSB were repaired by HDR using homology sequences present in the replacement donor plasmid pHD-DsRed-attP. Integration of 3xP3-DsRed, and consequent deletion of gsnor, was confirmed by sequencing using the primers P3'-F and P3'-R, and P5'-F and P51'-R. Schematic representation is not to scale.

After cloning, bothpCFD4-gRNA-*gsnor*3'/5' and pHD-DsRed-attP-*gsnor*3'/5' plasmids were propagated in *E. coli*, analysed by sequencing, and sent to Genetic Services, Inc. where they were co-injected into *vasa-Ca9* embryos that express Cas9 in the germline. Three independent transgenic flies expressing *DsRed* were recovered after injections, crossed with w^{1118} flies and sent back to our laboratory. All three stocks were made homozygous for *DsRed*, which we confirmed is inserted on the third chromosome as expected. The vasa-Ca9 construct was located on the X chromosome which was replaced by X chromosome from w^{1118} flies.

6.3 *DsRed* has integrated at the expected genomic region

To investigate whether the replacement of *gsnor* by *DsRed* has taken place, primers were designed to amplify and sequence the surroundings of the integration site. One primer of each pair was designed to hybridise with a sequence in the genome of *D. melanogaster* while the other should hybridise with a sequence within the replacement donor fragment (shown in Figure 6-2 - P5'-F / R and P3'-F / R). As presented in Figure 6-3, primers P5'-F/R and P3'-F/R amplified fragments of the expected sizes (1415 bp for P3' and 1037 bp for P5') from the genome of all three *DsRed* homozygous flies but did not amplify any fragments from the genome of wild type flies. These results indicate that *DsRed* has integrated in the correct genomic position in all three transgenic flies tested.

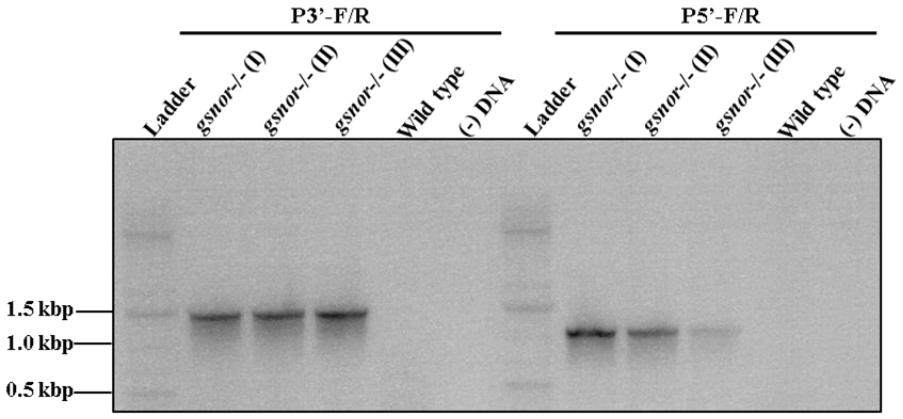


Figure 6-3: *DsRed* integration in the genome of *D. melanogaster*

Genomic DNA (10 ng) from *gsnor*$^{-/-}$ (I, II and III) and wild type flies were used as templates in PCRs to amplify both upstream (primers P3'-F/R) and downstream (primers P5'-F/R) regions of the integration site of DsRed. As expected, fragments of approximately 1415 bp and 1037 bp were amplified using P3'-F/R and P5'-F/R primers, respectively, when using the DNA of *gsnor*$^{-/-}$ (I, II and III) but not wild type flies as templates in the reactions.

To further analyse the integration sites, the PCR products shown in Figure 6-3 were purified, sequenced and aligned against the original sequence deposited in the *D. melanogaster* genomic database. These analyses suggest the insertion of two extra nucleotides in the intergenic region between *gsnor* and the adjacent gene *CG4596.* An adenine and a guanine do not match with the original sequence, being 54 bp and 84 bp from the Cas9-induced cleavage site towards *CG4596*, respectively. These insertions were seen in the genome of all three mutants, suggesting that this is a natural variation and not caused by the HDR process. Insertions in this intergenic region are unlikely to affect the transcriptions of the adjacent gene.

These results indicate that *DsRed* has integrated in the genome of *D. melanogaster*, and suggest that this integration was driven by CRISPR/Cas9-mediated HDR. It could be confirmed by PCR and sequencing of the 5' region that a recombination event, and consequent integration of *DsRed*, took place at the site

where a DSB was expected.

6.4 Off targets

In a recent work investigating the efficiency and specificity of CRISPR/Cas9 in *D. melanogaster*, Ren and co-workers, based on a robust screen with 104 gRNAs, suggested that the mutagenesis efficiency reduces to zero when three or more mismatches, even at the most distal end to the PAM, are introduced into the crRNA sequence of a gRNA (Ren et al. 2014). The *gsnor* 5' gRNA has 14 perfect matches, 12 in a PAM-proximal region and 2 in the PAM-distal region, to a sequence in an intron region of the *Glial cell line-derived neurotrophic family receptor-like* (*Gfrl*) gene, also on the third chromosome.

Even though, due to the presence of five mismatches in the PAM-distal region of the gRNA, an off target effect is very unlikely to happen, primers were designed to amplify and sequence this region from the genome of DsRed ($gsnor^{-/-}$) flies. Attempts to amplify this region from the genome have failed to generate single fragments and for this reason an off-target effect of this gRNA has not been ruled out yet.

6.5 Protein extract of *DsRed* flies present no GSNOR activity

Since the integration of *DsRed* seems to have taken place at the expected genome location, *gsnor* should have been deleted from the genome of these flies, and for this reason they should have no GSNOR activity. To confirm this hypothesis, protein extracts from these flies were assayed for GSNOR activity by measuring the rate of NADH oxidation. As presented in Figure 6-4, the activity of this enzyme was

detected in the protein extract of wild type flies but not in the protein extracts of *DsRed*-expressing flies.

Protein extracts from all three *DsRed*-expressing lines generated by CRISPR/Cas9 (I, II and III) showed similar conversion rates of NADH to NAD^+, and this was similar to the conversion rate seen in the protein extract of $gsnor^{-/-}$ overlapping deficiency flies. These results suggest that these animals are *gsnor* null mutants. The slow conversion rate of NADH seen for all $gsnor^{-/-}$ lines is not related to GSNOR activity since it is very similar to the conversion rate of NADH in samples with protein extracts of wild type flies but without added GSNO. Thus, GSNOR activity accounts for most of NADH oxidation when GSNO is added to the samples, however, to a less extent, other enzymes present in the protein extracts of *D. melanogaster* also oxidise NADH.

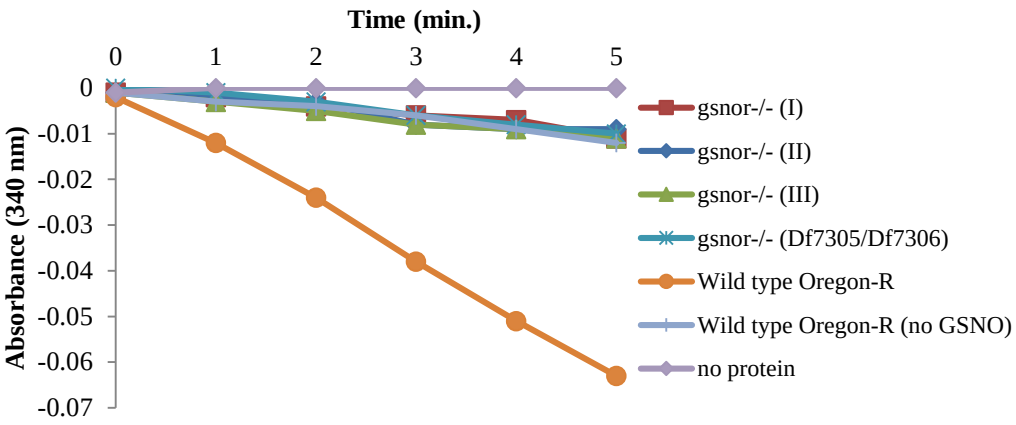


Figure 6-4: CRISPR/Cas9-generated*gsnor*$^{-/-}$ mutants present no GSNOR activity

Protein extracts from *DsRed* homozygous flies (*gsnor*$^{-/-}$ I, II and III) were assayed for GSNOR activity by spectrophotometrically measuring the rate of NADH oxidation in the presence of GSNO. *gsnor*$^{-/-}$ overlapping deletions (Df7305/Df7306) and wild type Oregon-R flies were used as controls. Omission of GSNO from samples containing protein extracts of wild type Oregon-R flies and samples without protein extracts were also used as controls for this experiment.

6.6 Deletion of *gsnor* reduces tolerance to the NO donor SNP

gsnor$^{-/-}$ overlapping deletion flies were shown to be less tolerant than wild type flies to treatment with the NO donor SNP. Having confirmed that the new *gsnor*$^{-/-}$ mutants are unable to metabolise GSNO due to lack of GSNOR activity, the tolerance of these animals to SNP was assessed.

As shown in Figure 6-5, these flies were less tolerant to SNP than wild type Oregon-R flies. While approximately 70% of the wild type flies survived 24 hours of feeding on a 5 mM SNP / 5 % sucrose solution, only approximately 30% of the mutants survived. Although Oregon-R wild type flies do not share an identical genetic background with CRISPR-generated *gsnor*$^{-/-}$ flies, which were generated in a W^{1118} background, differences in genetic background are not likely have such a

profound impact on resistance to SNP. In any case, experiments should be repeated using W^{1118} as control to rule out any interference of genetic background in the outcomes of these assays.

In accordance, these results are consistent with what has been shown before using the *gsnor*$^{-/-}$ overlapping deletion flies, indicating an important role for GSNOR activity in controlling NO homeostasis in *D. melanogaster*.

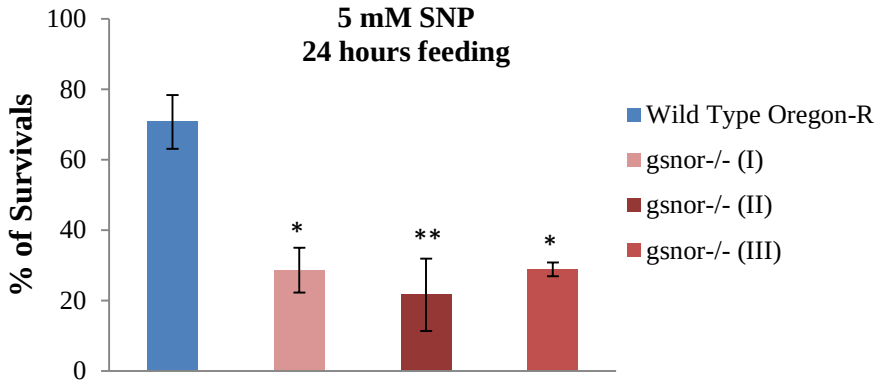


Figure 6-5: CRISPR/Cas9-generated *gsnor*$^{-/-}$ mutants are less tolerant to SNP than wild type flies

Groups of 20 female flies, aged between 3 and 6 days, were transferred to empty vials and starved for two hours at 25°C. After that, flies were transferred to vials containing a cotton roll soaked in 5% sucrose solution supplemented with 5 mM SNP. Vials were kept at 25°C and the number of survivors was recorded one day after the treatment. Based on one-way ANOVA ($F_{3,8}$ = 9.67 p< 0.005) and Tukey HSD test, * and ** indicate significant differences between treatments, p ≤ 0.05 and p ≤ 0.01 respectively. The data represent the mean of three independent experiments (±SE). The difference between the survivals of the three mutant lines is non-significant.

6.7 CRISPR/Cas9-generated *gsnor*$^{-/-}$ mutants are more susceptible to *B. bassiana* infections

One of the consequences of deleting *gsnor* from the genome of the *gsnor*$^{-/-}$ overlapping deletion flies was shown to be a higher susceptibility of these animals to infections caused by the entomopathogenic fungus *B. bassiana*. In support of that, complementation of these animals with a new copy of *gsnor* controlled by a UAS/GAL4 system restored the immunodeficiency phenotype of these flies to the pathogen (Kanchanawatee 2012).

To investigate whether the CRISPR/Cas9-mediated mutation of *gsnor* would also generate immunocompromised animals, the Kaplan–Meier method was employed to estimate the survival curve of these mutants in comparison to wild type flies after infection with spores of *B. bassiana*. The Log-rank test was used to test the null hypothesis that there is no difference between the populations in the probability of death at any time point. Null hypothesis were rejected and differences were considered statistically significant when *P*-values were lower than 0.05.

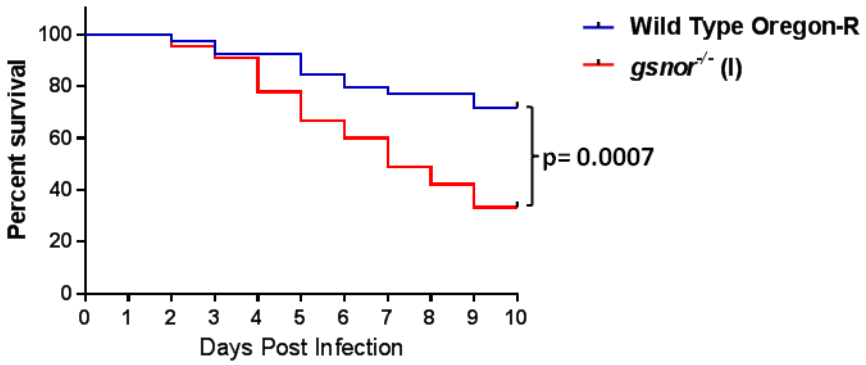
As presented in Figure 0-6 (a, b and c) the percentage of survivals of *gsnor*$^{-/-}$ mutants, compared to the wild type flies, was significantly reduced after infection for lines I ($p<0.0007$) and III ($p=0.0249$). Although line II (Figure 0-6-b) was also reduced in comparison to wild type flies, the difference was not statistically significant according to the Log-rank test ($p=0.1034$). However, it should be highlighted here that the Oregon-R is not the most appropriate control for these comparisons and W^{1118} flies should be included as wild type flies in further comparison.

Figure 0-6-d presents a Kaplan–Meier estimation of survival curve comparing

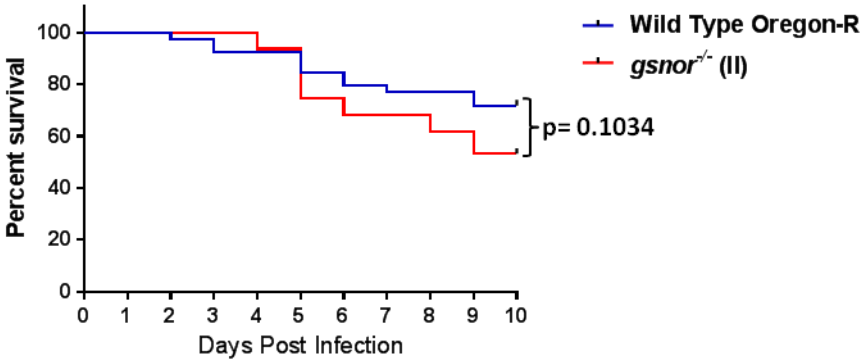
the survival of the three CRISPR/Cas9-generated *gsnor*$^{-/-}$ independent lines. According to the Log-rank test there is a significant differences in survival between lines I and II (p=0.0378), but no differences were detected between lines I and III (p=0.1628) and I and II (p=0.5101).

These results suggest that the site-directed CRISPR/Cas9-mediated *gsnor*$^{-/-}$ mutants, similarly to the *gsnor*$^{-/-}$ overlapping deficiencies (Kanchanawatee 2012), are more susceptible to infections caused by *B. bassiana* when compared to wild type flies. Although complementation experiments of these lines as well as a direct comparison to *gsnor*$^{-/-}$ overlapping deficiencies must be undertaken before drawing strong conclusions, these results, together with those presented by Kanchanawatee (2012), indicate that *gsnor* plays an important role in *D. melanogaster* immune defence against *B. bassiana*.

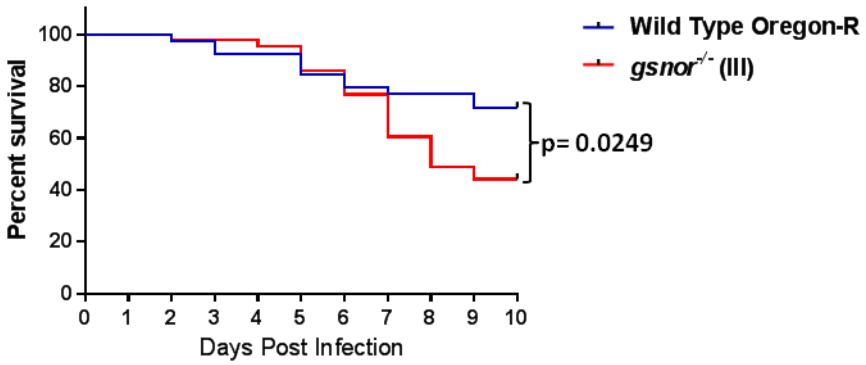
a)


Wild Type Oregon-R
gsnor-/- (I)
Percent survival
100
80
60
40
20
0
0 1 2 3 4 5 6 7 8 9 10
Days Post Infection
p= 0.0007

b)


Wild Type Oregon-R
gsnor-/- (II)
Percent survival
100
80
60
40
20
0
0 1 2 3 4 5 6 7 8 9 10
Days Post Infection
p= 0.1034

c)


Wild Type Oregon-R
gsnor-/- (III)
Percent survival
100
80
60
40
20
0
0 1 2 3 4 5 6 7 8 9 10
Days Post Infection
p= 0.0249

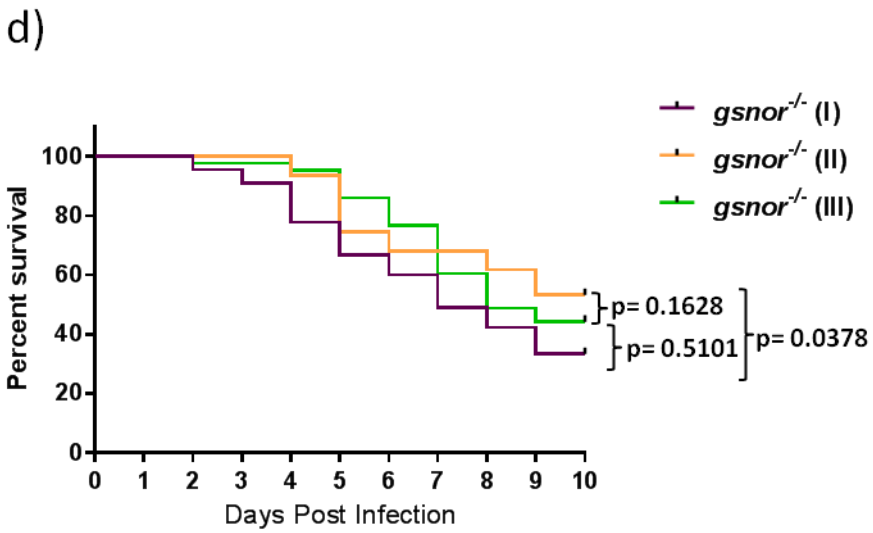


Figure 0-6: **CRISPR/Cas9-generated *gsnor*$^{-/-}$ mutants are more susceptible to *B. bassiana* infection than wild type Oregon-R flies**

Groups of 15 female flies, aged between three and six days, were transferred to 2 mL tubes containing spores of *B. bassiana* and gently shaken for two minutes. Flies were transferred to new vials containing standard cornmeal media and kept at 29°C. The number of flies surviving was recorded every 24 hours after infections and the flies that were alive were transferred to new vials every other day. The percentage of surviving flies (n=45) after infections of three CRISPR/Cas9-generated gsnor$^{-/-}$ transgenic lines (I, II and III) is compared to that of wild type Oregon-R flies. Log-rank analysis demonstrated a statistically significant differences in survival of wild-type and transgenic lines I (p=0.0007) and III (p=0.0249), graphs **a** and **c**, respectively. The difference in survival between line II and wild type flies was not statistically significant (p=0.1034) as shown in graph **b**. In graph **d** a comparison of the three mutants is presented. When individually compared by the Log-rank test, the survival of lines I and II present a statistically significant difference. (p=0.0378). However, no differences are detected between lines I and III (p=0.1628) and I and II (p=0.5101).

6.8 Conclusions

The CRISPR/Cas9 system has very recently emerged as a powerful tool for genome engineering and functional genomics studies. This new technology has already been shown to be highly efficient and specific in editing the genome of a variety of organisms, including plants (Belhaj et al. 2013; Jiang et al. 2013), yeast

(Dicarlo et al. 2013), flies (Gratz et al. 2013; Port et al. 2014; Ren et al. 2014; Gratz et al. 2014), zebrafish (Hwang et al. 2013), mice (Li et al. 2013; Wang et al. 2013) and human cells (Cong et al. 2013; Shalem et al. 2014).

By making use of this new technology, *gsnor*$^{-/-}$ knock-outs were successfully generated in the genome of *D. melanogaster*. The strategy employed here aimed to induce RNA-guided Cas9-mediated DSB in genomic regions flanking *gsnor*, and these DSB would be repaired by HDR using a donor plasmid as template. The donor plasmid carried *DsRed* flanked by sequences with homology to regions of both sides of *gsnor* to allow the integration of the marker *DsRed* during HDR.

Flies expressing DsRed were screened, made homozygous, and analysed for the site of integration by PCR and sequencing. These results confirmed that the integration took place at the expected genomic position.

GSNOR activity assays also confirmed that these mutants are not able to metabolise GSNO, suggesting the integration of *DsRed* generated *gsnor* knock-out mutants. These mutants, like the *gsnor*$^{-/-}$ overlapping deletion flies presented before, were shown to be less tolerant to SNP treatment, confirming the important role played by this enzyme in maintaining NO homeostasis in *D. melanogaster*.

Finally, underscoring a role for this enzyme in *D. melanogaster* immunity, CRISPR-generated *gsnor*$^{-/-}$ mutants were more susceptible than wild type flies to *B. bassiana* infection. These data resemble what has been shown for the *gsnor*$^{-/-}$ overlapping deletion flies (Kanchanawatee 2012), however a deeper investigation of the immune response of these animals should be done. This should include the complementation of the mutants with *gsnor*, infections with other pathogens, measurements of AMP expression in response to infections, and *S*-nitrosylation

assays of proteins within the Toll signalling pathway.

The immunodeficiency phenotype seems to be shared between the overlapping deletion flies and CRISPR-generated *gsnor*$^{-/-}$ flies. However, unlike the *gsnor*$^{-/-}$overlapping deletion flies, the fertility of CRISPR-generated *gsnor*$^{-/-}$ flies seems normal as they produce viable stocks. Since the overlapping technique generates heterozygous deletions of other genes, this phenotype could be attributed to that or to a sum of factors, including the loss of GSNOR activity.

Although further studies should be undertaken to better characterise the integration region and to analyse off-target effects caused by the gRNAs, the technique employed here efficiently generated flies with no GSNOR activity. A more detailed investigation of these new mutants will certainly help understand the roles played by *gsnor* in *D. melanogaster* physiology and immunity.

Chapter 7

General discussion

7.1 *gsnor* and redox homeostasis in *D. melanogaster*

GSNOR activity is conserved from bacteria to plants and humans. By catalysing the degradation of GSNO this enzyme regulates SNO homeostasis (Liu et al. 2001). As presented in Chapter 3, *D. melanogaster gsnor*$^{-/-}$ overlapping deficiencies present higher levels of total proteins *S*-nitrosylation compared to wild type flies. These results are in accordance with what has been shown in mice and plants, where mutations in *gsnor* lead to an increase in SNO levels (Liu et al. 2004; Feechan et al. 2005). In addition, the activity of this enzyme was shown to be necessary for *D. melanogaster* to cope with oxidative stresses induced by the NO donor SNP (nitrosative stress). Flies lacking GSNOR activity were less tolerant, while flies overexpressing the enzyme were more tolerant to treatments with this compound. These results demonstrated that GSNOR is important for maintaining NO homeostasis in *D. melanogaster*.

The activity of this enzyme was also shown to be important for flies to cope with oxidative stress induced by the herbicide paraquat (PQ). This phenotype resembles what has been seen in the plant model *A. thaliana*, where a mutation in *gsnor* induces higher tolerance to this herbicide (Chen et al. 2009). PQ produces oxidative stress by a redox-cycling mechanism. In this process, an electron is transferred from NADPH to PQ, forming the PQ cation radical (PQ^+), which rapidly reacts with oxygen to form superoxide (O_2^-). Thus, it has two major consequences,

production of ROS and depletion of NADPH (Bus & Gibson 1984; Suntres 2002).

A mechanism of PQ toxicity involving nitric oxide (NO) has be proposed by Day and co-workers (Day et al. 1999). The authors have shown that neuronal nitric oxide synthase (nNOS) can catalyse the transfer of electrons from NADPH to PQ. This process induces the production O_2^- at the expense of NO. Because the activity of endothelial NOS has been shown to be modulated by *S*-nitrosylation(Ravi et al. 2004), and *D. melanogaster* has only one NOS, it is possible to speculate that higher levels of *S*-nitrosylation caused by *gsnor* loss of function could also inhibit NOS-mediated PQ-induced O_2^- generation. However, this might not be extrapolated to plants as a NOS has not been identified in plants so far and the levels of PQ-induced O_2^- are similar in wild type and *gsnor* plants (Chen et al. 2009).

Another possibility that should be investigated is the direct involvement of GSNOR itself in PQ redox-cycling. If this enzyme is able to reduce PQ to PQ^+ using NADH as a source of electron, it could induce the generation of O_2^-. Thus, *gsnor* null mutants would generate less ROS when in contact with PQ. This could be done *in vitro* by measuring PQ-dependent production O_2^- in the presence of recombinant GSNOR.

It will be interesting to know whether the *gsnor* mice reported by Liu and co-workers (Liu et al. 2004) also present higher tolerance to PQ. This could be of great significance to public health and medical sciences as despite being shown to induce loss of nigral dopaminergic neurons in rodents (Mccormack et al. 2002; Cicchetti et al. 2005) and being associated with the development of Parkinson's disease (PD) in humans (Tanner et al. 2011), paraquat is still the most widely used herbicides worldwide.

Future studies should also clarify the relation between GSNOR and CAT activities. As presentenced in this work, a point mutation in *Cat* partially restores the immunodeficiency phenotypes of *gsnor* knock-out flies, a process similar to what has been reported in *A. thaliana* (Brzezek 2014). Thus, in the same way that higher levels of GSNO induced by *gsnor* loss of function seem to attenuate the effects of PQ-induced oxidative stress, higher levels of ROS caused by a mutation in *cat* also seem to attenuate the deleterious effects of *gsnor* loss of function. Mutations in *cat* increase the susceptibility of *D. melanogaster* to H_2O_2 (Mackay & Bewley 1989), indicating that CAT is responsible for the metabolism of this molecule in flies.

In order to confirm the role of CAT in reducing H_2O_2 in flies and the relation between the levels of this molecule and the phenotype of *cat/gsnor* double mutants, H_2O_2 measurements should be carried out. Indeed, ongoing work in our laboratory using the Amplex red / Horseradish peroxidase method (Votyakova & Reynolds 2004)to measure H_2O_2 in *D. melanogaster* and the KI method (Loreto & Velikova 2001) for *A. thaliana* suggest that the levels of H_2O_2 are increased in *cat* mutants and reduced in *gsnor* mutants. In addition, H_2O_2 content seems to be restored to wild type levels in in *cat/gsnor* double mutants. Thus, suggesting that a concomitant impairment in the activity of CAT and GSNOR re-balance the levels $H_2O_2$and ameliorates the immune response of the flies (unpublished work).

The production of O_2^-, which is readily converted to H_2O_2 by a class of superoxide dismutase (SOD) enzymes, is triggered in response to infections in a process called the oxidative burst and involves the activation of NADPH oxidases. ROS produced during the oxidative burst directly kills bacteria engulfed by neutrophils (Henderson & Chappell 1996) and also plays an important role in plant

immunity (Torres et al. 2002). Yu and co-workers have provided evidences relating excessive *S*-nitrosylation of the *A. thaliana* NADPH oxidase AtRBOHD, at Cys 890, with impairment in ROS production during immune functions (Yun et al. 2011). In addition, the author showed that this modification also occurs in protein homologs of humans and *D. melanogaster*, suggesting a conservation of the mechanism across different kingdoms.

Considering the results discussed above, it is possible to speculate that NADPH oxidase-mediated generation of O_2^-, is compromised in *gsnor* knock-out flies due to excessive *S*-nitrosylation of this enzyme. Impairment in O_2^- generation and H_2O_2 accumulation during immune responses contribute to the higher susceptibility of these flies to infections. An additional mutation in *Cat* could favour the accumulation of H_2O_2 and partially restore the ability of these flies to respond to infections.

Lozinsky and co-workers have shown that flies that emerged from larvae fed with 1.5 mM SNP presented higher SOD activity, being 76% higher in males and 84% higher in females compared to controls without SNP. On the other hand, CAT activity was lowered by 48% in males and 40% in females compared to controls (Lozinsky et al. 2012). These results suggest that increases in the levels of NO direct H_2O_2 accumulation by up-regulating SOD and down-regulating CAT activities in *D. melanogaster*.

7.2 *S*-nitrosylation and the Toll signalling pathway-mediated immunity

D. melanogaster relies on two NF-kappaB signalling pathways to fight off microorganism infections, the *Toll* and the *imd* (Ferrandon et al. 2007). The *Toll*

mediates the response against Gram-positive and fungal infections (Valanne et al. 2011), while the *imd* plays a similar role in the host defence against Gram-negative bacteria (Myllymäki et al. 2014). Mutations affecting these pathways lead to higher susceptibility to infections, and this is associated with lower production of the final products of these pathways, the anti-microbial peptides (AMPs) (Lemaitre & Hoffmann 2007).

D. melanogaster gsnor$^{-/-}$ flies were shown to be highly susceptible to infections caused by Gram-positive bacteria and fungi but not to infections caused by Gram-negative bacteria (this work and Kanchanawatee 2012). These results, together with evidence that the production of AMPs in response to infections were reduced in in these flies compared to wild type flies, led to the speculation of a compromised signal transduction through the Toll pathway in response to infection.

A deeper investigation looking for possible targets for *S*-nitrosylation within the Toll signalling pathway pointed to CLIP-serine proteases (SP) as potential candidates. Further i*n vitro* and *in vivo* studies have confirmed that at least two of these proteases, PSH and SPE, are indeed *S*-nitrosylated, and this process seems to control the formation of oligomers and affect responsiveness to infection. *S*-nitrosylation of PSH at C254 is also predicted to be a relevant process during the immune response of flies as a non-*S*-nitrosylable form of this protein, PSHC254S, partially restores the resistance of *gsnor*$^{-/-}$ overlapping deletion flies to infections caused by *B. bassiana*.

Extracellular SPs (E-SPs) are involved in a variety of physiological processes in vertebrates and invertebrates. In arthropods, for example, E-SP cascades control processes involved in embryonic development, coagulation, melanisation, and

immune signalling in response to infections (Jiang & Kanost 2000). In vertebrates, E-SP cascades also participate in the coagulation process, in fibrinolysis, and play important roles in immunity, inflammation and carcinogenesis. The complement system is one such example of the role played by E-SP cascades in immunity. The pathways of this system are triggered by proteins that act as pattern recognition receptors to detect the presence of pathogens. Upon activation, these detectors activate a proteolysis cascade that will generate the effector complement components necessary to aid the removal of the microorganisms (Murphy Kenneth 2011).

S-nitrosylation of serine proteases have been reported previously. Stamler and co-workers have demonstrated that the human serine protease tissue-type plasminogen activator (t-PA) is *S*-nitrosylated at C83, and this modification does not seem to affect the catalytic efficiency of this enzyme, but instead confers it with vasodilatory and antiplatelet properties (Stamler et al. 1992). PSH shares 33% amino acid identity and seven conserved cysteine residues, including C254, with the human t-Pa. This enzyme catalyses the activation of plasminogen to the fibrin-degrading protease plasmin, which is important not only for fibrinolysis, an anti-thrombotic coagulation-related mechanism (Miles & Plow 1988), but also involved in a wide variety of physiological and pathological processes, including angiogenesis, cell migration, tumour growth and metastasis (Myöhänen & Vaheri 2004).

Moreover, plasmin has recently been shown to bind, cleave and inactivate C3b and C5 , central proteins of the complement system, thus playing an important role in regulating the human immune system (Barthel et al. 2012). Future work looking at the implications of t-PA *S*-nitrosylation on binding of this enzyme to C3b and C5, and consequent inhibition of the complement system, would elucidate the

interactions between NO signalling and the complement-mediated immunity in mammals.

Another role for the t-PA/plasmin(ogen) cascade in immunity is related to its substrate, fibrinogen. This molecule is known to escape the vascular system and accumulates at sites of inflammation (Dvorak et al. 1985). Smiley and co-workers have provided data suggesting a function for fibrinogen in stimulating macrophage chemokine secretion through Toll-Like Receptor 4 (TLR4) activation (Smiley et al. 2001). Thus, fibrinogen could be considered a mammalian functional homolog of *D. melanogaster* pro-Spätzle. Both protein are final substrates of E-SP cascades and activate Toll-mediated immunity.

Results presented in this thesis suggest that *S*-nitrosylation of PSH, an E-SP structurally similar to human t-PA, regulates the immune system of flies. Considering the results presented by Stamler and co-workers (1992), which indicate that t-PA is *S*-nitrosylated, and the results presented by Smiley and co-workers, suggesting that the final substrate of the t-PA-triggered cascade, fibrinogen, activates TLR4 in mammals, it is plausible to speculate that NO, through *S*-nitrosylation of immune-related E-SPs, regulates the immune response of both insects and mammals, and perhaps other organisms.

S-nitrosylation of SPE, the enzyme responsible for processing pro-SPZ to SPZ in flies, also occurs *in vitro* and *in vivo,* suggesting that the Toll signalling pathway could be modulated at multiple targets in *D. melanogaster*. Future work should investigate whether the human plasminogen, which is positioned downstream of t-PA, is also modified by *S*-nitrosylation, and whether this process would affect processing of fibrinogen and activation of TLR4. It should also be investigated

whether *S*-nitrosylation of t-PA, and perhaps plasminogen, induces these proteins to form oligomers, a process that would resemble what has been shown to happen with *S*-nitrosylated PSH and SPE in flies.

The results presented and discussed in this thesis provide insight into the endogenous mechanism by which NO regulates E-SP cascades in animals. A better understanding of these mechanisms might help to delineate new pharmacological approaches to treat diseases and conditions where these cascades play important roles, such as coagulation and thrombosis, transplant rejection, inflation, asthma, tumour growth, metastasis, immune responses and auto-immune diseases.

7.3 Conclusions and implications

The involvement of GSNOR activity in controlling SNO homeostasis has been confirmed in *D. melanogaster*, a process conserved from plants to mammals. Absence of this enzyme results in flies with higher levels of *S*-nitrosylated proteins, lower tolerance to the nitric oxide donor SNP and higher susceptibility to certain classes of pathogens.

A relation between ROS and SNO homeostasis has also been explored. Higher levels of SNO apparently ameliorate ROS-induced stresses as $gsnor^{-/-}$ flies were shown to tolerate paraquat much better than wild type flies. As paraquat is a widely used herbicide and has been associated with the induction of neurons death in animals, the finding presented here could contribute to a better understanding of the mechanism involved in paraquat toxicity.

In addition, an interplay between *cat* and *gsnor* in regulating the immune responses of flies has also been proposed. A mutation in *cat* partially reversed the

immunodeficiency phenotypes of *gsnor*$^{-/-}$ flies and this was similar to what has been shown to happen in plants. These results suggest the existence of a mechanistic conservation between plants and animals in regulating H_2O_2 levels. H_2O_2, like NO, plays important roles as a signalling molecule in numerous processes in the cell. A better understanding of the mechanisms underpinning H_2O_2homeostasis could shed light on fundamental processes in the cell like the interaction of different PTM in controlling signalling cascades.

Work presented in this thesis has also uncovered a new mechanism by which NO regulates innate immunity. *S*-nitrosylation of CLIP-SPs of the Toll signalling pathway might be a way by which NO finely tunes the immune response of flies. This process was shown to be responsive to infections and involved in modulating oligomerisation/monomerization of CLIP-SPs. The conservation of the peptidase domain and *S*-nitrosylation site among SPs present in other animals suggest that this process might be evolutionary conserved. Future work will confirm whether *S*-nitrosylation of SPs links NO signalling and innate immunity in other organisms.

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