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The cytoplasmic purge as a resilience response to pore-forming toxins and xenobiotics in the *Drosophila* intestinal epithelium

ROS signaling and protective effects against sustained exposures

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Abbreviations

A

ABC: ATP-Binding Cassette
ADH: alcohol dehydrogenase
ADP: Adenosine diphosphate
AGO2: Argonaute 2
ALDH: aldehyde dehydrogenase
AMPs: Antimicrobial Peptides
ARE: Antioxidant Response Element
ATP: Adenosine triphosphate
AdoR: Adenosine Receptor
AhR: Aryl hydrocarbon Receptor
Atg1: Autophagy-related Gene 1

C

cAMP: Cyclic Adenosine Monophosphate
CAR: Constitutive Androstane Receptor
CGD: Chronic Granulomatous Disease
Cnc: Cap'n'collar
CNS: Central Nervous System
CycJ: Cyclin J
CYP: cytochrome P450 monooxigenase

D

DAP: meso-diaminopimelic acid
DH31: Diuretic Hormone 31
DIAP2: Drosophila inhibitor of apoptosis 2
DIF: Dorsal-related Immune Factor
DNA: Deoxyribonucleic acid
Dredd: death-related ced3/Nedd2-like protein
dsRNA: Double Stranded RNA
DSS: Dextran Sodium Sulfate
DTT: Dithiothreitol
DuOx: Dual Oxidase

E

EB: Enteroblast
EC: Enterocyte
EE: Enteroendocrine cell
E_{GSH}: glutathione redox potential
EGF: Epidermal Growth Factor
EGFR: Epidermal Growth Factor Receptor
ER: Endoplasmic Reticulum
ERK: Extracellular Regulated Kinase
ET: Eye Transformer
ETC: Electron Transport Chain
EtOH: ethanol

F

Fadd: Fas-associated death domain
FMO: Flavin-containing Monooxygenase
FtH: Ferritin Heavy chain

G

Gal4: Galactose 4 (transcription factor)
$Gal80^{ts}$: Galactose 80 (thermosensitive inhibitor)
GFP: Green Fluorescent Protein
GNBPs: Gram-negative bacteria binding proteins
GPCR: G protein coupled receptor
GPx: Glutathione Peroxidase
GR: Glutathione Reductase
Grass: Gram-positive specific serine protease
Grx: Glutaredoxin
GSH: Glutathione
GSSG: Glutathione Disulfide
GST: Glutathione S-Transferase

GULO: Gulonolactone oxidase

H

h: hour

Hop: Hopscotch

J

JAK-STAT: Janus Kinase-Signal Transducers and Activators of Transcription

JNK: Jun N-terminal kinase

I

IKK: inhibitor of NF-kB kinase

IMD: Immuno-Deficiency

IRC: Immune Regulated Catalase

ISC: Intestinal Stem Cell

L

lcs: lacosta

LPO: Lactoperoxidase

LPS: Lipopolysaccharide

M

MAMP: Microbe Associated Molecular Patterns

MAPK: Mitogen Activated Protein Kinase

ModSP: Modular Serine Protease

MyD88: Myeloid Differentiation primary response gene 88

N

NAC: N-acetylcysteine

NADPH: Nicotinamide Adenine Dinucleotide Phosphate

NEM: N-ethyl-maleimide

NET: Neutrophil Extracellular Traps

NF-kB: Nuclear Factor Kappa B

NK: Natural Killer

NOS: Nitric Oxide Synthase

NOX: NADPH Oxidase

Nrf2: Nuclear factor E2-related factor 2

O

OD: Optical Density

OMVs: Outer Membrane Vesicles

P

PBT: PBS-TritonX

PFT: Pore-Forming Toxin

PGN: Peptidoglycan

PGRP: Peptidoglycan Recognition Protein

PHD: Peroxidase-Homology Domain

PHH3: Phosphohistone H3

PKC: Protein Kinase C

PI3K: Phosphoinositide 3 Kinase

PIAS: Protein Inhibitors of Activated Stat

PLCβ: Phospholipase Cβ

PPO: Prophenol Oxidase

PRR: Pattern Recognition Receptor

Prx: Peroxiredoxin/Thioredoxin Peroxidase

Psh: Persephone

PTP1B: Protein Tyr Phosphatase 1 B

PXR: Pregnane X Receptor

R

RET: Reverse Electron Transport

RISC: RNAi Induced Silencing Complex

RNA: Ribonucleic acid

RNAi: RNA interference

RNS: Reactive Nitrogen Species

roGFP: Redox-sensitive GFP

ROS: Reactive Oxygen Species

RyR: Ryanodine Receptor

S

siRNA: Small Interfering RNA

Socs: Suppressor of Cytokine Signaling

SOD: Superoxide Dismutase

SPE: Spätzle Processing Enzyme

STING: Stimulator of Interferon Genes

T

T6SS: Type VI Secretion System

TAB2: TAK1-associated binding protein 2

TAK1: transforming growth factor beta-activated kinase 1

TEPs: Thioester containing protein

TISC: Toll Inducing Signaling Complex

TLR: Toll-Like Receptor

TNF: Tumor Necrosis Factor

TRPA1: Transient Potential Receptor A1

Trx: Thioredoxin

TrxR: Thioredoxin Reductase

U

UAS: Upstream Activating Sequence

Upd: Unpaired

UPR: Unfolded Protein Response

V

Vit. C: Vitamin C/ascorbic acid

W

WG: Wingless

WT: Wild Type

whe: what else

X

XBP1: X-box Binding Protein 1

XR: Xenobiotics receptor

Y

YFP: Yellow Fluorescent Protein

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PREFACE

During the lifetime, all metazoans encounter pathogenic microorganisms and contaminants presents in the environment. Therefore, organisms have developed defenses to fight these threats: physical barriers and immune system limit the onset of infections and pathologies. However, the attack of the immunity to the pathogens, known as resistance, is complemented by another arm of the host defense: the resilience. This is defined as the ability to endure and repair the damages inflicted by the pathogen or the immune response itself, and it constitutes a crucial determinant of host survival.

In our laboratory, we study the resilience in the model organism *Drosophila melanogaster*. The intestine represents a major exchange surface in the body, as many of the microorganisms and xenobiotics enter the internal milieu by this route by contamination of water and food. We have recently described a novel resilience mechanism in the fruit fly intestine, the cytoplasmic purge. The response caused by the Gram-negative bacterium *Serratia marcescens* and specifically by its pore-forming toxin hemolysin, led the enterocytes to extrude their cytoplasm in the intestinal lumen. Thereby, the damaged cellular components are expelled, and the host gets protection from the others bacterial virulence factors. This phenomenon morphologically translates into a spectacular transformation of the intestinal epithelium: the cells become thin and flat as a result of the purge and then gradually recover their original volume and shape.

While we started to understand some aspects of the recovery phase of the cytoplasmic purge, the mechanism that initiates it remains unclear. During my PhD, on one hand my objective was to investigate the signal required from the host side to trigger the extrusion, focusing on reactive oxygen species (ROS). On the other hand, I studied the conservation of the process with other compounds. This work led to the statement that the purge and recovery of enterocytes could also protect the body to some extent against certain xenobiotics, as ethanol and caffeine. Finally, I described a priming mechanism, during which the enterocytes become resistant to a second purge in the days following the first challenge.

General introduction

1. Drosophila melanogaster

1.1 *Drosophila* as a model organism: history & general features

Drosophila melanogaster, also commonly referred as the fruit fly or vinegar fly, is an insect belonging to the family of Drosophilidae, in the order of Diptera. This little fly has been used as a model organism for more than a century: the professor of Harvard William Ernest Castle was the first to introduce it in a laboratory and he was the author of the very first article on the genetics of *D. melanogaster* (Castle et al., 1906). In parallel, Thomas Hunt Morgan started his work at the Columbia University of New York in what became known as "the fly room". The discovery of a white-eyed male in Morgan's culture of red-eyed *Drosophila* paved the path to the formulation of the "Chromosomal Theory of Heredity". Even though initially he was skeptical about Mendel's inheritance laws, crosses and subsequent progeny inbreeding led Morgan to characterize the *white* gene and to the discovery of sex-linked inheritance (Morgan, 1910). For this important breakthrough he was awarded of the Nobel prize in 1933. Morgan's students further contributed to the study of genetics: Alfred Henry Sturtevant found that homologous chromosomes could interchange (the "*crossing-over*") and Hermann Joseph Muller discovered that radiations caused genetic mutations (Muller, 1928). For this discovery the latter won the Nobel prize in 1946. In parallel, *Drosophila* was also used for neuroscience and development, allowing the identification of the Notch transcription factor by Poulson (Poulson, 1937). Developmental studies were further carried on by Christiane Nüsslein-Volhard and Eric Wieschaus in the '80s, providing great knowledge about key genes controlling larval development such as Toll, Wingless and Hedgehog (Jurgens et al., 1984; C Nüsslein-Volhard et al., 1984; Christiane Nüsslein-Volhard & Wieschaus, 1980; Wieschaus et al., 1984). These pathways were found to be conserved in vertebrates, and earned them the Nobel prize in 1995, together with Edward Lewis. Two more Nobel prizes were given for researches conducted on the fruit fly: the last in order of time (2017) is the one awarded to Jeffrey C. Hall, Michael Rosbash and Michael W. Young for the discovery of molecular mechanisms of circadian rhythm, continuing the work started some time earlier by Seymour Benzer. The one that concerns us much closely is the 2011 Nobel prize for physiology and medicine given to Jules Hoffmann and Bruce Beutler for their discoveries concerning the activation of innate immunity (together with Ralph Steinman for the characterization of dendritic cells). Right here at the IBMC in Strasbourg, the team of Jules

Hoffmann showed that *Toll* mutant flies were more susceptible to an infection, and that the expression of the antimicrobial peptide Drosomycin was dependent on *Toll/Spatzle* signaling. The finding that this pathway was involved in immunity was rapidly followed by further characterization and separation of bacterial- and fungi-specific defense responses (Lemaitre et al., 1996, 1997). Finally, Beutler and his team proved the conservation of such receptors also in mammals, hence the name *Toll-like Receptors (TLR)* (Poltorak et al., 1998). The advent of the era of *-omics* meant important advances also for *Drosophila* research: collaboration between laboratories and company led to sequencing of the whole fly genome, the second among major complex organisms (Adams et al., 2000). Annotation and mapping of the genome sequence, together with individual gene information, expression levels, localization, mutant strain, related literature and more resources are publicly available on the FlyBase database (http://flybase.org/). Today, we know that *Drosophila* genome contains around 14.000 protein-coding genes; importantly, 75% of genes implicated in human's diseases have a functional homolog in flies (Pandey and Nichols, 2011). Therefore, the fruit fly represents a powerful tool to study a wide range of pathologies such as cancer, neurodegeneration, aging, diabetes, infections, drug-addiction and toxicity, inflammation and much more.

The well-annotated genome constitutes only one of the numerous advantages of *Drosophila* as a model organism. More than 100 years of research have been creating a deep knowledge of this organism, taking advantage of its many conveniences. Due to their small size, large amounts of flies can be stored in a small place; the rearing is relatively cheap, favored by high reproduction rates (~50eggs/day/female) and short lifecycle (Figure I1; 10 days at 25°C). These features facilitate high-throughput *in vivo* experimentation. The essential equipment to work with flies is minimal, including vials for rearing, incubators, carbon dioxide anesthetic pads, binocular stereoscopic microscope and dissection tools. As many organs even in the adult are transparent or semi-transparent, tissue-mounting on microscopy slides and observation do not require histological slicing: this allows to keep the organ intact, as well as to save time and material. However, the main advantage compared to other models is undoubtedly its genetics. The quantity and ease of use of multiple genetic tools aimed to alter or localize gene expression makes difficult to find equal in other metazoans. Physical and genetical mapping, whole-genome mutational screens, transgenesis techniques and the insertion of balancers chromosomes, which allows to maintain lethal or sterile mutations and prevents meiotic recombination, are all hallmarks of *Drosophila*'s genetic plasticity (Rubin and Lewis, 2000). Moreover, the introduction of the yeast's Upstream Activating Sequence (UAS)-Galactose 4 (Gal4)-thermosensitive Galactose80 ($Gal80^{ts}$) to either knockdown (UAS-RNAi) or overexpress (UAS-gene) a gene opened the doors to spatial and temporal control of transgene expression (Brand and Perrimon, 1993; Mcguire et al., 2004). Because of that, it is nowadays easy to modulate the expression or follow a gene of interest for numerous applications (Duffy, 2002). In addition, there are many other techniques to modulate *Drosophila* gene

expression besides UAS-Gal4 system: these include FLP/FRT system, tetracycline-responsive transcription factors (Tet-on/Tet-off), steroid hormones-responsive transcription factors (GeneSwitch or ER-Gal4) and CRISPR-Cas9 (Gratz et al., 2016; Kanca et al., 2019; Mcguire et al., 2004). Research is further facilitated by the creation of large stock centers (as Vienna Drosophila Research Center or Bloomington) that provides ready-to-use fly lines. The shipping is very practical, done in small tubes across many countries; this straightforward procedure also encourages communication and sharing between research teams, which is part of a culture of sharing stocks from the very beginnings.

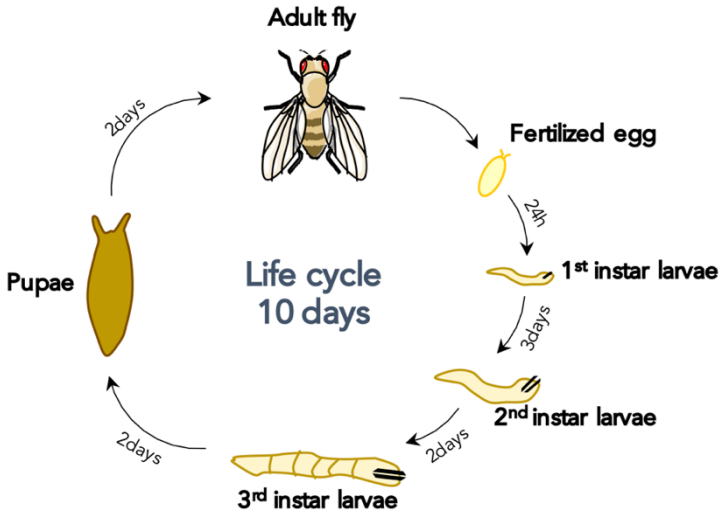


Figure I1. The life cycle of *Drosophila melanogaster*.

For all these advantages (and more), implementation of *Drosophila* in research is very versatile and has brought much knowledge to the scientific community. Obviously, mammalian models show a higher conservation for human health's purposes, but high costs, time delays and ethical laws represent real setbacks. The possibility to realize large forward genetic screens, to study responses from molecular to whole organism level and to perform crosses, infections and treatment without ethical restrictions have affirmed the fly as a very valuable model for host-pathogen/stressors interactions. Due to the high degree of conservation of many responses, the fundamental findings using *Drosophila* has been often used as a useful base of knowledge to perform experiments in mammals (Brunke et al., 2015; Pospisilik et al., 2010). For instance, *Drosophila* cells can be used to perform large screens to elucidate surface and intracellular mechanisms involved in infection. The key advantage of using fly cells for such purpose is the availability of libraries of dsRNAs for specific knockdown of every *Drosophila* gene (http://www.flyrnai.org/). This kind of approach has been used for characterization of cellular response face to pathogens as *Listeria monocytogenes* (Agaisse et al., 2005) and *Candida albicans* (Stroschein-Stevenson et al., 2006); it also led to the identification of important proteins such as PGRP-LC (Rämet et al., 2002) and Eater (Kocks et al., 2005). Besides cell culture experiments, the short lifespan, fast lifecycle and availability of ready-to-use fly collections open the possibility to do *in vivo* RNA

interference (RNAi) screens using flies. This approach provides a more pertinent vision of what happens in the organism during an infection: our team took advantage of this for the identification of hundreds of genes conferring resistance or susceptibility in the intestinal infection by *Serratia marcescens* (Cronin et al., 2009), in what was the first screen on intestine in a metazoan using survival as a readout. Specific inhibition of protein expression in flies allowed the recognition of key actors in the antiviral RNAi pathway such as Dicer-2 (Galiana-Arnoux et al., 2006) and Ago2 (Van Rij et al., 2006). Again, fundamental research on *Drosophila* provided a solid base to comprehend picornavirus entry in the cell (Cherry and Perrimon, 2004) and description of unknown bacterial virulence factors (Kim et al., 2008) or their mechanism of action (Guichard et al., 2010). This topic is also developed in our laboratory: we showed that *RhlR*, a component of *Pseudomonas aeruginosa* quorum sensing, is reducing TEP4-mediated opsonization and phagocytosis (Haller et al., 2018). Also, other team members are currently investigating *Serratia*'s virulence factors other than hemolysin, namely flagellar proteins and proteases.

1.2 The immune system of Drosophila melanogaster

As all invertebrates, the fruit fly only does not possess the adaptive immunity, thereby relying only on innate immunity. As mentioned above, this simplified immune system allowed the understanding of the molecular mechanisms that operate in innate responses (Lemaitre and Hoffmann, 2007), providing key insights on how this system works in mammals and on the collaboration between innate and adaptive immunity.

In nature, *Drosophila* lives in a microbe-rich environment and feeds on decaying fruits: hence, an efficient and diversified immune system is needed for an optimal survival in these conditions. The first line of defense is represented by physical barriers: an impermeable cuticle covers almost the totality of the fly body, and local responses take place in the entry sites of the organism, that is intestinal, trachea and genital barrier epithelia (Ferrandon et al., 1998). Here, antimicrobial peptides (AMPs) secretion and ROS production act as sentinels to protect the frontier. In most cases these defenses are sufficient to control the infections; however, some pathogens are able to penetrate the cuticle or pass through the epithelial barriers. In such circumstances, the systemic immune response must guarantee the sterility of the internal milieu (Bier and Guichard, 2012). On one hand we have the cellular response, which controls the pathogen burden via phagocytosis and melanization. On the other hand, the fat body, the fly's functional homolog of mammalian liver and adipose tissue, produces and releases AMPs in the hemolymph under the control of the Toll and Immune Deficiency (IMD) pathways (Ferrandon et al., 2007). Both are triggered by Microbe Associated Molecular Patterns (MAMPs) recognition by Pattern Recognition Receptors (PRRs), which activates NF-kB (Nuclear Factor Kappa B) family transcription

factors. AMPs-mediated defense is a very ancestral and conserved mechanism, from plants to humans. As pathogen's membranes are enriched in negatively charged lipids, positively charged AMPs selectively bind on them through electrostatic interactions mediating death by pore formation (Zasloff, 2002). In addition to that, NF-kB pathways also control the expression of small molecules (Drosophila immune induced molecules or DIMS) (Clemmons et al., 2015; Uttenweiler-Joseph et al., 1998), proteases, cytokines, structural elements and many other genes (De Gregorio et al., 2001; Irving et al., 2001). Other fundamental pathways, namely JAK-STAT and JNK, also contribute to immunity and stress responses.

Although *Drosophila* may look very different from mammals, in reality most of genes and pathways involved in host defense are identical or very similar (Hoffmann, 2003), therefore representing an excellent model for the study of innate immunity. Moreover, the fact that flies manage to live in microbe-rich environment without the adaptive system underlines the power and efficiency of innate immunity. In this section, I will summarize the mains components of fly immunity, starting from humoral systemic responses, cellular immunity and reaction to stress and viruses. Tissue-specific immunity, notably in the midgut, will be presented in the section dedicated to the digestive tract.

1.2.1 The Toll pathway

This pathway was the first NF-kB-dependent pathway described in *Drosophila*. As previously mentioned, Toll was initially identified as a determinant of the dorso-ventral polarity in embryos (Anderson et al., 1985) during the genetic screens conducted by Nusslein-Volhard. Around ten years later, the laboratory of Professor Hoffmann found an additional role for this gene in antifungal immunity (Lemaitre et al., 1996). The discovery of Toll role in immunity ultimately conciliated the two most accredited theories at the time for pathogen recognition: the detection of "non-self" by the MAMPs-PRR interaction (Medzhitov and Janeway, 2002), and the sensing of the "danger signals" (Matzinger, 1994). The MAMPs activating the Toll pathway are the Lys-type peptidoglycan (Lys-type PGN) of Gram-positive bacteria (Leulier et al., 2003) and the βglucans of fungi (Gottar et al., 2006). The Toll pathway PRRs are secreted protein circulating into the hemolymph, mainly the PGN recognition proteins (PGRPs) and Gram-negative bacteria binding proteins (GNBPs). GNBP1 and PGRP-SA recognize Lys-type PGN (Gobert et al., 2003; Leulier et al., 2003; Michel et al., 2001), whereas fungi are detected via GNBP3 (Gottar et al., 2006; Matskevich et al., 2011; Mishima et al., 2009). However, this MAMP-PRR interaction does not activate directly Toll. Instead, a proteolytic cascade involving the modular serine proteases ModSP, Grass (Gram-positive specific serine protease) and finally SPE (Spätzle Processing Enzyme) lead to the maturation of Spätzle, that is the Toll ligand (Buchon et al., 2009a; El Chamy et al., 2008; Jang et al., 2006) (Figure I2). The Toll pathway is also activated by

recognition of pathogen enzymatic activity. Indeed, fungi like *Beauveria bassiana* and *Aspergillus oryzae* or Gram-positive bacteria as *Bacillus subtilis* secrete proteases that cleave and activate the serine protease

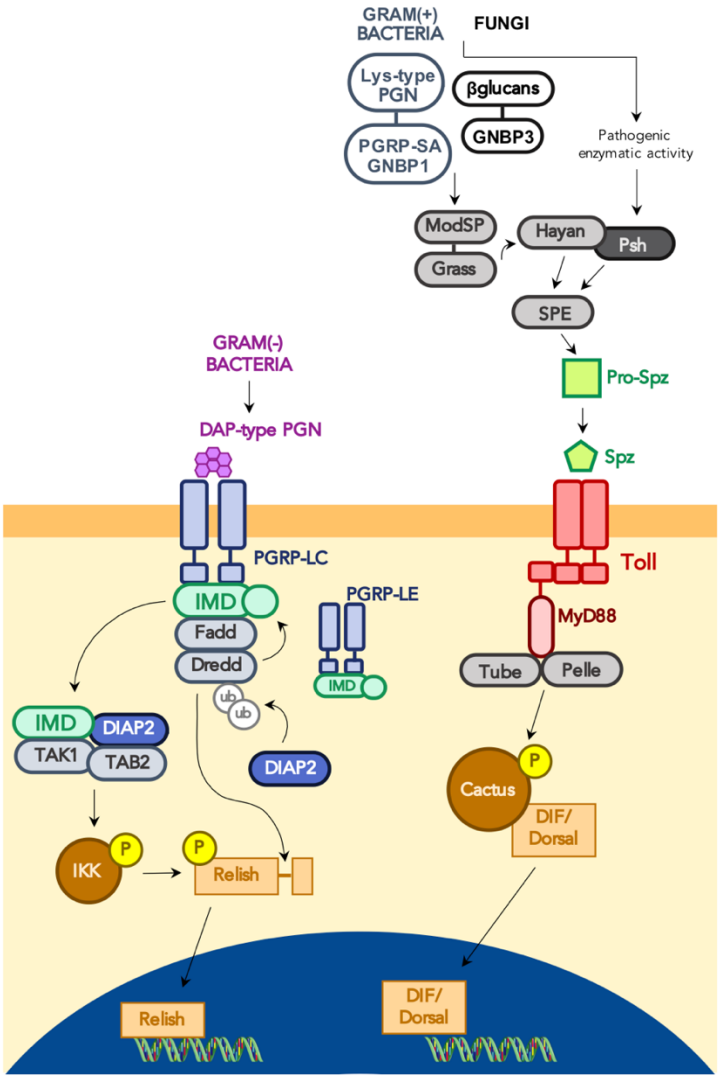


Figure I2. Simplified view of the IMD and Toll pathways. The IMD pathway (left) is activated when meso-diaminopimelic-type peptidoglycan (DAP-type PGN) of Gram(-) bacteria or *Bacillus spp*. is recognized by the Peptidoglycan Recognition Proteins PGRP-LC (membrane-bound) and PGRP-LE (intracellular). This leads to a signalization cascade which implies the recruitment of IMD, Fadd and Dredd. The protein DIAP2 activates Dredd by poly-ubiquitinylation, which subsequently cleaves IMD. A multi-protein complex formed by IMD, DIAP2, TAK1 and TAB2 then phosphorylates the IKK complex. The latter phosphorylates the N-ter of Relish, while Dredd cleaves the C-ter, allowing the translocation into the nucleus and transcription of immune mediators, notably AMPs such as Diptericin.

The Toll pathway (right) is activated upon sensing of Gram(+) bacteria and fungi. Unlike IMD, Toll does not recognize MAMPs directly: it needs the cleavage of Pro-Spatzle into active Spatzle (Spz). This occurs when lysine-type peptidoglycan (Lys-type PGN) or βglucans binds on the receptors GNBP1, GNBP3 or PGRP-SA, which activates a protease cascade involving ModSP, Grass and SPE. Alternatively, SPE can be activated by Psh, which senses enzymatic activity of the pathogen. Spz binding trigger Toll dimerization and signaling kinase cascade implying MyD88, Tube and Pelle. The phosphorylation of Cactus allows the release of NF-kB factors DIF and Dorsal, which are responsible for the expression of other AMP, e.g., Drosomycin. Adapted from Buchon et al., 2014, Ferrandon et al., 2007, Dudzic et al., 2019.

Persephone (Psh), which in turn cleaves SPE (El Chamy et al., 2008; Gottar et al., 2006). More recently, Psh has been shown to be an indirect sensor to a wide range of pathogens, including also Gram-negative bacteria (Issa et al., 2018). Therefore, the MAMP and the danger routes converges to Spätzle cleavage and its binding to Toll receptor. This triggers receptor dimerization and subsequent recruitment of the adaptor proteins MyD88 (myeloid differentiation primary response gene) and Tube, which in turn mobilizes the kinase Pelle (Horng and Medzhitov, 2001; Moncrieffe et al., 2008; Sun et al., 2002a). These proteins interact via their death-domains forming the Toll Inducing Signaling Complex (TISC). TISC activates the NF-kB transcription factors Dorsal and DIF (Dorsal-related immunity factor) by degrading Cactus, a negative regulator that holds them in the cytoplasm (Belvin et al., 1995; Fernandez et al., 2001). Dorsal and DIF translocation into the nucleus results in the transcription of many genes, including those for the AMPs *Drosomycin*, *Defensin* and *Metchnikowin*, which are typical of the Toll pathway (Ferrandon et al., 2007; Lemaitre & Hoffmann, 2007).

1.2.2 The IMD pathway

A mutation in the immune deficiency (IMD) gene was found to impair the fly's antibacterial response (Lemaitre et al., 1995, 1996). Indeed, this pathway, which presents several similarities with the mammalian Tumor-necrosis factor (TNF) pathway (Hoffmann, 2003), governs the AMPs production upon sensing of Gram-negative bacteria principally. The recognized MAMP is the meso-diaminopimelic-type (DAP-type) peptidoglycan, a main component of Gram-negative bacteria but also of some Gram-positive bacilli (Leulier et al., 2003). Therefore, the fly can orient its response according to the type of PGN recognized. DAP-type PGN is sensed by PGRPs: the transmembrane PGRP-LC (Choe et al., 2002; Gottar et al., 2002; Rämet et al., 2002), the cytosolic PGRP-LE (Kaneko et al., 2006) and the epithelia enriched PGRP-LA (Gendrin et al., 2013). Upon recognition, the receptor dimerizes and recruits the adaptor protein IMD (Choe et al., 2005; Georgel et al., 2001) (Figure I2). This leads to the subsequent recruitment and activation of several effectors, such as Fadd (Fas-associated death domain) (Naitza et al., 2002) and the caspase Dredd (death-related ced3/Nedd2-like protein) (Leulier et al., 2002). The latter requires poly-ubiquitinylation by DIAP2 (*Drosophila* inhibitor of apoptosis 2) in order to be active (Meinander et al., 2012). Consequently, Dredd cleavage of IMD (Kim et al., 2014) recruits in their turn the DIAP2 complex (Zhou et al., 2005), TAK1 (transforming growth factor beta-activated kinase 1) and TAB2 (TAK1-associated binding protein 2) downstream (Kleino et al., 2005). More recently, it has also been shown the formation of amyloid fibrils complexes downstream of IMD, which are required for signaling (Kleino and Silverman, 2019; Kleino et al., 2017). Expression of effector genes by Relish, the final player in IMD pathway, is achieved through two complementary mechanisms. From one part, IMD/DIAP2/TAK1/TAB2 complex phosphorylates the inhibitor of NF-kB kinase (IKK) complex (Silverman et al., 2003): this triggers the phosphorylation of Relish, which is then transcriptionally active (Ertürk-Hasdemir et al., 2009). From the other part, Relish has to be

translocated to the nucleus: this is accomplished by Dredd-mediated cleavage of C-terminal domain of Relish (Kim et al., 2014; Stöven et al., 2003). The latter finally binds to kB-responsive elements in nucleus, regulating the expression of hundreds of genes, mainly AMPs: hallmarks of IMD pathway are *Diptericin*, *Attacin* and *Cecropin* (Hetru and Hoffmann, 2009).

1.2.3 The cellular immune response

Drosophila cellular immune response is mainly mediated by macrophage-like cells, the hemocytes (Lemaitre and Hoffmann, 2007). These can be divided in different types: plasmatocytes, crystal cells and lamellocytes (Crozatier and Meister, 2007). The first are the most abundant and the only type found in adults. Their main function is phagocytosis, in order to internalize pathogens or altered self-molecules. This is done via phagocytic receptors, such as Eater (Kocks et al., 2005; Melcarne et al., 2019), Nimrod C1 (Estévez-Lao and Hillyer, 2014; Kurucz et al., 2007; Melcarne et al., 2019), PRGRP-LC (Rämet et al., 2002), Draper (Manaka et al., 2004), Croquemort (Franc et al., 1999; Stuart et al., 2005) and Peste (Philips et al., 2005). As mammalian macrophages, plasmatocytes engulf microbes or apoptotic bodies through invagination of cell membrane forming a phagosome. Progressive acidification and subsequent fusion with lysosomes ensure the degradation of internalized content (Kinchen and Ravichandran, 2008).

In larvae, crystal cells constitute around 5% of hemocytes (Crozatier and Meister, 2007). The function of these cells is melanization, that is deposition of black-pigmented melanin to support cellular functions like wound-healing, encapsulation or direct killing of pathogens (Dudzic et al., 2015, 2019; Söderhäll and Cerenius, 1998). Melanization is an important immune process, as flies lacking this response are more sensitive to Gram-positive and fungi infection (Binggeli et al., 2014). In the adults, melanization occurs through the prophenoloxidase (PPO) enzyme, which circulates in the hemolymph. Upon pathogen sensing, serine proteases cleave the prophenoloxidase (Dudzic et al., 2015; Söderhäll and Cerenius, 1998), converting it into active phenoloxidase that is responsible for melanin production. The last hemocyte type, lamellocytes, appear only in parasitic wasp infection (Crozatier and Meister, 2007), and they also have a role in melanization and encapsulation of wasp eggs (Dudzic et al., 2015).

In addition, insects also possess a batch of genes, the thioester-containing proteins (TEPs), which are the functional homolog of mammalian complement system. In mosquitoes, TEPs are crucial for anti-*Plasmodium* immunity (S. Blandin et al., 2004 & 2009) and phagocytosis (Levashina et al., 2001). In fruit fly, TEP family is composed by 6 members (TEP1-6) that are thought to have redundant function; they are principally expressed in hemocytes and specific hypodermic regions but can also be induced in the fat body (Bou Aoun et al., 2011). *Drosophila* lacking TEPs show increased sensitivity to Gram-positive and fungi infection (Dostálová et al., 2017), suggesting a role in the early stages of activation of the Toll pathway. Moreover, in flies TEPs can also act as an opsonin (Haller et al., 2018)

and mediate immune response against microsporidia, an intracellular parasite (Gaétan Caravello, unpublished results).

1.2.4 Antiviral immunity

Viruses are important natural pathogens of *Drosophila*: more than 25 viruses can infect flies in the wild (Lemaitre and Hoffmann, 2007), which therefore have evolved efficient antiviral defenses. The most well-characterized and the main arm of antiviral immunity is the RNAi pathway (Kemp et al., 2013). This defense strategy aims to hamper viral replication in the cell, by detection and subsequent degradation of the viral RNAs. Recognition of dsRNAs is operated by Dicer2, that then cleaves them into small interfering RNAs (siRNAs) of 21 nucleotides long. Cooperation with R2D2 allows these siRNAs to be charged into Argonaute2 (AGO2) (Marques et al., 2013). A mature RNA induced silencing complex (RISC) complex is formed when one of the two siRNA strands is ejected: the single strand siRNA serves as a guide to recognize complementary RNA sequences, which are subsequently degraded (Lamiable and Imler, 2014; Marques and Imler, 2016). In general, RNAi is a vital pathway that controls the replication of a broad range of viruses (Kemp et al., 2013). Nevertheless, cell can fight against viruses also with other weapons: apoptosis, phagocytosis, JAK-STAT and NF-kB are been shown to act in antiviral defenses (Lamiable and Imler, 2014; Lamiable et al., 2016; Marques and Imler, 2016; Mussabekova et al., 2017). Viral infection triggers the expression of many genes, including some AMPs (Huang et al., 2013) and other antiviral peptides such as *Vago* (Deddouche et al., 2008). More recently, *Drosophila* STING (Stimulator of interferon genes) has been identified as a viral sensor upstream of IKKβ and Relish that drives the responses against picorna-like viruses through the effector gene *Nazo* (Goto et al., 2018). JAK-STAT pathway can also provide some virus-specific immunity, controlling an antiviral program of gene expression (Kemp et al., 2013).

1.2.5 Stress responses

The mechanisms presented just above ensure the recognition and in most cases the elimination of invading pathogens. However, in natural conditions those defenses may not be sufficient to provide an optimal host fitness. Indeed, wild flies are not only threatened by pathogens: environmental stressors such as temperature, physical injury and exposure to xenobiotics and toxins are a critical determinant of survival. Here, I will summarize two of the main pathways controlling the stress response, Jun N-terminal kinase (JNK) and Janus Kinase-Signal Transducers and Activators of Transcription (JAK-STAT) in addition of the wound healing response.

1.2.5.1 JNK

JNK is one of the three *Drosophila* Mitogen Activated Protein Kinase (MAPK) besides ERK (Extracellular Regulated Kinase) and p38 pathways, and it is relevant for key developmental steps in the fly (Noselli, 1998). This is just one of the multitude of processes in which JNK is involved: this kinase cascade influences apoptosis, cell proliferation, metabolism, autophagy and tissue repair (Biteau et al., 2011; Ríos-Barrera and Riesgo-Escovar, 2013). UV irradiation, heat-shock, mechanical deformation, ROS, DNA damage, cytokine and hormones ligands, infection and inflammation are all stimuli that activate the first phosphorylation step (Biteau et al., 2011; Stronach and Perrimon, 1999) (Figure I3). The latter is mediated by a JNK Kinase Kinase (JNKKK), which can be MLK2 (Stronach et al., 2014), ASK1 (Kuranaga et al., 2002), MEKK1 (Ryabinina et al., 2006) or TAK1 (through IMD activation) (Silverman et al., 2003). The classical phosphorylation cascade switch on two JNK Kinase (JNKK), hemipterous (Glise et al., 1995) and dMKK4 (Sathyanarayana et al., 2003), and finally activate the Basket kinase, which targets the phosphorylation of the transcription factors AP-1 (Riesgo-Escovar and Hafen, 1997) and FOXO (Wang et al., 2005). The major negative regulator of the pathway is Puckered, a phosphatase that restricts Basket activity (Martín-Blanco et al., 1998). Given the broad range of activators and targets, JNK function varies depending on the context. In addition of genes involved in morphogenesis, JNK also stimulate cytoskeleton remodeling (Boutros et al., 2002), stress-dependent proteins (Biteau et al., 2011; Ríos-Barrera and Riesgo-Escovar, 2013) and proliferation (Biteau and Jasper, 2011). Importantly, JNK is part of the pathways triggered by infection (Boutros et al., 2002), activated by microbial stimulus or oxidative stress and mediating host's protection (Biteau et al., 2008; Buchon et al., 2009b).

1.2.5.2 JAK-STAT

JAK-STAT is a highly conserved pathway with a wide range of functions: in mammals was first described for its antiviral function through interferon production (Stark and Darnell, 2012), whereas in *Drosophila* it was identified as a player in embryonic segmentation and hematopoiesis (Hou et al., 1996; Luo and Dearolf, 2001). In the fruit fly, the pathway is less complex compared to mammals: signalization is started by the three cytokines Unpaired (upd1, upd2, upd3), which are the ligand for the unique receptor Domeless (Wright et al., 2011) (Figure I3). The signal travels intracellularly through the Janus Kinase (JAK) Hopscotch, which is constitutively associated with the intracellular part of Domeless (Binari and Perrimon, 1994). Dimerization and phosphorylation induce recruitment and binding of the Stat92E transcription factors (Myllymäki and Rämet, 2014). Another phosphorylation by JAK trigger the translocation into the nucleus, where Stat92E regulates the expression of many genes (Brown et al., 2003; Myllymäki and Rämet, 2014; Yan et al., 1996). JAK-STAT pathway possesses many negative regulators, including Socs (Suppressor Of Cytokine Signaling), PIAS (Protein Inhibitors of Activated Stat) and ET (Eye Transformer) (Agaisse and Perrimon, 2004; Myllymäki and Rämet, 2014). The

diversity of physiological processes in which JAK-STAT pathway is involved is remarkable. In *Drosophila*, it is involved in hematopoiesis and it controls the expression of several immune genes such as TEPs, Turandot, Listericin and Drosomycin-3 (Agaisse & Perrimon, 2004; Buchon, et al., 2009b; Goto et al., 2010; Lemaitre & Hoffmann, 2007). As previously mentioned, it also intervenes in antiviral immunity. JAK-STAT is also involved in the response against bacterial infections (Cronin et al., 2009), and it controls midgut epithelial renewal through ISCs compensatory proliferation that replace stressed and dead enterocytes (Buchon et al., 2009c; Jiang et al., 2009). The processes involving this pathway in the midgut will be described in more detail later.

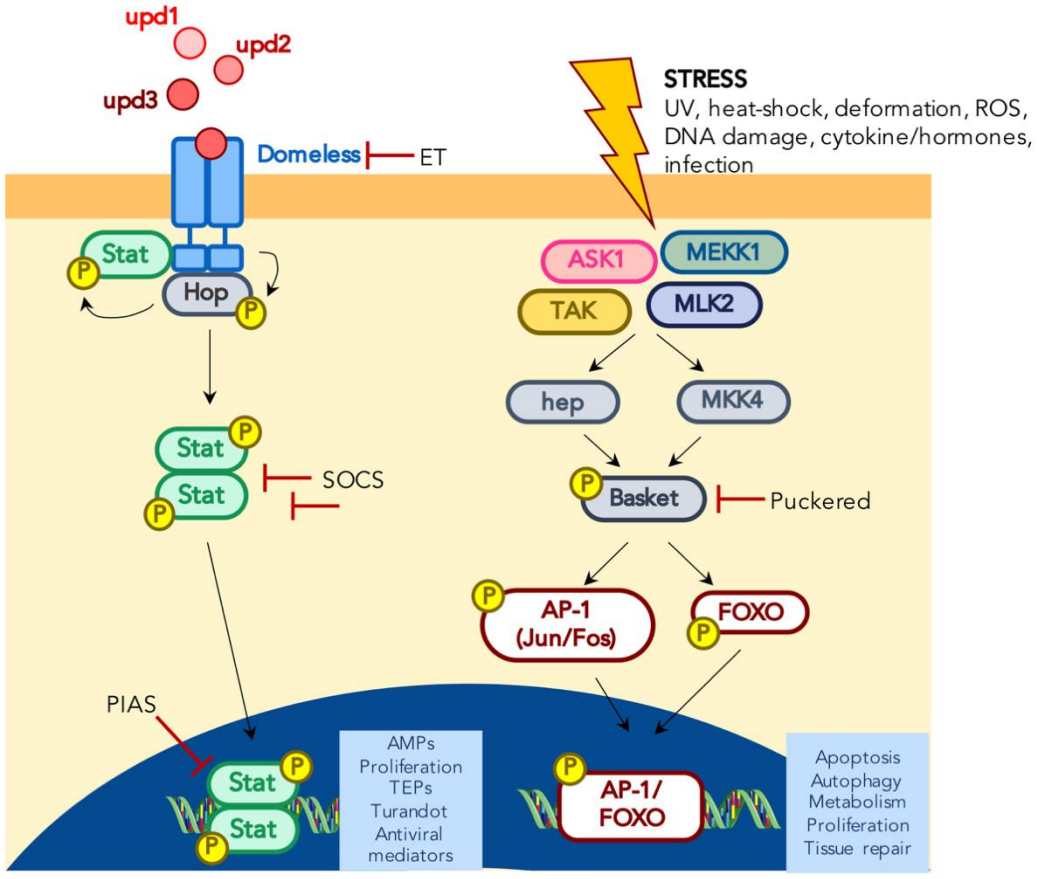


Figure I3. Schematic overview of the JAK-STAT and JNK pathways. The binding of Unpaired (upd) cytokines to the Domeless receptor activates the The Janus Kinase–Signal Transducers and Activators of Transcription (JAK-STAT) pathway (left). Upd are released upon bacterial or viral challenge, trigger Domeless dimerization and subsequent JAK Hopscotch (Hop) phosphorylation. The transcription factor Stat92E (or simply Stat) is recruited and phosphorylated. It forms a dimer, and then it translocates into the nucleus where it induces the transcription of several genes. Adapted from Chen et al., 2014.
The Jun N-terminal kinase (JNK) pathway (right) is activated by infection or various stress signals. Four potential JNK kinases kinases (JNKKK) are then activated: ASK1, MEKK, TAK or MLK2. The Mitogen Activated Protein Kinase (MAPK) cascades that follow converge on the phosphorylation of Basket (the *Drosophila* JNK homolog). Finally, Basket phosphorylates the transcription factors Activating Protein 1(AP-1), Jun and Forkhead box O (FOXO), which regulates the expression of many context-specific genes. Adapted from Biteau et al., 2011.

1.2.5.3 Wound healing

Wild *Drosophila* can be subjected to physical injury in their natural environment. Wound repair mechanisms are therefore needed to preserve the internal homeostasis and avoid systemic infections by pathogens. Tissue regeneration for wound repair are complex responses involving communication between the wound site and remote organs: epithelial movements, hemocyte recruitment, apoptosis, ROS generation, melanization and epithelial renewal all intervene in the process (Lee and Miura, 2014).

In embryos, the earliest step is an instantaneous Ca^{2+} flash that travels among the cells via gap junctions, activating H_2O_2 production by Dual Oxidase (DuOx) enzyme (Razzell et al., 2013): this step is important to trigger wound response genes (Juarez et al., 2011). In larvae, JNK signaling at the wound site promotes the expression of Upd cytokines, which in turn activate JAK-STAT pathway (Pastor-Pareja et al., 2008): this generates a systemic amplification loop through the induction of additional cytokines production by fat body and hemocytes. The latter proliferate and they are then recruited from the circulation to the wound site, where they phagocyte dead bodies (Babcock et al., 2008). Intracellular transport increase, cytoskeletal rearrangements and cell fusion are also consequence of JNK activation (Jasper et al., 2001; Lee et al., 2017; Thomas et al., 2009). ERK and the gene *grainy head* are equally required for the wound repair (Mace et al., 2005).

In parallel, melanin deposition at the wound site through circulating phenoloxidase activation (Cerenius and Söderhäll, 2004; Neyen et al., 2015) is also a step important for repair: melanin forms a stab at the damaged location, and together with the reactive oxygen intermediates generated during melanin synthesis it prevents microorganism's invasion (Tang, 2009). In addition to the local response at the site, in adults flies phenoloxidase-dependent ROS diffuse and trigger JNK activation in neurons, providing protection against physical injury and barrier disruption, demonstrating the importance of interorgan communication upon integumental wound (Nam et al., 2012). Of note, it is appropriate to make the distinction between injuries in embryos and coagulation in adults, which proceeds through peculiar mechanisms (Schmid et al., 2019; Theopold et al., 2014).

1.3 The digestive system of *D. melanogaster*

The intestine is the organ responsible for digestion, that is, the ability to absorb nutrients from food. At the same time, it is one of the most important epithelial barriers of the body, since most of the pathogens or xenobiotics enter through the digestive tract. Therefore, organisms have evolved physical barriers and biological defenses to preserve homeostasis. Moreover, the intestine continuously exchanges information with other organs, and is home to one of the most important symbiotic communities of the body, the gut microbiota.

The intestinal tract of *Drosophila* is very similar to that of mammals: the epithelium is monolayered, composed practically of the same types of cells, it houses a symbiotic microbial community and has a powerful local immune response. The possibility to combine genetic and functional approaches, together with the other advantages of the fruit fly, has allowed many laboratories to establish this insect as a model organism for the study of intestinal homeostasis and oral infections.

In this section I will describe some of the anatomical and functional properties of the fly intestine, the major mechanisms regulating its homeostasis and the key defenses present in the *Drosophila* digestive tract.

1.3.1 Anatomy and function

Besides the tubular monolayered epithelium, the *Drosophila* gut is encircled by two layers of visceral muscles, as well as tracheae and nerves (Lemaitre and Miguel-Aliaga, 2013; Miguel-Aliaga et al., 2018). Neurons control sensory and motor functions of the gut: connecting the epithelium and the muscles with the nervous system, this circuit regulates nutritional and reproductive status, which provide for many conserved physiological functions (Cognigni et al., 2011). Anatomically, the intestine can be divided in three main parts, enclosing cells from distinct embryonic origins: the foregut, the midgut and the hindgut (Miguel-Aliaga et al., 2018; Murakami et al., 1999) (Figure I4).

The foregut and the hindgut constitute the most anterior and most posterior part, respectively. They derive from ectoderm; thus, they are covered by cuticle. The foregut holds several structures. It starts with a drinking and feeding appendage, the proboscis, and continues with the pharynx and the esophagus, the first parts of digestive apparatus. Then we find the crop, a diverticulum unique to Dipteran species, which probably serve as a food storage compartment and for regulation of nutrient circulation, likely with some food-processing function (Stoffolano and Haselton, 2013). Finally, the last portion of the foregut is constituted by the proventriculus, a pear-shaped structure that regulates the passage of the food bolus into the midgut.

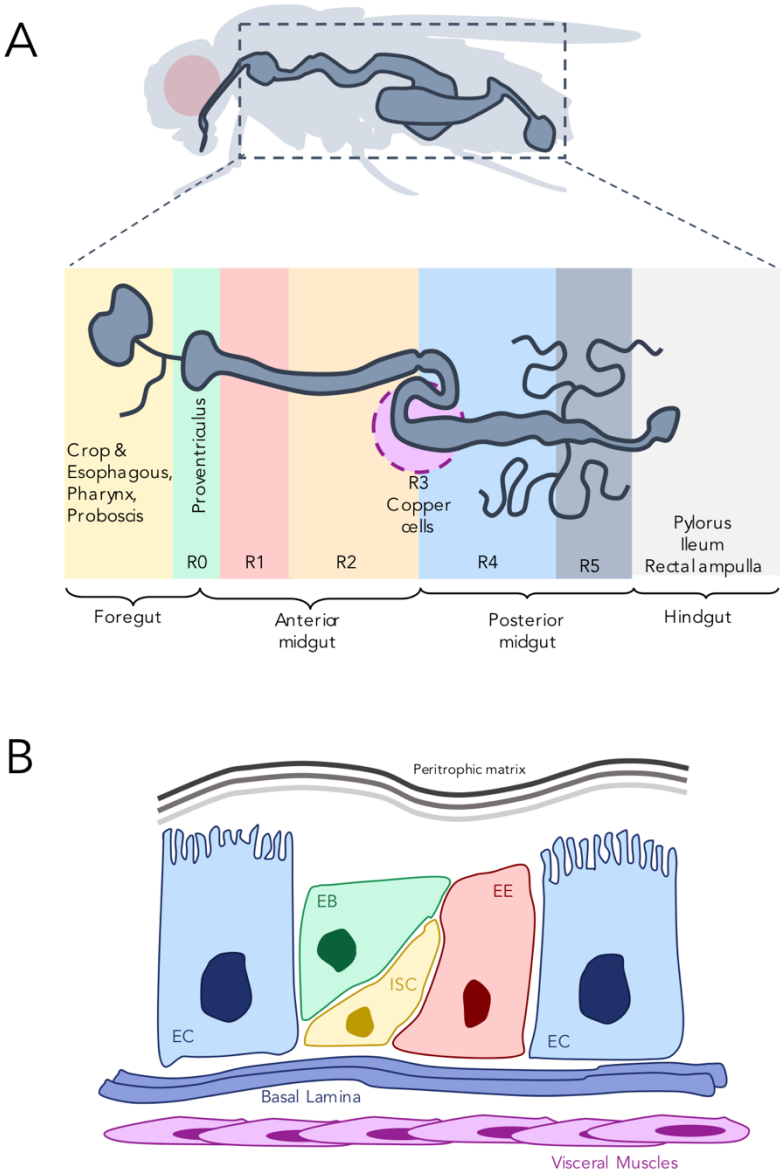


Figure I4. Simplified overview of the *D. melanogaster* digestive tract. (A) The gut of the fruit fly is divided in three broad anatomical regions: foregut, midgut and hindgut. The foregut is the entrance of the digestive tube: it is composed by the proboscis, the esophagus, the pharynx, the crop and the proventriculus. The midgut is composed by six regions named from R0 to R6. R0, R1 and R2 constitute the anterior midgut; the posterior midgut includes R4 and R5. In between, the acidic region R3, also known as copper cells region, represents alone the middle midgut. The hindgut is the most distal part of the intestine, made of pylorus, ileum and rectal ampulla. **(B)** The *Drosophila* digestive tract is surrounded by visceral muscles and covered with a glycoproteic multi-layer called peritrophic matrix. The intestinal epithelium includes four type of cells. The Intestinal Stem Cells (ISCs) are responsible for the regeneration of the organ: an asymmetric division give birth either to enteroendocrine cells (EEs) or enteroblasts (EBs). The latter will then develop into mature enterocytes (ECs). EEs play a neural-like function, integrating signals and communicating with the other cells, whereas ECs are principally devoted to digestion.

The final part of the digestive tract, the hindgut, is also divided in three sections: pylorus, ileus and rectal ampulla. This portion is devoted to ion exchange, water reabsorption and defecation (Cognigni et al., 2011), and also served as a micromodel for organogenesis (Lengyel and Iwaki, 2002). Additionally, numerous ramifications branch out from the digestive tract, especially in the pyloric region; they act as homologues of the mammalian kidneys and were given the name of Malpighian tubules.

1.3.1.1 THE MIDGUT

The largest and most studied part of the *Drosophila* intestine is the midgut. Unlike the foregut and the hindgut, the midgut originates from the endoderm, and its main function is digestion (Lemaitre and Miguel-Aliaga, 2013; Miguel-Aliaga et al., 2018). In nature, *Drosophila* feeds on decaying material: the fly evolved an incredible array of digestive enzymes to handle with the complex composition of this nourishment. Based on bioinformatic prediction, they are around 350: a complete list can be found here https://lemaitrelab.epfl.ch/resources, and their regulation is accompanied by an exhaustive description of how nutrients are absorbed are well reviewed in Miguel-Aliaga et al., 2018. It is also possible that *Drosophila* uses microorganisms colonizing rotten fruits and plants as food, since in the genome chitinases, glucanases and 15 lysozymes with no known immune function are present. Nevertheless, flies fed only on *E. coli* die from starvation (Arshad Ayyaz, unpublished). To obtain optimal absorption of nutrients, the gastrointestinal tract must specialize in specific regions for the processing of food at the various stages of digestion (Karasov et al., 2011). Given the total length (around 6mm) and the specialization of different segments, midgut has been divided broadly in anterior, middle and posterior midgut (Buchon and Osman, 2015). Furthermore, morphological and molecular analyses outlined a functional compartmentalization, with six different regions (named from R0 to R5) that follow from anterior to posterior, differing in anatomical boundaries, tissue histology and gene expression patterns (Buchon et al., 2013a). The study from Buchon and collaborators had a big impact for all researchers working on fly midgut. This compartmentalization, which is established as soon as two days post-eclosion, has a great importance in digestion, local immune response, and generally in host health, since its disruption impairs gut homeostasis and immunity. Even more importantly, based on those data a large database containing information on *Drosophila* adult midgut have been created (https://flygut.epfl.ch and its complementary resource http://flygutseq.buchonlab.com), providing a very useful tool to all drosophilist who explores intestine physiology. The second in particular provides the transcriptomic profile of each intestinal cell type sorted by FACS from the five regions R1-R5 (Dutta et al., 2015). The cellular profiling of gut cells has still had an important advancement: previously in this year, single-cell RNA sequencing contributes to grow the knowledge about gene expression in the midgut epithelium (Hung et al., 2020).

Refining the same parameters, the six major segments can be further divided in 10-14 subsets (Buchon et al., 2013a; Marianes and Spradling, 2013). For simplification, I will shortly describe here only the regions from R0 to R5 and the main cell type composing *Drosophila* digestive tract (Figure I4).

R0 correspond to the cardia, which is the endodermal part of the proventriculus. It is composed by three epithelial layers and it is responsible for the synthesis of the major part of the peritrophic matrix (King, 1988). The anterior region of the midgut continues with R1 and R2: these segments are responsible of breaking down macromolecules during digestion. In particular, the R2 region contains voluminous enterocytes with a remarkable dome-like structure: because of these characteristics we chose this portion for our studies in the laboratory. The R3 constitutes alone the middle midgut because of its peculiar features. It is also known as the copper cell region, an acidic compartment that is the functional homologue of the mammalian stomach. Indeed, these cells secrete H^+ creating an ideal pH environment for the function of certain digestive proteases (Dubreuil, 2004). Finally, the posterior midgut is composed by R4 and R5: here the epithelial cells are mainly devoted to nutrient absorption, they have a more irregular morphology and they are thinner approaching towards the hindgut.

We can distinguish four cell types in the midgut epithelium: the intestinal stem cells (ISCs), the enteroblasts (EBs), the enteroendocrine cells (EEs) and the enterocytes (ECs) (Figure I4). ECs are the most abundant cell type in the midgut; they are polyploid and their main function is to secrete digestive enzymes and absorb nutrients (Lemaitre and Miguel-Aliaga, 2013; Miguel-Aliaga et al., 2018). EEs are smaller, diploid, and they account for around 5-10% of the total midgut epithelial cells (Beehler-Evans and Micchelli, 2015; Micchelli and Perrimon, 2006). They regulate intestinal physiology by sensing nutritional state (Amcheslavsky et al., 2014; Song et al., 2017) and communicating with other cells and tissues by secreting neuropeptides hormones such as Bursicon, Allatostatin, Tachykinin, Neuropeptide F and DH31 (Beehler-Evans and Micchelli, 2015; Scopelliti et al., 2014), thus playing a neural-like function. The intestine is one of the organs with the higher regenerative capacities in an organism: it is constantly renewed through ISCs activity (Jiang and Edgar, 2011; Nászai et al., 2015). In *Drosophila* this process takes around 1-2 weeks (Micchelli and Perrimon, 2006). The small triangular-shaped ISC progenitor cells, which are localized at the base of the epithelium, as in mammals (Ohlstein and Spradling, 2006), divide asymmetrically generating another ISC and an EB. For a long time, the EB have been considered as the common precursor of both ECs and EEs. Yet, further studies demonstrated that ISCs commitment to EEs depend on the induction of the transcription factor *Prospero* in ISCs (Biteau and Jasper, 2014; Guo and Ohlstein, 2015; Zeng and Hou, 2015). Therefore, EBs are more like immature progenitors of ECs, and whether they represent a distinct type of cell is controversial. The EC fate of EBs is determined by high levels of Notch signaling via the expression of *Delta* ligand by ISCs (Micchelli and Perrimon, 2006; Nászai et al., 2015; Ohlstein and Spradling, 2007). In parallel, low levels of *Delta* released from EEs are required to maintain the identity of the neighboring ISCs (Guo and Ohlstein, 2015). Thus, Notch is a key determinant of ISC differentiation. Nevertheless, this pathway is

far from being the only one important for ISCs maturation into ECs. As we will see later in the part dedicated to resilience, the compensatory proliferation of ISCs is very complex and has been much studied in the last 15 years. Many pathways intervene following different stimuli: JAK-STAT, Hippo, JNK, EGF and Wingless, just to name some of them.

1.3.1.2 The microbiota

Nowadays, we cannot talk about intestine without talking about its microbiota. The symbiotic microbial community associated with the gut, previously known under the inappropriate name of intestinal flora, is a characteristic of all metazoans. It has been proven over the years that microbiota affects a myriad of physiologic processes such as digestion, immune response, disease severity, development, metabolism and much more (Bäckhed and Crawford, 2010; Fraune and Bosch, 2010; Round and Mazmanian, 2009). The impact of gut microbiota on the host is so deep that it can touch even apparently unrelated processes like neurodegeneration and allergy transmission (Brosseau et al., 2019; Roy Sarkar and Banerjee, 2019). In mammals, microbiota is very diverse and complex: more than 2000 species have been isolated from human digestive tract, where the most represented phyla are Firmicutes, Bacteroidetes, Proteobacteria and Actinobacteria (Thursby and Juge, 2017).

Once again, *Drosophila* provides a simplified model to study host-gut microbiome interactions. Its microbiota is limited, including 30 taxa at the most, with the genera *Lactobacillus* and *Acetobacter* that are the most represented (Broderick and Lemaitre, 2012; Chandler et al., 2011). Wild flies conserve a core composed by these two phyla, although the overall composition is obviously more diverse than *Drosophila* raised in laboratory. Diet is a crucial factor: for instance, high sugar fly medium promotes an enrichment in *Acetobacter* (Huang and Douglas, 2015), whereas starvation correlates with a general decrease in midgut microbial content (Obadia et al., 2017). Surprisingly, the *D. melanogaster* microbiome displays considerable individual variability, even within flies of identical genetic background and age raised in the same tube (Broderick, 2016; Broderick et al., 2014). This variation consists in both composition and density. The latter is correlated with abundance of food in the gut, which indicates that microbes are acquired with the food and/or that this one is an important factor for microbe-fly association (Broderick et al., 2014). Indeed, the establishment and maintenance of the microbiota is dependent on repeated reintroduction rather than a stable association (Blum et al., 2013). Frequent transfer on sterile food induces a decrease in *Lactobacillus* and *Acetobacter* densities, and germfree flies are more susceptible to *S. marcescens* and *P. aeruginosa* oral infection. The nature of this association can be at the basis of the variability observed inter-individually and across generations. Flies acquire their microbiome right after hatching, when the larva eat the chorion of the embryo which is contaminated with adult feces (Broderick and Lemaitre, 2012). Nevertheless, flies can also establish stable associations with microbes in certain cases. Recently, it has been shown that *D. melanogaster* builds a

lasting and beneficial mutualistic relationship with a wild isolated *Acetobacter* species, *Acetobacter thailandicus* (Pais et al., 2018). Also larvae sustain the association with *L. plantarum* by secreting factors that helps the bacteria to overcome the cost of the passage through the midgut (Storelli et al., 2018).
The other way around, it is also true that the microbiota influences host metabolism. Acetic acid, the product of *Acetobacter* fermentation, has been linked with lipid homeostasis (Chaston et al., 2016; Kamareddine et al., 2018), and affects larval development or adult fly behaviors like oviposition (Kim et al., 2018). In conditions of poor nutrient availability, both *Lactobacillus plantarum* and *Acetobacter pomorum* help host growth by acting on the central metabolic TOR and insulin pathways, respectively (Shin et al., 2011; Storelli et al., 2011). Nonetheless, microbiota is not essential for fly development, since with an adequate diet it is possible to raise axenic flies. Comparison between axenic and conventionally raised flies elucidated the multiple contributions fulfilled by the microbiota in many physiological functions: metabolism, through regulation of lipid storage and glucose levels; gut homeostasis by stem cell activity and activation of developmental and homeostatic pathways; behaviors such as mating preference and oviposition; disease susceptibility, via basal immune system and stress pathways activation (Broderick, 2016; Broderick et al., 2014). For instance, DuOx-derived ROS are key to control the over proliferation in the gut of *Saccharomyces cerevisiae*, an essential component of *D. melanogaster* diet (Ha et al., 2009a). Yeast provides amino acids, sterols, B vitamins and fatty acids, which are needed to complement a high-sugar diet (Broderick and Lemaitre, 2012). Bacteria gut community further improve nutritional supply, e.g., providing thiamine (Sannino et al., 2018) or decreasing glucose levels while favoring lipogenesis and lipid storage in high-sugar diet (Whon et al., 2017).

Compartmentalization of the gut also concerns gene expression, including AMPs genes, immune receptors and negative regulators of the immune response (Buchon et al., 2013a). Potential pathogens introduced with food are killed by AMPs, which are mostly expressed in the anterior midgut, whereas negative regulators are mostly expressed in the posterior midgut and hindgut, promoting colonization and persistence of the microbiota (Broderick, 2016). In fact, immune response and gut-microbe associations are in close relationship: commensal stimulate basal level of immune response (AMPs and ROS), but in turn high levels of immune mediators alter bacterial composition. Strikingly, old flies, which harbor higher bacterial density (Buchon et al., 2009b; Ren et al., 2007), also have stronger AMPs production and ROS production. This immune dysregulation is followed by an excessive proliferation of the ISCs, leading to accumulation of undifferentiated cells and intestinal dysplasia (Biteau et al., 2008; Buchon et al., 2009b). However, intestinal homeostasis alteration and immunity over-activation are just a consequence of the changes in microbial composition (and eventual dysbiosis); finally, intestinal barrier disruption and chronic inflammation lead fly to death (Clark et al., 2015). Intestinal acidity contributes to control microbial load as well as ROS and AMPs: chronic activation of JAK-STAT pathway in the copper cell region decreases the number of these cells, triggering epithelial metaplasia and loss of microbiota compartmentalization, which equally drive to

dysbiosis, homeostasis loss and death (Li et al., 2016). In fact, a certain number of homeostatic and developmental pathways are induced in addition to the immune genes, and account for the epithelial morphologic changes in response to microbiota. Axenic flies midguts are about 40% longer compared to their conventional raised counterparts, an effect due to alteration of size and shape rather than a raise in cell number (Broderick et al., 2014). Insulin and EGFR signaling are involved in those imbalances, as well as Notch: in the absence of microbiota, this pathway is dampened, causing an increase in EEs and a decrease in EBs.

1.3.2 The local immune response

In addition to be the organ responsible for digestion and the "home" of the intestinal microbiota, the gastrointestinal tract also constitutes an important barrier to invading pathogens. This is particularly important for *Drosophila*, that feeds and reproduces on decaying matter such as rotten fruit, an environment particularly rich in microorganisms. The fruit fly evolved multiple arms of defense to prevent the passage of potential harmful microbes into the hemolymph: a physical barrier, the peritrophic matrix, and a local immune response composed of AMPs and ROS.

1.3.2.1 The peritrophic matrix

While foregut and hindgut have an internal coating of cuticle, the midgut is devoid of it, which favors digestive functions in this segment. To avoid direct contact between the food bolus and the microbiota with the epithelial cells, a semi-permeable barrier composed by multiple layers of chitin and glycoproteins (mucins) is present. This is called peritrophic matrix, and it is mainly secreted from the proventriculus in the form of laminae, which are then compressed by visceral muscles to be unrolled into the midgut as two layers (King, 1988). However, Drosocrystallin, a protein expressed in midgut but not in cardia, is an essential component of the peritrophic matrix, showing that also ECs and EEs contribute to its structure (Kuraishi et al., 2011). Semi-permeability of this barrier is an important property, as allowing the digestive enzymes to reach the bolus and the immune mediators to the microorganisms. This molecular sieve has pores that preclude the passage of components with a diameter bigger than 10nm or with a molecular weight superior to 200kDa (Lehane, 1997). The peritrophic matrix is a first passive defense and its alteration makes the flies more susceptible to intestinal infection of pathogen like *Serratia marcescens* and *Pseudomonas entomophila* (Kuraishi et al., 2011).

1.3.2.2 The AMPs response

AMPs production in the midgut is vital for the flies upon bacterial challenge (Buchon et al., 2013b), since the absence of these molecules makes the flies susceptible to infection with *Ecc15* (Buchon et al., 2009b), *Pseudomonas aeruginosa* (Limmer et al., 2011), *Pseudomonas entomophila* (Liehl et al., 2006) and

Serratia marcescens (Nehme et al., 2007). This response relies almost exclusively on the IMD pathway (Tzou et al., 2000), which detects pathogenic bacteria through the membrane receptor PGRP-LC and the intracellular sensor PGRP-LE. The expression of these is compartmentalized: the first one is responsible of bacterial sensing in foregut and hindgut, the second one in the midgut (Bosco-Drayon et al., 2012; Neyen et al., 2012). This organization is accompanied by the expression of their respective negative regulators PGRP-LB, PGRP-SC, Pirk and Caudal (Guo et al., 2014; Lhocine et al., 2008; Paredes et al., 2011; Ryu et al., 2008). The regionalization of the PRRs with the negative regulators fine-tunes the immune response, fighting efficiently against pathogen but in parallel maintaining tolerance to the commensal community (Bosco-Drayon et al., 2012; Ferrandon, 2013; Lhocine et al., 2008; Ryu et al., 2008). The inability to mount an immune response in the intestine results in an activation of the systemic response (Bosco-Drayon et al., 2012). Conversely, sustained IMD activity and AMPs production in the midgut impair host survival (Ryu et al., 2008). Interestingly, a recent study showed that both isoforms (secreted and cytosolic) of the PGN-amidase are encoded by PGRP-LB; this limits NF-kB activation in ECs and the spreading into the body cavity of microbiota-derived PGN, preventing the activation of the systemic immune response (Charroux et al., 2018).

1.3.2.3 The ROS response

Reactive Oxygen Species (ROS) constitute the most primitive and conserved immune response among multicellular organisms. Their very high reactivity means that they can damage many biological molecules, namely proteins, DNA and lipids. This gives them an intrinsic microbicidal capacity, but at the same time they also pose a danger to the self as they cannot distinguish host cells from pathogens. ROS derive from oxygen metabolism and can be free oxygen radical or non-radical ROS: some examples are superoxide ($\cdot O_2^-$), hydrogen peroxide (H_2O_2) and hydroxide anion ($\cdot OH$). For long time perceived exclusively as toxic molecules, it is nowadays well established that ROS biology is more complex and plays crucial physiological roles. A more detailed presentation will be addressed later, since they are the subject of chapter 1. Here, I will focus only on their function in the immune response.

Generalities on the ROS-mediated immunity

The strong production of $\cdot O_2^-$ by professional phagocytes acting as a bactericide agent in humans (mainly neutrophils and macrophages) was reported around 50 years ago (Babior et al., 1973) and known under the name of "respiratory burst" or "oxidative burst". However, the primary product of this oxidative burst, that is $\cdot O_2^-$ and H_2O_2, are weakly microbicidal; instead, they are used to form more microbicidal oxidants such as oxidized halogens (as hypochlorite, OCl^-) and oxidizing radicals (as hydroxide anion, $\cdot OH$) (Babior, 1984). In professional phagocytes, the ROS production is one of the principal pathogen-killing mechanism: it occurs at the endosome membrane with the involvement of

NAPDH oxidase enzymes. These are complexes formed by subunits named "phox" (phagocyte oxidase). We recognize a membrane-bound catalytic core subunits ($gp91^{phox}$ and $p22^{phox}$) and cytosolic regulatory subunits ($p40^{phox}$, $p47^{phox}$, $p67^{phox}$ and Rac) (Kohchi et al., 2009; Roos et al., 2003; Yang et al., 2013). NAPDH oxidase converts NADPH into $NADP^+$ and transfers one electron through the catalytic core extracellularly, where they are caught by molecular oxygen forming $\cdot O_2^-$, which in turn is rapidly dismutated into H_2O_2 by Superoxide Dismutase (SOD). Mutation in any of the NAPDH oxidase subunits results in Chronic Granulomatous Disease (CGD), a severe genetic disorder where the neutrophils are unable to produce ROS and to eliminate microorganism (Roos et al., 2003; Segal, 1996). Consequently, those patients suffer from recurrent bacterial and fungal infections, primarily localized at epithelial barriers (digestive tracts, lungs, skin) (Roos et al., 2003). ROS production from NADPH oxidase is also tightly linked to formation of Neutrophil Extracellular Traps (NETs) (Amulic and Hayes, 2011; Fuchs et al., 2007; Wartha and Henriques-Normark, 2008); accordingly, CGD patient are unable to form them. Besides direct pathogen killing, ROS influence the immune system in multiple ways. They are involved in danger sensing through inflammasome activation, and they shape the response of many immune cells as NK cells, dendritic cells, T and B lymphocytes; also, mitochondrial ROS play a role in anti-viral and anti-bacterial signaling. All these dynamics are reviewed more in depth in Yang et al., 2013. Beyond host defense, NOXs enzymes intervene in multiple physiological processes as cellular signaling, protein processing, regulation of gene expression and cell differentiation (Bedard and Krause, 2007).

NOX2 is the major NADPH oxidase expressed in phagocytes, but in humans NOX family is composed by other six members as well (NOX1-5 and DuOx 1-2), differing in their cell type and tissue distribution (Bedard and Krause, 2007; Lambeth, 2004). DuOx enzymes are so called because besides their NADPH oxidase domain, they also possess a N-terminal Peroxidase-Homology Domain (PHD), which allows the formation of stronger microbicidal ROS such as HOCl from H_2O_2. Between the NADPH oxidase domain and the PHD domain there are two EF-hand motifs, indicating that enzymatic activity is regulated by calcium (Donkó et al., 2005). Both DuOx isoforms were initially identified in humans in thyroid, where they play a role in hormone biosynthesis (Carvalho and Dupuy, 2013; Donkó et al., 2005). Research on lower organisms, namely *C. elegans*, sea urchin and mosquitoes, established DuOx as an player in extracellular matrix modifications through ROS-mediated protein crosslinking (Edens et al., 2001; Kumar et al., 2010). Finally, DuOx is also found in mucosal surfaces, where it plays a role in defense. By working in tandem with lactoperoxidase (LPO), DuOx contributes to confer antimicrobial properties to mucosal fluids like saliva (but also tears, milk and airways secretions) (Geiszt et al., 2003). Actually, DuOx acts as H_2O_2 donor for LPO, which oxidizes thiocyanate and iodide generating the powerful microbicidal molecules hypothiocyanite and hypoiodite (Donkó et al., 2005). Topologically, DuOx is expressed at the apical part of the cell, in thyroid as well as in respiratory epithelium (Schwarzer et al., 2004). The same localization pattern is found in gastrointestinal tract,

where DuOx2 is found all along the intestine, particularly enriched in caecum and colon (Geiszt et al., 2003; El Hassani et al., 2005).

ROS RESPONSE IN *DROSOPHILA* MIDGUT

In *Drosophila* midgut, microbicidal ROS production relies on the enzymes Nox1 and DuOx2 (Jones et al., 2013). The former produces ROS in response to commensal bacteria, particularly *Lactobacillus*, avoiding dysbiosis and activating homeostatic mechanisms like stem cell mediated midgut renewal (Iatsenko et al., 2018; Jones et al., 2013). Also DuOx is indispensable for gut antimicrobial responses, but it is particularly important against pathogenic bacteria infections (Ha et al., 2005a). Its structure is very conserved between flies and mammals, with a $gp91^{phox}$-like oxidase domain, two EF-hand motifs, a PHD domain and seven transmembrane portions that insert it in the apical part of enterocyte plasma membrane. The DuOx pathway (Figure I5) has been largely studied in the laboratory of Professor Won Jae Lee, but the PRR responsible for pathway activation is still missing. However, they showed that the intracellular Ca^{2+} pulse needed for ROS production by DuOx requires the activity of phospholipase Cβ (PLCβ). In turn, this is activated by GTP-bound subunit of the Gq class of heterodimeric G proteins (Gαq) (Ha et al., 2009a). This suggest that the lacking receptor is possibly a G protein coupled receptor (GPCR). Instead, bacteria-derived uracil has been identified as the MAMP eliciting the DuOx pathway (Lee et al., 2013). They propose that the quantity of this ligand is at the basis of the host capacity to distinguish between pathogens and commensals, since each bacterial species has different levels of uracil release. Therefore, the absence of uracil-induced DuOx activation allows the maintenance of beneficial symbiotic gut microbes whereas an adequate ROS-mediated immune response is triggered by pathogens, which in most cases are cleared from the gastrointestinal tract achieving gut-microbe homeostasis. Nonetheless, some pathogens such as *Serratia marcescens* and *Ecc15* are resistant to ROS action.

On the one hand, DuOx requires a biochemical activation (Figure I5). Bacteria-derived uracil is crucial for both activation of Hedgehog signaling pathway and $Rab7^{+}$ endosomes formation in enterocytes (Lee et al., 2015). These endosomes work as a signaling platform recruiting PLCβ and protein kinase C (PKC) leading to Ca^{2+} release from ER. Consequently, the EF-hand Ca^{2+} sensing domain activates ROS production by DuOx (Ha et al., 2009b, 2009a; Lee et al., 2015). On the other hand, this enzyme is also transcriptionally regulated by the p38 MAPK pathway (Ha et al., 2009b). The activation of that is triggered again by PLCβ, which starts the MEKK1/MKK3/p38 kinase cascade leading to *DuOx* gene transcription by the transcription factor ATF2 (Figure I5). Nonetheless, DuOx transcriptional upregulation is triggered only in the case of an infection. Indeed, conversely to the PLCβ-controlled DuOx activity pathway, $p38^{-}$ mutant flies have a normal survival in conventional rearing conditions (Ha et al., 2009b). Additionally, p38 can also be activated by PGRP-LC and IMD in

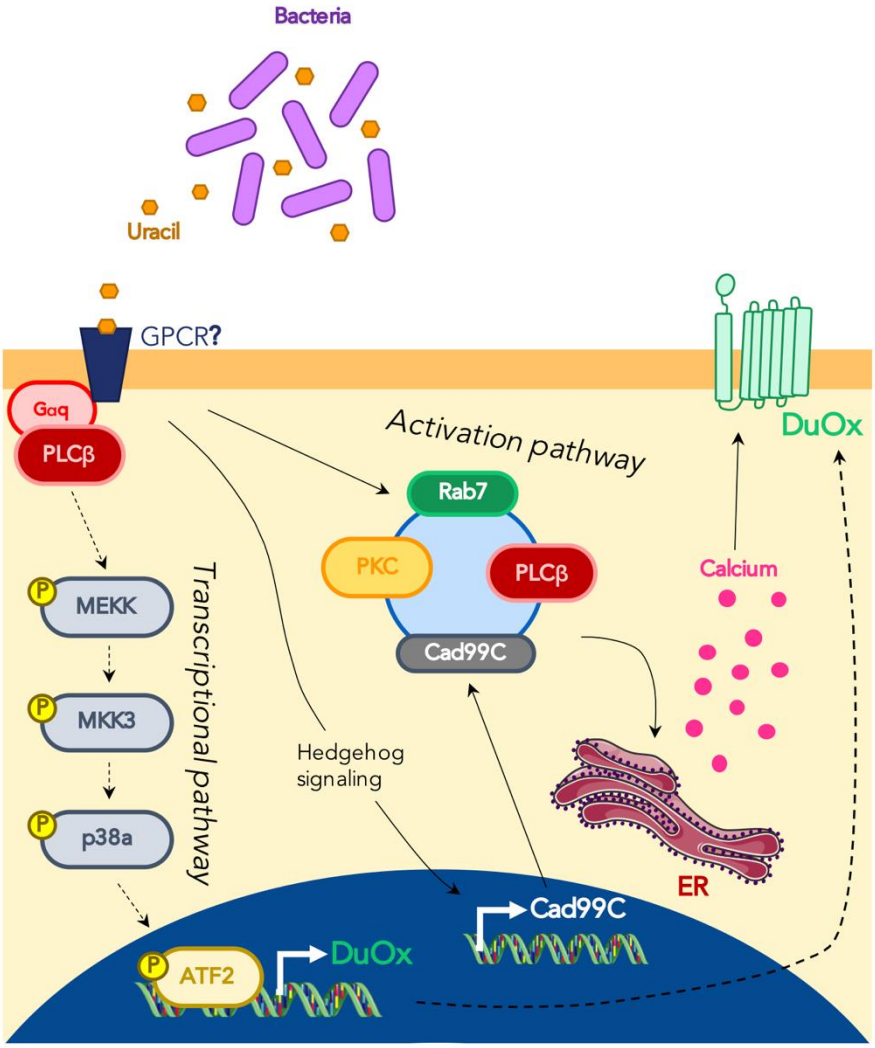


Figure 15. Simplified overview of the DuOx pathway. DuOx pathway has a double regulation in *Drosophila*, one transcriptional (left) et one biochemical (right). Ingested bacteria secrete uracil in the gut lumen, which is recognized by a still unknown GPCR. Then, a phosphorylation cascade initiated by PLCβ activates in sequence MEKK, MKK3, p38a and the Activating Transcription Factor 2 (ATF2). Once in the nucleus, ATF2 is responsible for the upregulation of the *DuOx* gene.
In parallel, the binding of uracil on the GPCR induce the expression of cadherin Cad99C, in a Hedgehog-dependent manner. At the same time, it promotes the formation of a $Rab7^{+}$ endosome, which needs Cad99C and further recruits Protein Kinase C (PKC) and PLCβ. The latter triggers a Ca^{2+} release from the endoplasmic reticulum: this activates DuOx through its EF-hand Ca^{2+}-sensitive domain. Activated DuOx secrete ROS in the gut lumen, notably HOCl and H_2O_2, which are responsible for bacterial killing but also for damage to the host cells.

a PGN-dependent manner. Since PGN also derives from commensals in absence of an infection, midgut epithelium must tolerate a certain amount of PGN without triggering excessive DuOx activity to avoid dysbiosis. Therefore, PLCβ is also involved in the negative regulation of the same p38 pathway by inducing the phosphatase MKP3, responsible of p38 inactivation. All these dynamics fine-tune the midgut ROS response, allowing the clearance of dangerous infections when needed but maintaining the permanence of gut microbiota in normal conditions. While PLCβ-controlled DuOx activity pathway is constitutively active at low levels to control commensal hyperproliferation (Ha et al., 2009a), DuOx transcriptional pathway is stimulated only in case of bacterial invasion (Ha et al., 2009b). Regulation of microbial community abundance in the digestive tract by DuOx immune response is fundamental for fly lifespan. Indeed, *Drosophila* lacking DuOx expression have a shortened survival if raised in conventional conditions, but not in germ-free conditions (Ha et al., 2009a). Finally, another degree of complexity is constituted by the fact that lipid metabolism influences the activity of DuOx. In fact, the same laboratory of Prof. Won Jae Lee claimed that lipolysis through the Autophagy-related Gene 1 (Atg1) is essential for the activation of DuOx; this is on the contrary blocked by lipogenesis (Lee et al., 2018). Besides the intrinsic regulation of DuOx, the amount of ROS produced and the relative oxidative damage is buffered by a secreted protein, the Immune Regulated Catalase (IRC) (Ha et al., 2005b). This effector is crucial to ensure host optimal survival during gastrointestinal infection, as adult flies with downregulated IRC expression showed high mortality rates caused by excessive oxidative stress-related damages, even when challenged with killed bacteria.

Aside from the direct immune function, DuOx-derived ROS has been recently shown to mediate gut peristalsis (Benguettat et al., 2018). During intestinal infection with ROS-inducing bacteria such as *Erwinia carotovora carotovora* or *Bacillus thuringensis*, ROS-sensitive transient potential receptor A1 (TRPA1) induces the release of neuropeptide DH31 from enteroendocrine cells. This provokes the contraction of visceral muscles surrounding the intestine, favoring bacterial elimination.

2. Serratia marcescens

2.1 History and general features

Identification of *Serratia marcescens* dates back to 1819 by the Italian pharmacist Bartolomeo Bizio and it is linked to curious facts. The bacterium was indeed established as the responsible of the "bloody polenta", where red spots appeared on polenta or other starch-rich foods, fueling people's superstition (Bizio and Merlino, 1924). Bizio named the new microorganism *Serratia* in honor of the Italian physicist Serafino Serrati, and chose the term *marcescens* since it decayed in a viscous mucilaginous matter. Analogously, *S. marcescens* may be the scientific explanation of the Corporal of Bolsena in 1263, where the consecrated host started to "bleed" during a mass, taking on a red color. Indeed, some *Serratia* species produce a red-colored pigment called prodigiosin. Its physiological role is not very clear, but it has been shown to possess broad antimicrobial activity against bacteria, fungi and parasites, in addition to immunosuppressant and anticancer properties (Bennett and Bentley, 2000; Petersen and Tisa, 2013). However, not all *Serratia* strains are red, especially the ones isolated from patients, that are usually non-pigmented. Simultaneously studied by several scientists, this microorganism has also been known under the name of *Zaogalactina imetrofa*, *Monas prodigiosa*, *Bacillus prodigiosus*, *Micrococcus prodigiosus* and *Erythrobacillus*, before returning to Bizio's original name of *Serratia marcescens* (Grimont and Grimont, 1978). Being considered as an innocuous saprophyte until the '50s, its red coloration was used in schools for tracing experiments; as biological marker, it was also used in some insidious medical tests to evaluate pathogenicity and transmission (Mahlen, 2011; Yu, 1979). Notably, it was used as a biological warfare tracer organism in experiments conducted by the U.S. government on military bases and general population in various areas of the country between 1940 and 1970. The case of sprays in San Francisco Bay in 1950 or the New York subway in 1966 has become dramatically famous: to test a potential bioterrorist attack, *Serratia* was released in the air causing an increase in pneumonia cases, severe urinary tract infection and even one death (Mahlen, 2011).

Taxonomically, *Serratia marcescens* belongs to the family of *Enterobacteriaceae*. It is a conditional anaerobic Gram-negative bacillus, that produce energy through respiration and fermentation and provided with a flagellum (Hejazi and Falkiner, 1997). *S. marcescens* is a very ubiquitous bacterium found in many environmental niches, and able to infect a very wide range of host from plant to humans including insects (Grimont and Grimont, 1978; Mahlen, 2011). It has particular relevance in nosocomial infections: although non-pathogenic for a healthy person, *S. marcescens* is a dangerous opportunistic pathogen in an immunocompromised human host. Remarkably, it is the third most common cause of

outbreaks in hospitals after *Klebsiella spp.* and *Staphylococcus aureus* (Gastmeier, 2014), with important incidence in pediatric units (Iosifidis et al., 2012; Maltezou et al., 2012). The success of *S. marcescens* as an infectious agent is due in part to the large variety of tissues that can be attacked: urinary, respiratory and digestive epithelia, pericardium, eyes, muscles and sub-cutaneous tissues can all be infected mainly by the use of contaminated solutions and medical material (e.g., contact lenses liquid and catheters) (Albers et al., 2001; Egebo et al., 1996; Mahlen, 2011; Marre et al., 1989; Riberi et al., 1997; Yu, 1979). We can cite the cases of 2005 and 2007, where outbreaks were triggered by contaminated bags of magnesium sulfate and heparin syringes, respectively. Sometimes the bacteria can also colonize the gut, that function as a reservoir further promoting infections (Jones et al., 1978; Knowles et al., 2000). The incidence of *Serratia* infection is worsened by the fact that this bacterium not only can grow in antiseptic and disinfectant solutions, but it also resistant to a wide range of antibiotics, mainly βlactams, tetracyclines and fluoroquinolones among others (Arakawa et al., 2000; Knowles et al., 2000; Stock et al., 2003; Traub, 2000). The sequences of two completes *Serratia* genomes were published so far: the one from the multidrug resistant clinical isolate SM39 and one from an insect isolate, Db11 (Iguchi et al., 2014). This study allowed the identification of a core genome of around 4.000 genes, but also underlined a high intraspecies variation. Moreover, numerous genes involved in antibiotic resistance were revealed, together with the plasmid pSMC1, containing multiple drug resistance determinants.

2.2 Virulence

On one hand, the capacity of *Serratia* to colonize such a broad range of hosts and natural habitats is due to the extraordinary resistance of this bacterium. In addition of the several antibiotics resistances cited above, *Serratia* also possesses a catalase that may confer protection against H_2O_2 (Zeng et al., 2011), and it can withstand very high doses of AMPs (100µM) *in vitro* (P. Bulet and C. Hetru, unpublished). On the other hand, its pathogenicity is mediated by various offensive molecular and cellular weapons. The flagellum gives it the ability to swim and swarm (Hejazi and Falkiner, 1997), and bacteria communicate between themselves through quorum sensing to develop biofilms (Labbate et al., 2004). The latter contributes to the bacterial virulence, since a strain lacking quorum sensing is less virulent during *C. elegans* infection (Coulthurst et al., 2004). LPS O-antigen and fimbriae are equally relevant to the bacteria adhesion capacity to cells and abiotic materials (Petersen and Tisa, 2013). Here below, I will discuss in more detail some of the *Serratia* virulence factors: the type VI secretion system (T6SS), outer membrane vesicles (OMVs) and a group of secreted effectors including proteases, nucleases, phospholipases, chitinases and the pore-forming toxin hemolysin.

2.2.1 T6SS

Many Gram-negative bacteria use the T6SS machinery to deliver effector proteins in target cells upon contact. It is a complex formed at least by 13 proteins (known as core components), and it presents structural similarities with a bacteriophage cell puncturing device (Bönemann et al., 2010; Cascales and Cambillau, 2012; Coulthurst, 2013). Interestingly, some studies pointed out that T6SS are mostly implicated in regulation of bacterial interactions and competition rather than delivery of virulence factors in eukaryotic cells (Jani and Cotter, 2010; Murdoch et al., 2011; Russell et al., 2011). The antimicrobial effectors delivered through *Serratia* T6SS attack and kill efficiently other bacterial species (Lazzaro et al., 2017; Mariano et al., 2018), even very close strains (Murdoch et al., 2011). Within the *Serratia* T6SS gene cluster, two antibacterial toxins, Ssp1 and Ssp2, act as peptidoglycan amidases targeting bacterial cell wall (English et al., 2012).

2.2.2 Outer membrane vesicles

OMVs are a characteristic of all Gram-negative bacteria. They arise from protrusions of the outer membrane, enclosing a pool of periplasm. This spheroid nanoparticles are about 20 to 300nm in diameter, and contains DNA, RNA, peptidoglycan, enzymes, periplasmic proteins, virulence factors and more (Pathirana and Kaparakis-Liaskos, 2016). OMVs production confers several advantages to bacteria: they enable the secretion of insoluble molecules, allowing their delivery in a protected and concentrated form even to distant targets; they favorize the communication between bacteria, mediating quorum sensing and biofilm formation; they are also important for nutrient uptake and overall survival (Kulp and Kuehn, 2010). The capacity of delivering high concentrations of toxins in a given area is a key feature of OMVs. For that reason, the host immune system mounts diverse innate and adaptive responses to counteract them, and in their turn OMVs can modulate immune cells (Kaparakis-Liaskos and Ferrero, 2015). In our team, a whole project is dedicated to the study of *Serratia*'s OMVs pathogenicity in *D. melanogaster*. The data collected show that injection of relatively small concentration of purified OMVs kill the flies faster than the bacteria itself. In parallel, a complex defense response is triggered in the host, involving IMD, melanization, phagocytosis and JNK (Sina-Rahme, Bruna et al., in preparation). Globally, the secreted protease PrtA has been identified as a key determinant of OMVs pathogenicity.

2.2.3 Secreted virulence factors

Different secreted factors contribute to the virulence of *Serratia marcescens*. The protease PrtA just mentioned above is one of the main effectors. This metalloprotease, also known under the name of serralysin, influences bacterial pathogenic capacity and biofilm formation, and it is transcriptionally regulated by the thermosensitive CpxAR system (Bruna et al., 2018). PrtA constitutes a very dangerous

virulence factor for the host: it has been shown to be cytotoxic (Marty et al., 2002; Shanks et al., 2015), able to degrade IgA, IgG and other serum proteins (Molla et al., 1986) and favoring bacterial adhesion and tissue colonization (Butterworth et al., 2014). Moreover in insects, studies on *Bombyx mori* revealed that PrtA induces the adhesion of circulating hemocytes, affects phagocytosis (Ishii et al., 2014a) and impairs the larvae coagulation response (Ishii et al., 2014).

Serratia genome also encode a nuclease, *nucA*, which is able to cleave DNA and RNA (Meiss et al., 2000). Nucleic acid degradation can promote bacterial growth by providing carbon sources (ribose and deoxyribose) and nucleic acid that can be recycled avoiding metabolically expensive synthesis (Benedik and Strych, 1998). *nucA* expression depends on the *flhDC* operon (Liu et al., 2000b), which regulates the flagellum, that is considered itself a T3SS. Therefore, the nuclease is possibly secreted via the flagellum export system, which is also mediating the phospholipase A secretion (Givskov et al., 1995). The latter is encoded by the PhlA gene, and it is able to lyse erythrocytes or to kill epithelial cells (Shimuta et al., 2009).

Finally, three chitinases are the product of the translation of *chiA*, *chiB and chiC* genes (Horn et al., 2006). This machinery degrades chitin, a N-acetylglucosamine polymer, which is the main component of the fungi cell wall and of the exoskeleton and peritrophic matrix of insects. Considering all these extracellular enzymes secreted by *Serratia*, we can see them altogether as an array of weapons to degrade large, complete organisms to readily access simple metabolites.

2.2.4 Hemolysin

Albeit it is also part of the secreted virulence factors, we present hemolysin separately because it represents the most crucial one in this study. As we will see later, this pore-forming toxin is a key inducer of the cytoplasmic purge resilience mechanism (Lee et al., 2016). Hemolysins are a type of pore-forming toxins (PFTs) that took their name for the ability to lyse red blood cells *in vitro* (Schiebel and Braun, 1989), although this property is not always relevant during infection. Hemolysins constitute a quite heterogenous group of toxins, generally cytolytic, produced by many bacteria (as *Staphylococcus aureus* and *Escherichia coli*) and also by some fungi (Vesper and Jo Vesper, 2004). *Serratia*'s PFT is probably considered as its most relevant virulence factor: its expression and regulation are under the control of the *shlAB* operon (Hertle, 2005). Hemolysin itself is coded by the *shlA* gene; however, this insoluble, short-lived protein (only few seconds of half-life in solution) of 165kDa is initially inactive. Indeed, hemolysin is secreted through T5SS (also known as two partner secretion system) and requires ShlB to be exported extracellularly and activated. ShlB is inserted in the outer membrane and mediates ShlA translocation across it: in this way ShlA gains its active conformation (Schiebel et al., 1989; Walker et al., 2004). Nevertheless, activation of ShlA is achieved only upon binding to

phosphatidylethanolamine, which is enriched in the external membrane of bacteria (Hertle et al., 1997). Accordingly, *Serratia*'s hemolysin is not truly secreted into the extracellular medium, but rather it requires direct contact or a delivery system to exercise its effect. In epithelial cells, sub-lytic doses of hemolysin induce the formation of tiny pores (2-3nm) without membrane disruption, which cause K^+ efflux and subsequent ATP depletion (Hertle, 2005; Hertle et al., 1999). At higher concentration, the toxin promotes cytoplasm vacuolization and lysis or apoptosis. Additionally, hemolysin is needed for the invasion of epithelial cells by *Serratia* through microtubules mobilization, and it is the major factor triggering their lysis (Hertle and Schwarz, 2004). Moreover, the bacterium is able to enter the cells via the formation of autophagosome-like vacuoles, which is triggered by hemolysin (Di Venanzio et al., 2014). Interestingly, *Serratia marcescens* is able not only to survive, but also to proliferate inside those compartments (Fedrigo et al., 2011). Indeed, the vacuoles do not become acidic/degradative vesicles, and the bacterium escapes from invaded cells by mobilizing Ca^{2+} and rearranging actin cytoskeleton (Di Venanzio et al., 2017).

2.3 *Serratia marcescens* Db11 strain

The large set of exoenzymes and toxin possessed by *S. marcescens* make it a serious threat to insects. The bacterium is pathogenic for around 70 species, and isolated from more than 30 (Grimont and Grimont, 1978). Among them we find also *Drosophila* spp., of which *S. marcescens* is a natural pathogen. In our laboratory we employ mainly the unpigmented strain Db11 (lacking prodigiosin), a streptomycin-resistant mutant derived from Db10, which was initially isolated from moribund flies (Flyg et al., 1980). As previously mentioned, this strain was fully sequenced (Iguchi et al., 2014) and a study in *C. elegans* allowed the identification of the genes required for full virulence *in vivo*, that consist principally in hemolysin production, LPS biosynthesis and iron uptake (Kurz et al., 2003). Previously in our laboratory, Nehme and colleagues established two different models of *Serratia* infection in the fly, depending on the route of infection: septic injury or oral feeding (Nehme et al., 2007).

2.3.1 Different ways, different infections

When directly injected in the hemolymph of *Drosophila*, *S. marcescens* Db11 is highly pathogenic, killing the flies in less than one day (Nehme et al., 2007). Despite the activation of the IMD systemic pathway, *Serratia* rapidly proliferate in the body cavity causing septicemia followed by death. Indeed, IMD-deficient flies are as sensitive as WT: the bacterium eludes the AMPs response through a mechanism involving its LPS-O antigen (Kurz et al., 2003; Nehme et al., 2007).

Conversely, Db11 is much less virulent when ingested. Flies resist the infection for days when they fed a sucrose solution containing the bacteria. In this case, a local IMD immune response is elicited, and IMD-deficient flies succumb faster to the infection. The decreased virulence of Db11 in not due to the confinement into the digestive tract, as bacteria are found in hemolymph. Actually, a moderate number of bacteria cross the gut barrier and reach the systemic circulation. Contrary to the septic injury model, here *Serratia* does not proliferate nor induce a systemic immune response, because it is controlled by phagocytic hemocytes (Nehme et al., 2007). Therefore, not only *Serratia* adopts different infective programs depending on the entry route, but it also seems clear that the passage from midgut to hemolymph attenuates its virulence.

In order to find key genes involved in the host defense after Db11 ingestion, a genome-wide RNAi *in vivo* screen has been performed (Cronin et al., 2009). 790 genes came out as determinant of fly's survival. They found among them genes involved in typical immune responses, as IMD and phagocytosis, but also others that regulates processes as vesicle trafficking, proteolysis, signaling, transport and transcription. Some of these hits were further tested using enterocyte- and hemocyte-specific drivers, as they are the major cell types implicated in the oral infection model. 98 genes conferring sensitivity were identified in hemocytes, 129 in enterocytes, with 79 genes in common. JAK-STAT pathway was found as a negative regulator of fly's survival and crucial determinant of midgut homeostasis (Cronin et al., 2009). Notably, this study underlines the importance of many genes that have no function in classical immune pathways. Thus, besides resistance mechanisms, the defense during Db11 oral infection relies largely on resilience processes to ensure an optimal host survival.

3. The concept of resilience in immunity

Immunity indicates the capacity of an organism to protect itself and avoid falling into a state of disease (Soares et al., 2014). In the study of the host's defenses, much attention has been placed on resistance mechanisms. These are defined as the ability of the immune system to detect, neutralize, destruct or expulse the pathogen. Therefore, those processes reduce the microbial load with the aim to eradicate it completely. AMPs secretion, phagocytosis and ROS production are typical examples of resistance mechanisms. However, the pathogens or the immune system itself cause damages to tissues: in order to ensure an optimal survival, the host must endure and repair these injuries. The totality of these homeostatic responses is known as resilience (Ferrandon, 2013). By reducing the impact of a given infection, resilience mechanisms represent the complementary face of resistance with respect to the organism's defenses. Ayres and Schneider used the fortress metaphor to describe the two aspects working together: if we consider our body as a castle, resistance would be the walls and the weapons to defend against invaders (the microbes). Meanwhile, citizens must maintain the fortress, produce and distribute food, repair damages to buildings, educate and train younger generations and decide when it is time to fight (Ayres and Schneider, 2012), representing the resilience. The latter is not a totally new concept, since it was previously known as tolerance or disease tolerance (Ayres and Schneider, 2012; Medzhitov et al., 2012; Soares et al., 2014). This notion was initially employed in plant biology, to identify species that remained healthy and continue to produce crop even when infected or preyed on by herbivores (Ayres and Schneider, 2012; Caldwell et al., 1956; Soares et al., 2014). Nevertheless, this term can be confusing since it is already used in other contexts, such as immunological tolerance for example (Ferrandon, 2013). Moreover, we can pinpoint another nuance of meaning between the two terms: while tolerance defines an important pathogen burden without important consequences for the host, resilience is more intended as active response to damages.

Yet, the word resilience is employed in many domains. The first definition was given in materials science, where resilience is the physical property of a material to absorb energy when it is deformed elastically and release that energy upon unloading. Resilience is also a common term in psychology, defined as an individual's ability to properly adapt to stress and adversity; resilience is one's ability to bounce back from and react to a traumatic experience. This conception outruns the simple definition of recovery and is seen more as the ability to maintain a stable equilibrium (Bonanno, 2004). Emmy Werner was one of the first scientists who used the term resilience back in the '70s in her study on cohort of children exposed to traumatic experiences in the Kauai island (Werner, 1997). In ecology, resilience means the capacity of an ecosystem to respond to a stochastic perturbation (fire, earthquake

or windstorm) or to an artificial disturbance (such as deforestation), by enduring damages and recovering rapidly. In the informatic field, it is the readiness to provide and maintain an acceptable level of service face to faults and challenge to normal operation; and we could still cite many other fields where this term is adopted.

In this current context, that is, that of an infection, resilience can be distinguished from resistance because it does not cause a decrease in the charge of the pathogen, although it is crucial for host's survival. Albeit less studied than the functioning of the immune system, some resilience mechanisms have been described in both mammals and *Drosophila*.

3.1 Examples of resilience mechanisms

Being linked to multiple homeostatic processes, resilience mechanisms can be very diversified. In mammals, systemic inflammatory response to an infection can damage tissues and organs. This condition, known as sepsis, is a major cause of hospital mortality (Ulloa and Tracey, 2005). In the most severe form, septic shock, overproduction of inflammatory mediators (notably the cytokine storm) provokes an exaggerated response which is characterized by a strong mortality (Takeuchi and Akira, 2010). In this background, it has been shown that in a mouse model of sepsis, anthracycline treatment confers substantial protection without altering the bacterial load and without host immunosuppression (Figueiredo et al., 2013). Here, the increase in tolerance to infection is rather due to the activation of DNA repair mechanisms and autophagy, controlling tissue damage especially in lungs.

The same group recently showed that doxycycline, a molecule frequently prescribed and not as toxic as anthracyclines, drastically improves the survival of mice in a sepsis model (Colaço et al., 2019). The authors also described that mice recovered faster from a sublethal challenge with the influenza virus. A strong inflammation and the associated cytokine storm is also involved in the second phase of the disease caused by the now famous SARS-CoV-2 (Oberfeld et al., 2020; Zhang et al., 2020). As some of the patients suffering from viral infections also undergo sepsis (Lin et al., 2018), this approach might be worth testing in order to develop new therapeutic strategies.

Interestingly, resilience in mice has been found also in the case of viral infection. A mutagenesis unbiased approach allowed the identification of Kir6.1, a constituent of a potassium channel conferring hyper susceptibility to mouse cytomegalovirus, LPS, polyI:C and CpG DNA when mutated (Croker et al., 2007). The increased susceptibility is not suppressed by mutations in genes as *MyD88*, *Tnf* or *Stat1*.

Conversely, the complete channel (composed by Kir6.1 and SUR2) enables the adaptation to the vasoconstriction provoked by the induced cytokines during antiviral response. Therefore, an ATP-dependent potassium channel expressed in the smooth muscles and in the coronary arterial endothelium helps in the homeostasis during infection, enhancing host survival independently from viral titer and immune mediators.

Examples also exist in lower organisms: in the nematode *C. elegans*, the activation of the Unfolded Protein Response (UPR) via XBP1 (X-box Binding Protein1) has a protective role when the worms are challenged with *Pseudomonas aeruginosa* (Richardson et al., 2010). The UPR has an important role in regulating protein synthesis and their structure in the case of ER stress. In this particular situation, activation of immune response upon infection in XBP1-deficient animals induces disrupted ER morphology which eventually leads to lethality.

Tolerance mechanisms also intervene during *Plasmodium* infection. Disease severity greatly varies among different mice strains, and more importantly genetic variations in resilience traits are not correlated to genetic variations in resistance, demonstrating that tolerance is independent from it and genetically encoded (Raberg et al., 2007). To give an example, in mice infected with *Plasmodium chabaudi chabaudi* the iron sequestering protein ferritin heavy chain (FtH) reduces tissue damage caused by induction of JNK and apoptosis (Gozzelino et al., 2012). Always without affecting pathogen loads, also heme oxygenase 1 (HO-1) has a protective effect in the case of malaria, by reducing free heme and subsequent apoptosis (Seixas et al., 2009).

In *Drosophila*, a transcriptomic study comparing the responses to ten different bacteria identified the transcription factor *CrebA* as a regulator of host tolerance face to infections (Troha et al., 2018). In the presence of a pathogen, Toll and IMD pathways induce the upregulation of *CrebA*, which in turn controls the increment of secretory pathways in the fat body. These are crucial to adapt the fat body to infection, preventing ER stress and improving host survival without variations in pathogen burdens (Troha et al., 2018). Combined with the fact that in unchallenged conditions loss of *CrebA* does not trigger ER stress and host mortality, these findings identify *CrebA* as a resilience factor which alleviates the stress produced in response to the infection. Moreover, this study is a very good example of how resistance and resilience work together. Similarly, a tolerance mechanism has been reported in the case of infection by the intracellular bacterium *Salmonella typhimurium* (Shinzawa et al., 2009). Here, the p38 MAPK enhances host survival by phagocytic encapsulation, that is sequestration of bacteria into hemocytes. Although the bacterial burdens are not changing, encapsulation by immune cells (hemocytes) decrease the bacterial virulence (Ferrandon, 2009; Shinzawa et al., 2009), thereby placing this mechanism at the interphase between resistance and resilience.

Therefore, albeit sometimes processes differ between fruit fly and mammals, *Drosophila* still represent a good model to investigate resilience. The growing interest in tolerance mechanism during the past twenty years has shred a light on this important aspect of host defense. Despite it is often seen as an oversimplified model especially for immunity, the fly has provided great knowledge to the field and proven to be more complex than expected. For instance, a single mutation in a protease involved in the melanization cascade triggers wide changes both in resistance and tolerance against diverse pathogens (Ayres and Schneider, 2008). These same authors identified in a genetic screen upon *Listeria monocytogenes* infection 18 genes that were previously unknown in *Drosophila* host defense (Ayres et al., 2008). The 18 genes were further divided in genes that when mutated alter microbial load and host survival (resistance) and those that alter survival without affecting bacterial burden (resilience). This type of unbiased approach is very useful to discover new resilience mechanisms which remain still much less studied than resistance ones. The key advantage they bring is the advancement in alternative therapeutic approaches aiming to directly improve the host survival and tolerance to the infection rather than acting on the pathogen itself, decreasing the risk of development of resistance to drugs and escaping strategies.

3.2 Resilience in the midgut

The *Drosophila* intestine is a good model for the study of resilience. As previously mentioned, this organ is constantly exposed to environmental stressors that enter the organism mainly through the digestive tract. Moreover, fly's midgut owns a great plasticity and renewal capacity and more than one resilience mechanism has been discovered in the intestine. For instance, the secretion of IRC during an oxidative burst can be seen as one of them. Indeed, this enzyme protects the enterocytes from the deleterious effect of ROS produced by DuOx during a bacterial challenge, and the absence of IRC makes the flies susceptible even to heat-killed bacteria (Ha et al., 2005b).

An excellent example of resilience is the compensatory proliferation of ISCs following high cell death caused by virulence factors or ROS-induced damage (Figure I6). To counterbalance the important loss of enterocytes, ISCs divide and differentiate rapidly to restore intestinal homeostasis, and a lack of this proliferation makes flies more sensitive to intestinal infections (Biteau et al., 2008; Buchon et al., 2009c, 2009b; Cronin et al., 2009; Jiang et al., 2009; Lee, 2009). JNK and Hippo pathways play a major role upon damage of midgut epithelium. They are activated in response to stimuli like *Pseudomonas entomophila* and *Erwinia carotovora carotovora* infection and after ingestion of *Pseudomonas*

aeruginosa or DSS (Buchon et al., 2009c; Jiang et al., 2009; Karpowicz et al., 2010; Shaw et al., 2010). Activation of these signaling lead in turn to secretion of various ligands such as unpaired cytokines

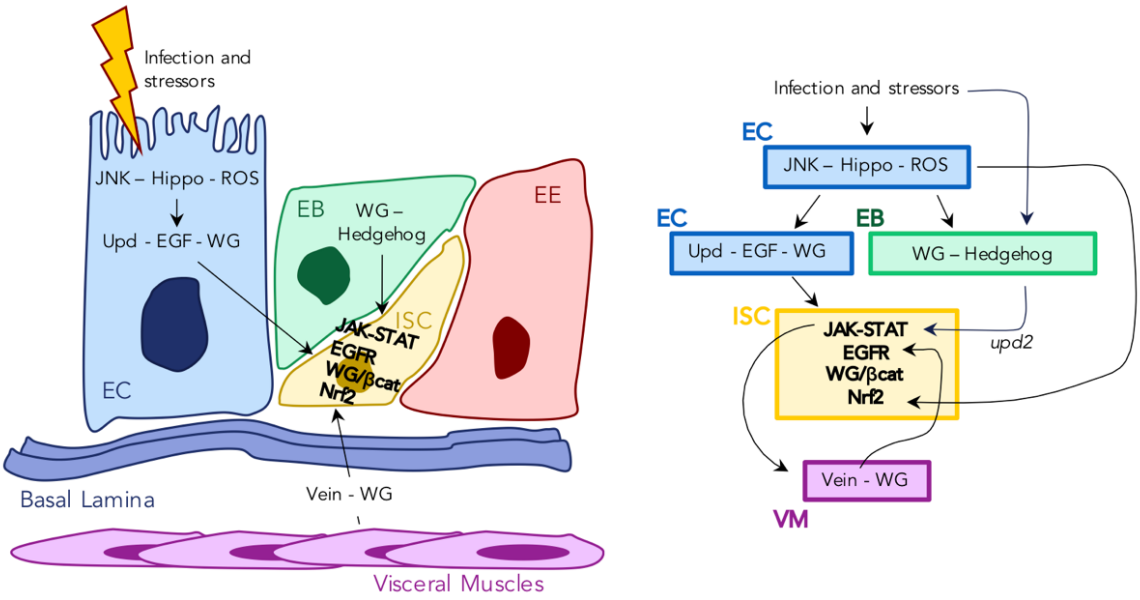


Figure I6. Simplified representation of ISCs compensatory proliferation. Infection and stressors attack enterocytes (EC), causing damage and cell death directly or through ROS production. Stressed ECs activate pathway such as JNK and Hippo and secrete ligands (unpaired, EGF, wingless) that support intestinal stem cells (ISCs) proliferation upon binding on the respective receptors. Wingless (WG) and Hedgehog signaling from enteroblasts (EBs) also contribute to the process and require JNK activation. WG is produced at basal levels from visceral muscles (VM) as well, which additionally secrete the EGFR ligand *Vein* after JAK-STAT activation. Moreover, ROS production regulates Nrf2, which is constitutively expressed by ISCs and is further mediates proliferation. This complex network of mitogenic pathways induces the division and differentiation of ISCs into EBs that give rise to new ECs to compensate the loss caused by infection.

(upd), epidermal growth factors (EGF) and wingless (WG), which upon binding on their respective receptors on ISCs induce the compensatory proliferation mechanism (Cordero et al., 2012; Jiang et al., 2009; Ren et al., 2010; Shaw et al., 2010). JAK-STAT activation consequent to upd binding on Domeless leads not only to ISCs proliferation (Buchon et al., 2009c) but also to the expression of the EGFR ligand *Vein* in visceral muscles (Xu et al., 2011; Zhou et al., 2013). Epithelial cell injury equally induces other two ligands of this pathway, Keren and Spitz (Jiang et al., 2011; Xu et al., 2011). All these contributes to ISCs proliferation via the EGFR/Ras/MAPK pathway (Buchon et al., 2010; Jiang et al., 2011; Xu et al., 2011). WG/βcatenin pathway is active at a basal level to maintain epithelial renewal as a result of *wingless* ligand production by visceral muscles (Lin and Xi, 2008). In addition, following stress or damage, wingless is also expressed by EBs, which signal to ISCs to activate Myc-dependent hyperproliferation (Cordero et al., 2012). WG signaling requires JNK activation, a property that is shared by the Hedgehog pathway (Tian et al., 2015). The latter is activated by stressors such as Dextran

Sulfate Sodium (DSS) as well and mediated by EBs. The final outcome is the secretion of upd2 cytokine that contribute to proliferation stimulating JAK-STAT (Tian et al., 2015). Damage induced by bacterial infection trigger the secretion of Bone Morphogenic Proteins homologs (*Decapentaplegic* and *Glass Bottom Boat*) by hemocytes recruited to the gut, which support ISCs compensatory proliferation (Ayyaz et al., 2015). High ROS production has also been shown to be an activator of two receptors triggering Ca^{2+} release form ER, TRPA1 and Ryanodine Receptor (RyR) (Xu et al., 2017). Ca^{2+} signaling is required for oxidative-stress induced Ras/MAPK pathway, that we have seen to be important for ISCs proliferation. Additionally, the antioxidant master regulator Nrf2 (Nuclear factor E2-related factor 2) is constitutively expressed in ISCs, and control their proliferation according to the intracellular redox state (Hochmuth et al., 2011). Midguts suffer a redox stress also when peroxisomes are compromised (Di Cara et al., 2018). Disrupting peroxisomal function in enterocytes triggers TOR-dependent autophagy, which increases cell death. Furthermore, the epithelial instability created alters host-microbe interaction, with increased bacterial titer in the midgut, modification of the microbiota and impaired immune response against intestinal infection with *Ecc15*. The organism tries to face these unbalances by an increase of ISCs divisions (Di Cara et al., 2018). In addition to all these molecular signals, ISCs differentiation is also activated in response to mechanical stress by the stretch-activated cation channel Piezo, which also works through Ca^{2+} signaling (He et al., 2018). In conclusion, the complexity and the large number of molecular pathways that induce this resilience mechanism is undoubtedly a sign of its importance for host intestinal homeostasis.

3.3 The cytoplasmic purge

Our laboratory recently described a novel resilience mechanism in *Drosophila* midgut (Lee et al., 2016). Oral infection with *Serratia marcescens* Db11 triggers a rapid response in enterocytes, which extrude their cytoplasm in the gut lumen from their apical part. This results in a very thin epithelium three hours after bacterial ingestion: the intestinal cells lose their thickness and typical dome-like structure. However, no cell death is observed during this process; the permeability of the intestine is not altered either and cells show no signs of stretching. This process, that has been called the cytoplasmic purge, is dependent from Db11 hemolysin since the *Serratia* hemolysin-deficient strain 21C4 induce this mechanism only marginally, conversely to a genetically modified *E. coli* able to express Db11 hemolysin. Nevertheless, 21C4 kills the flies more rapidly than Db11, demonstrating that the enterocytes purge is a protective mechanism for the host. The rationale behind this is that extrusion would allow to alleviate pathogen-related stress by getting rid of other bacterial virulence factors and damaged organelles. Likely, the cytoplasmic purge could also help to clear the bacteria faster from the organism. Together with the fact that the purge does not affect the bacterial load but limits the passage from the

intestine to the hemolymph, we can certainly classify the enterocytes purge as a new resilience mechanism.

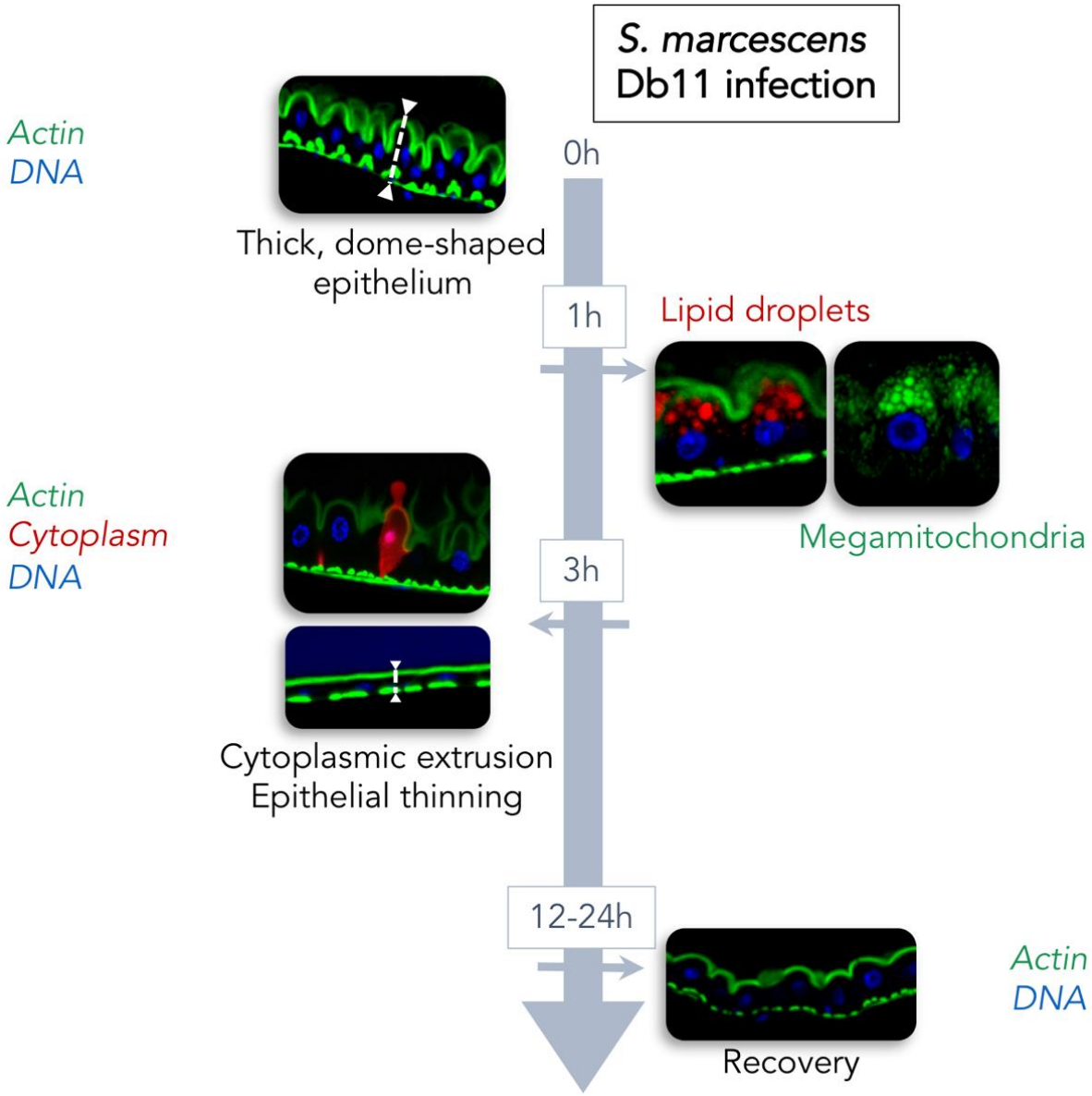


Figure I7. The enterocyte purge and recovery process after Db11 infection. From the top to bottom. At time 0, actin staining of enterocyte brush border reveals a thick epithelium with dome-like structure. Within the first hour after Db11 ingestion and hemolysin attack, lipid droplets and megamitochondria become evident in the apical part of the enterocytes. These events precede the enterocyte purge, that is the formation of an aperture at the top of the cell through which cytoplasm and damaged components are expelled into the intestinal lumen. This results in a thin and flat epithelium three hours post infection. Then, without cell death and compensatory proliferation, enterocytes start a process of progressive recovery that is completed in 12/24 hours, culminating in an epithelium morphologically very similar from an uninfected one.

In the very early phases of infection, the first events that takes place in enterocytes is the transient presence of lipid droplets, accompanied by formation of megamitochondria by fusion (Figure I7). Both these organelles are founded in the apical portion of the epithelial cell. Around one hour after bacterial ingestion, we observe consistent part of cytoplasm containing damaged organelles going out through a large aperture in the actin brush border. The thinning reaches its peak around three hours post ingestion, but cells do not remain flat for long: a recovery process quickly begins, and after 12/24 hours enterocyte completely recovered their morphology, making indistinguishable an epithelium that has recovered from an uninfected one. Previous team members have identified *CycJ* as an important factor for this recovery phase. *CycJ*-deficient strains do not recover the original enterocyte volume and architecture and possess an epithelium that remain thin. Accordingly, they are more sensitive to Dbl1 infection. We also identified four short peptides which are strongly induced in a *CycJ*-dependent manner. Strikingly, the ectopic expression of two of them, *what else (whe)* and *lacosta (lcs)*, rescues the *CycJ* mutant phenotype, even if knocking down one of them does not prevent midgut recovery.

Remarkably, although *whe* and *lcs* are found only in *D. melanogaster*, the enterocyte purge and recovery is not a mechanism specific of flies but it is conserved through evolution. Exposure to *Serratia* hemolysin or to the whole bacteria in honeybees, mice and human intestinal Caco2 cells induce a process reminiscent of what we observe in *Drosophila* (Lee et al., 2016).

AIM OF THE STUDY

More than ten years ago, Nadine Nehme with the rest of the team established an oral infection model with the bacterium *Serratia marcescens* (Nehme et al., 2007). In the years that followed, other researchers including Kwang-Zin Lee, Matthieu Lestradet, Richard Bou Aoun, Stephanie Limmer, Catherine Socha and Samuel Liegeois described a completely new resilience mechanism in the fly midgut, the cytoplasmic purge (Lee et al., 2016). Their work allowed not only the identification the central role of the pore-forming toxin hemolysin in the induction of cytoplasmic extrusion by enterocytes, but also the characterization of the *Cyclin J* and *whe* genes.

On my arrival in the laboratory, if on the one hand it was well established that the hemolysin of *Serratia* was a necessary and sufficient factor to trigger the whole process, the signal responsible for the induction of cytoplasmic extrusion in the fly was still unknown. Aiming to identify the host's factor required to start the cytoplasmic purge, we focused our attention on the events that precede the epithelial thinning. As detailed in the article, the first extrusion events take place around 1h after bacterial ingestion. Prior to that, we were able to image a transient lipid droplets accumulation and megamitochondria formation. As reported in the literature, these two processes maybe aimed to alleviate the oxidative stress within the cell (Bailey et al., 2015; Liu et al., 2015a; Nagaraj et al., 2012; Shutt et al., 2012). Moreover, in our RNA sequencing experiment, we found the induction of some antioxidant genes (*peroxiredoxin2540*, *jafrac1*, *jafrac2*, *GST-D10*, *GST-E8*) at 6h and 9h post-infection. Finally, searching in the genomic data available on FlyBase, we remarked that some metals (Cu, Zn, Cd) and paraquat, well-known oxidizing agents, also induce the *whe* genes (as well as other xenobiotics like ethanol and caffeine). These cues may point the ROS as signaling molecules in the very early phases of the cytoplasmic purge. Previous results also indicated that some of those xenobiotics, notably Cd, paraquat and caffeine seemed to alter the midgut morphology with prior megamitochondria formation. This may suggest that the enterocyte purge is a common cellular response to exposure to pore-forming toxins or xenobiotics. The case of caffeine and ethanol is particularly intriguing, as they are the xenobiotics to which man is (voluntarily) most exposed. Therefore, we also aimed to understand whether there is a crosstalk between the bacterial and the xenobiotic challenge, in what could be a "priming" response of enterocytes against repeated exposures to these stimuli.

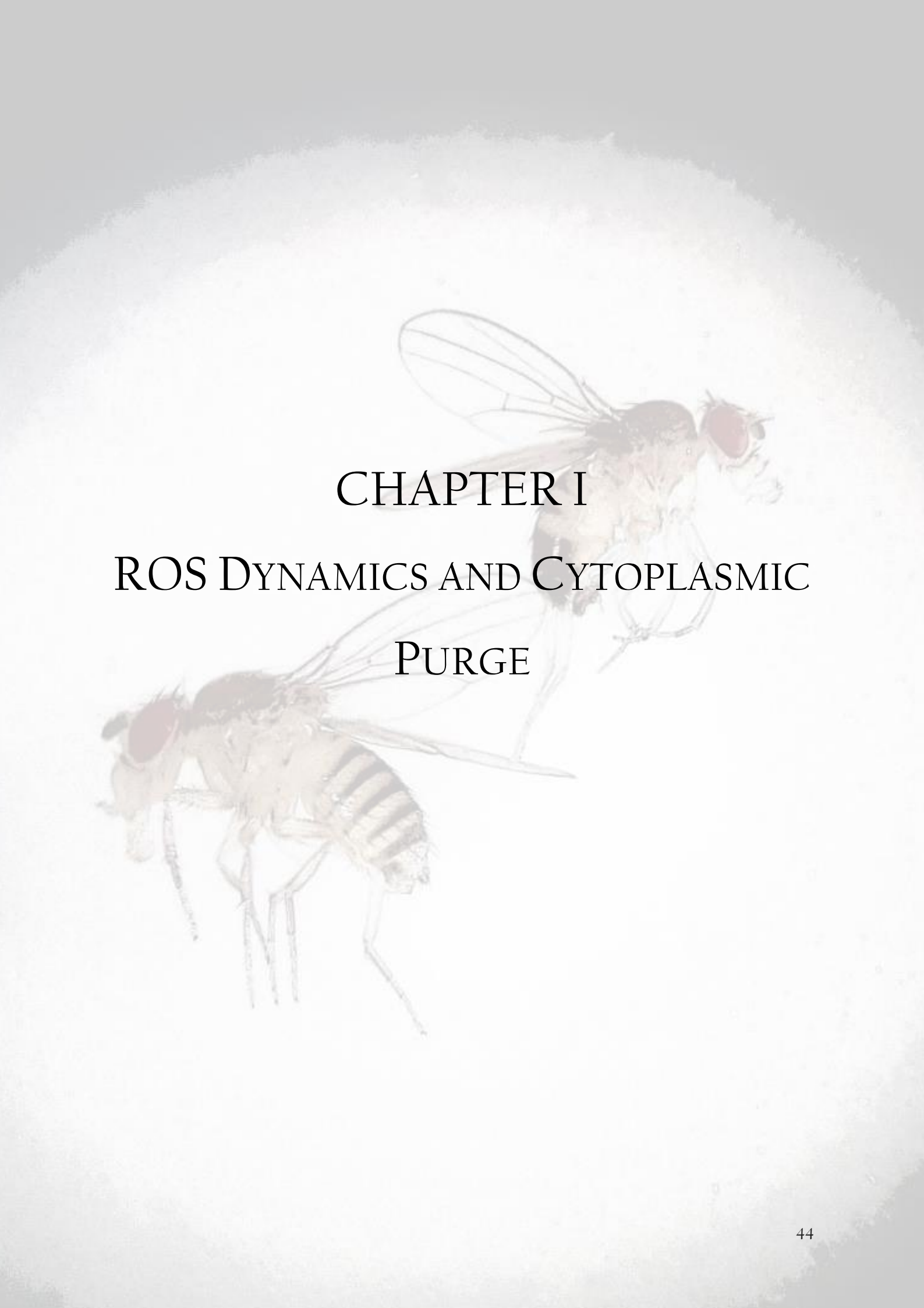
To that end, the purpose of my PhD can be summarized in three main questions:

Are the ROS involved in the induction of the cytoplasmic purge and what are their dynamics?
(Chapter 1)

Is the cytoplasmic purge a conserved response face to xenobiotic stimuli such as caffeine and ethanol?
(Chapter 2)

Is there a priming response or a cross-priming, which prepares the enterocytes to a second challenge and prevents continuous extrusions to occur?
(Chapter 2)

Finally, in a short annex at the end of the manuscript, I will summarize the main points of a side project on which I worked during my PhD.



CHAPTER I

ROS Dynamics and Cytoplasmic Purge

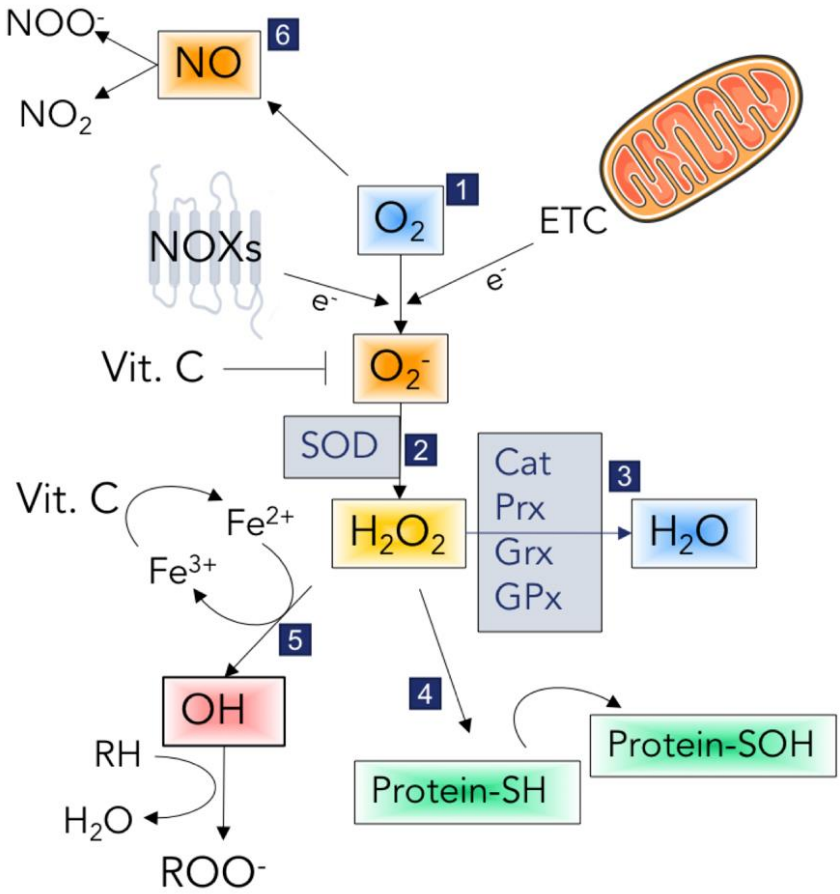


Figure 1.1. Basis of ROS dynamics. Most relevant species with relative scavenging molecules. Reactions are not balanced and do not include all the production and detoxification systems. **(1)** Molecular oxygen (O_2) can be oxidized to superoxide anion (O_2^-) by electrons (e^-) coming from NADPH oxidases (NOXs) or mitochondrial electron transport chain (ETC), and it can be scavenged by vitamin C (Vit. C). **(2)** Superoxide dismutase (SOD) catalyze the dismutation of O_2^- into hydrogen peroxide (H_2O_2). **(3)** H_2O_2 is scavenged by catalase, peroxiredoxins (Prx), glutaredoxin (Grx) or glutathione peroxidases (GPx). **(4)** Alternatively, H_2O_2 mediates the oxidation of thiol groups (SH) on the Cys of target proteins. **(5)** Through Fenton's reaction, it can lead to the formation of hydroxyl radical (·OH), that is involved in further oxidation of biological molecules such as lipids, proteins and nucleic acids, with the appearance of peroxyl radicals (ROO^-). **(6)** NOS enzyme catalyzes the formation of nitric oxide (NO) from O_2. NO can produce peroxynitrites (NOO^-) or undergo autoxidation into nitrite (NO_2), which also damages macromolecules.

1. Introduction

1.1 Oxidation

Oxygen (O_2) is a central molecule for the life of most organisms. It was the first known oxidizing agent, described in a reaction that forms an oxide, hence the name oxidation. Nowadays, redox reactions are identified as all those reactions where an exchange of electrons takes place, and the oxidation state of the atoms changes. As the term *Red-Ox* says, one chemical species undergoes reduction, that is the gain of electrons and a decrease in its oxidation state; simultaneously, another species goes through oxidation, which is defined as the loss of electrons and an increase of the oxidation state. These two processes cannot happen independently: the oxidizing atom or molecule reduces itself causing the oxidation of a reduced species and vice versa.

O_2 is a bi-radical in its ground state; however, it is a stable molecule, because the two unpaired electrons in its outer shell are in parallel spins. This uncommon configuration prevents the direct reaction with many molecules, but partial reduction by gain of a single electron brings to the formation of the radical superoxide $\cdot O_2^-$. A dismutation of superoxide anion forms hydrogen peroxide (H_2O_2); this molecule further reacts with metal ions such as Fe^{2+} producing the hydroxide radical ($\cdot OH$), in what is called Fenton's reaction. In addition, superoxide can also be protonated to form perhydroxyl radicals ($HOO\cdot$). All these species are known under the name of reactive oxygen species (ROS), thus indicating all those responsive compounds that derive from oxygen metabolism (Figure 1.1). All ROS are highly reactive and therefore short-lived, nevertheless there are substantial variations regarding their stability and reactivity (Pryor, 1986). The most short-lived is $\cdot OH$, with an estimated half-life of 10^{-9} s; it is so unstable that it reacts within 1 to 5 molecular diameters from its site of production. This means that $\cdot OH$ is the most dangerous ROS, because it is quite unselective on its targets, oxidizing all potential reactive species in its neighborhood; moreover, it cannot be detoxified by any specific enzyme. $\cdot O_2^-$ is also very reactive, arising principally from the electron transport chain (ETC) in mitochondria (Han et al., 2001; Murphy, 2009). The ETC consists in a series of protein complexes in the inner mitochondrial membrane that transfer electrons from NADH to the final acceptor oxygen, creating a transmembrane electrochemical gradient by pumping protons into the intermembrane space (Sousa et al., 2018); this gradient is then used for ATP production. $\cdot O_2^-$ formation occurs when single electrons leak from the chain reactions and are captured by O_2 (Figure 1.2). There are seven site described in the mitochondrion producing ROS, although the ones with greatest importance are complexes I and III (Brand, 2010; Murphy, 2009). Concerning the topology of this production, complex I seems to generate ROS toward

the mitochondrial matrix, whereas complex III towards both matrix and intermembrane space (Han et al., 2003) (Figure 1.2A). Another mechanism that has been considered as an artifact in the past but is

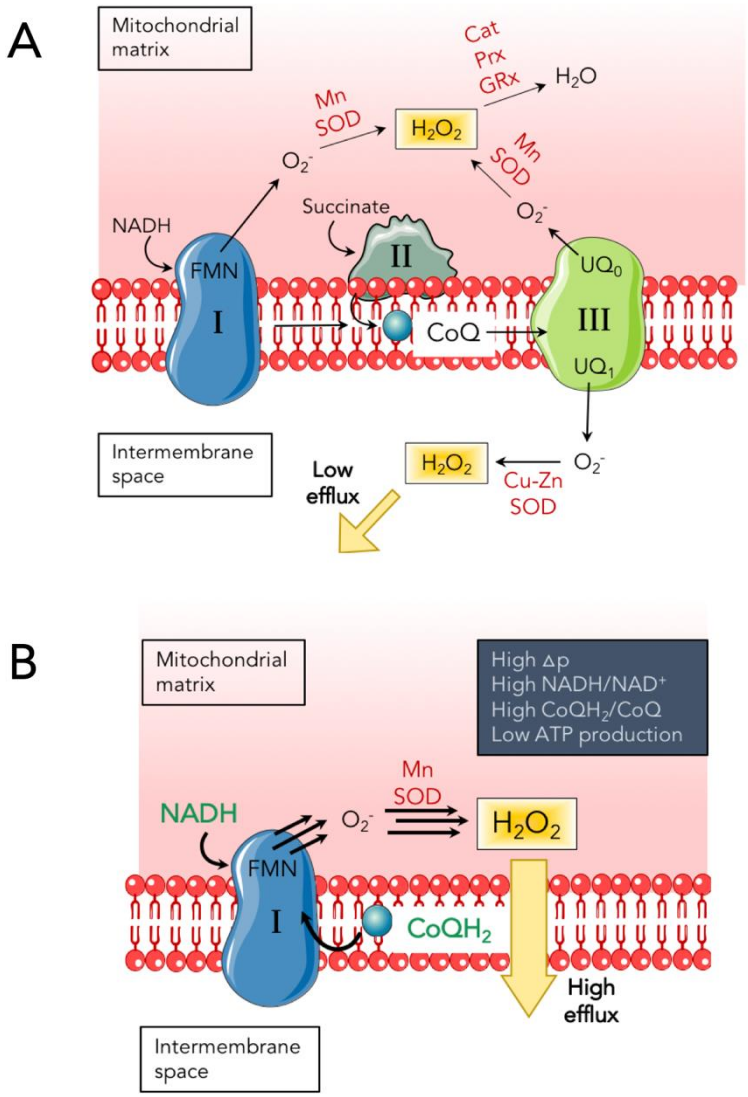


Figure 1.2. ROS production from mitochondria. Arrows indicate the electron flux. **(A)** During electron transport, complex I receives electrons from reduced NADH. Electrons can leak from the flavoprotein fraction (FMN) of complex I, and they react with molecular oxygen forming superoxide (O_2^-) towards the mitochondrial matrix. Complex III acts similarly through the ubisemiquinone site (UQ_0). Mn-SOD rapidly transforms it into H_2O_2, which is further scavenged by other mitochondrial antioxidant enzymes. In addition, O_2^- and H_2O_2 are also generated into the intramembrane space by another ubisemiquinone site (UQ_1). Here the dismutation is operated by Cu-Zn SOD. With mitochondria actively making ATP, the production of O_2^- and the ralative H_2O_2 efflux into cytoplasm is moderate. **(B)** When the NADH pool is highly reduced and there is low ATP demand, O_2^- production by complex I is intensified. Moreover, highly reduced coenzyme Q ($CoQH_2$) favors reverse electron transport (RET) further enhancing ROS accumulation into the matrix. In these conditions ROS production and H_2O_2 efflux are greater than in (A). Adapted from Murphy, 2009 and Han et al., 2003.

now acquiring more relevance is reverse electron transport (RET), well reviewed in Scialò et al., 2017. RET arises when an over-reduction of coenzyme Q from complex II is added to a high proton motive force (Δp). Then, the electron flux is inversed, transferring electrons from coenzyme Q to complex I, and generating important quantities of superoxide inside the mitochondria (Figure 1.2B). Understanding the source of ROS is not only important for academic matters, as ROS generated at different sites affect diversely how and which proteins are modified (Bleier et al., 2015), implying medical relevance.

H_2O_2 is a relatively stable and unreactive molecule; indeed, despite being considered a ROS, it is uncharged, as all its electrons are paired, resulting in a higher lifetime. Nevertheless, H_2O_2 remains a strong two-electron oxidizing agent, highly reactive with proteins containing metal centers or thiols. Moreover, it can pass across membranes by gradient (Antunes and Cadenas, 2000) or through some aquaporins (Bienert et al., 2007). These peculiar characteristics make it an optimal second messenger, a function that will be discussed later.

ROS do not only arise from mitochondria: 31 ROS-generating enzymes have been identified in the human genome (Go et al., 2015), meaning that their production is not only a collateral effect of ATP production but instead a regulated process in response to a stimulus. Among this group, the most relevant in this context are NADPH oxidases (NOX) (Lambeth, 2004; Panday et al., 2015), all located to the plasma membranes. In human, we can distinguish 5 "true" NOX enzymes and 2 Dual Oxidases (DuOx). The former have six transmembrane domains with an heme cluster that transfers electrons through the membrane into the extracellular space (or into the phagosomes) (Finegold et al., 1996). The latter own a peroxidase-like domain more than an additional seventh transmembrane domain, allowing the direct generation of H_2O_2. The product of NOX is $\cdot O_2^-$ instead. Besides cellular sources, ROS can also be induced by exogenous factors, such as UV light (Heck et al., 2003), ionizing radiation (Riley, 1994), pollutants (Gurgueira et al., 2002) and chemicals (Winterbourn, 2008).

Apart from mitochondria and NADPH oxidases, many other enzymes produce ROS as part of their function: cytochrome p450, xanthine oxidase, lipoxygenases, cytooxygenase are just a few examples. We also have to mention oxidoreductases, a large enzyme family catalyzing redox reaction generally using $NADP^+$ or NAD^+ as cofactors.

In addition to ROS, reactive nitrogen species (RNS) play an important role as oxidizing agents in biological systems as well (Figure 1.1). The radical species nitric oxide (NO·) is produced by a reaction between arginine and molecular oxygen catalyzed by Nitric Oxide Synthases (NOS) enzymes. In case of hypoxia, NOS activity is decreased, and NO· production is compensated by the nitrate-nitrite-NO pathway (Lundberg et al., 2008). This two complementary systems ensure the pivotal functions exerted by NO in vascular homeostasis, neurotransmission and also host defense (Moncada & Higgs, 1993).

Historically, ROS have been thought to be collateral and harmful products associated with many diseases (Carroll & Cross, 1987). The imbalance between the ROS quantity and the organism's capacity of detoxifying them create a condition known under the name of oxidative stress. In these conditions and given their high reactivity, ROS can cause damage to many biological molecules such as DNA (Dizdaroglu and Jaruga, 2012), lipids (Reed, 2011; Steinberg, 1997) and proteins (Butterfield et al., 1998; Stadtman and Berlett, 1998). This harmful effect towards macromolecules and mitochondria is the basis of a theory elaborated more than 60 years ago by Denham Harman, the free radical theory of aging (Harman, 1956). Since then, a great debate has been going on in the scientific community to establish this theory with certainty or disprove it, and the literature on the subject is very vast. On one side is undeniable that damaged mitochondria that produce a big amount of ROS impair cell function and viability provoking aging and disease (Balaban et al., 2005; Beckman & Ames, 2003; Muller et al., 2007). On the other, some authors claim that the mitochondrial free radical theory of aging is incomplete and need more proof (Sanz et al., 2006). Indeed, much evidences has been collected that do not support this theory: among the most relevant, we can cite studies in which knockdown of ETC proteins increase lifespan in *C. elegans* (Dillin et al., 2002; Lee et al., 2003b). Also in *Drosophila*, literature is controversial (Scialo et al., 2016 & 2020; Sun et al., 2002), making it difficult to draw a definitive conclusion. In addition, clinical trials based on antioxidant treatment have led to disappointing results, and no antioxidant-based drugs are in widespread use today, with the exception of ascorbic acid.

However, research in the last decades clearly indicates that response to ROS displays hormesis, since opposite effects are observed depending on their levels (Schieber and Chandel, 2014). Actually, if high levels of ROS generate oxidative stress that is linked to countless pathologies, it has become evident that ROS act both as immune molecules and as well as messengers that regulate many biological processes (Finkel, 2011). These roles will be discussed in detail in the next paragraphs.

1.2 Antioxidants

To conterbalance the potential destructive effect of ROS, cells have developed antioxidant systems that fine regulate ROS-dependent outcomes. The fine modulation of ROS levels to exercise beneficial physiological responses is termed ROS biology or ROS homeostasis. This is the antithesis of oxidative stress. Therefore, maintaining an equilibrium between oxidants and antioxidants is key for cell survival.

The term "reductant" refers to any species that gives electrons to an oxidizing agent in a redox reaction; more precisely, reductants are nucleophiles that do not form a bond with the oxidant. When these electrons are transferred to a radical species, the reducing molecule is often called a "scavenger".

In biology, the term "antioxidant" describes any molecule preventing oxidation, regardless of its mechanism of action. Thus, reductants are antioxidants, but not the other way around. For instance, compounds that block the activity of NADPH oxidases, such as apocynin and DPI, are antioxidants (i.e. prevent the formation of ROS), but not reductants.

1.2.1 Endogenous antioxidants

1.2.1.1 Superoxide dismutase

The discovery of superoxide dismutase (SOD) (McCord and Fridovich, 1969) opened the field of research in redox biology. The discovery of this enzyme that can break down reactive species suggested other roles of ROS outside their implication in pathologies. SOD catalyzes the dismutation of $\cdot O_2^-$ to H_2O_2 (Figure 1.1). It exists in multiple isoforms and it is present in different subcellular localizations (Huseynova et al., 2014; Zelko et al., 2002). CuZn-SOD is found in cytosol, Mn-SOD exclusively in mitochondria, and EC-SOD is secreted in extracellular spaces. They are also called SOD1, SOD2 and SOD3 respectively, and all the three are found in insects (Parker et al., 2004). Despite this spatial separation, there are interactions between the isoforms that affect ROS sensitivity (Blackney et al., 2014), and these enzymes support host's immunity during fungal infection (Gretscher et al., 2016).

1.2.1.2 Catalase

Another important enzyme is catalase, responsible for the transformation of H_2O_2 into water and O_2 (Figure 1.1). As in mammals, in *Drosophila* this enzyme localizes into peroxisomes (Jules et al., 1989), and its loss of activity appear to be critical for survival (Mackay and Bewley, 1989). As previously said, more recent studies identified a secreted catalase involved in immune defenses (Ha et al., 2005b). This protein, named Immune Regulated Catalase (IRC), presents sequence similarity to heme-peroxidases and owns putative signal peptides which made it secreted outside the enterocytes. Although it is not upregulated in oral infection model, IRC knock-down flies showed higher susceptibility due to increased ROS content in the midgut (Ha et al., 2005b).

1.2.1.3 Glutathione

Glutathione (GSH) is a tripeptide composed by glycin, cysteine and glutamate. Present in millimolar concentrations in the cell, it is a reductant and therefore counted as the major cellular antioxidant. GSH is mostly present in cytosol (90%), but it also localizes to mitochondria, endoplasmic reticulum and nucleus. Despite this compartimentalization, GSH is present in all cell compartments travelling through specific membrane transporters (Oestreicher and Morgan, 2019). It exists also in its oxidized form GSSG, better named glutathione disulfide; however, other possible GSH oxidized

species exist, although their biological relevance remains very ambiguous. GSH/GSSG ratio has been extensively used as a biochemical measure of the general oxidative state of the cell or tissue. Nevertheless, this kind of general biochemical quantification has recently been questioned as it does not take into account cell type, subcellular localization and rapid kinetics events which might be crucial in glutathione redox homeostasis.

GSH is synthetized *de novo* in cytosol in a two steps process requiring ATP. The first is the formation of a peptide bond between glutamate and cysteine by the enzyme glutamylcysteine synthetase; the second is catalyzed by GSH synthetase that form another peptide bond adding glycine to glutamylcysteine. Degradation of GSH is mediated by specific enzymes (Bachhawat and Kaur, 2017), the most well-known are glutamyl transpeptidases, which cleave the γ-glutamyl group and transfer it to peptides and amino acids or water. These enzymes are located into the plasma membrane with the catalytic site facing the extracellular side. This suggests their main role might be the recovery of the constituent amino acids from extracellular pool and implies that GSH has to be transported outside the cell to be degraded. There are also cytoplasmic enzymes called GSH-specific γ-glutamylcyclotransferases (GGCT) that exclusively degrade GSH through hydrolysis of GSH into Cys-Gly and 5-oxoproline.

Three major parameters determine the GSH homeostasis (Oestreicher and Morgan, 2019): i) the concentrations of free GSH and GSSG; ii) the molar ratio GSH:GSSG; iii) the glutathione redox potential (E_{GSH}). It has been shown that in the cytosol, which account for the vast majority of the GSH cellular content, the GSH pool is remarkably reduced (Gutscher et al., 2008). The development of YFP- (Østergaard et al., 2001) and GFP-based redox probes (Dooley et al., 2004) further revealed that the GSH:GSSG ratio in this compartment is estimated around 50.000:1, much higher than previously believed (Morgan et al., 2013). In this context, very small changes in GSSG concentration elicit important changes in cytosolic E_{GSH} and therefore in cellular responses. Accordingly, it is crucial to tightly regulate this ratio: on one side this is made by transporters that redistribute GSSG across different compartments, like *Ycf1* that mediates vacuolar import in yeast (Morgan et al., 2013). Otherwise, the GSSG is reduced back via the enzyme glutathione reductase (GR), or the proteins glutaredoxin (Grx) and thioredoxin (Trx) (Figure 1.3). The complexity of the system greatly complicates the understanding of GSH homeostasis. Indeed, the depletion of both *Ycf1* and GR does not result in an increase of oxidation state of the cytosol or the vacuole even in presence of oxidant stressors; conversely, overexpressing *Ycf1* in a GR^{Δ} mutant background strongly increase oxidation in the vacuole but not in cytosol. These findings revolutionize the perception of GSH homeostasis. The large increment of whole cell GSSG levels cannot be used as a universal measure for oxidative stress, as they are very dependent on transporters that sequestrate GSSG from cytosol to other organelles (Morgan, 2014); moreover, the reduction of the GSSG back to GSH relies on several pathways that compensate each other in the case of the lack of one of these (Figure 1.3).

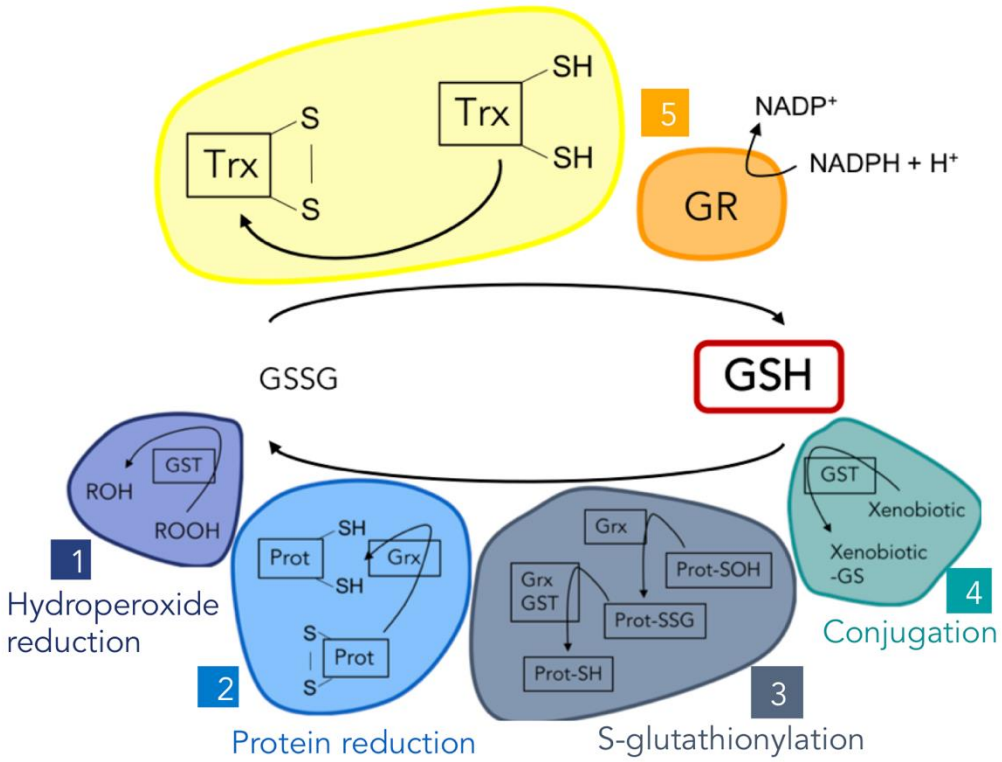


Figure 1.3. Glutathione (GSH) system. Reduced GSH exercise its antioxidant activity in many ways. **(1)** It can be used by Glutathione S-transferases (GSTs) to reduce organic hydroperoxides or **(2)** by glutaredoxin (Grx) to resolve disulfide bridges within proteins. **(3)** It is also employed by Grx and GSTs to protect damaged proteins with S-glutathionylation. **(4)** Lastly, it is used for xenobiotic detoxification in the conjugation reactions catalyzed by GSTs. **(5)** Oxidized GSH (GSSG) is reduced back by glutathione reductase (GR) using NADPH as electron donor, or alternatively by Trx. The latter is particularly important in insects, that lack the GR gene.

1.2.1.4 GSH peroxidases, Glutaredoxins and Glutathione-S-transferases

Despite its central role in redox homeostasis, GSH is not a good direct scavenger of ROS. Its reaction rates with H_2O_2 or $O_2^{\cdot-}$ in physiological contexts are very low, so it needs the help of enzymatic catalysis (Flohé, 2013). These are glutathione peroxidases (GPx), a group of related proteins that transfer electrons from GSH to H_2O_2. The reactive group in these proteins is a seleno-cysteine residue (Sec) in vertebrates; however, in insects Sec is absent, and the electron transfer is therefore entrusted to cysteine (Maiorino et al., 2007). In this case, the catalytic mechanism involves the formation of an internal disulfide and a subsequent interaction with Trx, making the activity of these enzymes identical to that of a thioredoxins peroxidase.

Glutaredoxin (Grx) constitutes another family of small enzymes that catalyze the glutathionylation/deglutathionylation of proteins and the reduction of disulfide bridges (Figure 1.3). Di-thiol Grx (Cys-X-X-Cys) are implicated in important redox biology processes such as cell death, proliferation and gene expression (Hanschmann et al., 2013), whereas monothiol Grx (Cys-X-X-Ser) seems more to be involved in iron metabolism (Berndt and Lillig, 2017).

Glutathione S-transferases (GSTs) are phase II detoxification enzymes that catalyze the conjugation of GSH to compounds that have electrophilic carbon, nitrogen or sulphur atoms (Figure 1.3). They are divided in three families based on their localization: cytoplasmic, mitochondrial or microsomal. Their main function is to detoxify xenobiotics or organic hydroperoxides (Hayes et al., 2005), thus being useful for the neutralization of chemotherapeutic drugs, insecticides, herbicides, carcinogenic agents and oxidative stress by-products. The conjugation of these xenobiotics with GSH generates less reactive products which are easier to eliminate. GSTs are a very large superfamily: we can distinguish seven different classes only among the cytosolic forms in mammals (alpha, mu, pi, zeta, theta, sigma and omega). In rodents and humans, cytosolic GSTs share more than 40% of sequence identity within a class, while between classes identity is less then 25% (Hayes et al., 2005). In other species, including *Drosophila*, other classes have been described (beta, delta, epsilon, lambda, phi, tau and the "U" class). In the fly, 11 genes are assigned to the delta (D) class, 14 genes to the epsilon (E) class, 4 genes to the omega, 2 to the zeta, 4 for the theta and one gene alone for the sigma class (Tu and Akgül, 2005). Although not yet fully clarified, this great diversity is due in part to tissue and developmental stage specificity. The most studied implication of GSTs is in the context of insecticide resistance: indeed, an elevated biochemical GSTs activity is associated with resistance to the main insecticides classes (Prapanthadara et al., 1996; Vontas et al., 2002). Nevertheless, in most cases the individual enzyme responsible have not been identified, and resistance have been inferred on the basis of increased expression levels of enzymes (Enayati et al., 2005). Outside of their detoxification role, GST (and GSH biosynthesis) are also implicated in development, being required for ecdysone biosynthesis (Enya et al., 2017).

1.2.1.5 Thioredoxin system

Thioredoxins (Trxs) are other key actors in ROS homeostasis. These small thiol-proteins are ubiquitous across organisms, and they play roles in multiple vital functions. For instance, they participate in deoxyribonucleotide synthesis (alternatively to Grx) (Zahedi Avval and Holmgren, 2009) and in protein folding (Berndt et al., 2008). Naturally, Trxs also have a role in ROS homeostasis, not only in the defense against oxidative stress, but also regulating other redox dependent processes such as transcription factors activity, growth and apoptosis (Arnér and Holmgren, 2000), as well as immunity and cancer (Lu and Holmgren, 2014). They can resolve disulfide bridges in proteins and reduce GSSG back to GSH acting as glutathione reductase (Figure 1.4). The latter ability is particularly

crucial for insects, in which GR is lacking (Bauer et al., 2002; Kanzok et al., 2001). Seven putative Trxs have been found in the fruit fly (Corona and Robinson, 2006), three of which have been well characterized. Trx1 or deadhead and TrxT are sex specific for the ovaries and the testis, respectively; Trx2 localizes in the cytoplasm of somatic tissues and appears to be the most important one for development, longevity and whole-body redox homeostasis (Tsuda et al., 2010). Mutation of this gene decreases lifespan, while overexpression provides increased resistance to oxidative stress (Svensson and Larsson, 2007). It is also actively involved in stress/immune pathways, e.g., as a negative regulator of ASK1/p38 pathway (Liu et al., 2000a).

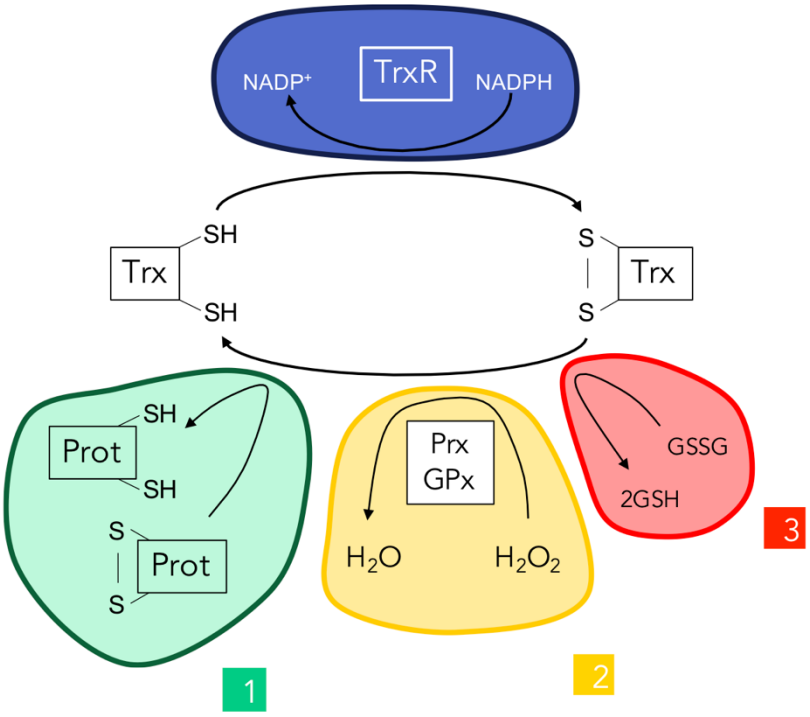


Figure 1.4. Thioredoxin (Trx) system in insects. (1) Thioredoxin directly reduces disulfide bridges in proteins or (2) gives electrons to peroxiredoxin (Prx) and glutathione peroxidase (GPx) that scavenge H_2O_2. (3) As seen in figure 1.3, Trx also reduce GSSG to 2GSH, interacting with the GSH system. (4) Thioredoxin reductase (TrxR) reduces oxidized thioredoxin using NADPH as a cofactor.

Trxs direct antioxidant function is wielded as electron donors of thioredoxins peroxidases (TPxs or simply peroxiredoxins, Prxs), which detoxifies hydrogen peroxide and organic peroxides (Figure 1.4). Prxs are very abundant in the cell, and they are divided in two subfamilies, 1-Cys or 2-Cys Prxs. The latter have a peroxidatic Cys and a resolving Cys, while 1-Cys Prxs lack the resolving one and therefore need a cofactor for the catalysis. In *Drosophila* genome five homologs have been initially identified (Radyuk et al., 2001), with a sixth gene discovered and characterized later (Corona and Robinson, 2006; Michalak et al., 2008), and their functions in longevity and immunity are well

described (Odnokoz et al., 2017; Radyuk et al., 2009, 2010). Prx can also operate in a Trx-independent manner (Radyuk et al., 2001). Trxs are then reduced from the disulfide to the di-thiol form by Trx reductase (TrxR), in a NADPH dependent reaction (Figure 1.4). TrxR are very relevant, since they can directly reduce H_2O_2 (Zhong and Holmgren, 2000), lipid peroxides (May et al., 2002), ascorbyl free radicals (May et al., 1998) and Grx2 (Johansson et al., 2004). The TrxR1 gene encode three splice variants, two cytoplasmic and one mitochondrial, all essential for viability (Missirlis et al., 2002). The other TrxR gene, TrxR2, has an unknown function. TrxR mutants activate the UPR and generate more ROS in HeLa cells (Kritsiligkou et al., 2018), a direct consequence of the accumulation of oxidized Trxs.

1.2.1.6 Nrf2 transcriptional response

Nuclear factor E2-related factor 2 (Nrf2) is at the center of a complex regulatory network. This transcription factor is not only pivotal for antioxidant response but play a role also in a myriad of other processes such as xenobiotics defense (Kwak et al., 2001; Xu et al., 2005), autophagy (Komatsu et al., 2010), metabolism (Hayes and Dinkova-Kostova, 2014), stem cell homeostasis (Hochmuth et al., 2011) and ER stress (Cullinan and Diehl, 2004). Under homeostatic conditions, Nrf2 is kept in the cytoplasm by its negative regulator Keap1 (Itoh et al., 2003). A Keap1 dimer acts as an adaptor for the ubiquitin ligase Cul3 E3, which is responsible for the continuous ubiquitinoylation of Nrf2 and therefore its degradation via the proteasome. During oxidative stress, Cys residues on Keap1 are oxidized and the protein inactivated, resulting in the nuclear translocation of Nrf2. Here, it dimerizes with the small Maf proteins and then binds to the antioxidant response elements (ARE) represented by the consensus sequence 5'-TGACXXXGC-3'. However, Keap1 is far to being the only regulator of Nrf2: on a transcriptional level, its expression is regulated by factors as NF-kB, PI3-AKT, Notch, Myc, AhR-ARNT, epigenetic modifiers; post-transcriptional regulation and protein stability are made by microRNAs, PKC, Brca1, p21, just to name a few (Tonelli et al., 2018).

Nrf2 was initially identified as a factor which binds to ARE for the transcription of phase II detoxification enzymes (Itoh et al., 1997). These are mainly transferases (GST included) which are in charge of detoxifying the toxic products derived from phase I detoxification enzymes. Later on, it was shown that Nrf2 is the mediator coupling the two phases (Miao et al., 2005). In addition of regulating GSTs expression, Nrf2 affects ROS homeostasis in multiple ways. It is known to be responsible of the upregulation of the glutamylcysteine synthetase, a key enzyme in GSH biosynthesis (Moinova and Mulcahy, 1999; Wild et al., 1999), thereby regulating GSH levels. Moreover, it also activates the gene responsible for Cys import into the cell (Sasaki et al., 2002). Regulating the activity of GR gene (Harvey et al., 2009; Thimmulappa et al., 2002), Nrf2 also affects the GSH oxidative state. In addition, also Trx and TrxR promoters contain AREs (Hawkes et al., 2014), and therefore are controlled by Nrf2 (Sakurai et al., 2005; Wakabayashi et al., 2004). Again, many ROS-detoxifying enzymes such as GSTs and GPxs are upregulated (Chanas et al., 2002; Thimmulappa et al., 2002). NADPH is a major electron donor in

reducing reactions and a cofactor for many metabolic enzymes and antioxidant systems; Nrf2 boosts its production by upregulating NADPH producing enzymes (Lee et al., 2003a; Mitsuishi et al., 2012; Thimmulappa et al., 2002; Wu et al., 2011). Even iron metabolism is regulated by Nrf2, via the induction of heme oxygenase and ferritin genes (Alam et al., 1999; Wu et al., 2011).

It is not surprising that this protein is highly conserved across the evolution, given its central importance in so many processes. As we have just described it is called Nrf2 in mammals and zebrafish, but in other organisms the homologous gene takes other names: *Yap1* in yeast, *skinhead-1* in *C. elegans* and *Cap'n'collar (Cnc)* in *Drosophila.* Here, it was named after the typical expression pattern observed during development, in the mandibular segment and hypopharyngeal region (Mohler et al., 1991). Then, it was identified that this gene encodes for three splicing variants (McGinnis et al., 1998), and that only the longest isoform *CncC* owns the domain to bind Keap1 and then represents the true Nrf2 homolog, being involved in anti-stress responses (Sykiotis and Bohmann, 2008). *CncC* target genes are the same as in mammals: phase I and II detoxification enzymes and antioxidants proteins, thereby mediating oxidative stress resistance and longevity.

1.2.2 Other endogenous and exogenous antioxidants

In addition to the numerous antioxidant defenses present in the cell, some dietary micronutrients can also have relevant effects on redox equilibrium. Vitamins, precisely A, C and E, are among them.

1.2.2.1 Vitamins

Vitamin C (Vit.C) or ascorbic acid is a hydro soluble molecule discovered back in 1920s by Albert von Szent Györgyi, who found that it prevents and cures scurvy. Scurvy is a serious pathological condition created by a long-term shortage of fruit and vegetables: vitamin "C" was then named ascorbic acid to mean "anti-scurvy". Vitamin C became over the century one of the most used drugs by humans. Ascorbic acid is an essential molecule for life for animals and plants; nevertheless, some species including humans have lost the ability to synthesize it. Indeed, human cell cannot catalyze the last step of the biosynthesis, the transformation of L-gulono-G-lactone into ascorbic acid. This because the gene coding for the enzyme responsible for this reaction, the gulonolactone oxidase (GULO), accumulated several mutations across the evolution and it turned into a pseudogene (Nishikimi and Yagi, 1991). Regardless, the ability to synthetize ascorbate appears only with amphibians (Chatterjee, 1973), and it is unclear whether insects need this molecule (Kramer & Seib, 1982): vitamin C is found in some tissue or whole-body lysates, but several species can be reared on synthetic media without it. As the other insects, *Drosophila* lacks the GULO enzyme, but interestingly it seems to possess an alternative pathway to synthetize Vit. C, mainly in females (Henriques et al., 2019).

The essential nature of this molecule relies first in the fact that it is an important co-substrate for many enzymes and a regulator of important transcription factors such as HIF1α (Knowles et al., 2003). Second, after the discovery of ROS, vitamin C was soon identified as an antioxidant molecule. Ascorbate can directly scavenge ROS such as H_2O_2, $\cdot O_2^-$ and ROO· (Buettner, 1993; Foyer and Noctor, 2011), but it can also be an indirect pro-oxidant in a dose-dependent manner (Figure 1.1). In fact, Vit. C at high concentration also reduces metallic ions like Fe^{3+} and Cu^{3+}, promoting the Fenton reaction that generates ·OH (Putchala et al., 2013).

Vitamin E (tocopherol or tocotrienol) antioxidant activity is very similar to ascorbic acid's one, with the only difference to be liposoluble instead, therefore localizing mainly to the membranes. The two vitamins can interact, in order to regenerate reduced vitamin E. As the latter, also vitamin A (retinol or βcarotene) is liposoluble, mainly protecting lipids, and they both have to be acquired through the diet.

1.2.2.2 Dithiothreitol

Dithiothreitol (DTT) is a small reductant molecule currently used in molecular biology and biochemistry. Its properties are due to the thiol groups at his extremities; once both thiol groups are oxidized, it forms a six-member ring with an internal disulfide bond. As a potent reducing agent, it has been used for many years to resolve intramolecular and intermolecular disulfide bridges in proteins. However, its redox potential is very dependent on pH (Lukesh et al., 2012). DTT also activates ER-stress, a condition that is closely related to redox equilibrium (Xiang et al., 2016).

1.2.2.3 N-acetylcysteine

N-acetylcysteine (NAC) is maybe the most popular antioxidant compound concerning clinical trials, as well as *in vitro* and *in vivo* studies. Composed by the amino acid cysteine with an acetyl group attached to the nitrogen atom, it has a very low molecular weight (163,1951 g/mol). The number of studies and reports in scientific literature using or concerning NAC is immense. It has been used since the end of the '60s as a mucolytic agent (Hurst et al., 1967), and a few years later it was started to use also as for the treatment of paracetamol poisoning (Prescott, 1983), a method that is still applied today (Green et al., 2013). NAC was also widely employed in cancer research (Elamin et al., 2014a; Gal et al., 2015; Piskounova et al., 2015; Schafer et al., 2009), in the study of some anti-tumor drugs (Wang et al., 2014) and in the context of psychiatric and neurodegenerative disorders (Minarini et al., 2017; Tardiolo et al., 2018). Both *in vitro* and *in vivo* studies demonstrated the antioxidant effect of NAC (Dhanda et al., 2013; Dodd et al., 2008; Kondo et al., 1997; Ortiz et al., 2016; Sueishi et al., 2014). Similar to vitamin C, NAC has been shown to be a potential oxidant in certain cases (Sagristá et al., 2002). Notwithstanding this broad use, the ways in which NAC counteracts ROS is not straightforward. Its antioxidant activity

can be related to two different mechanisms: i) a direct effect toward reactive species or direct resolution on proteins disulfides; ii) an indirect one, since NAC provides Cys, which is a component and the limiting-step in GSH biosynthesis, therefore acting as a GSH metabolic precursor.

The NAC mucolytic power can be attributed to its direct effect on molecules: breaking the disulfide bonds in cross-linked mucus glycoproteins results in a reduced viscosity (Aldini et al., 2018). This property has been extensively exploited for the therapy of pathologic mucus and elimination of bacterial biofilms (Dinicola et al., 2014). NAC pre-treatment on *Helicobacter pylori* colonized patients inhibits biofilm formation or destructs developed ones, which greatly improves the bacteria eradication after antibiotic administration (Cammarota et al., 2010). The diminished mucus thickness is not due to polysaccharide degradation, but rather to a decreased production (Olofsson et al., 2003). This effect is not *Helicobacter pylori*-specific; NAC has been shown to negatively affect the growth and biofilm formation of many bacteria, including *Escherichia coli*, *Enterococcus faecalis*, *Stenotrophomonas maltophilia*, *Burkholderia cepacia*, *Streptococcus pyogenes*, *Staphylococcus epidermis*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and *Lactobacillus salivaris* (Aslam et al., 2007; El-Feky et al., 2009; Eroshenko et al., 2017; Marchese et al., 2003; Moon et al., 2016; Olofsson et al., 2003; Pollini et al., 2018; Quah et al., 2012). Like vitamin C, NAC has also been used in combination with drugs for therapeutical approaches against *Mycobacterium tuberculosis* and *Staphylococcus aureus* (Khameneh et al., 2016).

Although it is theoretically possible that NAC detoxifies ROS directly through its thiol groups, it is very unlikely that this process is relevant in physiological conditions. Indeed, the rate constants for reactions with H_2O_2, $\cdot O_2^-$ and NO· are too low (or even null) to be competitive in comparison to other ROS scavenging systems (Benrahmoune et al., 2000; Samuni et al., 2013). NAC scavenges ·OH, $\cdot NO_2$ or $CO\cdot^-_3$, but this is likely caused by the very high reactivity of these ROS. NAC antioxidant activity could play a more important role in the reduction of the disulfide bridges and sulfenic acid derivatives in proteins: compared to other thiol proteins such as Trx and Grx, which are more efficient but also much larger, NAC is a very small molecule that can act in sterically less accessible spaces.

The fact that NAC constitutes a GSH metabolic precursor is supported by the observation of incorporated radioactive-labelled Cys into GSH (Ono et al., 2017). This can be important in conditions of critical GSH depletion like paracetamol overdose (Green et al., 2013), but may not be relevant in normal conditions. Actually, some authors described that NAC fails to replenish GSH store, albeit it continues to provide oxidant protection (Patriarca et al., 2005); others proved wrong that NAC is used for GSH biosynthesis *in vivo* (Zhou et al., 2015). Therefore, there is still no global agreement on how NAC performs its antioxidant and protective functions, as highlighted before by some authors (Parasassi et al., 2010). An interesting insight involving NAC catabolism has been proposed recently (Ezeriņa et al., 2018). In this paper authors demonstrated that NAC catabolites generate hydropersulfides (RSSH) in mitochondria, and these mimic NAC protective effect against oxidative stress. These hydropersulfides scavenge H_2O_2 directly better than thiols (RSH) even at low

concentrations. Moreover, protein hydropersulfidation may protect protein thiols against oxidation. This study advocates another mechanism for NAC-induced oxidative protection, suggesting a careful interpretation of NAC treatments outcomes and a refinement of therapeutic strategies.

1.3 Physiological relevance of ROS network

Evolution is not just a theory, but an evidence if we look at living organisms: from plants to humans, we have developed adaptations to the environment surrounding us. These macroscopic changes are due to the accumulation of small adjustments at the cellular scale. Just as the external world is not static, neither is the extracellular environment: cells are exposed to countless stimuli and immense combination of cases. To integrate all these inputs, cells must shape adequate responses: hence, the necessity to communicate and transmit messages correctly. The first trigger is a molecule that binds to specific receptors on the cellular or the organelle surface. This generates the rapid intracellular increase of a second messenger, which regulates multiple effectors, achieving amplification, distribution and diversification of the primary signal. Moreover, second messenger levels can be affected simultaneously by more sources, finely adjusting an adequate and punctual response of the cell. Second messengers harbor the following characteristics: they are enzymatically produced or delivered in a regulated manner into the cytosol; they are degraded by proteins or their concentration is downregulated by channels, pumps, diffusion or consumed during the reactions; their quantity vary within a short delay, generating gradients; their effect is specific. Some examples of molecules acting in this way can be cAMP, DAG or ions such as Ca^{2+}. Yet, it is evident that ROS can embrace all these characteristics. Indeed, nowadays ROS and antioxidants are no longer seen as two forces perpetually in battle fighting for supremacy, but instead as parts that collaborate to modulate important signaling processes (Holmström and Finkel, 2014).

1.3.1 Reactivity of thiol groups

The ability of ROS to act as signaling molecules lies in the ability to react with the side chains of the cysteines present in proteins (Figure 1.5A). Oxidation of thiol groups (SH) causes allosteric changes that activate, deactivate or influence the links of the target proteins (Figure 1.5B). Despite reactivity of free cysteine thiol group is not so high, it can greatly raise depending on the amino acid environment (Finkel, 2011). These reactive thiols are then readily oxidized to the sulfenic form (SOH), which is quite unstable and can encounter two destinies: be reduced back by Trx and GSH-pathways or be subjected to further oxidation into sulfinic form (SO_2H). Resolution of this high oxidation state requires the specialized enzyme sulphiredoxin, which is however capable of acting only on 2-Cys Prxs (Jeong et al., 2012). Under greater oxidative stress conditions, the sulfonic form is produced (SO_3H).

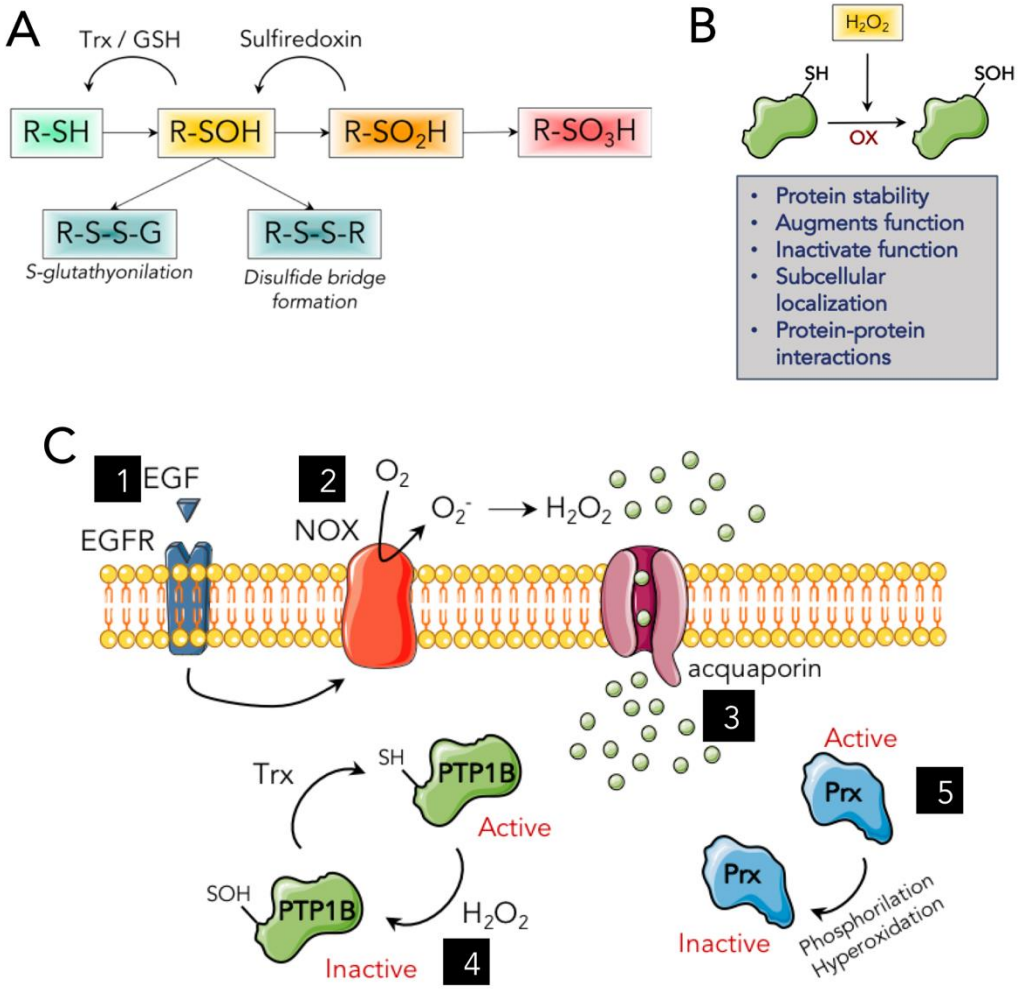


Figure 1.5. Thiol switches and protein regulation. (A) From the left to the right: thiols groups (SH) form sulfenic acid (SOH) upon non-radical oxidation. This reaction can be reversed by the action of Trx or GSH. Otherwise, SOH group is protected by S-glutathyonilation or it forms an intra- or inter-molecular disulfide bridge with another thiol. Alternatively, it undergoes further oxidation into sulfinic acid (SO_2H). Sulfiredoxin can reverse this reaction in some proteins. Under high oxidative conditions, sulfonic acid (SO_3H) is formed. SO_2H and SO_3H may cause permanent damage to proteins.

(B) Synthetic representation of the different ways in which the oxidation of thiols groups in proteins can affect their activities.

(C) Example of redox mediated protein regulation during epidermal growth factor (EGF) signaling. **(1)** Upon binding of EGF on its receptor (EGFR), NOX activity is stimulated **(2)**: the superoxide formed extracellularly is transformed into H_2O_2 by cytoplasmic SOD. Accumulated H_2O_2 enters the cell mainly through aquaporin **(3)** and inactivates the protein tyrosine phosphatase PTP1B by oxidizing its exposed thiols **(4)**, allowing the downstream signal to take place. The local Prx pool, that normally scavenge H_2O_2 with high reaction rates, is inactivated by phosphorylation or hyperoxidation **(5)**, making H_2O_2's reaction competitive on PTP1B. Adapted from Finkel, 2011.

This provides a first degree of specificity: under mild concentration of ROS (principally H_2O_2) oxidation of thiolate modifies protein properties in reversible reactions via antioxidants systems, whereas sulfenic and sulfonic modification can be irreversible and generate permanent protein damage and oxidative stress (Schieber and Chandel, 2014).

1.3.2 Specificity of redox reactions

Proteomics approaches identified hundreds of possible Cys targets within proteins. How is then possible to obtain any specificity in the messages to be sent? As in the case of GSH:GSSG ratio, a solution is achieved through compartmentalization. This is a key factor which determines whether the outcome will be oxidative stress or signaling. To allow ROS signaling to take place, it is logic to think that H_2O_2 targets localize to the proximity of the site of production. Indeed, proteins activated by NADPH oxidases are also located in the plasma membrane, e.g., protein Tyr phosphatases. This particular case was important to open the field of investigation on redox biology (Figure 1.5C). Until then, the increase in H_2O_2 that occurred after epidermal growth factor (EGF) binding to the receptor was not fully explained. It has been described that after the binding, sequential activation of PI3K and then Rac1 stimulated the activity of NOX (Holmström and Finkel, 2014). Next, H_2O_2 mediated oxidation of Cys residues in the protein Tyr phosphatase PTP1B was required for the inactivation of this protein (Bae et al., 1997). Since PTP1B dephosphorylates the EGF receptor, its inactivation by H_2O_2 grants the downstream growth signaling. Oxidized PTP1B is then reduced back by Trx, ensuring the reversibility of the signal (Lee et al., 1998; Salmeen et al., 2003). This mechanism is not exclusive of PTP1B, but is also true for other phosphatases (Denu and Tanner, 1998). Regularly, Prxs rapidly scavenge H_2O_2 before it oxidizes less reactive proteins, making the reactions towards phosphatases uncompetitive. This is prevented by a local inactivation of a Prx pool close to the membrane by phosphorylation (Woo et al., 2010), allowing H_2O_2 PTP1B inhibition. Prx phosphorylation is not the only way to inhibit their function: based on the "floodgate" model (Figure 1.6A), low fluxes of H_2O_2 are scavenged by Prxs, but high quantities trigger their inactivation (Wood et al., 2003). In this way, less reactive substrates undergo oxidation even in the presence of powerful scavengers, enabling the right signaling protein to be targeted.

Diffusion distance is also an important parameter to take into account. For this reason, H_2O_2 is the ROS with the greatest relevance in signaling: being relatively stable, it can diffuse for many cell diameters, always depending on the concentration of the related antioxidant systems. Peroxiredoxins and peroxidases are very abundant in the cell and their reaction constant is very high. However, the signal also propagates far from the production site, since the oxidized thiols on peroxiredoxins and peroxidases can in turn oxidize other target proteins, often involved in transcriptional or post-translational responses (Zeida et al., 2019). This oxidoreduction sequence is known as redox relay (Figure 1.6B).

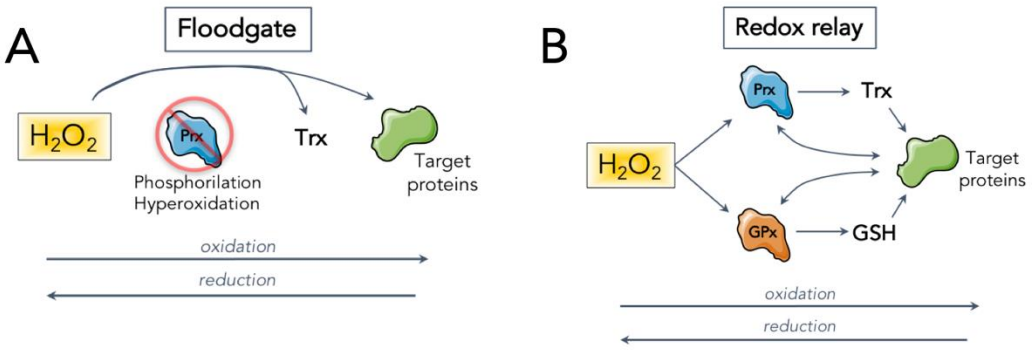


Figure 1.6. Redox signaling. (A) Prx are very abundant and rapidly react with H_2O_2. In the floodgate model, the inactivation of Prx by hyperoxidation or other post-translational modifications allow less competitive substrates to be directly oxidized by H_2O_2. **(B)** The redox relay model describes sequential oxidation from one protein to another. Oxidized peroxidases (Prx or GPx) can oxidize thiol groups on secondary targets directly or via their typical partners Trx and GSH. If the secondary targets are proteins involved in signaling pathways, their redox state constitutes an on/off signal.

Furthermore, although hydrogen peroxide can diffuse through membranes, its entry seems preferably mediated by specific aquaporins, as in the case of the growth factors NOX signaling (Miller et al., 2010). This represents another mechanism through which signaling can be regulated and oriented towards specific targets. H_2O_2 can be directly produced from many enzymes (Go et al., 2015), but many others, precisely NOXs, produce mainly $\cdot O_2^-$. Nevertheless, $\cdot O_2^-$ is a less relevant second messenger as it is rapidly dismutated into H_2O_2; this is a consequence of the high affinity of SODs for this substrate as well as the fact that the SOD concentration in cytosol largely exceeds the concentration of $\cdot O_2^-$ (Forman and Fridovich, 1973), rapidly generating H_2O_2. Therefore, it is likely that superoxide itself act as a signaling molecule only in a very short range from its site of production to avoid dismutation. However it is possible that $\cdot O_2^-$ becomes a more relevant signal when the cell state shifts to a more oxidizing profile, where SOD could be inactivated (Yim et al., 1990). Once more, this underlines the importance of the microenvironment in which ROS are produced (Hancock and Whiteman, 2018). Even more extreme is the case of ·OH: as previously said it is highly unstable, therefore not specific, and the cell does not have *ad hoc* detoxification systems. It is therefore more evocative of oxidative stress than signaling. Although, in the context of a strong oxidative state, its action is possible, assuming a close proximity between the targets and the production site. In fact, there are studies that show the implication of ·OH in cell differentiation in plants (Smirnova et al., 2014) and human cells (Nagy et al., 1993). It is also known that mitochondria are not static and isolated organelles, but instead they go

through fusion, fission and they form complex networks. They also actively move towards their target (Al-Mehdi et al., 2012), thereby modifying local redox equilibrium.

In the light of this complexity, it becomes evident why antioxidants enzymes exist in multiple variants specific for each compartment. In addition, each organelle displays peculiar ROS dynamics. In ER oxidized GSH (GSSG) is more abundant than in cytosol (Birk et al., 2013), and the GSH pool is regulated differently in mitochondria (Kojer et al., 2012). It has been observed that the mitochondrial GSH pool is more sensitive to H_2O_2 oxidation than the cytosolic GSH pool (Calabrese et al., 2019). In some cases, it is clear how there is a need for a specific ROS species coming from a certain compartment, as in the case of mitochondrial H_2O_2 regulation of OxyR (Brunelle et al., 2005).

Taken together, all these aspects highlight how the type of ROS, its source, the cellular environment and specific antioxidants and redox couples differentially shape ROS signaling, making any generalization meaningless either at the level of single cells, tissues, organs or whole organisms.

1.4 ROS balances and crosstalk

Redox modifications and dynamics are commonly represented as a balance, on which on one side we put the pro-oxidant species and on the other the antioxidant defenses. Oxidative stress is the condition that occurs when pro-oxidant levels increase and/or antioxidant levels decrease, and is associated with countless pathological conditions, such as neurodegeneration (Butterfield et al., 2006; Chinta and Andersen, 2008), cancer (Giles, 2006), cardiovascular diseases (Misra et al., 2009) and aberrant cell death (Ott et al., 2007). Research over the past two decades is completely revolutionizing this concept. Redox changes are now established as physiological, rapid, reversible and specific signals that regulate the activity of precise proteins to obtain adequate cellular responses. We have seen in the previous paragraphs how ROS homeostasis is localized and diverse in different organelles or in given cellular regions at a precise time point. Moreover, the reactions are not global but specific, given that in physiological conditions certain species are reactive only with few substrates and the vast majority of reactions are catalyzed by enzymes which are, by definition, specific. This new view of a "compartmentalized specific redox signaling" (Hanschmann et al., 2013) completely denies the conventional belief that all cellular redox couples respond in the same way to the same stimulus, and confute that the total balance between oxidants and antioxidants determines if the outcome is oxidative stress or redox biology (Jones, 2006). It is instead more plausible that oxidative stress derives from a disruption of specific redox signaling pathways. Therefore, this disequilibrium can be specific for a given cell or cellular compartment, representing natural and localized fluctuations rather than a general balance (Figure 1.7). Nevertheless, it is true that ROS display hormesis in multiple processes (Schieber and Chandel, 2014): at certain homeostatic doses they are beneficial and essential, but at the

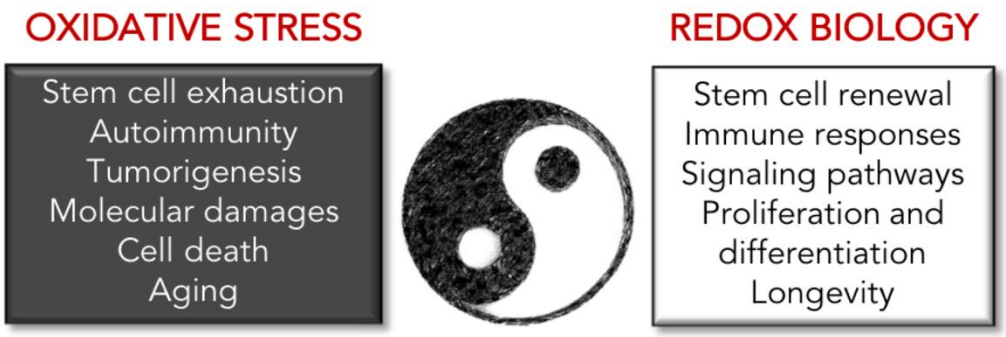


Figure 1.7. New concept of redox homeostasis. Although often represented as a balance opposing oxidants and antioxidants, the concept of redox homeostasis has been evolving in these last years. Excessive ROS accumulation is harmful (oxidative stress), but the same molecules are indispensable for multiple physiological processes (redox biology). These two aspects are part of the same universe composed by specific interactions and backup systems regulated in time and space and continuously flowing.

two extremes they impair several vital functions. Indeed, too low levels lead to immunosuppression and incapacity of proliferation and tissue renewal, resulting in hypersensitivity to infection, stress and ageing; uncontrolled high levels induce autoimmunity, tumorigenesis, cytotoxicity and stem cell exhaustion.

Much evidence indicates that the regulation of antioxidants is linked to the intracellular amount of ROS, as in the case of Nrf2. The increase in oxidative pressure breaks the bond with the inhibitor Keap1 and triggers the antioxidant response. A similar mechanism is adopted by the transcription factor FoxO (De Keizer et al., 2011). Mitochondrial ROS can also be a signal which down-regulate the biogenesis of mitochondria in yeast (Chevtzoff et al., 2010). Also in mammals new mitochondria production and antioxidant response are linked by the transcription factor PGC1α (St-Pierre et al., 2006) in what looks like a mitochondria quality control feedback. Moreover, even if separated and each one having its own redox balances, there is crosstalk between compartments. It has been shown in yeast that disrupting Prx-mediated GSH oxidation in the mitochondrial matrix upregulate the levels of a cytosolic catalase (Calabrese et al., 2019). This study also underlines other important points: i) cytosolic Prxs limit the quantity of H_2O_2 reaching the mitochondrial matrix and detoxify mitochondria derived H_2O_2; ii) the matrix GSH oxidation upon H_2O_2 stress is mediated by Prx1. This clear example of crosstalk between the Trx and the GSH system shows how the various antioxidant systems, although described individually before, are in close relationship with each other. Either Trx and GSH can directly scavenge ROS or participate in signaling pathways, sharing similarities in mechanisms and functions. In addition to the example of Prx1, it has been observed that Trx reduces Grx2 (Johansson et al., 2004) which in turn reduces Prx3 (Hanschmann et al., 2010). Furthermore, they act as a mutual backups (Du et al., 2012; Reichheld et al., 2007; Tan et al., 2010), and this collaboration is even more

obvious in insects: to supply the lack of GR, Trx directly reduces GSH (Kanzok et al., 2001), and therefore TrxR is the only enzyme responsible of reducing both the Trx and GSH systems.

1.5 ROS detection

Measurement and imaging of ROS in live tissues have proved to be extremely challenging. Undoubtedly, this is due to the extreme instability and reactivity of these molecules. Given the difficulties in detecting them directly, most of the measurements were carried out indirectly. Common procedures were the quantification of oxidative markers, or estimation of the GSH:GSSG ratio in cell or tissue lysates. However, these approaches bring a poor comprehension of what is the cellular redox biology. This is because we have seen how ROS cannot be classified as a single identity, and the same reasoning must be done with antioxidants. The specificity of reaction, the localization, the recycling and the metabolism of these compounds are parameters that must be taken in consideration in a biological context. Therefore, estimation of side products derived from oxidation such as protein carboxylation, lipid peroxidation or DNA breaks is deeply influenced by repair and turnover of those, as well as by alternative causes of these alterations (Murphy et al., 2011). Moreover, H_2O_2 acts as a signaling molecule modifying proteins without generation of oxidative damage. GSH quantification by HPLC or spectrophotometric method are also widely used (Hazelton and Lang, 1983; Hou et al., 2018; Sahoo et al., 2017). However, they are not useful for dynamic measurements, as they imply disruption of the cell/tissue. Extractions average the differences between cells, compartments or specific redox couples, in addition to creating artifacts. Meanwhile, a great number of ROS sensitive probes have been developed over the years. Although this represented a step forward towards more pertinent tools to study redox biology, many difficulties still had to be addressed for *in vivo* application and to draw firm conclusions. Side reactions, specificity towards a ROS species without need of a catalyst, cellular uptake and accessibility to non-transparent tissues are just some of those. Dichlorofluorescein (DCF), dihydroethidium (DHE), mitoSOX and AmplexRed are examples of probes that can undergo side reactions and generate experimental artifacts (Owusu-Ansah et al., 2008; Winterbourn, 2008; Zhao et al., 2005). Although the use of these probes can still be useful, the application in some systems *in vivo* is not always feasible, and the results obtained require a critical and prudent interpretation.

The study of the properties of aequorin (Shimomura et al., 1962), a bioluminescent protein of the jellyfish *Aequorea victoria*, led Shimomura to the discovery of the green fluorescent protein (GFP) (Shimomura, 2009). Only 30 years after its discovery, the protein was cloned (Chalfie et al., 1994) and enhanced (Helm et al., 1995), revolutionizing the biological research. Since then, the applications of GFP imaging of living organisms are uncounted (actually, 252.586 articles up of today). In the redox biology field, the conception of redox sensitive YFP (Østergaard et al., 2001) and GFP (Dooley et al.,

2004; Hanson et al., 2004) allowed to overcome many technical issues. The wild type GFP has a beta-barrel structure with an alpha helix containing the chromophore running through the center. This chromophore is the tripeptide Ser65-Tyr66-Gly77, which cyclizes when the protein is matured and folded correctly, requiring an oxidative step. The excitation peak depends on the protonation state of the Tyr66 side chain: the neutral form has its maximum at 395nm, while in the deprotonated state the excitation peak shift to 475nm. Nevertheless, once excited the Tyr66 transfers one proton to Glu222, switching in the anionic form. For that reason, both forms display the same excitation maximum at 509nm. Despite this protein was fluorescent and stable at room temperature, it presented imperfections: the dual excitation spectrum, pH sensitivity, poor photostability and fluorescence quantum yield. Many of those issues were resolved with the point mutation Ser65→Thr introduced by Roger Tsien, paving the way for the development of enhanced GFP (eGFP; λ_{ex}=488nm, λ_{em}=510nm).

Redox-sensitive GFP (roGFP) represented a breakthrough in the study of redox homeostasis, because they are a non-invasive method for visualization of the oxidation state (Dooley et al., 2004; Hanson et al., 2004) and present numerous advantages over the methods hitherto used. In roGFP (derived from WT GFP) and roGFP2 (derived from eGFP), two Cys residues have been inserted at the position Ser147 and Gln204, facing the external side of the βbarrel. These two residues are in proximity to the chromophore: the formation of the disulfide bond between them upon oxidation affects the protonation state of the chromophore and favors the excitation peak at 405nm at the expense at the excitation peak at 488nm (Schwarzländer et al., 2016). This oxidation is reversible allowing to follow redox dynamics in living cells. The fluorescence ratio between the two excitation peaks is an indicator of the degree of oxidation and redox potential, avoiding problems related to photobleaching or the absolute quantity of the probe. The state of the probe is also independent from pH in the physiological range (Schwarzländer et al., 2008). Moreover, these proteins can be expressed in different intracellular compartments and coupled to other proteins to raise their specificity. Coupling roGFP2 with human Grx1 results in a probe that is catalytically autonomous (independent from endogenous Grxs) and way more sensitive and specific to study GSH dynamics (Gutscher et al., 2008). Thiol switch on roGFP2 cysteines is a three-step process starting when the Cys23 of Grx1 specifically react with GSSG and form a mixed Grx1-glutathione disulfide (Figure 1.8A). Then, this intermediate S-glutathionylates one of the Cys of roGFP2, which favors a reorganization to form an internal disulfide bridge (Meyer and Dick, 2010). The same principle was used soon after to create probes for H_2O_2 exploiting the properties of peroxiredoxins (Figure 1.8A') and the redox relay concept. These enzymes rapidly scavenge H_2O_2 through thiol groups, with the formation of a sulfinic acid group and afterward an internal disulfide bond. This oxidation can be transferred to Trxs, Grxs, or to a target protein. Therefore, the genetic fusion of the peroxidase Orp1 with the roGFP2 ensures close proximity, and the thiol disulfide exchange between the Cys groups of the two protein can happen resulting in modification of the roGFP2 excitation spectrum (Figure 1.8B).

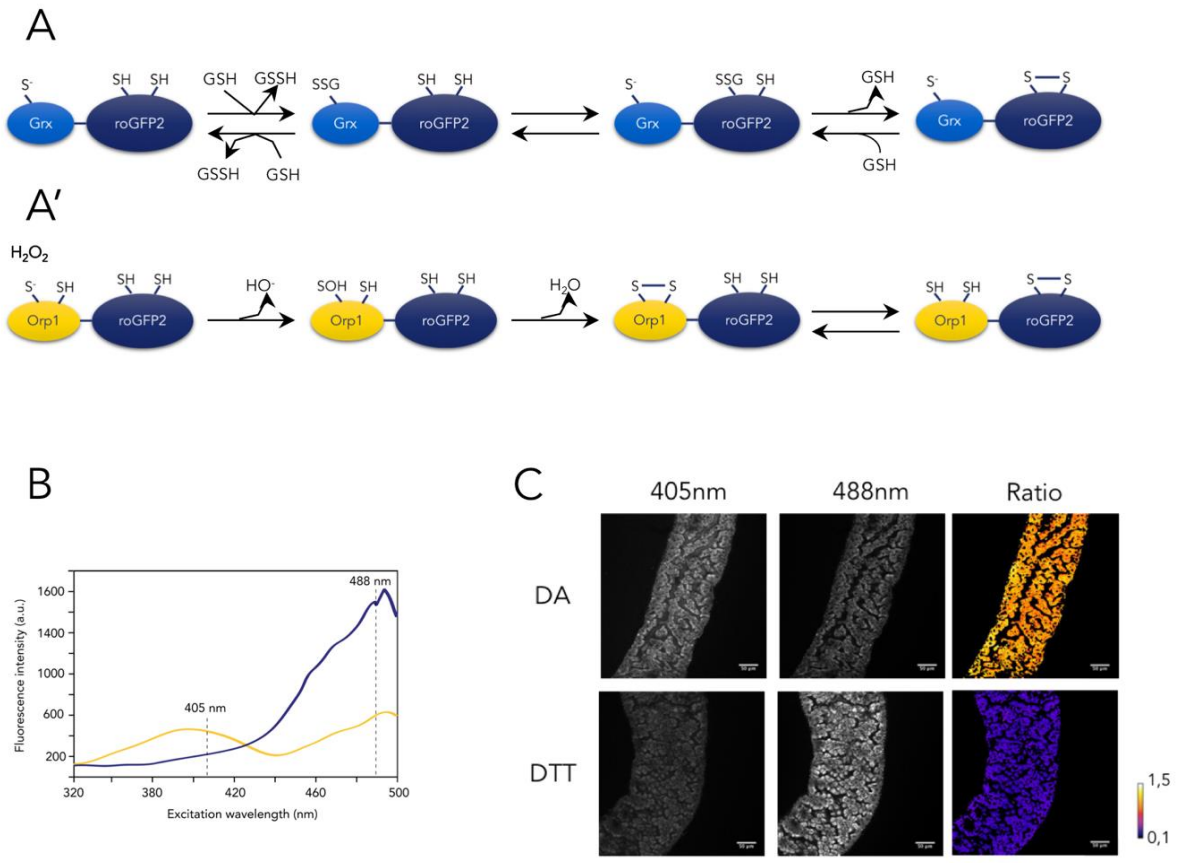


Figure 1.8. The ratiometric probe roGFP2. (A & A') Molecular mechanism of Grx1-roGFP2 and Orp1-roGFP2 probes. Adapted from Albrecht et al., 2011. **(A)** The active thiol group of cysteine on Grx1 reacts with GSH to form a mixed disulfide. The glutathyonilation can be transferred to one of the cysteines exposed on the surface of roGFP2. In the last step, the two cysteines can reorganize forming an internal disulfide bridge, provoking a shift in the probe's excitation peak. All steps are reversible. **(A')** The peroxidase Orp1 mediates the roGFP2 oxidation by H_2O_2. H_2O_2 scavenging leads to a formation of a sulfenic acid (SOH) on Orp1, which can lead to an internal disulfide bond. Based on the redox relay model, this disulfide is then transferred to the two cysteines of roGFP2, given their spatial proximity. **(B)** Excitation spectrum of roGFP2. The reduced form of the probe presents an excitation peak at 488nm (blue). In the oxidized form, the excitation spectrum changes, gaining excitability at 405nm (yellow). **(C)** Examples of fly's midguts expression the Orp1-roGFP2 probes. Fully oxidized samples were obtained incubating tissues in diamide (DA) 2mM, whereas fully reduced ones were attained after incubation in dithiothreitol (DTT) 20mM. Ratio was calculated by dividing the emission values after 405nm and 488nm excitation (405nm/488nm).

Of note, the disulfide bonds on Orp1 can also be reduced by endogenous antioxidants (Trx, GSH) present in the cellular environment. Although this can be seen as a limit in the measurement of H_2O_2, it actually represents a more accurate readout of what redox dynamics are in the cell (M. Schwarzländer et al., 2016).

Using specific genetic constructions, these probes were used to generate transgenic animals, notably *Drosophila melanogaster*, bringing interesting insights on redox differences within cell and tissues, in physiological behaviors and aging (Albrecht et al., 2011).

2. Results

2.1 Antioxidant response following the infection

In order to confirm the RNAseq data and verify the implication of an antioxidant response in the stages following the cytoplasmic purge, we used ARE-GFP reporter flies (Sykiotis and Bohmann, 2008). These flies express the GFP under the control of enhancers containing antioxidant response elements (ARE) sequences. After an oxidative stress, the transcription factor *Cap'n'collar (Cnc)* binds to these AREs presents in the enhancer of antioxidant response genes. This transgene allows the quantification of *Cnc* activity by GFP fluorescence measurement. For the experiment, we used paraquat and H_2O_2 as oxidizing agents to have a positive control. We chose 3h, 6h and 16h exposure as a time point, with the aim of monitoring the antioxidant response in the phases between the thinning and the recovery. Paraquat produces a peak of *Cnc* activity starting from 6h after feeding (Figure 1.9), even though we were expecting a stronger response. ARE activation following H_2O_2 exposure may follow a different kinetic, because the strongest induction is measured at 16h time point. We found that Db11 ingestion cause an induction of the *Cnc* targets mostly at 6h after infection. At this time point, the GFP fluorescence is stronger not only compared to the sucrose condition, but also to the earlier and later times of bacterial infection. Therefore, we confirmed that a group of potential antioxidants genes is induced by the *Drosophila* Nrf2 homolog after the thinning provoked by Db11 ingestion.

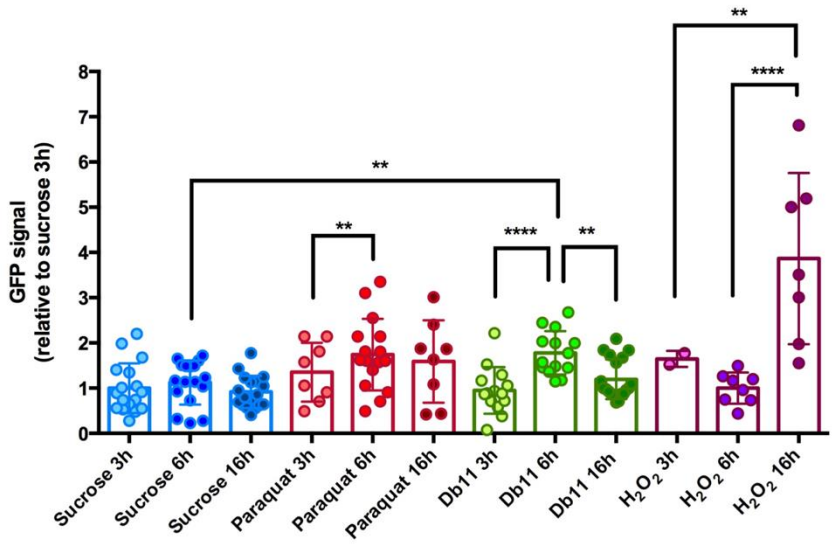


Figure 1.9. Cap'n'collar (Cnc) dependent ARE (Antioxidant Response Elements) are activated after Db11 ingestion. (A) ARE-GFP flies were exposed by feeding with sucrose 100mM, Paraquat 5mM, Db11 OD_{600}=10 and H_2O_2 5%, then dissected after 3h, 6h or 16h. Images of single intestines were taken by confocal microscopy, then the fluorescence intensity was measured using the ImageJ software and normalized to sucrose 3h. Each point represents a single midgut. Statistical analysis was performed using lmer linear mixed model.

2.2 ROS inhibition and impact on the cytoplasmic purge

Based on the evidences collected so far, we reasoned that ROS might play a crucial role in initiating the process of cytoplasmic extrusion. To test this hypothesis, we decided to buffer the effect of ROS by adding the widely used antioxidant N-acetylcysteine (NAC) at the time of infection. First, we verified that adding NAC to sucrose did not alter the epithelium morphology: the enterocytes remained thick and with dome-like structures (Figure 1.10A&B). Second, we tested the effect of this compound at different concentrations upon cytoplasmic extrusion, therefore on the thickness of the intestinal epithelium. For this we realized a dose-response alongside the Db11 infection. Feeding the flies Db11 supplemented with NAC led to an important reduction of the epithelial thinning (Figure 1.10A&B). Compared to the exposure to Db11 alone, where the enterocytes are completely flat, midgut epithelium of flies fed on Db11+NAC led to enterocytes that generally conserved an important thickness and the typical dome shape, although they were slightly flattened compared to the non-infected condition. The effect seemed to be dose-dependent but reached a plateau at the concentration of 10mM. Thus, we concluded that the use of NAC significantly reduces the cytoplasmic extrusion caused by Db11 hemolysin, possibly by decreasing the effect of ROS.

2.3 Phenotype validation with other antioxidants

To further support the outcome obtained with NAC, we tested further other antioxidants. We chose to use dithiothreitol (DTT) which neutralizes oxidizing agents through its thiol groups (-SH) to neutralize oxidizing agents, and ascorbic acid/vitamin C (Vit. C) which instead have hydroxyl group (-OH), to investigate potential differential mechanisms. To our surprise, repeating the same protocol as above by adding DTT or Vit. C to the Db11 infection solution did not lead to the same results observed with NAC (Figure 1.11A). Indeed, none of them affected importantly the enterocytes thickness, since adding one or the other to the infection solution did not significantly ameliorate the thickness score of the gut. Of note, we remarked a large proportion of semi-thin midguts in flies infected with Db11+Vit. C, but still not even a single thick epithelium. Hence, we tried to change the way of administration of antioxidant compounds, feeding the flies with antioxidant for 2 days before the infection instead of adding them to the bacteria. Even with this procedure, we did not observe major changes regarding epithelium thinning (Figure 1.11B). As a matter of fact, infected flies pre-treated with antioxidant presented a thin epithelium as much as the control condition. Collectively, these results suggest that beside its reducing capacity NAC presents properties differing from DTT and Vit. C, raising questions about the mode of action previously observed on the cytoplasmic purge.

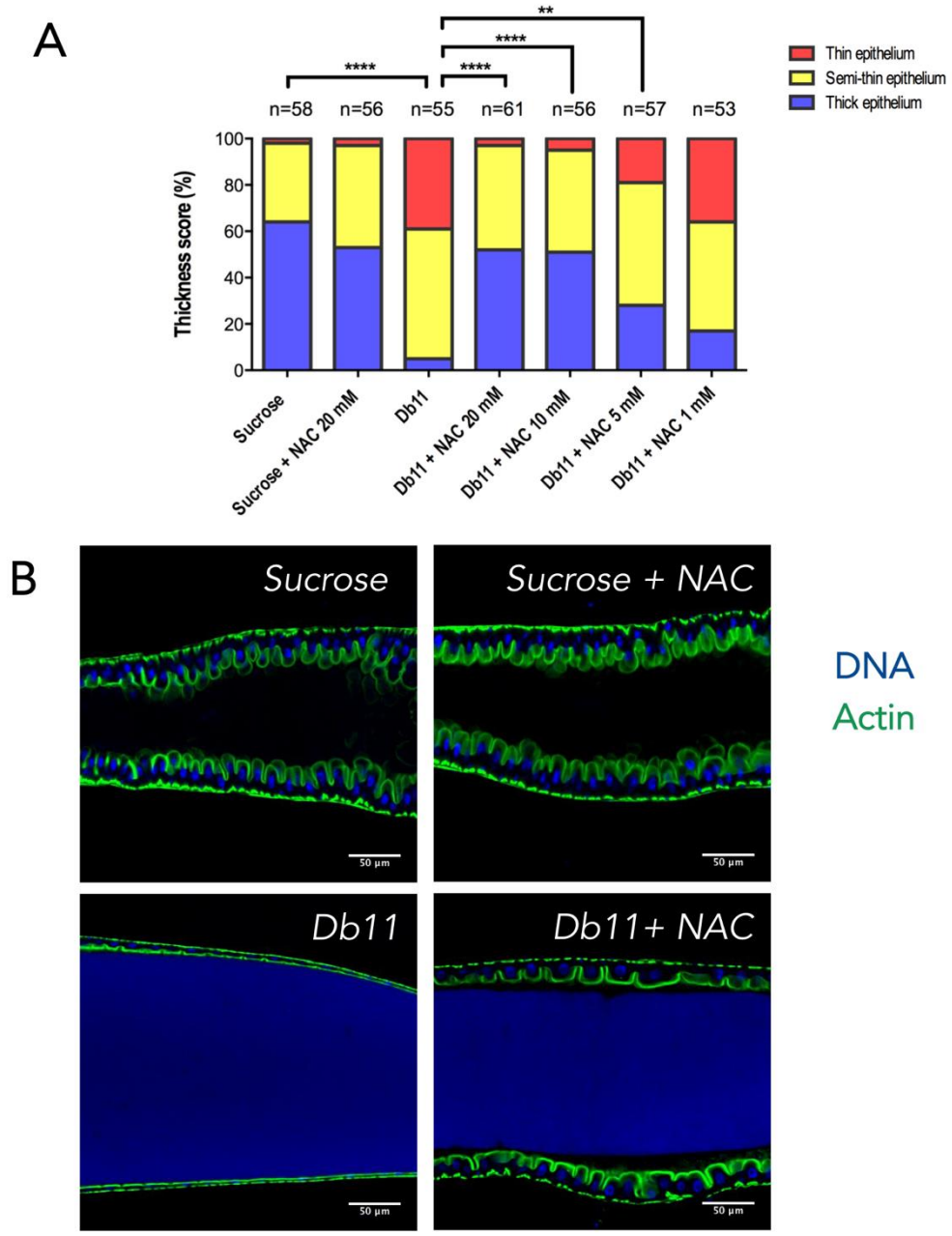


Figure 1.10. N-acetylcysteine inhibits the epithelial thinning. WT flies (w^{A5001}) were exposed to Db11 or Db11 mixed with different concentrations of NAC (from 1mM to 20mM). Flies were fed at 29°C for 3h with a bacterial OD_{600}=10. Dissected midguts were stained with phalloidin to visualize actin and with DAPI to visualize DNA. **(A)** Midguts were scored according to their epithelial thickness: thick, semi-thin or thin. The graph represents pooled results of four independent experiments. Statistical tests were performed using χ^2 test. **p=0,0025; ****p<0,0001. Only significant comparisons are shown. **(B)** Confocal pictures of R2 region of the midgut of flies exposed to sucrose, Db11, or each one supplemented with NAC 20mM.

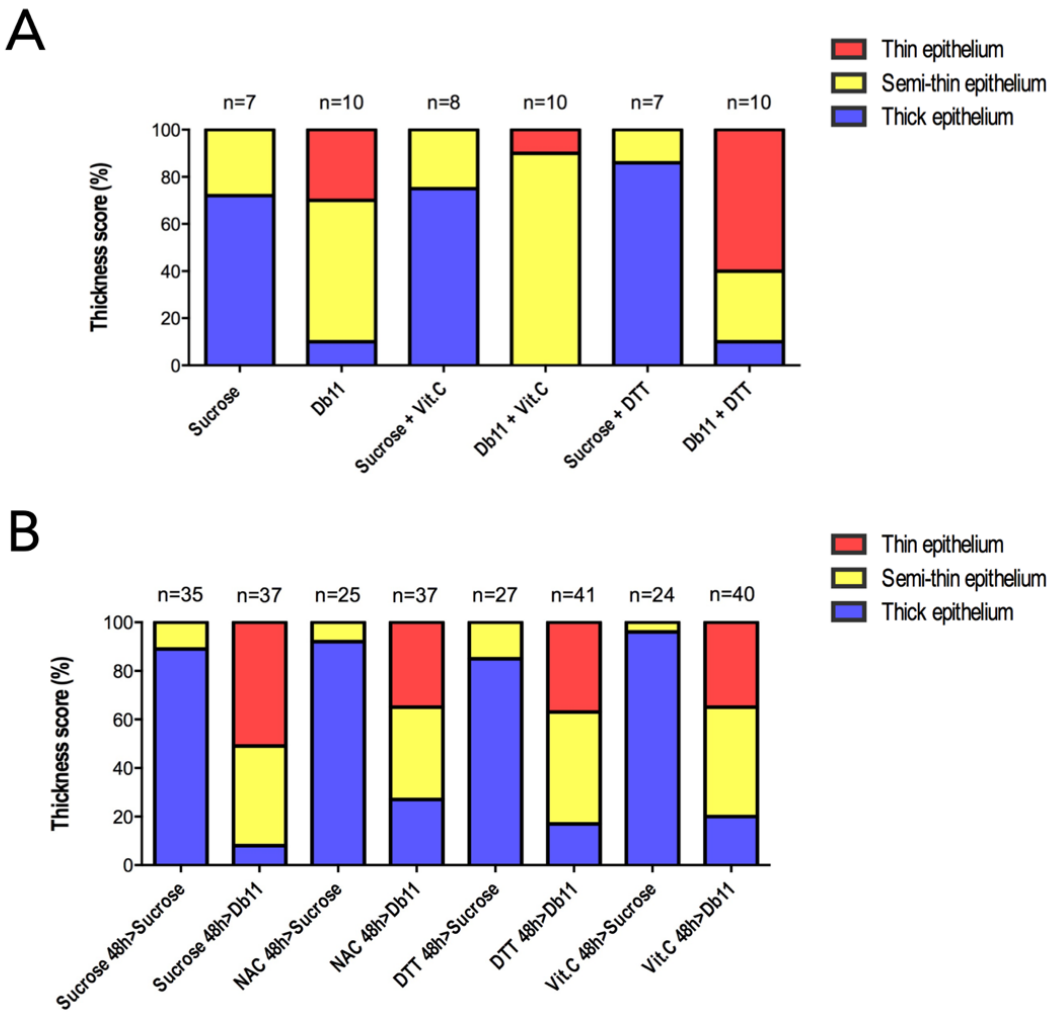


Figure 1.11. DTT or Vitamin C treatment does not produce significant changes in epithelial thickness. (A) w^{A5001} flies were exposed to Db11 or Db11 OD_{600}=10 mixed with DTT 20mM or Vitamin C 20mM. Intestines were dissected after 3h of infection and stained with phalloidin in order to visualize actin. They were scored according to their epithelial thickness: thick, semi-thin or thin. **(B)** w^{A5001} flies were pre-fed at 25°C for 48h with NAC 20mM, DTT 20mM or Vitamin C 20mM. Then they were exposed to sucrose 100mM or Db11 OD_{600}=10. Intestines were dissected after 3h of infection and stained with phalloidin in order to visualize actin. They were scored according to their epithelial thickness: thick, semi-thin or thin. The graph represents the pooled results of four independent experiments. (A&B) Statistical tests were performed using χ^2 test. Only significant comparisons are shown.

2.4 Side effect of NAC

Several examples of how NAC can affect bacteria can be found in the literature (Dinicola et al., 2014; Eroshenko et al., 2017; Moon et al., 2016; Pollini et al., 2018). Nevertheless, if growth inhibition and biofilm disruption by this molecule have been well studied, much less is known about its direct bactericidal activity. We then hypothesized that the considerable epithelium thinning inhibition was maybe due to a direct effect of this compound on bacteria. To test this possibility, we initially made a simple *in vitro* test to check bacterial growth (Figure 1.12). The outcome was very clear, showing that bacteria did not grow in a LB medium containing NAC. We then analyzed the bacterial content in the gut. The experiment revealed that within the digestive tract, all three bacterial species tested (Db11, *E. coli* and *Ecc15*) had a much lower burden in the presence of NAC (Figure 1.13A). We also evaluated whether DTT and Vit. C have similar bactericidal properties. We found that in contrast to NAC, these two antioxidants have no effect on bacterial load (Figure 1.13B). Next, we determined whether NAC has a direct bactericidal activity, checking the bacterial viability in presence of NAC. We co-fed the flies with GFP-expressing bacteria and propidium iodide (PI) to stain dead bacteria. In this way, we were able to discriminate live bacteria that are only stained in green from dead bacteria that are stained in red. Pictures in Figure 1.13C & 1.13D show that only PI staining is visible when NAC is present in the infection solution. Of note, we remarked that, without NAC, *E. coli* was killed while passing through the fly's intestine, precisely into the acidic copper cell region, while Db11 survived (Figure 1.13C & 1.13D upper lines). Taken together, these results indicate that NAC not only inhibit Db11 growth, but also has a powerful bactericidal activity *in vivo*. In addition, we speculate that *E. coli* is vulnerable to acidic pH, albeit Db11 is not.

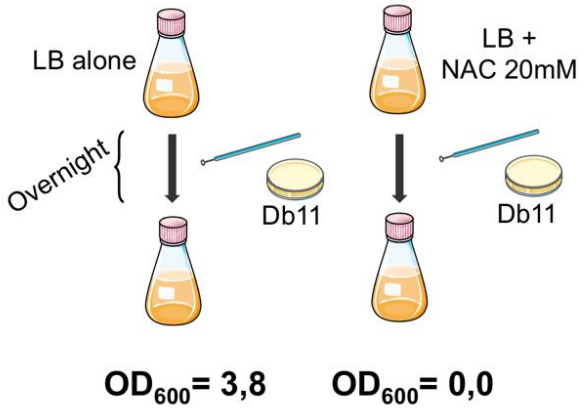


Figure 1.12. N-acetylcysteine affects bacteria *in vitro*. (A) Db11 single colony was inoculated in LB medium alone or containing NAC 20mM. The culture was grown at 37°C overnight. The OD_{600} was measured the following morning.

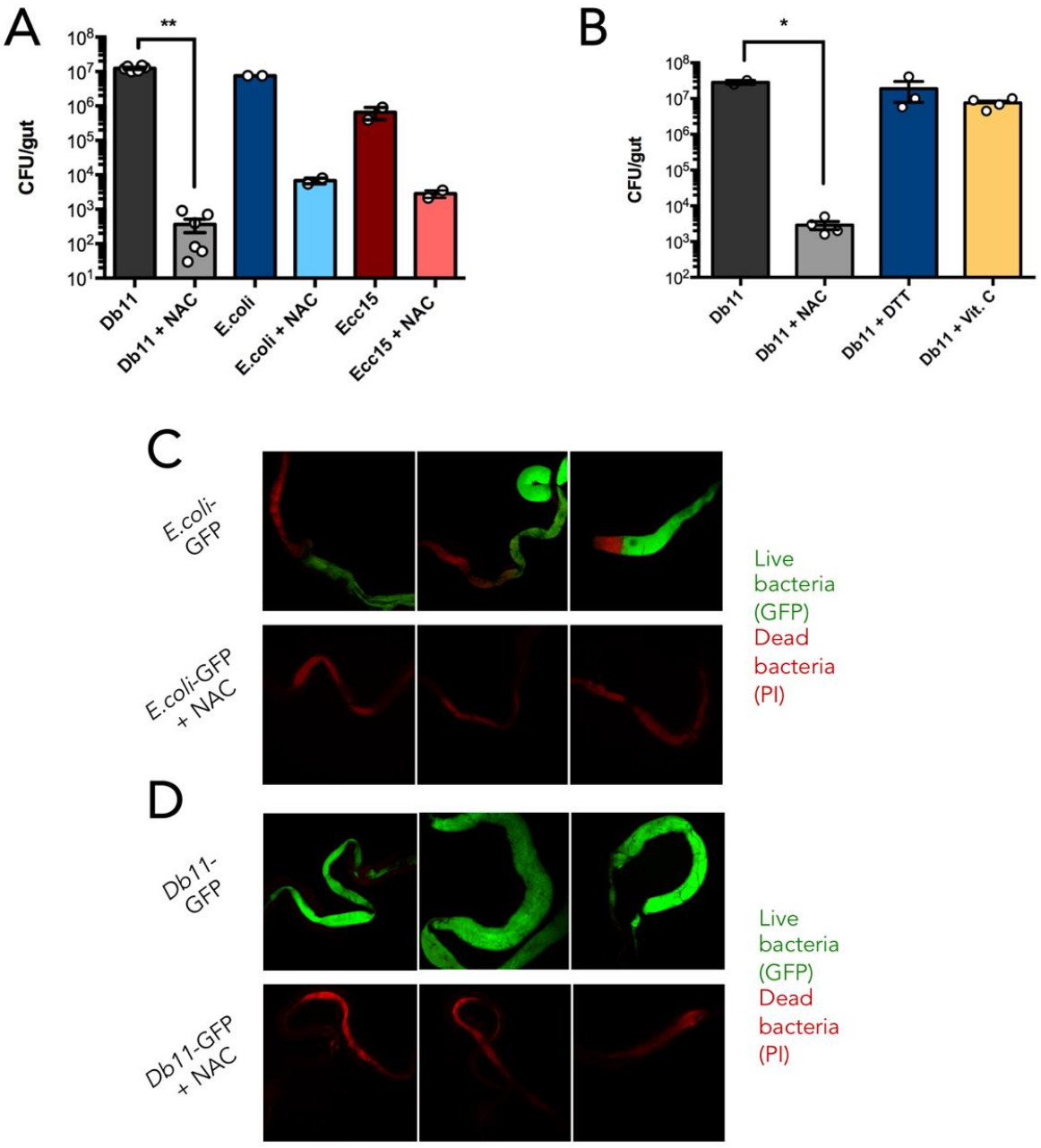


Figure 1.13. N-acetylcysteine is toxic for bacteria *in vivo*. **(A & B)** CFU count of bacteria in midguts of w^{A5001} flies 3h after infection with bacterial OD_{600}=10. Each point represents one biological replicate composed of 5 midguts. Error bars represents mean with SEM. Only significant comparisons are shown. **(A)** Flies were infected either with Db11, *E. coli* or *Ecc15* with or without NAC 20mM. For pair-wise comparison, statistical analysis was performed using Mann-Whitney test. **p=0,0022. **(B)** Infection with NAC 20mM, DTT 20mM or Vit.C 20mM added to Db11 OD_{600}=10. For multiple comparisons, Kruskal-Wallis test was used for statistics. **(C & D)** Confocal pictures of midguts infected with GFP-expressing *E. coli* (C) or GFP-expressing Db11 (D). NAC 20mM was added to bacterial solution before the infection. Bacteria were also stained with Propidium Iodide (PI) to revel dead cells.

In the light of these discoveries, we also studied the impact of NAC on host's survival. When we fed *Drosophila* with Db11+NAC, we noted that we rescued the slight mortality induced by Db11 alone (Figure 1.14). Moreover, and surprisingly, we noted how the simple fact of adding NAC to the solution of sucrose causes a sharp decrease in the survival of the flies. Thus, this molecule induces noxious effects on the host as well, although this toxicity disappears when Db11 is fed alongside NAC.

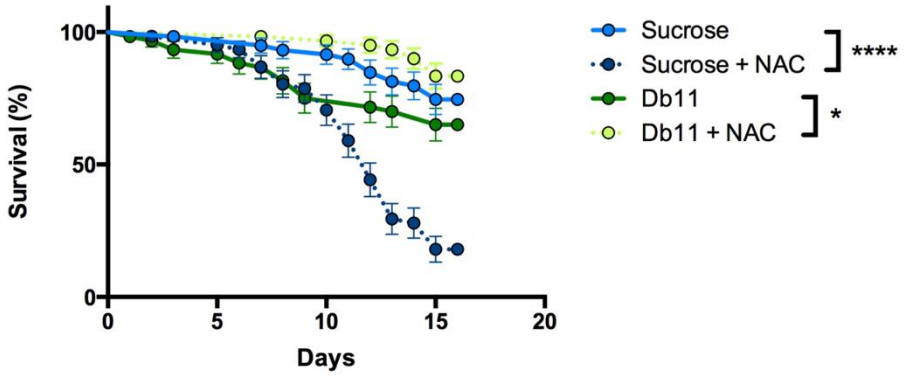


Figure 1.14. N-acetylcysteine is harmful to the flies but alleviates Db11-induced mortality. Survival curves of w^{A5001} flies that have been exposed to sucrose, sucrose + NAC 20mM, Db11 OD_{600}=1, or Db11 OD_{600}=1 + NAC 20mM. Statistics were performed using Logrank. *p=0,0117, ****p<0,0001. The graph represents one out of three experiments yielding similar results.

2.5 ROS measurement in the early phases of infection

The results above showed the limitation of a functional approach and in drawing conclusions based on treatments with antioxidants. Then, we opted for a direct method, measuring ROS right after Db11 infection. We used first the genetically encoded roGFP2 probes in two different variants, that is Grx-coupled or Orp1-coupled. Additionally, the cellular probes can be expressed in two different subcellular localizations, cytoplasm or mitochondria, raising the number of combinations to four (cytoplasmic-Grx, mitochondrial-Grx, cytoplasmic-Orp1, mitochondrial-Orp1). To monitor ROS levels changes in the phase that precedes the cytoplasmic extrusion, midguts were dissected at very early time points after Db11 ingestion. As we can see in Figure 1.15A, we did not detect changes with cytoplasmic-Grx, mitochondrial-Grx or cytoplasmic-Orp1, even with the positive control *Ecc15*. It is relevant to remark that the cytoplasmic-Orp1 probe present a very narrow dynamic range compared to the others. Conversely, with mitochondrial-Orp1 roGFP2 a significant increase is detected both after *Ecc15* and Db11 infection. This result indicates an increment of H_2O_2 production in mitochondria starting from 30

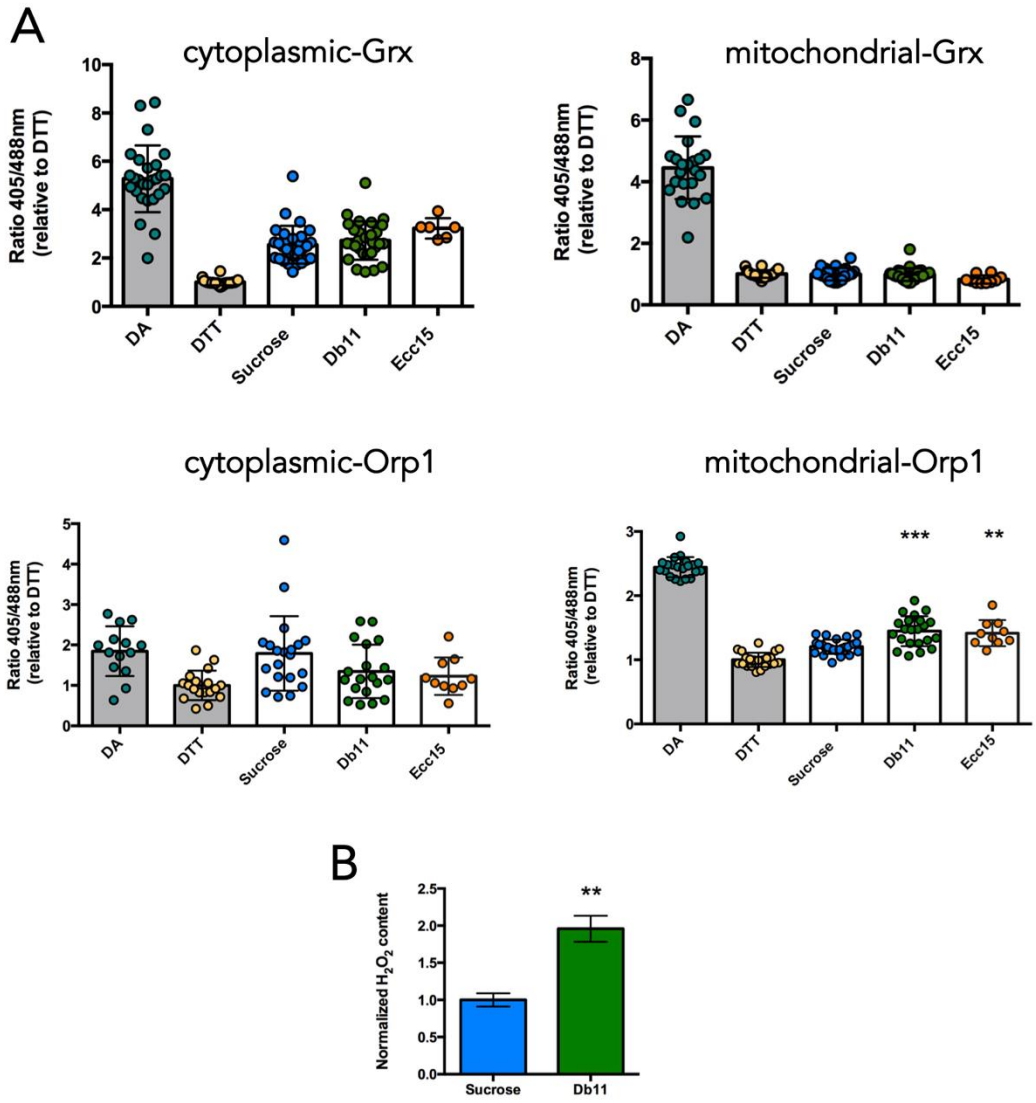


Figure 1.15. Db11 slightly increases H_2O_2 levels in mitochondria in the very early phase of infection. (A) Four different roGFP2 fly strains were treated with sucrose 100mM or Db11 and *Ecc15* OD_{600}=10. Midguts were dissected 30min after the ingestion. Each dot on the graph represents one intestine. Midguts were also exposed to DTT 20mM or DA 2mM post dissection to estimate their responsiveness to reduction and oxidation, respectively. Columns and error bars represent the mean and the SD. For pair-wise comparison, statistical analysis was performed using unpaired t test with Welch's correction, comparing each condition to sucrose treatment (except for cytoplasmic-Orp1, where Mann-Whitney test was used since sucrose column did not pass the normality test). **p=0.0097, ***p=0.0001. *Grx=glutaredoxin, Orp1=Orp1 peroxidase.* **(B)** Flies were exposed to sucrose 100mM or Db11 OD_{600}=10, then dissected 30min later. H_2O_2 content in midguts is measured by fluorometric H_2O_2 quantification assay. The graph represents pooled results of 3 independent experiments. Columns and error bars represent the mean and the SD. Statistical analysis was performed using Mann-Whitney test. **p=0,0022

minutes after Db11 feeding. To further confirm this data, we measured H_2O_2 in an independent manner using a biochemical detection kit. The H_2O_2 induction was confirmed, with about a 2-fold increase following Db11 ingestion (Figure 1.15B). In summary, these results suggest a production of ROS signals coming from mitochondria before the cytoplasmic extrusion occurs.

We also decided to genetically investigate other possible ROS sources in *Drosophila* midgut. As previously described, the NADPH oxidase *Duox* can generate high levels of ROS especially during a bacterial challenge. Then, we asked if Db11 was able to induce ROS production in a *Duox*-dependent manner. For this purpose, we knocked down by RNAi the expression of *Duox* with two different lines (VDRC^{GD} or one generously given to us by Prof. Won Jae Lee (Ha et al., 2005a)) using the *NpGal4Gal80^{ts}* driver to have a specific knock-down in the enterocytes. We will call these lines *Duox^{RNAi}* GD and *Duox^{RNAi}* WJL from now on. Surprisingly, the two lines showed a different outcome (Figure 1.16A). Indeed, if in *Duox^{RNAi}* WJL the H_2O_2 production is compelling after Db11 infection, in *Duox^{RNAi}* GD flies the induction is less important and not significant. Nevertheless, it is important to note that we still observe a trend: albeit not supported by statistics, Db11 infection increases the H_2O_2 levels in *Duox^{RNAi}* GD midgut. Moreover, in *Duox^{RNAi}* WJL flies the H_2O_2 levels were slightly higher after sucrose treatment as well. Thus, there is a lesser induction of H_2O_2 also in this line compared to controls (Figure 1.16C). Given that *Duox* enzyme works in close relationship with the secreted IRC (Ha et al., 2005b), we also genetically overexpressed this protein in enterocytes to elucidate this ambiguous result. In parallel, we also overexpressed the intracellular catalase *Cat*. The latter strongly decreased H_2O_2 content after sucrose treatment and abolished the induction provoked by Db11 ingestion (Figure 1.16B). In contrast, IRC overexpression did not affect Db11 mediated H_2O_2 production. Nevertheless, we noted an unexpected lack of responsiveness of the UAS-GFP line, which was used as a control in this experiment. Despite in several lines bacteria induced H_2O_2 production (Figure 1.16A&B), we remarked that the variation between infected and non-infected is less than in Control^{RNAi} line (Figure 1.16C). Collectively, these results seem to confirm the mitochondrial origin of ROS in the early phase of the purge, but also indicate a possible contribution of *Duox*, although this latter point remains tentative at this stage.

2.6 Localized ROS scavenging

Results described above point out the importance of mitochondrial-derived ROS (Figure 1.15). With the purpose of proving their importance in triggering the extrusion, we used the antioxidant mitoTEMPO, a compound already used for *in vivo* studies (Nazarewicz et al., 2013). This molecule is a

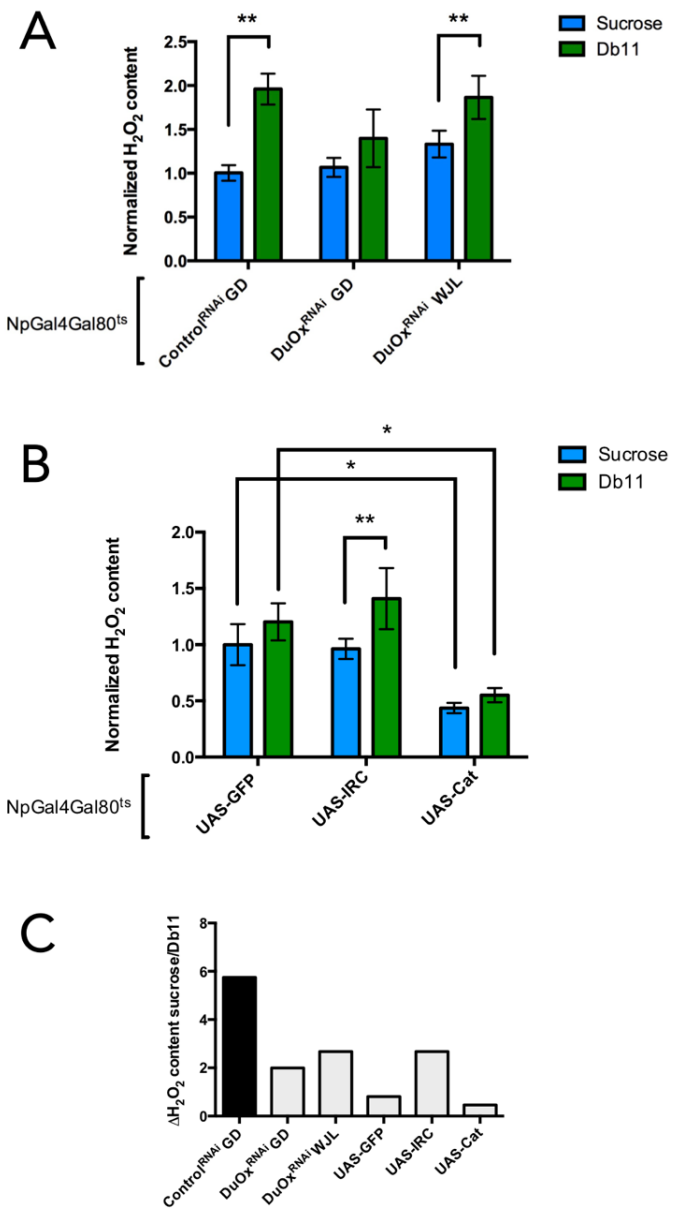


Figure 1.16. H_2O_2 production only partially depends on DuOx/IRC tandem. RNAi **(A)** or overexpression **(B)** lines were crossed to *NpGal4Gal80*^{ts} driver to provide a knock-down or a supplementary copy of the gene specifically in the enterocytes. *DuOx*^{RNAi} WJL strain is generous courtesy by professor Won Jae Lee. Flies were exposed to sucrose 100mM or Db11 OD_{600}=10, then dissected 30min later. H_2O_2 content is measured with the fluorometric H_2O_2 assay kit. In each experiment two biological replicates of ten midguts per condition were used. The graph represents pooled results of three independent experiments. Columns and error bars represent the mean and the SD. Statistical analyses were performed using Mann-Whitney test. Control^{RNAi} GD, sucrose VS Db11: **p=0,0022; DuOx^{RNAi} WJL, sucrose VS Db11: **p=0,0079; UAS-IRC, sucrose VS Db11: **p=0,0043; UAS_GFP VS UAS-Cat: *p=0,0286. **(C)** Difference in H_2O_2 content between infected (Db11) or uninfected (sucrose) flies of lines used in (A) and (B).

specific scavenger of mitochondrial superoxide composed by a combination of the antioxidant piperidine nitroxide TEMPO with the lipophilic cation triphenylphosphonium; this gives mitoTEMPO the ability to pass through lipid bilayers with ease and accumulate in mitochondria. We fed flies with this antioxidant for two days, then we performed a Dbl1 infection and checked the response of the midgut (Figure 1.17A). We observed that flies that ingested mitoTEMPO have a slight but still significant decrease of Dbl1-induced thinning. Since we measured decreased H_2O_2 total content in flies overexpressing the peroxisomal catalase *Cat*, in parallel we tested if the epithelial thinning was affected. Although this experiment was performed only once, these flies displayed a thinning score similar to WT flies (Figure 1.17B). Unfortunately, we remarked that the cross between the *NpGal4Gal80^{ts}* driver and the UAS-GFP line produces midgut that are less responsive to thinning. However, we assessed that the use of a mitochondrial ROS scavenger improved the epithelial thickness, whereas enhancing the catalase activity did not affect the thinning of enterocytes. Thus, this results further corroborate the possibility that is the mitochondrial ROS that contribute to trigger the cytoplasmic purge.

2.7 Effect of *Duox* knock-down on host physiology

To determine the involvement of *Duox* in the resilience mechanism to Dbl1 infection, we knocked-down the expression of this gene using the same genetic lines as above. First, we evaluated the effect of *Duox* lacking expression on the epithelial thickness after 3h of Dbl1 feeding (Figure 1.18). First, we remarked that the thinning was slightly less pronounced when we crossed the *NpGal4Gal80^{ts}* with the RNAi^{GD} control line compared to the driver alone. Nevertheless, we observed that *Duox^{RNAi}* WJL displayed a great improvement of enterocytes thickness compared with both strains. As shown in the pictures (Figure 1.18C), intestinal cells conserved their typical dome-shaped morphology, while the gut lumen is filled with bacteria. On the contrary, *Duox^{RNAi}* GD did not present any variation of the thinning phenotype, exhibiting thin and flat cells upon Dbl1 ingestion. The phenotype of *Duox^{RNAi}* WJL line was confirmed in further experiments (Figure 1.18B). These results with the *Duox^{RNAi}* lines were unexpected in that we observed similar differences in the H_2O_2 production upon infection (Figure 1.16C), but unlike *Duox^{RNAi}* GD, *Duox^{RNAi}* WJL line did not display an epithelial thinning upon Dbl1 infection (Figure 1.18A).

Next, we examined the survival of these same flies upon Dbl1 oral infection (Figure 1.19A). RNAi^{GD} flies succumbed earlier to the infection compared to WT flies (*w^{A5001}*), even if they showed a decreased lifespan also upon sucrose treatment. Survival curve for infected *Duox^{RNAi}* GD was very similar to the respective control, with a better endurance to sucrose feeding indeed. Conversely, *Duox^{RNAi}* WJL were more resistant to bacterial infection compared to all other lines tested.

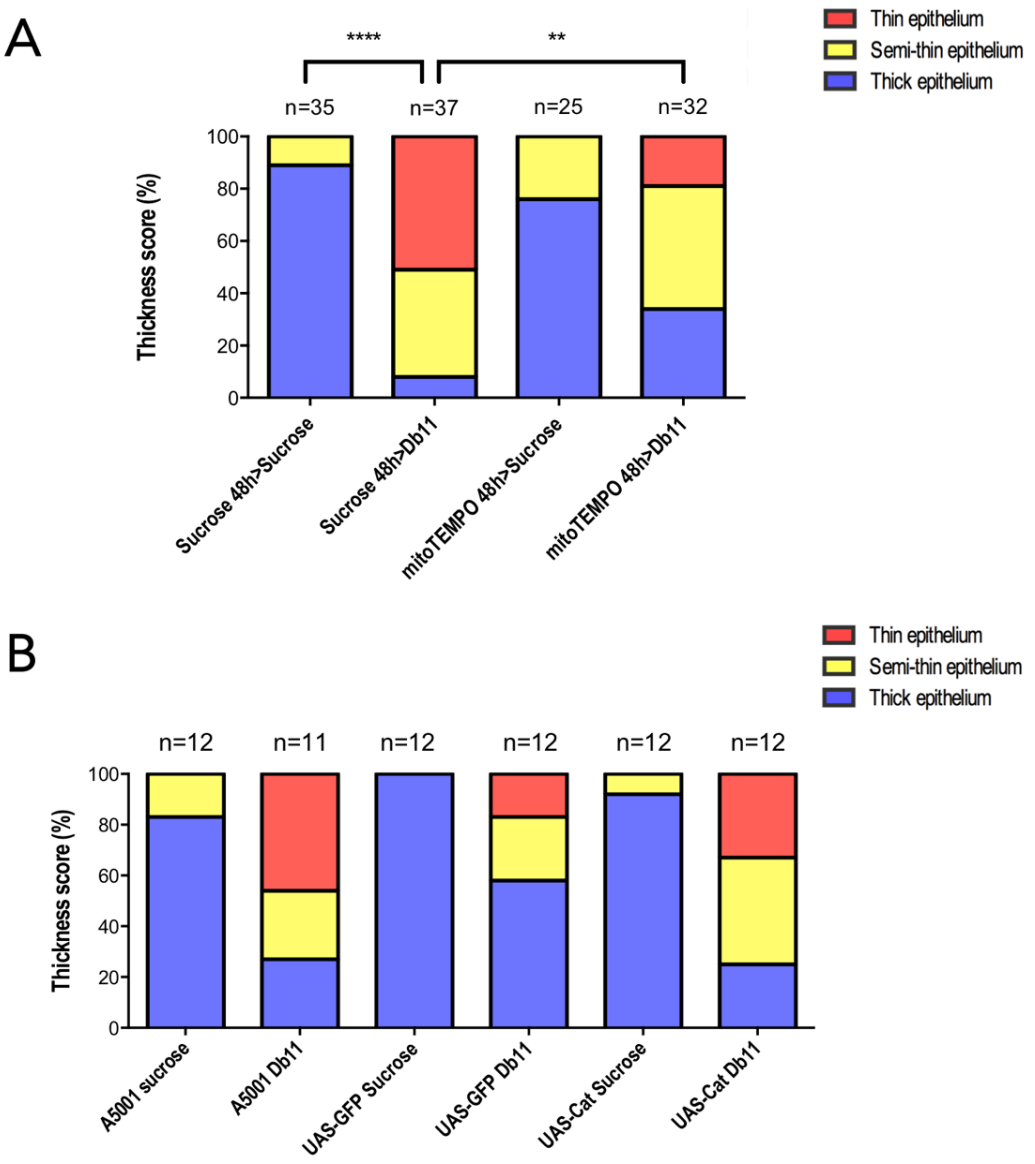


Figure 1.17. A mitochondrial antioxidant, but not the intracellular catalase, reduces the epithelial thinning. (A) w^{A5001} flies were fed at 25°C for 48h with mitoTEMPO 20µM. Then they were exposed to sucrose 100mM or Db11 OD_{600}=10. Intestines were dissected after 3h of infection and stained with phalloidin in order to visualize actin. They were scored according to their epithelial thickness: thick, semi-thin or thin. The graph represents the pooled results of four independent experiments. Statistical tests were performed using χ^2 test. Only significant comparisons are shown. **(B)** Catalase or GFP were overexpressed specifically in enterocytes using the *NpGal4Gal80*^{ts} driver. w^{A5001} flies were used as an additional control. Flies were fed with Db11 OD_{600}=10 and then dissected 3h after infection. Intestines were stained with phalloidin in order to visualize actin. They were scored according to their epithelial thickness: thick, semi-thin or thin.

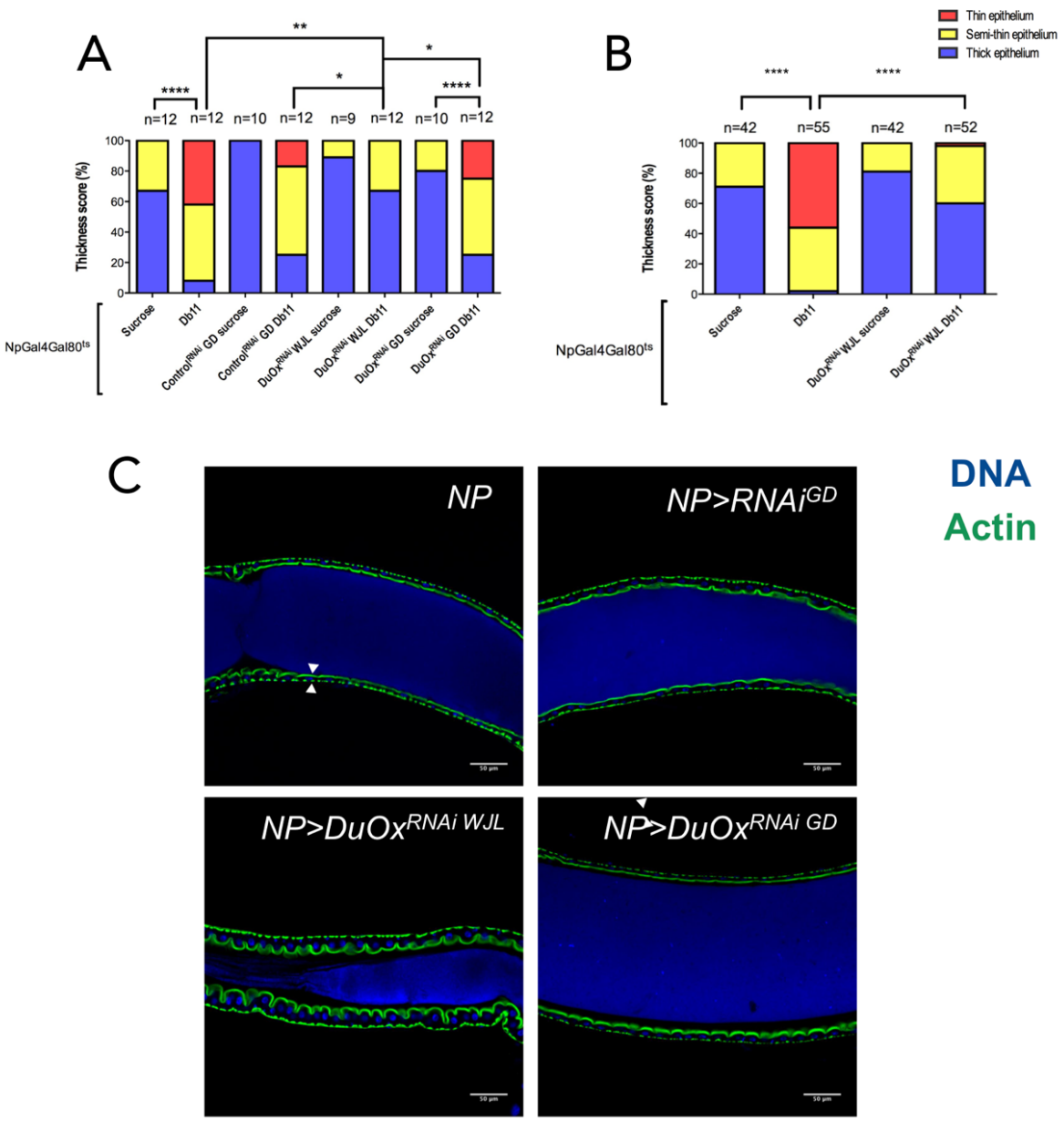


Figure 1.18. *DuOx*^{RNAi} *GD* strain, but not *DuOx*^{RNAi} *WJL*, displays a classical epithelial thinning 3h after Db11 ingestion. The expression of *DuOx* was knocked down by RNAi specifically in enterocytes using *NpGal4Gal80*^{ts} driver. Flies were fed with Db11 OD_{600}=10 and then dissected 3h after infection. **(A)** Intestines were stained with phalloidin in order to visualize actin. They are scored according to their epithelial thickness: thick, semi-thin or thin. Statistical tests were performed using χ^2 test for trends. Control^{RNAi} GD VS DuOx^{RNAi} WJL *p=0,0241, DuOx^{RNAi} GD VS DuOx^{RNAi} WJL *0,0175, **p=0,0010, ****p<0,0001. **(B)** The expression of *DuOx* was knocked down using RNAi WJL line specifically in enterocytes using *NpGal4Gal80*^{ts} driver and the intestines scored as in (A). The graph represents the pooled results of four independent experiments. Statistical tests were performed using χ^2 test. ****p<0,0001. **(C)** Confocal pictures of infected R2 midgut region of the different lines scored in (A).

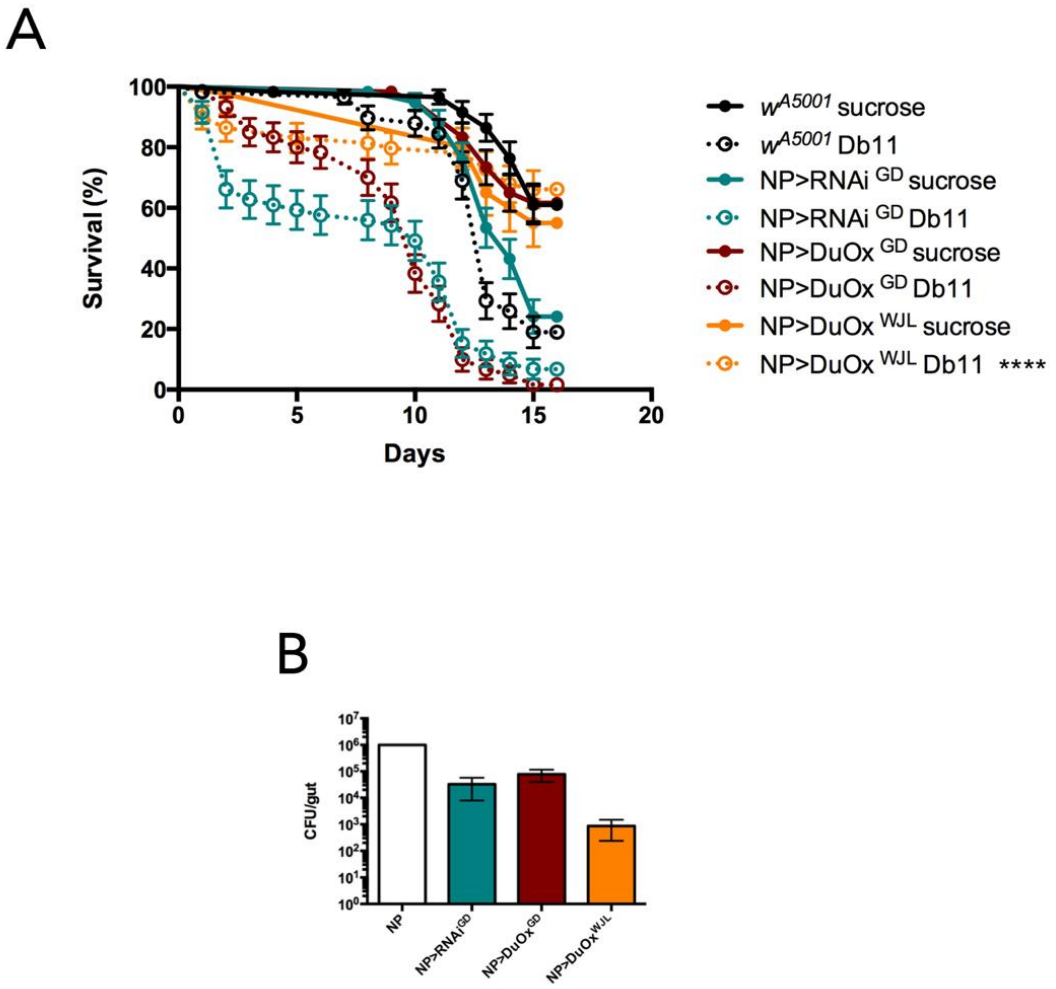


Figure 1.19. Some *DuOx* RNAi strains are more resistant to Db11 ingestion most likely because of a reduced bacterial burden. (A) Survival curves of flies exposed to sucrose or to Db11 OD_{600}=1. NP stands for *NpGal4Gal8o*^{ts}. Statistics were performed using Logrank, comparing each *DuOx* RNAi strain to the control RNAi^{GD}. ****p<0,0001. **(B)** CFU count of bacteria in midguts at day 10 of flies infected as in survival run in (A). Each bar represents three biological replicates composed of 5 pooled midguts, except *NpGal4Gal8o*^{ts}. Error bars represents mean with SEM.

To understand the fallout of the survival experiment, we measured the bacterial content at day 10 in the same experimental condition. We found that the Db11 titer of $Duox^{RNAi}$ GD flies was comparable to $RNAi^{GD}$, whereas $Duox^{RNAi}$ WJL displayed reduced bacterial load in their midguts (Figure 1.19B). Altogether, these results indicate that the increased survival of $Duox^{RNAi}$ WJL may be explained with a reduced Db11 burden along the survival, although the experiment need to be repeated.

2.8 Immune regulated catalase and epithelial thinning

After what we observed with $Duox^{RNAi}$ WJL flies, we asked about the effect of IRC. Using the *NpGal4Gal80ts* driver, we either overexpressed or knocked-down this enzyme in the enterocytes and we analyzed the Db11 induced thinning after 3h of ingestion (Figure 1.20A). Reducing IRC expression by RNAi did not affect the cytoplasmic purge, while its overexpression resulted in a thick epithelium with domed enterocytes, resembling the uninfected ones. The observations made using the UAS-IRC line were repeated in more experiments (Figure 1.20B). These data support the implication of *Duox* as a possible ROS source after Db11 ingestion. However, the survival of IRC overexpressing flies was not increased after bacterial infection (Figure 1.20D). Of note, these flies perished at the same rate as WT flies (w^{A5001}); unexpectedly, the control flies overexpressing GFP showed increased resistance to the infection. Overexpression of *Cat* resulted in a moderate early mortality that stayed stable after the first 9 days of exposure. We conclude that enhancing the buffering capacity of ROS in the midgut via IRC affects the enterocytes purge, although this does not translate into a change of survival rate. A better understanding of the results of those experiments awaits the determination of bacterial titer and the effect of *Cat* overexpression on the thinning.

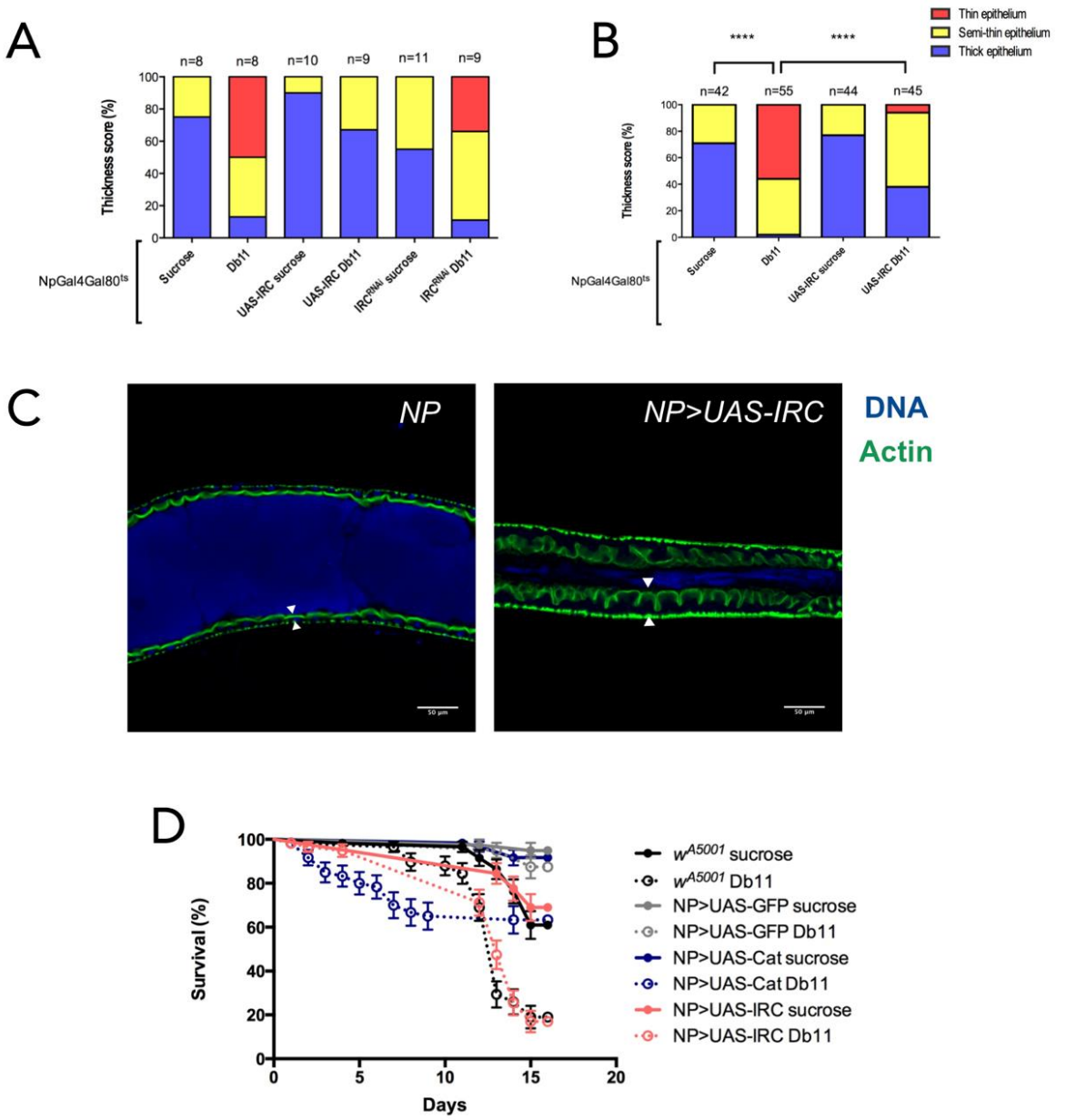


Figure 1.20. Overexpression of Immune regulated catalase (IRC) significantly reduces the epithelial thinning 3h after Db11 ingestion. *NpGal4Gal8o*^{ts} driver was crossed with UAS-IRC strain to overexpress this protein specifically in enterocytes or with IRC^{RNAi} to knock down the expression in the same tissue. Flies were fed with Db11 OD_{600}=10 and then dissected 3h after infection. **(A)** Intestines were scored according to their epithelial thickness: thick, semi-thin or thin. **(B)** *IRC* was overexpressed crossing UAS-IRC strain with the *NpGal4Gal8o*^{ts} driver and the intestines scored as in (A). The graph represents the pooled results of four independent experiments. Statistical tests were performed using χ^2 test. ****p<0,0001. **(C)** Confocal pictures of infected R2 region of the midgut of lines scored in (B). **(D)** Survival curves of flies exposed to sucrose or to Db11 OD_{600}=1. NP stands for *NpGal4Gal8o*^{ts}. Statistics were performed using Logrank, comparing each *DuOx* RNAi strain to the control RNAi^{GD}.

3. Discussion

3.1 Background

In our oral infection model with the Gram-negative bacterium *Serratia marcescens*, kinetics of cellular processes leading to epithelial thinning are well established. Within the first hour after Db11 ingestion, we observe the rapid and transient formation of lipid droplets in enterocytes accompanied by megamitochondria formation by fusion, which is detectable already in the first 30 minutes of feeding. After these early events, large apertures in the apical part of enterocytes become visible, from which the cytoplasm leaks out in the ectoperitrophic space and/or in the lumen. The thin epithelium that we see 3h post ingestion is the consequence of this cytoplasmic escape, since increasing the solution's osmotic pressure with the addition of dextran prevents the thinning. However, how this extrusion occurs is not clear yet. We do not know if the cytoplasm is pushed out through an active process or if it is just a passive osmotic leaking derived from the cell's breach. From the bacterial side, we proved that hemolysin is a necessary and sufficient factor to induce a massive extrusion event that leads to a very flat and thin epithelium (Lee et al., 2016). Nevertheless, infection with the hemolysin-deficient strain 21C4 also causes a limited thinning, and few cells extruding apical cytoplasm are also visible upon sucrose feeding. Indeed, there must be pathways in the host cell that are regulating the process, since we observe neither lysis nor necrosis; even in enterocytes bearing a large aperture, the cell remains alive, and activates a recovery process that is completed within 24 hours.

3.2 Relating ROS to cytoplasmic purge

ROS seemed to be a good candidate to fulfill the role of first actor initiating the cytoplasmic purge mechanism. First of all, *S. marcescens* have been reported to induce the secretion of ROS in *Drosophila*'s midgut (Lee et al., 2013). In neuronal tissue, ROS have been shown to trigger lipid droplets accumulation (Liu et al., 2015a): by sequestering oxidation-sensitive polyunsaturated fatty acids, lipid droplets can assume a protective role for the lipids of the cytoplasmic membrane by avoiding harmful peroxidation chain reactions (Bailey et al., 2015). GSSG and H_2O_2 at low concentrations promote mitochondrial fusion (Shutt et al., 2012), which recalls our observation of mitofusin-dependent (*Marf*) megamitochondria formation. Moreover, others showed that fused mitochondria have reduced ROS levels (Nagaraj et al., 2012). Thus, it may represent a process aimed to alleviate oxidative stress. In certain conditions, mitochondrial fusion has been proposed to be a way to avoid mitophagy and cell death (Gomes et al., 2011; Rambold et al., 2011). Both droplets and mitochondria are highly dynamic

organelles and regulate numerous cellular processes (Farese and Walther, 2009; Gomes et al., 2011; Murphy, 2009; Walther and Farese, 2012). Interestingly, we localized both of them in the apical part of the enterocytes, the area which is most exposed to hemolysin and nearby the sites where we later observe the extrusions. Besides, enterohemorrhagic *Escherichia coli* hemolysin have been shown to directly target mitochondria through OMVs (Bielaszewska et al., 2013), and we can speculate that Db11 hemolysin may work in a similar way. If we do not know the destiny of the lipid droplets, we demonstrated that mitochondria, that are likely damaged and non-functional, are extruded into the gut lumen. Apart from mitochondria, the apical membrane of the enterocytes also hosts another important ROS source that we have investigated during my PhD: the Dual Oxidase Duox. H_2O_2 secreted extracellularly by this enzyme can be transported in the cell via aquaporins or simple diffusion, generating a local H_2O_2 pool just underside of the plasma membrane. Therefore, kinetics and topology of the early steps leading to the extrusion event may fit well with ROS involvement. Additionally, ROS may be involved in the formation of apical aperture in enterocytes, since they have been implicated in cytoskeletal dynamics by acting directly on actin, non-muscular myosin and proteins responsible of cytoskeleton reorganization (Acevedo and González-Billault, 2018; Valdivia et al., 2015). A better understanding of these aspects is the object of a current project in the team. At present, we know that cytoskeletal dynamics involving non-conventional myosins in a potential blebbing process regulated by caspases may be required for thinning.

3.3 An antioxidant response is activated

An RNA sequencing experiment allowed the identification of genes in the midgut that are differentially regulated after the ingestion of Db11. Among the induced genes, we found some implicated in the antioxidant response, upregulated 6 and 9 hours after bacterial infection. The most significant were Peroxiredoxin2540, Jafrac 1 & Jafrac 2, GST-D10 and GST-E8. This suggests that an antioxidant response is activated after the thinning phase. Interestingly, all those genes are under the transcriptional control of CncC (Nrf2). In support of this hypothesis, we measured an increased fluorescence using an ARE-GFP reporter 6h after bacterial ingestion (Figure 1.9). At this time point, upregulation of genes containing ARE in their promoters or enhancers is significantly higher compared not only to controls but also to earlier and later time points of Db11 infection. Therefore, we could speculate that this antioxidant response is activated after extrusion and the consequent thinning (3h p.i.) to buffer the ROS produced. Nevertheless, this response is not sustained, as the induction is no longer observed at 16h post infection (Figure 1.9), suggesting that the infection does not provoke strong or chronic oxidative stress. We may hypothesize that after the cytoplasmic purge occurred, the ROS required for signaling are scavenged, Keap1 is no longer inactivated by oxidation and returns to

sequester CncC in the cytoplasm. Exposure to paraquat caused induction of ARE-containing genes induction with a similar kinetics, despite a more dispersed distribution of the data points. In contrast, CncC antioxidant response is delayed in flies fed on H_2O_2. However, this last condition must be interpreted with caution, since we had fewer data points and fluorescence intensities at 16h p.i. were very scattered.

3.4 N-acetylcysteine: a bactericidal antioxidant

3.4.1 Antioxidant function

As the role of ROS seemed increasingly likely, one of the first questions we asked was what happens if we inhibit ROS before extrusion. For that purpose, we added NAC to the infection solution and we actually observed that at certain concentrations NAC supplementation prevented the epithelial thinning (Figure 1.10). If initially this result was received with much excitement, it became puzzling after trying to reproduce the same phenotype with other antioxidants, precisely ascorbic acid (Vit. C) and DTT (Figure 1.11). First, we thought that the inefficiency of Vit. C and DTT was maybe due to a reduced or slower uptake by the enterocytes, therefore we changed the experimental protocol by pre-treating the flies prior to bacterial challenge. Nonetheless, this antioxidant-conditioning also failed to rescue the thickness of the midgut epithelium, which was globally thin or semi-thin. Even NAC was less efficient in inhibiting the thinning following this protocol. Possibly, the inefficacy of Vit. C and DTT may derive from some parameters that we have not explored, such as kinetics, feeding or concentrations. However, the results we obtained suggested that NAC action relied in something else besides its antioxidant properties. Indeed, the antioxidant properties of NAC have been questioned, and the biological effect maybe due to indirect ROS modulation or even nonradical mechanisms (Murphy et al., 2011; Parasassi et al., 2010). Furthermore, feeding NAC caused even an increase of H_2O_2 in larval fat body and gut, and raised H_2O_2 and GSSG levels in adults Malpighian tubules (Albrecht et al., 2011). Therefore, the assumption that this compound acts as an antioxidant *in vivo* should be considered very cautiously. For this reason, it is likely that the thinning inhibition observed in presence of NAC is provoked by an alternative process.

3.4.2 Bacterial viability

NAC powerful action in bacterial growth inhibition and destruction of biofilms is largely documented in the literature (Dinicola et al., 2014; El-Feky et al., 2009; Eroshenko et al., 2017; Quah et al., 2012). Nevertheless, it is still used as antioxidant alongside bacterial infection to conclude about ROS-related phenotypes (Ha et al., 2005b; Xie et al., 2018). In this work we showed a direct bactericidal effect of NAC that, to our knowledge, has not been reported so far. When this small molecule was added

in a bacterial solution used for an oral infection, it strongly decreased viability not only of *S. marcescens*, but also of other Gram-negative species such as *Escherichia coli* and *Erwinia carotovora carotovora* (Figure 1.13A). This aspect has to be considered carefully, since in the past the same concentration of NAC that we used was administered alongside *Ecc15* infection to draw conclusions about infection-induced ROS and host lifespan (Ha et al., 2005b).

In absence of NAC, we observed that Db11 were not killed throughout the passage in the midgut, at least at 3h p.i., indicating that this strain withstands both the IMD and ROS responses (Figure 1.13D). If resistance to IMD was known although we observed that *key* mutants die faster upon Db11 ingestion (Lee et al., 2016; Nehme et al., 2007), it has been shown that *Serratia* expresses a catalase that may confer resistance to ROS (Zeng et al., 2011). In contrast to *E. coli*, which is neutralized by the acidic pH in the copper cells region, Db11 resist well the passage across this area (Figure 1.13C&D). Thus, *S. marcescens* is a very resistant bacterium that greatly defies the immune system in the midgut: in such cases, resilience mechanisms such as cytoplasmic purge acquires primary importance in the host's defense. Taken together, these findings suggest that the reduction of thinning provoked by NAC supplementation is most likely caused by the decreased bacterial viability rather than an antioxidant effect. If the majority of Db11 is dead, they are not secreting hemolysin thereby preventing the extrusion to take place, so the midgut epithelium remains thick. This is also supported by the fact that Vit. C and DTT, which have no effect on the cytoplasmic purge, did not affect bacterial viability (Figure 1.11 & 1.13B).

3.4.3 Impact on survival

Despite the fact that Db11 resist well the immune response in the midgut, it is not extremely lethal in an oral infection, killing around 50% of the flies after 15 days on average. We were surprised to find that adding NAC to sucrose solution was more lethal to the flies than bacterial infection (Figure 1.14). We can speculate that NAC supplementation in normal conditions impairs some basic homeostatic functions such as ISCs activity, resulting in a decreased survival. Indeed, ROS have been shown to be a direct or indirect regulator of stem cell proliferation and differentiation (Biteau et al., 2008; Buchon et al., 2009b; Lee, 2009; Owusu-Ansah and Banerjee, 2009; Xu et al., 2017). Moreover, basal level of ROS are activated by microbes naturally present in the gut and are required to limit their uncontrolled proliferation (Ha et al., 2009c). Therefore, NAC can unpair this control on the microbiota, generating a dysbiosis. The latter can also arise from the bactericidal effect of NAC, which may kill some key commensal bacteria provoking potentially dangerous imbalances. It would be worth checking if we can detect variations in the fly's microbial community and monitoring stem cell activity after NAC feeding. In this light, the increased survival in flies fed with Db11+NAC was unforeseen in a certain way. We might expect that two stimuli that decrease host's lifespan would have an additive phenotype, further increasing mortality. Conversely, we hypothesized that NAC and Db11 neutralized each other:

the chemical compound kills the majority of bacteria, that in their turn can metabolize it to less harmful molecules.

3.5 Mitochondrial involvement in the early stages of Db11 infection

3.5.1 H_2O_2 levels in mitochondria

A functional approach by means of exogenous antioxidants could be useful to evaluate the effects on the thickness of the epithelium. However, conclusions about an implication of ROS in cytoplasmic purge can only be obtained by direct measurement of these. During my PhD we have been testing many probes before arriving at reliable and reproducible results. The fluorometric H_2O_2 assay gave us a stable and straightforward method to estimate H_2O_2 total content. This allowed us to detect an increase in hydrogen peroxide in midguts of flies exposed for 30 minutes to Db11 (Figure 1.15B). Moreover, this protocol can be easily applied to different genetic backgrounds, permitting to address contribution of specific proteins and pathways in H_2O_2 production (Figure 1.16). Still, this procedure presents some limitations, since it is a disruptive method that homogenizes putative differences between cellular compartments and various gut regions, in addition to intermix cells with the gut content. So far, the most insightful method to quantify ROS dynamics has been the use of roGFP2 probes. Being ratiometric and genetically encoded, they overcome key issues as probe absolute quantity, cellular uptake and photobleaching. Secondly, they can be expressed in distinct cell compartments and coupled to different proteins to provide specificity towards a given redox couples or species. These represent crucial advantages for accurate *in vivo* experiments. In our study, roGFP2 probes empowered not only the discrimination of cytoplasmic- or mitochondrial-localized ROS increase, but also a clearer view of ROS dynamics in the early phase of cytoplasmic purge. The glutathione pool is not altered in either of these two compartments, since Grx1-roGFP2 showed no increase in oxidation after the bacterial challenge (Figure 1.15A). In contrast, Orp1-roGFP2 pointed-out an increase in the 405nm/488nm ratio in mitochondria but not in cytoplasm. This observation is supported by the fact that cytosolic and mitochondrial H_2O_2 levels are differently regulated: in general, cytosol is less sensitive to changes, most likely because of its larger volume and abundance of scavengers (Morgan et al., 2016). The slight increase rather than an important change in H_2O_2-mediated oxidation fits well with a signaling function and not an oxidative stress. The latter would be expected during infection with *Ecc15* (Buchon et al., 2009b; Ha et al., 2009c) but we did not detect it most likely because we were looking at a very early time point. Of note, we use *Ecc15* as an additional positive control for bacterial infection, and this condition contained less data points than the others, therefore interpretations must be made with caution. Also, we encountered issues with the cytoplasmic-Orp1-roGFP2 probe, since it did not

respond very well to oxidation: the dynamic range is narrower compared to the others roGFP2 versions (Figure 1.15A). Collectively, we conclude that there is a quick raise in H_2O_2 levels after Db11 feeding, which is occurring in mitochondria and without altering the E_{GSH}. Megamitochondria formation might therefore be linked to this, although mitochondrial fusion seemed regulated by an increase in GSSG (Shutt et al., 2012) that we did not observe. At present, we cannot affirm if in our conditions megamitochondria formation is a direct consequence of increased ROS in the matrix: we should decrease oxidation in a chemical or genetic manner and check if giant mitochondria are still present. Complementarily to this, a time course with more time points might reveal an alteration of GSH pool later on. However, it has been reported that megamitochondria formation is aimed to reduce the intracellular ROS content by decreasing oxygen consumption (Wakabayashi, 2002). Strikingly, mitochondrial fusion is not necessary to trigger the epithelial thinning (Lee et al., 2016), and ROS alone are not sufficient. In fact, *Ecc15* infection does not result in a thin epithelium, and this observation is further supported by the fact that also H_2O_2 feeding does not cause any change in the morphology of the enterocytes (data not shown). A possible explanation might be that ingested H_2O_2 is scavenged in the intestinal lumen or in the cytosol without reaching the mitochondria. In parallel, giant mitochondria may represent just a by-product of an increased oxidative state and not a real determinant in the onset of cytoplasmic extrusion.

3.5.2 Mitochondrial ROS scavengers

Beyond mitochondrial fusion and H_2O_2 increase in these organelles, additional results further support their implication in the enterocyte purge response. Supplementation of infection solution with the mitochondrial superoxide scavenger mitoTEMPO reduced the epithelial thinning in a significant manner (Figure 1.17A). This represent a quite direct evidence of mitochondrial ROS requirement for an effective cytoplasmic extrusion. To strengthen this conclusion, we should play with overexpression of mitochondrial matrix antioxidants such as Mn-SOD, mitochondrial-targeted catalase or peroxiredoxins and observe the consequences on enterocytes thinning. Also, it would be worth to verify if fusion still occurs upon administration of mitoTEMPO. There is also proof of a relationship existing between mitochondria and Nrf2 system (Itoh et al., 2015): since they both are involved in the cytoplasmic purge, we could perform some epistatic analysis to investigate this crosstalk. Finally, the fact that *DuOx* knockdown or IRC overexpression in the enterocytes did not fully abolish the H_2O_2 increase (Figure 1.16) further support the hypothesis of a mitochondrial redox signalization.

3.6 A role for DuOx-IRC tandem?

3.6.1 DuOx influences epithelial thinning and host survival

In *Drosophila* midgut, Dual Oxidase (DuOx) play an essential role in activating an oxidative burst aimed to eliminate pathogens or control dietary and commensal microbes (Ha et al., 2009b, 2009c; Kim and Lee, 2014). Since NADPH oxidases can be protagonist in redox signaling mechanisms (Figure 1.5C), we asked whether DuOx can be also involved in redox signaling leading to cytoplasmic extrusion. Remarkably, we found that one RNAi strain, *DuOx^{RNAi} WJL*, greatly abolished the epithelial thinning induced by Dbl1 (Figure 1.18), and the same phenotype is obtained upon overexpressing IRC (Figure 1.20). These results confirmed that these two proteins have complementary roles in ROS homeostasis (Ha et al., 2005a), and point out a possible implication of DuOx in our resilience mechanism. Moreover, another member of our team showed that knockdown of *Atf2*, which control *DuOx* expression pathway (Ha et al., 2009b), also abolished the enterocyte thinning (Bechara Sina-Rahme, personal communication). However, the conclusions cannot be so simple, because other data seem to indicate otherwise. Indeed, another RNAi strain, *DuOx^{RNAi} GD* (VDRC #2593) presented an enterocyte thinning similar to controls. Of note, the *Control^{RNAi} GD* displayed a better thickness score than the *Np* driver alone. In any case both *DuOx^{RNAi} GD* and *Control^{RNAi} GD* displayed a majority of semi-thin epithelia with a relevant part of thin ones (Figure 1.18A). Additionally, *DuOx^{RNAi} WJL* and *DuOx^{RNAi} GD* behaved differently in a survival assay (Figure 1.19A). While the first showed an improved survival upon Dbl1 ingestion compared either to WT flies or to *Control^{RNAi} GD*, *DuOx^{RNAi} GD* was not more resistant to *S. marcescens* infection, and to some extent even more sensitive compared to *w^{A5001}*. A factor that can explain this difference in terms of survival is the Dbl1 load, which was reduced in the *DuOx^{RNAi} WJL* line (Figure 1.19B). Nevertheless, results obtained with *DuOx^{RNAi} GD* have to be considered as preliminary and interpreted with caution, since they have not been repeated many times.

3.6.2 Differences between *DuOx^{RNAi}* lines

Notwithstanding, this apparent paradox prompted us to meditate about possible explanations. Both lines were functionally validated, since they were equally more sensitive to *Ecc15* infection and embryonic lethal (data not shown). Therefore, the difference resides elsewhere than in RNAi efficiency. *DuOx^{RNAi} GD* has a putative off target in the gene coding for *rotund*, a developmental transcription factor (Del Alamo and Mlodzik, 2008; Li et al., 2013), but we do not know whether this could affect the cytoplasmic purge and Dbl1 survival. Off-targets in *DuOx^{RNAi} WJL* line also remain a possibility: sequencing of both lines would elucidate these inconsistencies. Another prospect are variations in the microbiota of the two RNAi lines, which might to some extent be related to the difference in the Dbl1 midgut titer. It would be interesting to characterize the respective intestinal bacterial communities and

their relative abundance, by plating and sequencing. Actually, past PhD student in the team demonstrated that microbiota did influence the cytoplasmic purge, hence the presence/absence of given commensal may account for the difference between *DuOx*^{RNAi} *WJL* and *DuOx*^{RNAi} *GD*. Since the individual microbiota bacterial species able to affect the response to Dbl1 have yet to be identified, this kind of experiments would further elucidate the relation between cytoplasmic purge and microbiota.

3.6.3 The role of Immune Regulated Catalase

IRC overexpression did not increase host survival (Figure 1.20D); this could mean that Dbl1-induced lethality is not due to oxidative burst related damage. However, the role of this protein deserves to be discussed in more detail. The name IRC (Immune Regulated Catalase) was given by the laboratory of professor Won Jae Lee based on: i) the expression of this protein is under the control of immune pathways (from here the name "immune regulated") and ii) the purified protein is able to degrade H_2O_2 (hence the name "catalase"). Nevertheless, sequence and phylogenetic analysis revealed that IRC is a heme-peroxidase rather than a catalase (Hughes, 2012). Indeed, heme-peroxidases also react with H_2O_2 causing its reduction into water, but unlike catalases they generate a radical substrate. In this light, how do we interpret the results with the IRC overexpression? If we assume that IRC scavenges H_2O_2, that is required for signaling, then we can have an explanation for the lack of thinning. Also, a secreted heme-peroxidase (IMPer or Hpx15) have been shown in mosquito midgut to collaborate with *DuOx* in the formation of a barrier that limits the contact between host's cells and pathogens (Kumar et al., 2010). If this were true also in *Drosophila*, the reduction of hemolysin attack on enterocytes may represent an alternative explanation for the absence of thinning of the *UAS-IRC* flies. Indeed, cooperation between NADPH oxidases and heme-peroxidases have been shown in various metazoans: hydrogen peroxide produced by the first is used by the second to exert a given biological function (Sirokmány and Geiszt, 2019). Therefore, this opens the possibility that DuOx and IRC may work together and not imperatively as antagonists as in (Ha et al., 2005b). On the other hand, H_2O_2 detoxification by the heme-peroxidase may result in formation of harmful radical, and this would justify why we did not see an improvement of flies' survival. We remarked that H_2O_2 is still induced in infected *UAS-IRC* flies (Figure 1.16B), but the difference with uninfected ones is less pronounced (Figure 1.16C; the same is observed for *DuOx*^{RNAi}, see paragraph below). This means that IRC scavenges H_2O_2, at least in part, or that this strain presents unpaired ROS production. In parallel, it is worth to note that the overexpression control line (*UAS-GFP*) displayed an odd phenotype: they did not have the H_2O_2 increase that we observed in the other lines (Figure 1.16B), they displayed a reduced epithelial thinning (Figure 1.17B) and were also extremely resistant to bacterial infection (Figure 1.20D). We speculate that again this can be linked to microbiota and/or to the different genetic background. Obviously, it will be important to clarify the influence of those potential perturbators to obtain less variability and unequivocal readouts.

3.6.4 ROS levels in *DuOx*^{RNAi}

In a similar manner, result from H_2O_2 measurement in DuOx strains are equally puzzling (Figure 1.16A). It seemed that all strains still produce H_2O_2 to some extent during Dbl1 infection: it is clear for *DuOx*^{RNAi} *WJL*, and also *DuOx*^{RNAi} *GD* showed a trend although the difference with sucrose treatment is not statistically significant. This may indicate that mitochondria, rather than DuOx, are the source of H_2O_2, supporting the data obtained with the roGFP2. In fact, it is very unlikely that DuOx-derived H_2O_2 would affect the probe in mitochondria; instead, we should have expected an oxidation increase in the cytoplasmic-Orp1-roGFP2 or cytoplasmic-Grx1-roGFP2. Still, we noted that the difference between infected and non-infected condition is less important in both *DuOx*^{RNAi} than in *Control*^{RNAi} (Figure 1.16C). Hence, we cannot totally exclude that DuOx contributes somewhat to overall H_2O_2 production, also considering the striking thinning phenotype of *DuOx*^{RNAi} *WJL* and *UAS-IRC*. It is an open possibility that both mitochondria and DuOx contributes to ROS signaling (Figure D1), and a good experiment to figure that out would be to monitor H_2O_2 content in presence of mitoTEMPO in a *DuOx*^{RNAi} and WT background. Alternatively, we can also hypothesize another role for DuOx outside the microbicidal oxidative burst.

3.6.5 Catalase: a possible target to explore

Finally, peroxisomal catalase (*Cat*) also provides food for thoughts. We observed that overexpression of this protein in enterocytes drastically reduced H_2O_2 content in enterocytes either in infected or non-infected condition (Figure 1.16B). *Cat* mainly reduce cytosolic H_2O_2, so potentially also the mitochondrial ROS secreted into the intermembrane space by complex III (Figure 1.1 & 1.2A). At present we cannot be certain whether *Cat* has a direct role in the epithelial thinning. We observed that flies overexpressing *Cat* in the enterocytes underwent a normal epithelial thinning, but the experiment was performed only once (Figure 1.17B). Therefore, there is need to confirm this result also having a reliable positive control beside. In the survival assay (Figure 1.20D) infected flies underwent a first phase of death that reaches a plateau after the first week. In a pure hypothetical way, we may think that the initial lethality is perhaps due to a lack of cytoplasmic purge, that renders the bacteria more harmful, followed by a stabilization in survival curve once the infection is cleared. Also, *Cat* might be beneficial at the beginning of the infection possibly limiting ROS-derived damages, but in the long term this action turns noxious altering normal host homeostasis.

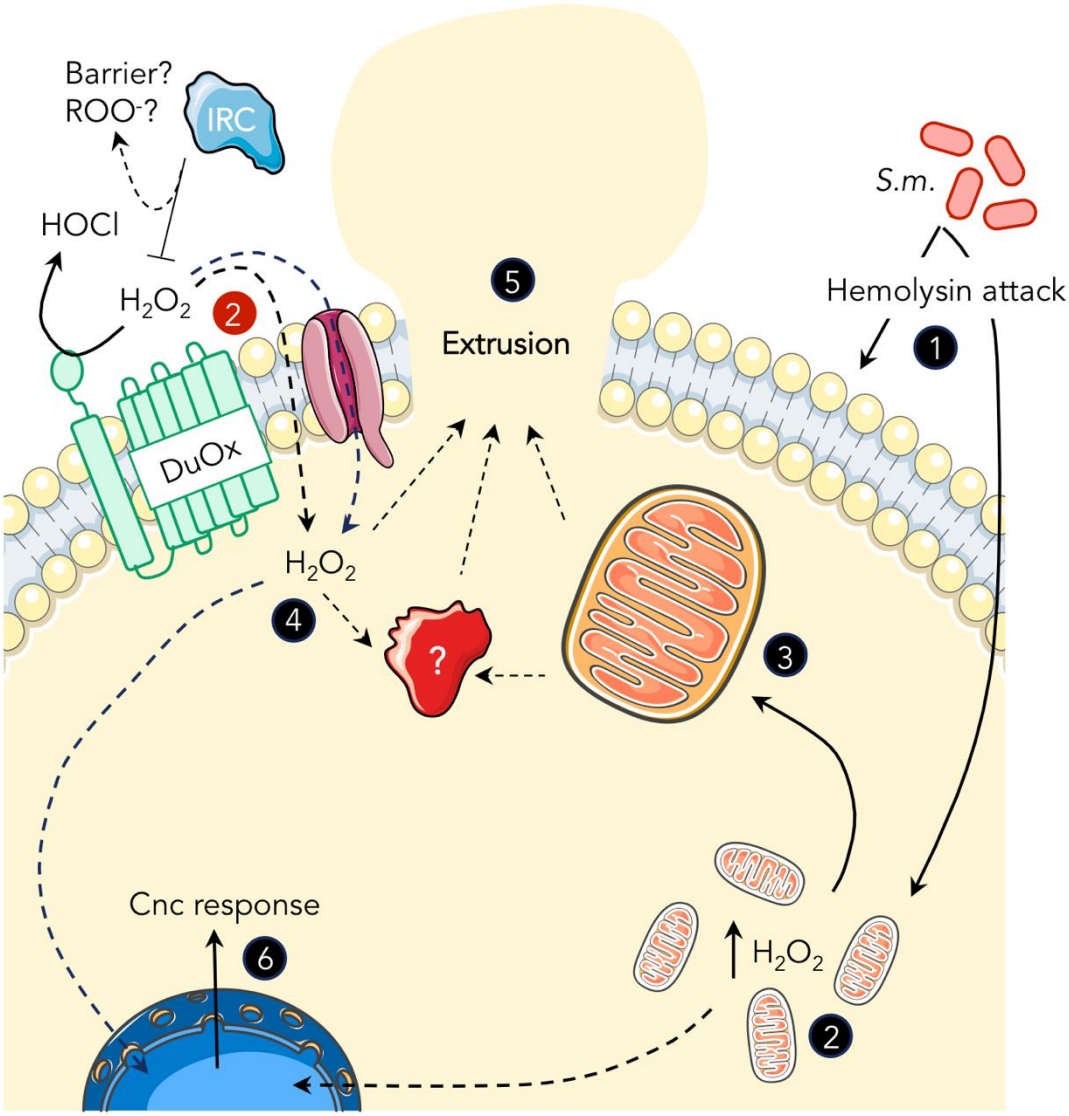
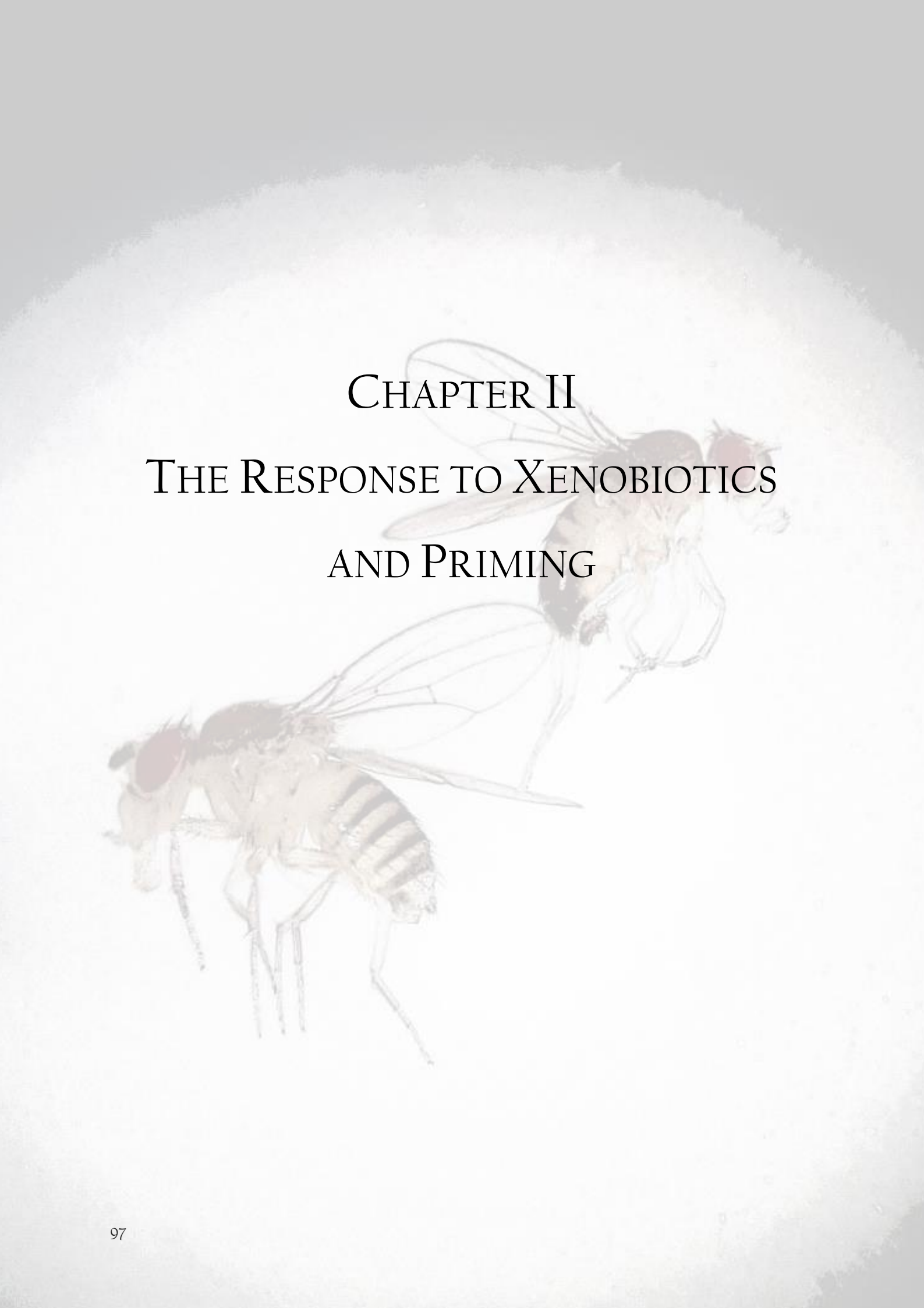


Figure D1. Schematic hypothesis of ROS dynamics during the early stages of *S. marcescens* infection. After Db11 ingestion, the pore forming toxin hemolysin attacks the enterocytes of the host **(1),** forming tiny pores in the plasma membrane and possibly reaching the cytoplasm and the mitochondria through OMVs (Bielaszewska et al., 2013). Bacterial infection causes rapid H_2O_2 increase in mitochondria **(2),** which accumulates in the apical part of the enterocytes and fuse forming megamitochondria **(3)**. Alternatively or simultaneously, ROS generation is induced by the Dual Oxidase DuOx **(2)**. H_2O_2 produced by this enzyme can be transformed in HOCl or enter the cell by diffusion or via aquaporins. Immune Regulated Catalase (IRC) scavenges hydrogen peroxide but may also produce some peroxyradical ($ROO^{\cdot}$) or strengthen the intestinal mucus barrier. The H_2O_2 gradient formed beneath the membrane **(4)** by one or both pathways can induce cytoskeletal and membrane reorganization, directly or indirectly, leading to aperture formation and subsequent cytoplasmic extrusion **(5)**. Lately, an antioxidant response is activated around 6h after Db11 ingestion **(6)** to restore cellular ROS homeostasis.

3.7 Conclusions and future directions

In conclusion, ROS, precisely H_2O_2-mediated signaling, appeared to be important in the steps preceding the cytoplasmic extrusion. Data obtained with the roGFP2 probe indicate mitochondria as the source of this signaling. Megamitochondria formation by fusion, mitoTEMPO effect on the epithelial thickness and RNAi knockdown experiment can be seen as further confirmation of mitochondria involvement. However, at present we cannot totally exclude an implication of DuOx during the cytoplasmic purge. Indeed, it is true that we still see H_2O_2 induction in *DuOx*^{RNAi} and in *UAS-IRC* flies, but the difference in H_2O_2 content between infected and uninfected is less remarkable. We also established that *DuOx*^{RNAi} *WJL* and *UAS-IRC* flies do not undergo epithelial thinning. Interestingly, they both are localized around the apical part of the enterocytes, where the extrusions subsequently take place. This supports the hypothesis that a local signal via H_2O_2 triggers the formation of the apertures, directly or indirectly, which allow the leaking of cytoplasm (Figure D1). To better understand the whole process, we consider expressing the roGFP2 probe in a DuOx mutant background, and to target catalase expression in mitochondria: this will provide decisive information about the site of H_2O_2 production. We could also check if there is a transcriptional activation of DuOx after Db11 challenge. It would also be interesting to investigate in depth the role of antioxidants systems. Since GSH dynamics does not seem to be involved, Prxs presumably have a great relevance: their genetic modulation may deeply affect the epithelial thinning extent. Prxs have been shown to enable thiol switches in proteins (Stöcker et al., 2018): this imply that they are not only scavengers, but also effective transmitters of H_2O_2 signaling. This provides a relevant alternative to direct H_2O_2 action in orchestrating protein thiol oxidation. In addition, Prxs in mitochondria (Prx3 & Prx5) are important to regulate immune hyperactivation, apoptosis and thereby lifespan (Odnokoz et al., 2017). Recently, a more sensitive H_2O_2 sensor has been developed coupling roGFP2 to the yeast Prx Tsa2 (Morgan et al., 2016). Generation of transgenic flies carrying this sensor may allow us to detect finest changes in H_2O_2 homeostasis during the first steps of the cytoplasmic purge. Additionally, microbiota and ROS dynamics are related (Ha et al., 2009b; Lee and Lee, 2014), a possibility that we can explore through the generation of axenic roGFP2 flies. It is also urgent to comprehend the discrepancies between *DuOx*^{RNAi} *WJL* and *DuOx*^{RNAi} *GD*, which may reside in the midgut microbial composition. Finally, NAC has to be used very cautiously for antioxidants purposes, especially in studies involving bacterial infections. Although NAC is quite commonly used, its bactericidal effect that we highlighted here can bring to misleading conclusions.



Chapter II

The Response to Xenobiotics and Priming

1. Introduction

1.1 Definition and risks for human health

Xenobiotics are defined as chemical compounds foreign to or not produced by an organism. The word derives from the Greek ξένος (xenos)=foreigner and βίος (bios)=life, plus the suffix -τικός, -ή, -όν (-tikos, -ē, -on). This definition encompasses a very vast range of compounds, that can be beneficial or harmful for animals or plants, although the negative connotation of this term is more common. Indeed, many of them constitute a danger for living species and related ecosystems, having direct or indirect consequences on human health and well-being: pesticides, environmental pollutants, drugs, metals and toxins produced by other organisms (biological toxins) are some examples. However, a small fraction of these toxicants is part of the normal metabolism. For instance, the endogenous histamine is not much different from the one that is found in the bee venom, and iron, which is an essential atom for life, it is not considered a xenobiotic despite that it can cause intoxication in infants. Xenobiotics are introduced in the body by ingestion of contaminated food and water (absorption in the case of plants), or by air, in the case of volatile compounds. Major sources are industrial waste (pharmaceutical, fossil fuels, paper bleaching), sewage overflow, sewage treatment plants, resource extraction, land disposal, agriculture, urban runoff, atmospheric deposition, construction and habitat modification (Ritter et al., 2002). Living organisms can also constitute a relevant source: bacteria, fungi and plants secrete biological toxins such as botulinum toxin, T-2 toxin and ricin, which are a real danger for health (Janik et al., 2019). Obviously, this is not an exhaustive list. Many xenobiotics resist to degradation and accumulate in the environment, and because of their toxicity, they pose a danger to human health. Indeed, they can be cytotoxic or trigger diverse pathologies (Bjorklund et al., 2018; Cave et al., 2016; George et al., 2017). Xenobiotics ingestion is also intimately related to the microbiota, influencing their metabolism and their detoxification, thereby affecting the onset of gut pathologies and cancer (Clarke et al., 2019; Nogacka et al., 2019). The intestinal microbial community interacts with xenobiotics in multiple manners: activation/inactivation of compounds, preventing their absorption and affecting the expression of genes involved in detoxification (Collins and Patterson, 2020). The relationship is also true the other way around, since xenobiotics can modify the composition of the microbiota with the consequences attached to it.

1.2 Detoxification

Xenobiotics introduced in the body may have four possible destinies: unchanged elimination, unaffected retention, spontaneous chemical transformation or enzymatic metabolism (Caldwell et al., 1995). Highly polar compounds are often eliminated untouched with urine, whereas hydrophobic compounds are eliminated unchanged in the feces. A few compounds such as mirex and cadmium can be retained unchanged in the adipose tissue and in the bones, respectively (Croom, 2012). Even if spontaneous dismutation is possible, the mechanism that accounts for the elimination is through enzymatic pathways (Figure 2.1).

In mammals, the major organ designed to noxious xenobiotic detoxification is the liver, even if minor transformation occurs also in digestive tract, lung, kidneys and brain. Here, the hepatic enzymes take care of the deactivation and subsequent excretion of the dangerous molecules via urine, feces, breathe or sweat. As mentioned in the previous chapter, xenobiotic metabolism is divided in three stages (Figure 2.1). Phase I reactions aim to make toxic molecules less harmful or more soluble, and usually the first reaction is performed by an enzyme catalyzing redox reactions. The most studied are cytochrome P450 monooxygenases (CYPs). This superfamily of hemeproteins includes numerous subfamilies and isoforms; they are ubiquitous from bacteria to humans where they are found in all organs, with a particular enrichment in liver and small intestine. Functionally, CYPs can be divided in two groups: the ones that detoxify xenobiotics such as pharmaceutical drugs, pesticides, carcinogenic agents and pollutants, and the ones that are involved in biosynthesis processes, e. g. bile acid and steroid hormones (Manikandan and Nagini, 2017). Flavin-containing monooxygenases (FMOs) are other phase I enzymes catalyzing oxygenation reactions; unlike CYPs, they display more specificity for the substrate and are not regulated transcriptionally by the xenobiotics (Croom, 2012). In addition to these two superfamilies, we can list other phase I enzymes: hydrolases, amine oxidases, proteases, and dehydrogenases such as alcohol dehydrogenase (ADH), aldehyde dehydrogenase (ALDH) and glucose-6-phosphate dehydrogenase.

The activity of phase I enzymes can generate products that are still toxic and difficult to excrete. Therefore, there is need of phase II enzymes for the inactivation of pharmacologically active substances and for the transformation into more excretable forms. This biotransformation, catalyzed by transferases, is made via conjugating reactions, including glutathionylation, glucuronidation, methylation, acetylation, sulfation, transpeptidation. Phase II enzymes have been much less considered than phase I enzymes for clinical and pharmacological purposes, despite reducing phase II detoxification leads to increased drug intoxication (Jancova et al., 2010). A very important group in phase II enzymes, GSTs, has been presented in the previous chapter. Besides glutathione conjugation, GSTs also catalyze isomerization, nucleophilic aromatic substitutions and hydroperoxides reduction. This antioxidant role is key to buffer the oxidation products derived from CYPs and from more oxidase's activity. Finally, the conjugated products are excreted through passive or active transport in

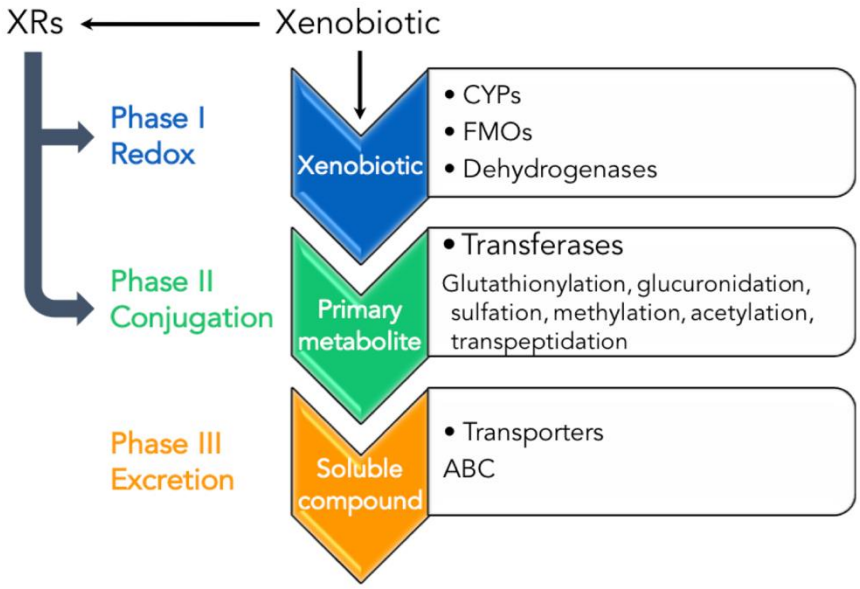


Figure 2.1. General features of enzymatic xenobiotic detoxification. Once they have entered the cell, xenobiotics are metabolized in a three-step process. The goal of the phase I is to generate products that are less harmful for the cell. These reactions are generally redox reactions, and they are catalyzed by large enzyme superfamilies, mainly cytochrome P450 (CYPs), flavin-containing monooxigenase (FMOs) and dehydrogenases. Nevertheless, the secondary products can still be toxic. Therefore, phase II enzymes inactivate these molecules by addition of chemical group. This is the task of transferases, that make the xenobiotic-derived products less reactive and more soluble. Finally, those compounds are excreted outside the cell and the organism. The main role in this phase III is therefore carried by transporters.
Xenobiotics also bind to the xenobiotics receptors (XRs) on the nuclear surface. This recognition activates cellular processes that aim to improve the detoxification; therefore, they upregulate phase I (CYPs) and II enzymes.

the urine, bile or air. This represents the phase III of detoxification process, and it is mediated by transporters: this function is principally fulfilled by ABC transporters (Nakata et al., 2006). Transcription of phase I and II detoxification enzymes and transporters are under the control of a subset of nuclear receptors called xenobiotics receptors (XRs). XRs have been attracting attention during the last two decades for their importance in the coordination of detoxifying responses in humans following pharmaceutical drugs and environmental pollutants accumulation (Wang and LeCluyse, 2003). The best known and most studied are the Constitutive Androstane Receptor (CAR), the Pregnane X Receptor (PXR), and the Aryl hydrocarbon Receptor (AhR). Despite the fact that each one of these can act individually, a substantial crosstalk occurs between them (and in general between all XRs) in multiple ways: the activation of one influences the activity of the other, and they share ligands and target genes (Mackowiak and Wang, 2016).

1.3 In insects

Looking at insect biology, detoxification in these species acquire relevance in two main contexts: adaptation towards feeding and breeding sites and pesticide metabolism.

Many insects have direct interactions with plants, and this implies the need to tolerate not only potential harmful microorganisms, but also toxic compounds produced by the plant for its own defense. The principal detoxification pathways in *Drosophila* are the same as in mammals, with the conservation of key phase I and II enzymes such as CYPs, GSTs, UDP glycosyltransferases and esterases (Good et al., 2014; Low et al., 2007; Luque and O'Reilly, 2002; Oakeshott et al., 1993). Some *Drosophila* species have evolved adaptation mechanisms towards new plant hosts, by overexpressing not only the xenobiotic detoxification genes but also others related to energy production, carbohydrate metabolism, structural elements and mRNA binding (Matzkin, 2012). In some cases, a single gene is required for detoxification of a given compound, like the gene *Osi6* conferring resistance against the toxic defense molecule octanoic acid (Andrade López et al., 2017). The central role of CYPs and GSTs has also been described in honeybees (Berenbaum and Johnson, 2015; Gong and Diao, 2017). Many studies have supported the correlation between CYPs upregulation and development of insecticide resistance (Liu et al., 2015b). In a few cases, the precise enzyme that confers resistance to a known insecticide has been found within this large family, as in the case of CYP6G1 for DDT resistance in *D. melanogaster* field populations (Le Goff and Hilliou, 2017). Understanding of these precise interactions between single xenobiotics and CYPs/GSTs genes has a great importance in control of insect pest or in slowing down the destruction of insect communities as in the colony collapse disorder (Glavan and Bozic, 2013; Gong and Diao, 2017). In addition to phase I and II detoxification enzymes, resistance to xenobiotics is also due to their accumulation and to the target sites sensitivity. In this light, a relevant role is played by ABC transporters superfamily (Buss and Callaghan, 2008; Wu et al., 2019), which have been associated with resistances in various insect species. In *D. melanogaster*, they are crucial both in multiple pesticide metabolites elimination (Denecke et al., 2017; Sun et al., 2017), and in plant secondary metabolite detoxification (Groen et al., 2017). Nuclear hormone receptors count 18 members in *Drosophila*, some of which are orphans receptors (Hoffmann and Partridge, 2015). Many of them have role in development and metamorphosis (Fisk and Thummel, 1995; King-Jones et al., 2005; Kozlova et al., 1998). The most studied in the context of xenobiotic detoxification is DHR96, the *Drosophila* ortholog of human SXR and CAR (Afschar et al., 2016; King-Jones et al., 2006). Of note, as previously said in the former chapter, also Nrf2/CncC is a master regulator of detoxification genes (Harvey et al., 2009; Hoffmann and Partridge, 2015; Itoh et al., 1997, 2015; Misra et al., 2011).

1.4 Caffeine

Caffeine (1,3,7 trimethylxantine) is a purine alkaloid, which are secondary metabolites of many plants (Ashihara et al., 2008); the most famous belong to the genus *Coffea*. First isolations of caffeine were made in independent manners by Friedlieb Ferdinand Runge in 1819 (who called it *Kaffebase*) and by Pierre-Jean Robiquet, Pierre-Joseph Pelletier and Joseph Bienaimé Caventou in 1821. The term caffeine derives from the French word for coffee, *café*, as it was used for the first time by Pelletier in the "Dictionnaire de médecine" in 1822. Although the coffee plant is the most notorious source, caffeine occurs naturally in around 60 species of plant. Among the other best-known sources, we can mention tea leaves, cocoa beans, kola nuts (from which the term "cola" derives), yerba mate and guarana berries, representing an extraordinary example of convergent evolution (Denoeud et al., 2014). The caffeine content in beverages fluctuates widely according to the source used and the preparation method; generally, a 30mL expresso contains around 60mg of caffeine.

Legends tell that the first uses of caffeinated drink date back to the Chinese Empire around 3000 BC, although historical evidence appeared only in the 15th century in the city of Mocha in Yemen. From there, coffee drinking spread to Middle East, then to Italy and the rest of the Europe, which bring it all over the world through colonization. Today, caffeine is the most widely consumed psychoactive drug in the planet, with diverse country-level consumption based on customs and traditions (Reyes and Cornelis, 2018). Considering only the US, nearly 90% of the population consume food or drinks containing caffeine, with an approximate average intake of 180mg/day (Frary et al., 2005; Fulgoni et al., 2015). Caffeine is mostly consumed for its booster properties, to enhance attention, wakefulness, focus and memory. Behavioral modifications and neuropsychic symptoms are observed after an exaggerated use or in sensitive subjects. For this high impact on human's life, caffeine has been extensively studied: searching the entry word "caffeine" in PubMed results in more than 30.000 publications.

1.4.1 Caffeine metabolism

CYP1A2 is the main enzyme accounting for caffeine metabolism (Figure 2.2), catalyzing the demethylations that assure the majority of its clearance (>90%) (Miners and Birkett, 1996). This isoform further contributes to the transformation of these primary products (paraxanthine, theobromine, theophylline), although other enzymes (CYP3A4, N-acetyltransferase2, xanthine oxidase) can support in minor part the formation of secondary metabolites. Finally, these products are degraded via the classical purine catabolism and excreted in urine.

1.4.2 Molecular targets

The caffeine molecule has a purine structure; therefore, it is related to adenine and guanine bases present in DNA and RNA (Figure 2.2). In addition to being a component of nucleic acids, adenosine (adenine + ribose) is also a neuromodulator in the CNS, with sedative-like properties. Four receptors that have been identified so far (A_1, A_{2A}, A_{2B}, A_3), each one with particular tissue distribution, signaling pathway and pharmacological profile (Landolt, 2008). Adenosine is an upstream inhibitor of the release of many others neurotransmitter such as glutamate, serotonin, acetylcholine, noradrenaline and dopamine (McLellan et al., 2016). Therefore, the physiological effect of caffeine depends largely on the action of these neurotransmitters (Figure 2.3). Due to structural similarities, caffeine works as an antagonist blocking A_1 and A_{2A} receptors at low to moderate doses (Fredholm, 1995). Moreover, those receptors also form functional heterodimers with the dopamine receptors D_1 and D_2, respectively (Ferré, 2016). A single nucleotide polymorphism (C→T) in the gene coding for the A_{2A} receptor (*ADORA2A*) has been associated with caffeine consumption, sensitivity and development of insomnia and anxiety (Childs et al., 2008; Cornelis et al., 2007; Yang et al., 2010).

At higher concentrations, methylxanthines also inhibits phosphodiesterase's activity (Boswell-Smith et al., 2006; Daly, 2007), blocks $GABA_A$ receptors and induces intracellular Ca^{2+} release through ryanodine receptors (RyR) in muscles and neurons (Daly, 2007; Guerreiro et al., 2008, 2011) (Figure 2.3). Those effects may be independent from adenosine receptors binding (Guerreiro et al., 2008), even if *in vivo* those consequences are likely influenced by neuromodulation that occurs at lowers concentrations of substrate. Nevertheless, some molecules (ATP, cyclic ADP ribose, acyl-CoA esters) can sensitize RyR to other agonists, reducing the activation threshold (Guerreiro et al., 2011).

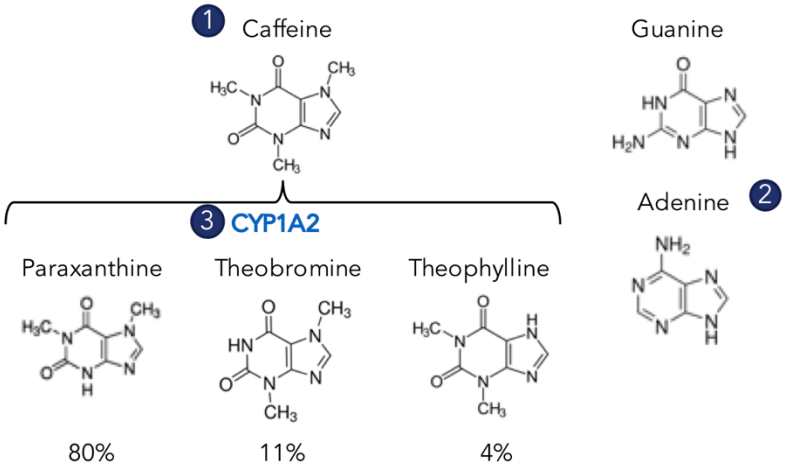


Figure 2.2. Caffeine metabolism. (1) 1,3,7-trimethylxanthine (caffeine) is an alkaloid with a purine-like structure, very similar to guanine and adenine **(2)**. It is rapidly absorbed through the midgut epithelium, entering the circulation and spreading all over the body. **(3)** It is principally metabolized in the liver by the enzyme CYP1A2. This CYP isoform catalyzes the demethylation of caffeine in three different positions, generating either paraxanthine, theobromine or theophylline. The relative abundances in humans are shown below. CYP1A2 is equally responsible of further demethylation of this secondary metabolites, that are then excreted or recycled by the cell via the purine metabolism.

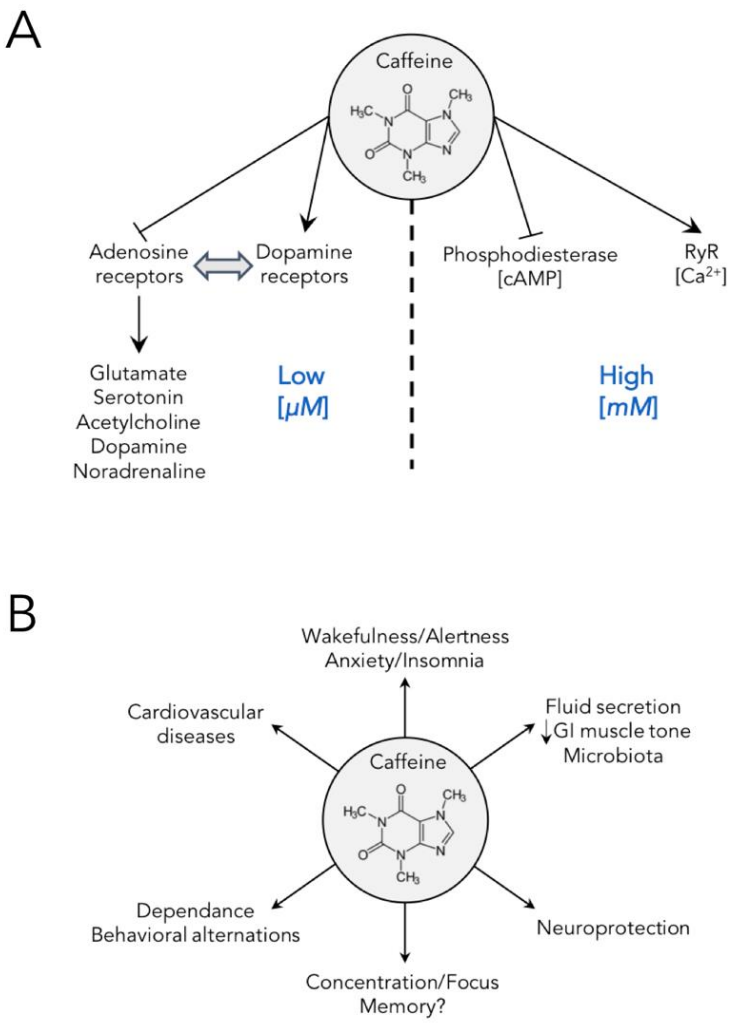


Figure 2.3. Molecular and physiological effects of caffeine. (A) Molecularly, caffeine acts primarily as an antagonist on the adenosine receptors at very low concentrations (left panel). This prevents the endogenous ligand, adenosine, to carry out its neuromodulator function. Indeed, adenosine is the upstream regulator of many neurotransmitters. It can also act directly on the dopamine receptors, which in their turn forms heterodimers with adenosine receptors. At higher concentrations (right panel), caffeine inhibits the function of phosphodiesterases, thereby provoking an increase of cAMP, an important second messenger. At the same time, caffeine activates ryanodine receptors (RyR), inducing a release of Ca^{2+} from the ER into the cytoplasm. This can have important physiological consequences especially in neural transmission and muscle contraction. **(B)** Physiologically, effects of caffeine on adenosine receptors produce many neural outcomes. Caffeine-containing beverages are principally consumed for their enhancement of wakefulness and alertness sensations, or their positive impact in cognitive functions such as concentration and memory. However, in susceptible individual caffeine can cause unpleasant side-effects as anxiety and insomnia. Generally, caffeine produces many behavioral troubles that are different from one individual to another, and in some cases can generate dependence. Nevertheless, caffeine is considered as a positive element to introduce in the diet, as it can be neuroprotective and brings benefits also in many cardiovascular troubles, in addition to the neural enhancement cited just above. In the digestive tract, it may induce fluid secretion and a reduced muscle tone, however it seems to modify the microbiota promoting growth of beneficial species.

1.4.3 Relevance to human health

Caffeine consumption has been widely explored in relation to cardiovascular diseases, although the conclusions are puzzling (Cornelis and El-Sohemy, 2007; Riksen et al., 2009). The difficulty to draw a definitive verdict is maybe due to a background noise brought by other key factors that are associated with caffeine consumption. Indeed, the latter can be viewed as a marker of lifestyle, and it is often intimately associated with potential confounders such as smoking, sex, age, diet, medical history, physical activity and residence area (Doepker et al., 2016). However, as in the case of adenosine receptors, genetic polymorphisms can account for individual differences relating caffeine and cardiovascular diseases. A→C SNP in the position 163 of the *CYP1A2* gene is associated with reduced metabolism (Castorena-Torres et al., 2005). A risk of myocardial infarction is associated with the 163C allele (Cornelis et al., 2006) and the slow metabolizers have also more risk to develop hypertension (Palatini et al., 2009). Of note, it also significant to remark that caffeine-containing beverages such as coffee and tea also have antioxidants compounds, which can be beneficial for the cardiovascular system (Cornelis and El-Sohemy, 2007). The influence of caffeine on pregnant women was also widely debated, but moderate consumption does not seem to be harmful for reproductive and perinatal outcomes (Brent et al., 2011; Bull et al., 2015). Given the influence of 1,3,7-trimethylxantine on CNS, it has also been studied in the context of neurological syndromes, showing a potential neuroprotection against Parkinson's disease (Soliman et al., 2018) and other neurodegenerative diseases as well (Oñatibia-Astibia et al., 2017).

In summary, the mechanism of action of caffeine by antagonizing the adenosine A_1 and A_{2A} receptors plus the interaction with dopamine receptors is well established, and the positive effect of a moderate consumption (~300mg/day) in healthy individuals on physical and cognitive functions are widely accepted (Davis et al., 2003; Lieberman et al., 1987; McLellan et al., 2016; Pickering and Grgic, 2019; Pray et al., 2014). In relation to the large variety of health outcomes (Figure 2.3), integration of caffeine in the diet is generally seen as a positive factor (Grosso et al., 2017). To observe some toxicity of caffeine intake, it is necessary to reach levels higher than 1.5g per day; caffeine overdose occurs at really high doses, around 10g per day, that would be the equivalent of 100-200 cups of coffee (Higdon and Frei, 2006; Nawrot et al., 2003).

1.4.4 Effects on the gastrointestinal tract

Despite the great interest received for its neuromodulator properties, the effect of this purine alkaloid on the intestine has been much less studied. Nevertheless, it deserves more attention, since oral administration is the only way by which caffeine is taken. Following ingestion, it is rapidly absorbed through the gastrointestinal tract, as soon as 45 minutes (Arnaud, 2011), rejoining the concentration peak in plasma within two hours (Kamimori et al., 2002; Skinner et al., 2013). It can cross the blood brain barrier, and its half-life in circulation is about 3-5 hours (McLellan et al., 2016). Early

studies reported an increase in small intestine secretions as soon as 15 minutes after caffeine ingestion (Wald et al., 1976). These observations are likely to correlate with a direct effect on the intestinal epithelium rather than the consequences of caffeine absorption and distribution throughout the body. In young rodents, caffeine treatment promotes gastric smooth muscle relaxation via RyR-mediated Ca^{2+} release, decreasing gastrointestinal muscle tone and motor function (Tokutomi et al., 2001; Welsh et al., 2015). According to Cui et al., 2020, caffeine also interacts with many pathways that play a role in colorectal cancer, influencing intestinal homeostasis and immune system. These effects are attributable to three cellular mechanisms induced by caffeine exposed above (Figure 2.3), that is binding on adenosine receptor, stimulation of intracellular Ca^{2+} efflux and accumulation of cyclic nucleotides due to phosphodiesterase inhibition. Caffeine, together with polyphenols found in many caffeinated beverages, also improves diet-induced metabolic syndrome (Gao et al., 2018). This is caused by the reduction of local inflammatory markers but especially by the modulation of the gut microbiota. Notably, caffeine containing drinks decrease *Firmicutes/Bacteroidetes* ratio that is associated with obesity and high-fat diet; moreover they also increase the numbers of *Bifidobacterium*, which is reputed for its beneficial properties (Jaquet et al., 2009; Pan et al., 2016). However, despite several studies report the effect of caffeinated beverages on the microbiota mainly in various pathological conditions, there is almost no report that describes the direct effect of caffeine on the intestinal microbial community.

1.4.5 Relevance in insects

The physiological role of caffeine has long remained unclear. Since it is present in high concentrations in leaves, fruits and flower buds and its degradation is slow, it has been proposed that this molecule serves as a defense against herbivores and pathogens (Ashihara et al., 2008). Indeed, it was previously reported that caffeine was toxic to insects by inhibition of phosphodiesterase activity (Nathanson, 1984). In support of this, caffeine feeding has been showed to increase the cAMP levels in *Drosophila* brain (Wang et al., 1998). Despite toxicity, in insects caffeine shows phenotypes similar to those observed in mammals. Accordingly, the best characterized phosphodiesterase gene in *Drosophila*, *dunce*, has been established as key actor in memory and learning (Qiu and Davis, 1993; Scheunemann et al., 2018). In addition, caffeine acts as a competitive inhibitor of phosphodiesterases in many other insects (Mustard, 2014). *Drosophila* exposed to caffeine decreased the visual learning performance (Folkers and Spatz, 1984; Wang et al., 1998). However, lower doses (µM range) enhance the responses of olfactory and memory neurons in honeybees, improving the capacity to remember and locate floral scent (Wright et al., 2013). The development of olfactory long-term memory has been associated with a Ca^{2+} increase (Perisse et al., 2009), which might be mediated by RyR, present in insects as well (Collet, 2009; Ebbinghaus-Kintscher et al., 2006; Lehmberg and Casida, 1994). This double effect is reminiscent of what is observed in mammals, where caffeine intake on learning and memory has conflicting and not fully clarified effects (Nehlig, 2010). Another aspect in common with mammals is the boost of activity

and alertness, which are reflected in phenotypes linked to locomotion and sleep cycles. Correspondingly, caffeine potentiates motility, sensory responses and behaviors in *Vespa orientalis* and *Apis mellifera* (Ishay, j. Paniry, 1979) and alters sleep patterns and circadian rhythms by strengthening wakefulness in fruit flies (Hendricks et al., 2000; Shaw et al., 2000; Wu et al., 2009).

Since binding to adenosine receptors occurs in the micromolar range, it is thought that this is the main molecular pathway that explains the caffeine effects in mammals (Fredholm, 1995; McLellan et al., 2016). A unique adenosine receptor (AdoR) has been identified and characterized in *Drosophila* (Dolezelova et al., 2007). Interestingly, using a *Drosophila* neuroblast cell line, other authors confirmed the increase in intracellular cAMP but not the raise in Ca^{2+} levels, and they showed that caffeine did not antagonize AdoR signaling (Kucerova et al., 2012). Furthermore, caffeine still induced sleep disorders in AdoR knockout flies, and a non-selective phosphodiesterase inhibitor mimicked caffeine action on sleep and circadian period (Wu et al., 2009). However, others observed that sleep loss is equally induced after adenosine receptor agonists (Andretic et al., 2008). Although partially controversial, these results suggest that in insects caffeine may act mainly through phosphodiesterases rather than adenosine receptors. Nevertheless, it may play a role in the nervous system via dopamine receptors. We said above how adenosine and dopamine receptor directly interacts in mammals (Ferré, 2016), and they can also share second messengers (cAMP) downstream. It has been showed that dopamine receptor DA1 mediates caffeine-induced wakefulness and arousal (Andretic et al., 2008); here, it seems that caffeine decreases the DA1 expression in the mushroom bodies in order to induce the psychostimulants effects, and DA1 overexpression result in caffeine resistance. Conversely, in honeybees dopamine receptor D2 is overexpressed after caffeine treatment (Kucharski and Maleszka, 2005). Further evidence of dopaminergic neurons involvement for caffeine induced wakefulness in an adenosine-independent manner has been reported more recently (Nall et al., 2016). In summary, these observations suggest that caffeine interferes with the insect nervous system, although further research is needed to draw clearer conclusions.

Compared to mammals, information about caffeine absorption and metabolism are more limited in insects, yet the importance of CYPs is well established (Coelho et al., 2015). Theobromine appears to be the major metabolite in flies, whereas it is paraxanthine in humans. It has also been shown that caffeine upregulates some CYPs isoforms (*Cyp12d1*, *Cyp6a8*) and some GSTs (*GstD2*) (Bhaskara et al., 2006; Willoughby et al., 2006); one isoform of CYP, *Cyp9f2*, is induced also in honeybees. A large study aimed to identify QTLs determining xenobiotics resistance, used caffeine as a model compound (Najarro et al., 2015): screening of more than 1700 genotypes led to identification 10 QTLs, among which *Cyp12d1* was the most relevant and was further functionally validated. Besides, some studies underline the importance of the gut microbiota for caffeine degradation. The coffee berry borer *Hypothenemus hampei* complete all its life cycle into the coffee fruit, and it has been demonstrated that this is possible

by virtue of a core microbiota composed by Pseudomonadales, Rhizobiales, Enterobacteriales and Actinomycetales (Ceja-Navarro et al., 2015). Indeed, it was already shown that bacteria and fungi (mainly *Pseudomonas* and *Aspergillus*) can degrade caffeine through the action of demethylases and oxidases (Gokulakrishnan et al., 2005). Also *Serratia marcescens* owns this capacity (Mazzafera et al., 1996).

Finally, parameters to be carefully considered in all studies is the concentration of caffeine used and the exposure time. Many of the apparently contradictions cited before may arise from biphasic effects at low versus high concentration, that in turn maybe caused by the different doses at which caffeine acts on neurons, phosphodiesterases or intracellular Ca^{2+} release. A further issue of studies in insects is represented by the quantity of caffeine ingested. Since they perceive caffeine as repellent (Glendinning, 1996; Sellier et al., 2011), protocols involving oral feeding may lead to concentrations low than expected.

1.5 Ethanol

Ethanol (EtOH or simply alcohol) is a two-carbon alcohol with the chemical formula C_2H_6O. It is volatile, flammable and colorless liquid at room temperature, with a wide range of applications: chemical solvent, antiseptic, fuel. Here we discuss the importance of EtOH as a relevant xenobiotic for humans, since it is the psychoactive substance present in alcoholic drinks. Voluntary alcohol intake is perhaps second only to caffeine: according to World Health Organization, in 2016 the worldwide consumption was equal to 6,4L of pure alcohol per person older than 15 (Figure 2.4). This is the equivalent of 53 bottles of wine per year per person, that is almost a liter per week (*ourworldindata.org/alcohol-consumption*). There is a marked geographical difference in alcohol consumption (Figure 2.4) and in the preferred EtOH source, mainly spirits, beer or wine, and man are more likely to drink than women. In alcoholic drinks, EtOH is the result of microbial fermentation (mainly yeast, Figure 2.5A) of sugar sources (generally grains, fruit or vegetables). The history of alcoholic drinks goes back to very ancient times: the existence of intentionally fermented beverages is traced back to the discovery of stone age jugs in Neolithic period. First scientific evidences of alcoholic drinks are dated seven thousand years BC in northern China (McGovern et al., 2004). Medieval Arabs used and described the distillation process in detail, and from Middle East alcohol consumption spread throughout Europe around the 14th century. Nowadays, despite its legality in almost all countries and widespread use as a recreational drug, alcohol consumption is one of the most relevant risk factors for premature death. The institute of Health Metric and Evaluation estimates to 2,84 million the number

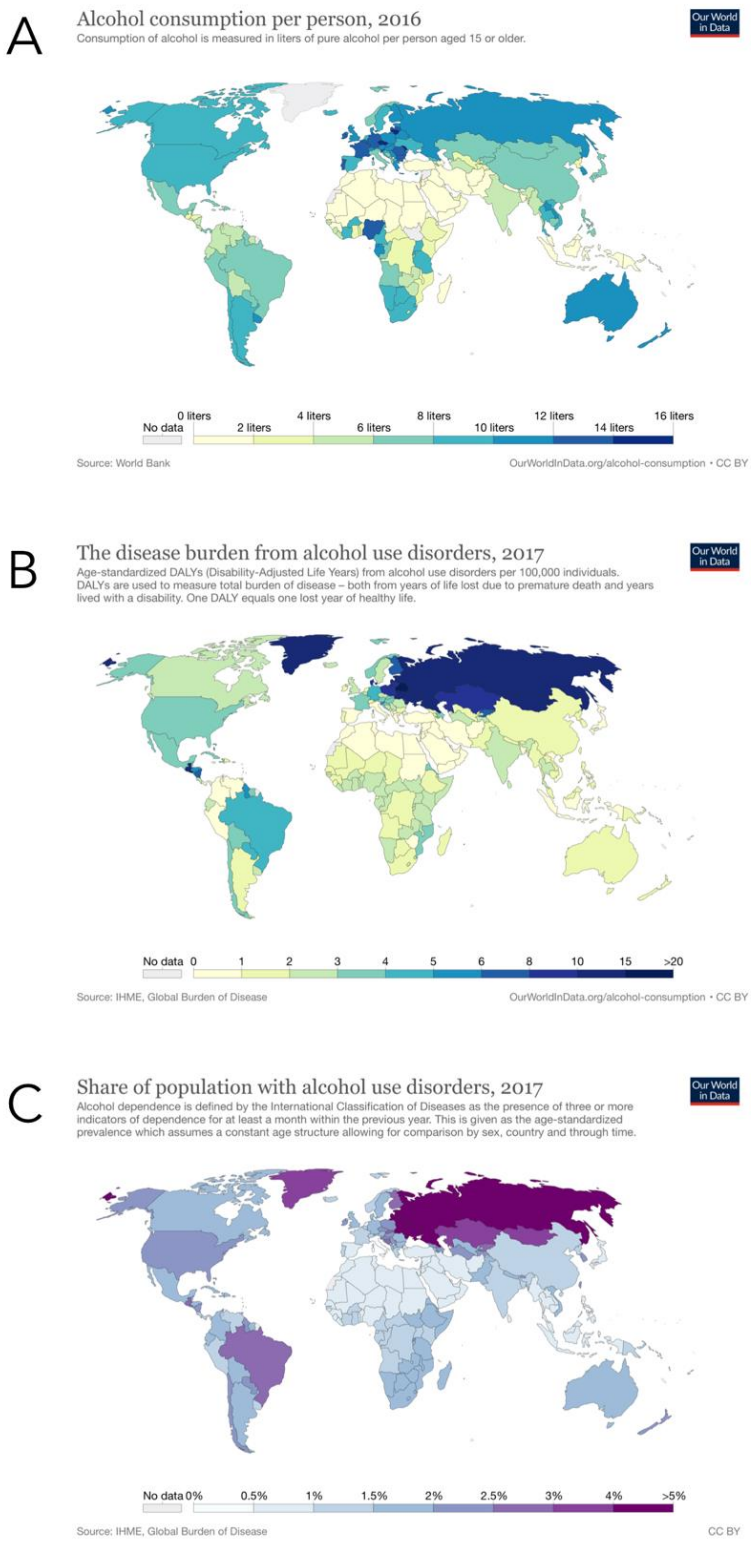


Figure 2.4. Alcohol consumption and associated disorders worldwide. (A) In 2016, the global average of alcohol consumption is 6,4 liters per person. This is reported in liters of pure alcohol to consider the differences in alcohol content of different drinks. Whereas the consumption is close to zero in North Africa and Middle East, Western countries are the major consumers, especially Europe. **(B)** This chart displays the total impact of alcohol consumption on health, taking into account premature deaths and years lived with a disability/disease. **(C)** It is estimated that around 1,4% of population deal with an alcohol use disorder. The prevalence is higher in people aged between 25 and 34.

of deaths due to alcohol use (and abuse) in 2017 (Stanaway et al., 2018). Around 1,4% of the world's population have an alcohol-related disorder (Figure 2.4).

EtOH is a CNS depressant: at low doses it causes symptoms like euphoria, decreased anxiety, improved sociability, sedation and impairment of cognitive, motor and sensory functions; at higher doses, it provokes drunkenness, unconsciousness, stupor and even death. Prolonged use in humans can lead to dependence, as in alcoholism. Although some authors described protective effects for light alcohol consumption (Bergmann et al., 2013; Le Daré et al., 2019), there is a general consensus about the harmfulness of high and long term EtOH intake (Le Daré et al., 2019).

1.5.1 Effect on CNS

Acute exposure to alcohol is well described, and the mechanisms are closely related of those of chronic intoxication (Figure 2.5B). EtOH directly affects ligand-gated ion channels and voltage dependent calcium channels on neuron's surface, thereby modulating the excitability of these. For instance, EtOH potentiates GABA gated currents by increasing the frequency and the duration of channel opening (Davies, 2003). Moreover, the putative sites for EtOH binding on $GABA_A$ and glycine receptor have been identified (John Mihic et al., 1997). Since $GABA_A$ have inhibitory function, the enhancement induced by alcohol accounts for the depressant effect on CNS. EtOH action on $GABA_A$ integrates many of alcohol induced physiological effects (Allen et al., 2018). However, this is far to be the only consequence of EtOH in nervous system. It is able to modify synaptic plasticity in many brain's regions (Lovinger and Abrahao, 2018), and to act as an antagonist of different glutamate receptors (NMDA, AMPA, kainate) (Lack et al., 2008). Given that glutamate is an excitatory neurotransmitter, this contribute to EtOH depressant action on CNS (Le Daré et al., 2019).

1.5.2 EtOH consumption and diseases

Chronic alcohol exposure can result in a very vast array of pathologies (Figure 2.5B), affecting various organs such as the liver, the nervous system, the digestive tract and the cardiovascular system. As the majority of xenobiotics, EtOH is metabolized principally in the liver. Therefore, it is not surprising that alcohol severely affects this organ. Liver injury progresses in three phases: in the first, chronic EtOH intake induces accumulation of free fatty acid in hepatocytes (steatosis), as a consequence of increased lipolysis in the adipocytes (Wei et al., 2013). In the second phase, steatohepatitis, an inflammatory state is established in the liver: the resident macrophages (Kupffer cells) are activated, immune cells (mainly neutrophils) infiltrate in the tissue, pro-inflammatory cytokines are released, lipids are subjected to oxidative stress and undergo peroxidation (Osna et al., 2017). These circumstances trigger important cellular damage and eventually hepatocytes cell death. The last phase is the fibrotic progression, where scar tissue replaces liver cells that have lost the ability to regenerate. This condition is also known as cirrhosis and can lead to further complications such as

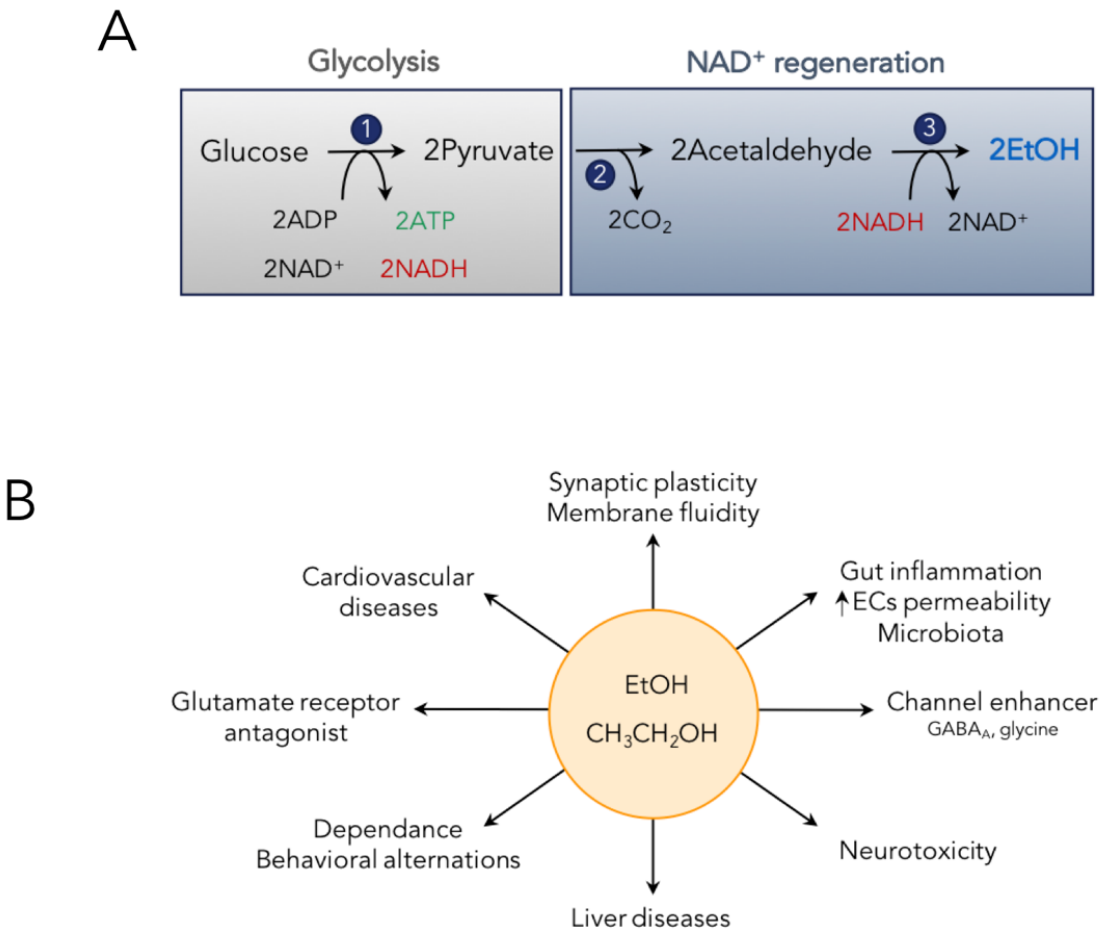


Figure 2.5. Alcoholic fermentation and molecular and physiological effects of EtOH. (A) Alcoholic fermentation is a biochemical process present in yeast, some bacteria and a few other microorganisms. The overall process converts sugars into EtOH, producing CO_2 and regenerating oxidized cofactor NAD^+. (1) One glucose molecule is converted in two pyruvates during glycolysis, with net production of two ATP molecules and two reduced NADH. In the second step (2), the enzyme pyruvate decarboxylase allows the exit of CO_2 from pyruvate, with the formation of acetaldehyde, that is finally reduced into EtOH by ADH (3), regenerating oxidized NAD^+. **(B)** Given the high solubility, low molecular weight and the high permeability through biological membranes, EtOH consumption has a broad range of consequences. Acute EtOH intoxication is biphasic, with excitatory effects at low doses but sedative when the concentration increases. These phenotypes, together with neurotoxicity, dependence and behavioral alterations result from neuronal modulation. EtOH acts as an agonist for some neurotransmitter receptors, influences the neuron excitability by strengthening the opening of ion channels or modifies synaptic connections. As it is almost exclusively metabolized in liver, it can damage this organ to different extents, including steatosis, steatohepatitis or ultimately fibrosis. Chronic high EtOH consumption equally affects cardiomyocytes, provoking cardiovascular diseases. Alcohol promotes an inflammatory state in the gastrointestinal tract. EtOH or its metabolites stimulates immune cells, and they disrupt epithelial integrity through direct effect on enterocytes, promoting loss of mucosa, apoptosis, and destabilization of cellular junctions. Modifications of microbiota are also a consequence of prolonged alcohol intake, often inducing dysbiosis and additional side effects on immune system and body homeostasis.

hepatocellular carcinoma (Osna et al., 2017). Given the broad impact of EtOH on neurons, it is not surprising that alcohol and its metabolites cause neurotoxicity, reducing grey and white matter volume (Bühler and Mann, 2011) and impairs neuropsychic performance (Konrad et al., 2012). Neurotoxicity is also enhanced when the liver function is impaired (Chen et al., 2012), as a consequence of reduced EtOH detoxification in hepatocytes. Alcohol-induced neurodegeneration is closely related to this liver-brain axis, as inflammatory mediators and lipid toxic by-products derived from hepatic misfunction directly impact neurons and glia (De La Monte et al., 2009). Although low or moderate consumption is associated with lower risk of heart failure, heavy drinking has been correlated with cardiomyopathy, cardiac arrythmia, hypertension, atherosclerosis and heart failure (Obad et al., 2018). Albeit not fully understood, chronic high EtOH intake impairs cardiomyocyte function and promote myopathies characterized by hypertrophy and myofibrillar abnormalities, and can also trigger cardiomyocytes apoptosis (Fernández-Solà et al., 2006). Hallmarks of alcohol induced tissue damage are increase of inflammatory markers, oxidative stress, mitochondrial dysfunction and ER stress (Obad et al., 2018). `

1.5.3 Action in gastrointestinal tract

Intestinal responses to EtOH consumption are particularly relevant, as intestinal epithelium is the first tissue in contact with anything ingested. It has been shown that alcohol disrupt intestinal homeostasis in different manners, provoking an inflammation state locally but also systemically (Patel et al., 2015). The inflammation derives from EtOH metabolism, that leads to increased permeability of the gut, microbiota modifications with bacterial overgrowth and dysbiosis, and alteration of the mucosal immune system (Bishehsari et al., 2017). Inflammation and increased permeability have been detected in non-cirrhotic alcohol-dependent patients (Leclercq et al., 2012). EtOH and its metabolites alters the expression levels and promotes redistribution of tight junction proteins such as ZO-1, occludine and claudin, in addition to cytoskeletal components, via pathways involving phosphatase 2A or MAPKs (Dunagan et al., 2012; Elamin et al., 2013a, 2014b; Ying et al., 2014). This higher permeability allow the passage of LPS and peptidoglycan through the gut barrier, activating a systemic immune response (Leclercq et al., 2014). Furthermore, alcohol consumption directly affects enterocytes, by inducing apoptosis (Pijls et al., 2013) or epithelium loss at the villi and damage of the mucosa (Rocco et al., 2014). Another important consequence of prolonged high EtOH exposure is the alteration gut microbiota. It has been shown by many that alcoholics have perturbations in the microbiome, with general increase of titer, changes in bacterial equilibrium and dysbiosis (Canesso et al., 2014; Engen et al., 2015; Mutlu et al., 2012). Decrease of "beneficial" species like *Lactobacillus* and *Bifidobacterium* contributes to local and systemic inflammation; moreover, since intestinal bacteria can metabolize alcohol, the overall increase in titer may rise the quantity of further pro-inflammatory metabolites (Zhong and Zhou, 2014).

1.5.4 Metabolism

EtOH is rapidly absorbed in the upper digestive tract (stomach, duodenum, jejunum) as it crosses passively biological membranes down its concentration gradient. Therefore, the higher the concentration of alcohol, the faster is the absorption (Cederbaum, 2012). EtOH metabolism begins already in the stomach, where some forms of alcohol dehydrogenase (ADH) are present. This step is important because it regulates the bioavailability of alcohol in circulation; with an empty stomach, alcohol absorption and passage into duodenum is more rapid, consequently concentrations in plasma are higher (Cederbaum, 2012). However, alcohol metabolizing enzymes are much more abundant in liver than in stomach, indicating that most of the alcohol metabolism takes place in that organ.

The oxidative pathway accounts for the majority of EtOH metabolism (Figure 2.6). The phase I detoxification enzymes involved in the process are ADH, CYP2E1 and catalase (Elamin et al., 2013b). ADH is the master alcohol metabolizing enzyme: it exists in ten isoforms grouped in five classes, and it catalyze the oxidation of EtOH to acetaldehyde using $NADP^+$ as cofactor (Cederbaum, 2012; Elamin et al., 2013b). The different isoforms differs in cellular localization and kinetic properties, and even though polymorphisms have been described, they do not seem to be related to any disease or metabolic change (Le Daré et al., 2019). CYP2E1 is responsible for about 10% of alcohol metabolism at low concentration, but becomes more active when EtOH levels raise (Cederbaum, 2012; Lieber, 1997). This pathway is mainly localized in hepatocytes ER, using NADPH and oxygen to oxidize EtOH to acetaldehyde, and producing ROS as a by-product (Lu and Cederbaum, 2008; Rashba-Step et al., 1993). Like many CYPs, CYP2E1 is not specific to EtOH but it has many substrates (acetone, benzene, other alcohols). However, chronic EtOH treatments promotes its upregulation via decrease in proteolytic proteasome activity (Lu and Cederbaum, 2008). Peroxisomal catalase may also detoxify alcohol in presence of H_2O_2, although this reaction does not appear relevant in physiologic conditions (Cederbaum, 2012). To a minor extent, EtOH is also metabolized by non-oxidative pathways (Figure 2.6), by conjugation (glucuronidation and sulfation) and formation of phosphatidylethanol and fatty acid esters by phospholipase D and fatty acid ester synthase respectively (Cederbaum, 2012; Le Daré et al., 2019; Schröck et al., 2018). Acetaldehyde produced from alcohol oxidation is further oxidized into acetate by acetaldehyde dehydrogenases isoforms. The mitochondrial ALDH accounts for the majority of the reaction, but the cytosolic form also contributes (Sophos and Vasiliou, 2003). Generally, most acetaldehyde is removed and its levels in circulation are very low, but when accumulated it causes unpleasant effects typical of alcohol intoxication. Effective acetaldehyde removal prevents this symptoms and cellular toxicity, and keeps efficient alcohol detoxification as acetaldehyde is itself an inhibitor of ADH (Cederbaum, 2012). Additionally, it can cause several types of tissular damages, by mitochondrial dysfunction, formation of protein and DNA adducts, impaired redox state and increase in gut permeability and leakiness (Dunagan et al., 2012; Ekstrom et al., 1986; Malaguarnera et al., 2014; Marshall et al., 2018; Rao, 2008).

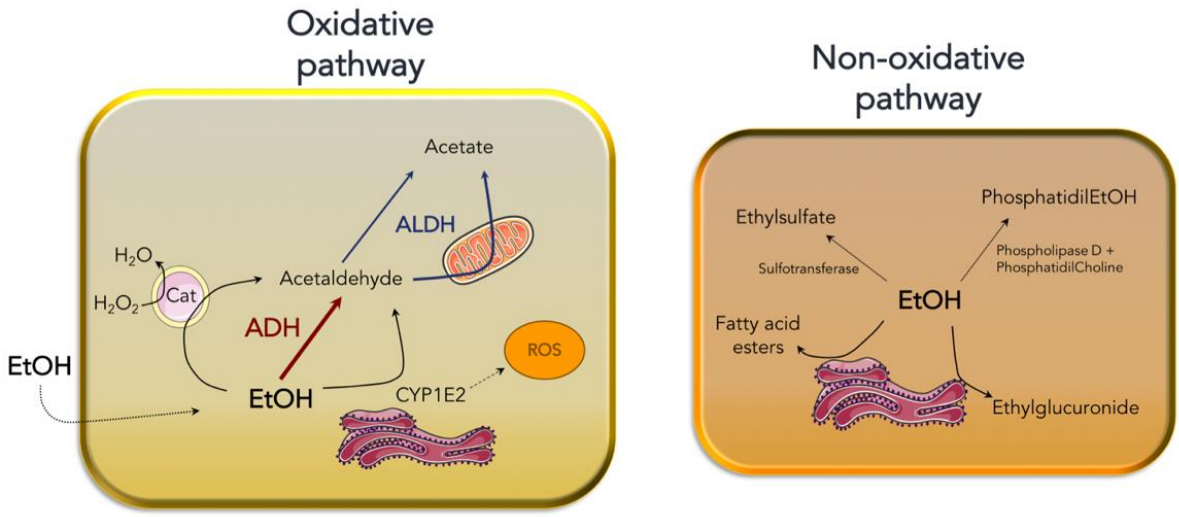


Figure 2.6. Alcohol metabolism. EtOH metabolism occurs essentially in the liver, and it can be divided into oxidative and non-oxidative pathways. In the former (left panel), that accounts for the majority of EtOH detoxification, the alcohol that has spread to the cell is mostly oxidized (90%) into acetaldehyde by alcohol dehydrogenase (ADH). To a lesser extent, this oxidation is made by CYP1E2 in the ER, a process that may generate ROS as by-product. In presence of H_2O_2 also peroxisomal catalase maybe a catalyst, although this reaction has very little physiologically relevance. Acetaldehyde is then further oxidized to acetate in the cytoplasm or in the mitochondria by acetaldehyde dehydrogenase (ALDH). Acetate is majorly converted into acetyl-CoA by acetyl-CoA synthetase, which enters the Krebs cycle, or it is used in fatty acid and cholesterol biosynthesis. The non-oxidative pathway (right panel) canalizes EtOH into conjugation reactions. Phosphatidylation, sulfation or glucuronidation form products that are readily excreted. Fatty acid esters are formed in the ER by fatty acid ethyl ester synthase. When oxidative metabolism is blocked, there is an increase of fatty acid esters, that are toxic to the cell. They are removed through binding to lipoproteins or albumin and released into the circulation.

1.5.5 Relevance to *Drosophila melanogaster*

Because of the facilities available in *Drosophila*, and the conservation of both metabolic, molecular and behavioral responses to alcohol, this animal have been a very valuable model to study these dynamics (Guarnieri and Heberlein, 2003; Kaun et al., 2012; Scholz and Mustard, 2013).

Since naturally they feed on rotten fruits, flies are exposed to EtOH very often, as a result of microbial fermentation in those substrates. Moreover, EtOH vapor is used by flies as a long-distance cue to trace fruit crops (Dudley, 2002). *D. melanogaster* is well adapted to this ecology, since it displays a high alcohol tolerance compared to other species: females prefer to lay eggs in alcohol containing media, and larvae can use EtOH as a food (Geer et al., 1993; McKenzie and Parsons, 1972). Indeed, conversely to caffeine, alcohol is an attractant to flies, which prefer to consume EtOH containing food over regular one, showing several features of alcohol addiction (Devineni and Heberlein, 2009; Ogueta et al., 2010). Moreover, EtOH protects flies from parasitic wasp infection: contaminated larvae actively seek out for alcohol-containing food, which enhance the chances of survival working as an anti-wasp medicine (Milan et al., 2012). However, despite the high tolerance, EtOH increases larval mortality when it reaches a critical concentration (Heinstra et al., 1987); these effects are in close relationship with the ADH genotype. Like almost all organisms, also in flies alcohol is metabolized mainly by this enzyme, and ADH mutants accumulates more EtOH than WT flies (Wolf et al., 2002). ADH accounts for 91-93% of EtOH degradation in flies, and this enzyme can also catalyze the oxidation of acetaldehyde into acetate, in parallel to acetaldehyde dehydrogenase (Geer et al., 1993). EtOH-induced phenotype in fruit fly is evocative of the one observed in mammals: low doses of alcohol stimulates locomotion and hyperactivity in a first phase, whereas EtOH accumulation (by higher doses, prolonged exposure or impaired detoxification) are sedative and impair coordination (Singh and Heberlein, 2000; Wolf et al., 2002). Impaired ADH function suppresses the locomotor activating effects, increases the sensitivity and sedative effects, and abolishes the preferences for low EtOH containing food (Ogueta et al., 2010; Wolf et al., 2002). Repeated exposure to alcohol lead to resistance to the locomotory symptoms, in mammals as in *D. melanogaster*, in what is called alcohol tolerance (Scholz et al., 2000). Interestingly, tolerance did not seem to be mediated by absorption or metabolism, but rather by responses taking place in nervous system. It has been shown that honeybees also readily consume EtOH and they can withstand quite high doses, showing behavioral alterations analogous to flies (Abramson et al., 2000; Maze et al., 2006).

Some of the molecular and cellular mechanisms involved in these changing have been elucidated. For instance, EGF and cAMP pathway confers resistance to the EtOH sedation (Corl et al., 2009; Moore et al., 1998), whereas PI3K/Akt/insulin pathway increase sedation sensitivity (Corl et al., 2005). Further evidence came from the identification of *arouser*, a gene that promotes EGF pathway activation and PI3K inhibition, promoting sedation resistance (Eddison et al., 2011). Moreover, mutation in *arouser*, as well as mutation in the neuropeptide *amnesiac*, expands the number of synapses,

another mechanism that correlate with increased sensitivity (Eddison et al., 2011). In support of this hypothesis, it has been found that the gene *hangover*, which is required for development of EtOH tolerance (Scholz et al., 2005), also negatively regulates synapses formation in larvae (Schwenkert et al., 2008). Abundant findings in the literature further support the importance of synaptic transmission in alcohol-induced behaviors, since modulation of key genes as *syntaxin1A*, *shibire*, *synapsin* and *homer*, determines sensitivity or tolerance (Kaun et al., 2012). Transcriptional and translation regulators, such as the previously mentioned *hangover*, *dLmo* or *krasavietz* are involved in these phenotypes as well (Devineni and Heberlein, 2009; Lasek et al., 2011). Neuronal excitability involved in EtOH-induced behaviors is linked with K^+ channels, as demonstrated by inhibition of the gene *slowpoke* (Cowmeadow et al., 2005) or by the use of an antagonist and an RNAi knockdown of the $GABA_B$ receptor, that is metabotropically coupled to K^+ channels (Dzitoyeva et al., 2003). Octopamine, a neuromodulator analog to noradrenaline, is equally a key mediator to develop tolerance (Scholz et al., 2000, 2005). Also the dopamine system is central governor of many EtOH-related behavior, in mammals as in flies (Kaun et al., 2011; Kong et al., 2010; Söderpalm et al., 2009). In those neurons, microtubules dynamics orchestrated by the protein Tao are required for alcohol-induced hyperactivity (King et al., 2011). Microtubules are not the only cytoskeleton element that is important for responses to EtOH. *krasavietz* governs actin organization by interacting with the crosslinking protein Short Stop (Lee et al., 2007), and in parallel actin regulation by the Rho GTPase activator protein 18B have been involved in both EtOH-induced hyperactivity and sedation (Rothenfluh et al., 2006).

1.6 Priming the intestinal epithelium against sustained attacks

As described in the paragraph 3.3 of the general introduction, in the laboratory we have discovered a new resilience mechanism in the *Drosophila* midgut, the cytoplasmic purge (Lee et al., 2016). During the work that led the team to the description of this phenomenon, some interesting features have been remarked. First, we obtained evidence that *CycJ* mutants do not recover the initial epithelial morphology after 24h or even more after Db11 ingestion; second, we know that this gene mediates the recovery through the expression of *whe* class of genes (Lee et al., 2016). With the intention of inducing continuous cycles of purge, we realized that the WT flies did not undergo epithelial thinning if infected a second time in the days following the first challenge.

Moreover, large-scale genomic data available on Flybase indicate that the top four *CycJ*-dependent genes induced by hemolysin are all strongly induced in flies exposed to xenobiotics such as soft metals (Cu, Zn), heavy metals (Cd), caffeine, EtOH, and paraquat. Following the ingestion of caffeine, preliminary data showed megamitochondria formation and some cytoplasmic extrusion, which was then followed by recovery. We observed *CycJ*-dependent induction of *CG1678* and *lcs* by

caffeine, hence the name *what else (whe)* we gave to *CG1678*. *CycJ* mutants succumbed earlier to caffeine ingestion. We have also observed similar dynamics after exposure to the other xenobiotics, including EtOH. Thus, enterocyte purge and recovery may also protect the organism to some extent against some noxious xenobiotics. Importantly, these observations suggest that a common process is triggered by xenobiotics and hemolysin and thus initiates enterocyte purge. Based on all these observations, we hypothesized the existence of a priming mechanism, a form of memory of prior exposures to infectious stress. This might also be true for a first exposure to xenobiotics. There is controversy on the use of the term "memory" to describe the ability to use information on a previous encountered stimulus upon a secondary exposure, since this term was until recently restricted to the immune memory of the adaptive immunity in vertebrates. Actually, the existence of "adaptive" immunity in invertebrates is controversial since the discovery of the Dscam gene (Ziauddin and Schneider, 2012). Having many thousands of possible splicing variants, this gene presents an incredible plasticity that allows the generation of pathogen-specific effectors (Dong et al., 2012). Therefore, to avoid confusion the term "priming" is most used in phenomena described in invertebrates, where some forms of recall of a previous exposure have been shown (Milutinović and Kurtz, 2016). Specific primed immune responses have been shown in *Drosophila* upon infection with *Streptococcus pneumoniae* (Pham et al., 2007) and *Pseudomonas aeruginosa* (Christofi and Apidianakis, 2013): the mechanism is dependent on phagocytes in both cases, with a contribution of the Toll or the IMD pathway, respectively. Nevertheless, those researches placed emphasis mostly on resistance mechanisms (immune mediators) specific to a pathogen or on transmission of the priming across generations (Milutinović et al., 2016). To our knowledge, a priming event involving broad resilience mechanism has not been described yet.

2. Results

2.1 Xenobiotics ingestion and cytoplasmic purge

Concentrations initially chosen for the first series of experiments were based on a large study on the *Drosophila* transcriptome after several stimuli (Brown et al., 2014). Despite our preliminary data include a broad range of compounds, we found lately that some of them induced responses that seemed unlikely to be associated with the resilience mechanism of purge and recovery. Those include metals such as Cadmium, Copper and Zinc, and drugs as rotenone and paraquat. For this reason, we decided for the moment to put aside the study of these compounds, even if they present common points with the process induced by hemolysin, which will be discussed later.

We focused our attention on the analysis of two compounds with important human interest: ethanol and caffeine. From here on, with the general term of xenobiotics we will refer only to these two molecules. Our previous data showed megamitochondria formation, thin epithelium and *what else* class of genes induction upon caffeine exposure; thinning was also observed after ethanol treatment. To better define whether ethanol and caffeine trigger the cytoplasmic purge, we performed a time-course on midguts of WT w^{A5001} flies in parallel with Db11 infection. We remarked a thin epithelium starting as soon as 1 hour after feeding (Figure 2.7); ethanol and caffeine caused a loss of enterocytes volume and shape to a similar extent than Db11. Nevertheless, it is important to note that thinning seemed less homogeneous than in the case of bacterial infection: neighboring cells did not present exactly the same morphology and the same degree of flattening (Figure 2.7A). Also, at 3 hours post ingestion, when Db11 induced thinning is at its peak, flies that ingested xenobiotics had enterocytes which were already lightly thicker (Figure 2.7B). Yet, a full recovery was registered only 24 hours after feeding. Therefore, we conclude that ethanol and caffeine affect the enterocyte shape and provoke a thinning of the intestinal epithelium faster than Db11 but in a less homogenous manner.

Hereafter, we verified that xenobiotics-induced thinning was due to a cytoplasmic extrusion from the enterocytes. For this, we used flies overexpressing GFP in their cytoplasm in order to image the leaking into the gut lumen (Figure 2.8). Following ethanol and caffeine ingestion, a disruption of the actin cytoskeleton was observed, creating numerous apertures in the apical part of enterocytes in correspondence of the extrusion sites. This phenomenon was observed only rarely upon sucrose feeding and is evocative of what was observed after hemolysin attack (Lee et al., 2016). Therefore, we can conclude that after xenobiotic exposure, epithelial thinning is likely due to the cytoplasmic purge.

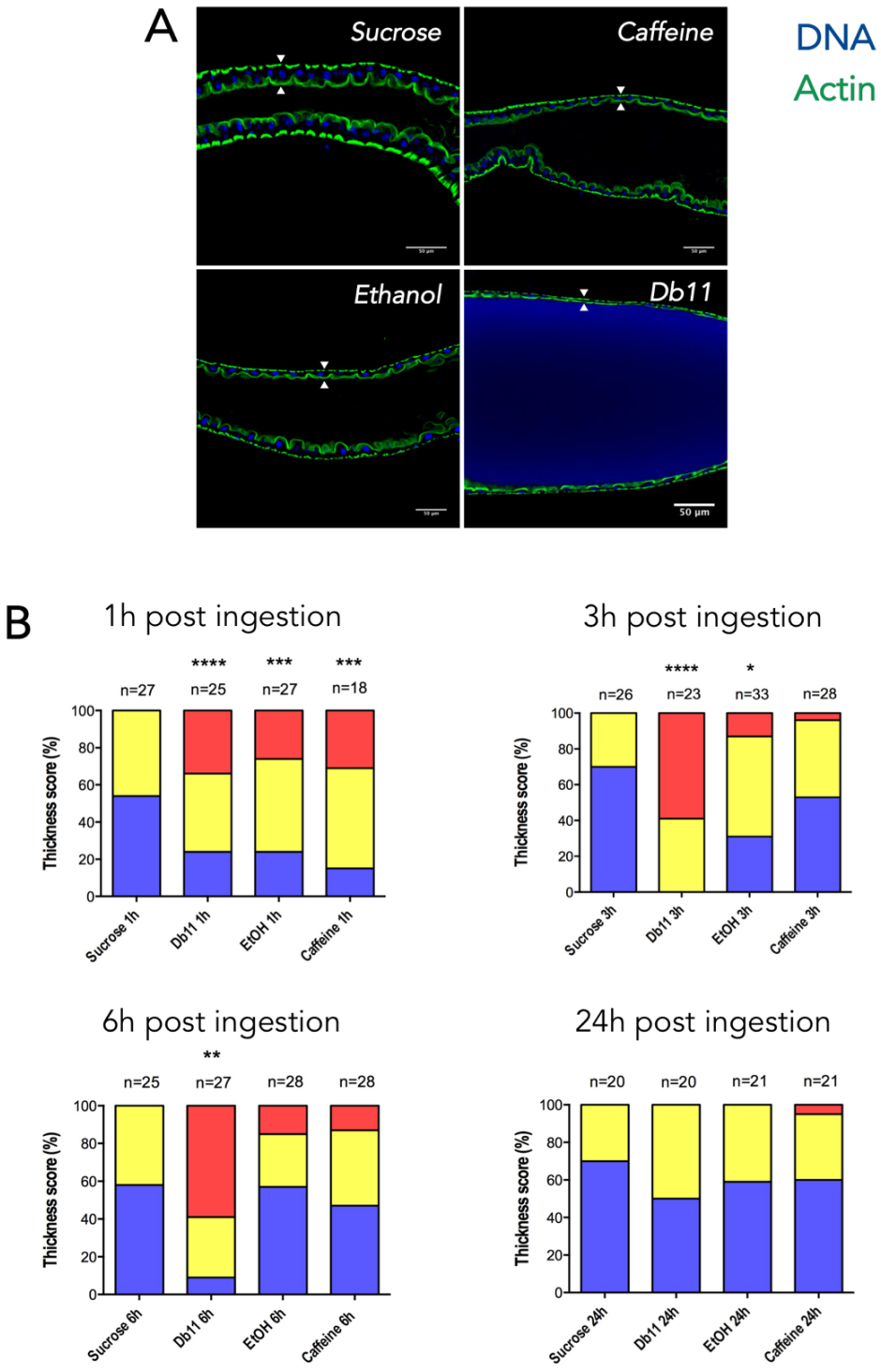


Figure 2.7. Ethanol and caffeine also induce epithelial thinning. (A) Confocal pictures of midguts of w^{A5001} flies fed with sucrose 100mM, Ethanol 2,5%, Caffeine 2,5mg/mL, or Db11 OD_{600}=10. Flies were dissected 1h after ingestion, unlike Db11 dissected 3h post ingestion. Intestines were stained with phalloidin in order to visualize actin. Scale bar: 50µm. **(B)** Corresponding scores of pictures shown in A. Intestines were graded according to their epithelial thickness: thick, semi-thin or thin. The graphs represent the pooled results of two independent experiments. Statistical tests were performed using χ^2 test.

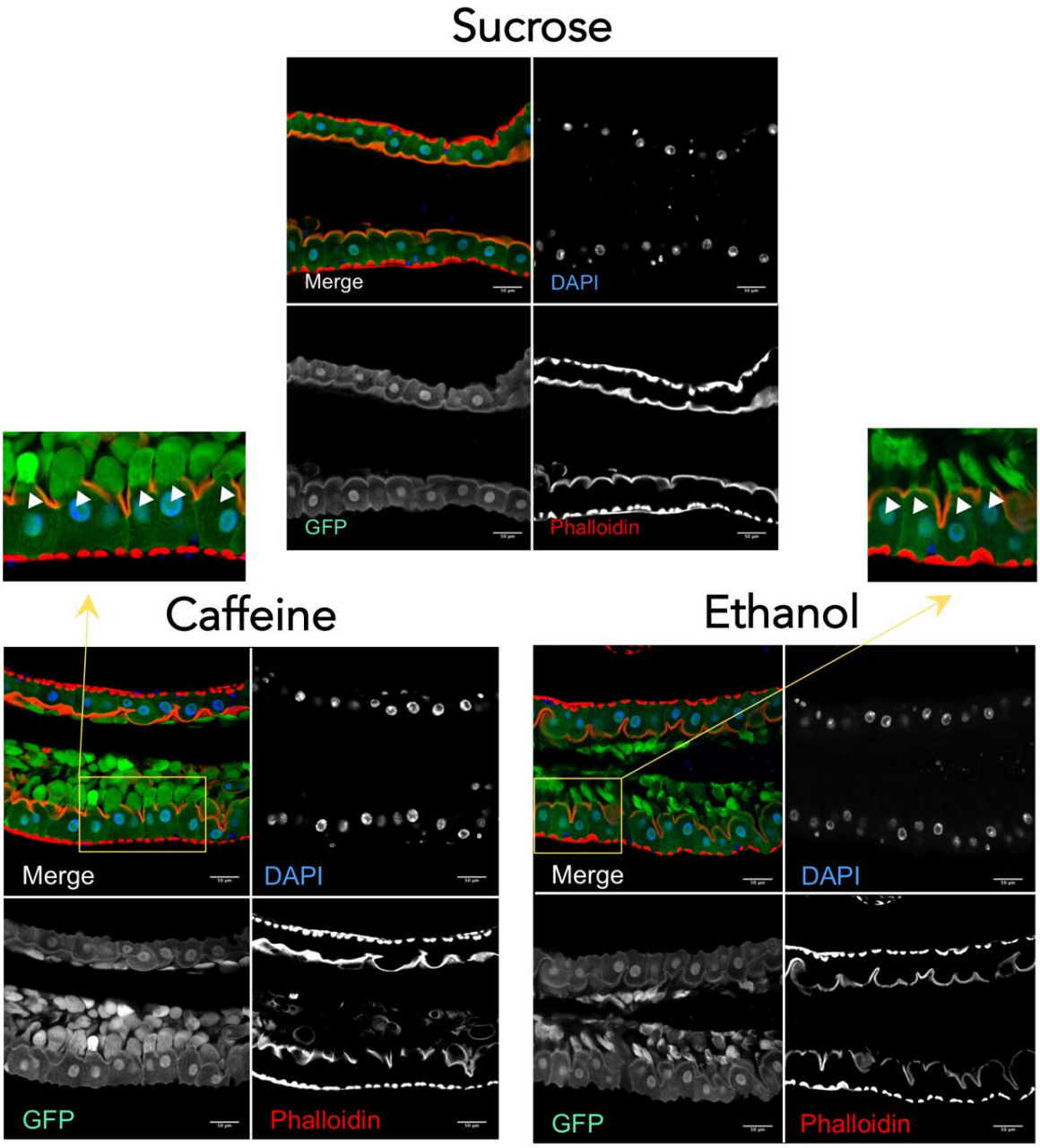


Figure 2.8. Ethanol and caffeine mediated thinning is due to cytoplasmic extrusion. Confocal pictures of midguts of *NpGal4Gal80o*^{ts}*>UAS-GFP* flies fed with sucrose 100mM, Ethanol 2,5% or Caffeine 2,5mg/mL. Dissection started 30minutes after ingestion. Numerous points can be seen where the plasma membrane is open and the cytoplasm is leaking into the lumen. Scale bar: 50µm.

2.2 ROS response during xenobiotic challenge

To understand whether the cytoplasmic purge induced by caffeine and EtOH follows the same dynamics as for Dbl1 exposure, we measured changes in ROS levels using the ratiometric roGFP2 probe. As in the case of Dbl1, no variations were observed using the Grx1-roGFP2 probes (data not shown). In contrast, we registered a slight increase in H_2O_2 levels 30 minutes after caffeine exposure using the mitochondrial-Orp1-roGFP2 (Figure 2.9A). Surprisingly, this was not the case for EtOH treatment, after which H_2O_2 levels remained similar to the control treatment sucrose. We noticed that the distribution of oxidative state levels of cytoplasmic-Orp1-roGFP2 is wider and more diverse compared to the mitochondrial one (Figure 2.9B). Analogously to what was observed after infection with Dbl1 (Figure 1.15A, chapter 1), we did not detect any significant change in cytoplasmic H_2O_2 content. We must also take into account the reduced dynamic range of this version of the probe. Nevertheless, we noticed that the intestines exposed to EtOH globally show a higher oxidative state of the cytoplasmic-Orp1-roGFP2. Despite statistically it passed normality test, caffeine treatment caused a bimodal-like distribution in H_2O_2-mediated cytoplasmic Orp1 oxidation. This experiment highlights a possible similar mechanism of action for Dbl1 and caffeine, consisting of a raise in H_2O_2 levels in the mitochondrial matrix. Although we did not observe a similar response following EtOH feeding, fluctuations of H_2O_2 cytoplasmic content may also be relevant for ROS dynamics after xenobiotic ingestion. On the other hand, ARE were not activated after xenobiotic challenge (Figure 2.9C). Despite we recognized a slight trend at 16h post ingestion, EtOH and caffeine did not seem to activate a *Cnc* antioxidant response, at least not in the same way Dbl1 did.

2.3 Xenobiotics effect on host survival and intestinal homeostasis

We performed survival assays to determine the toxicity of xenobiotics. A single exposure to ethanol 2,5% did not affect the lifespan of the flies which survived as well as controls (Figure 2.10A). Raising the concentration to 5% did not increase the mortality either. Consistent with studies published long ago that report a potent insecticide effect for caffeine (Nathanson, 1984), we confirmed that this compound severely decreased the survival of w^{A5001}, as soon as 2-3 days of feeding. Similarly, administration of paraquat was very noxious and caused the rapid death of the flies. An important load of Dbl1 killed all flies within 15 days, with an LT_{50} around day 8. We equally performed a similar survival by adding alcohol chronically (Figure 2.10B); we found that in these conditions mortality slightly increased. At the same time, we observed that WT flies tolerate well a smaller quantity of Dbl1 in the oral infection model.

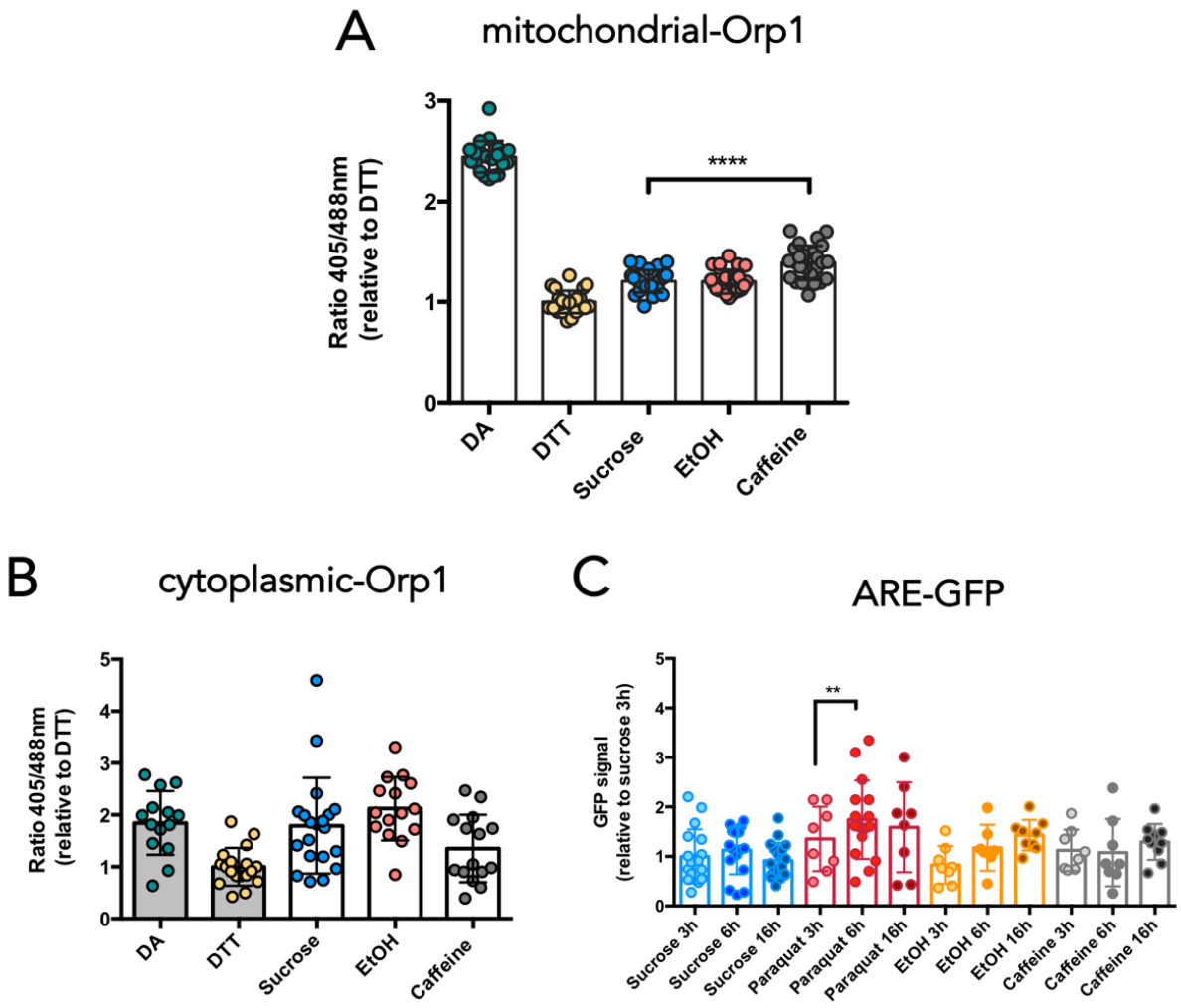


Figure 2.9. Caffeine, but not EtOH, increases H_2O_2 levels in mitochondria in the very early phase of infection. Mitochondrial-Orp1-roGFP2 **(A)** and cytoplasmic-Orp1-roGFP2 **(B)** strains were treated with sucrose 100mM, EtOH 2,5% or caffeine 2,5 mg/mL. Midguts were dissected 30min after the ingestion. Each dot on the graph represents one intestine. Midguts were also exposed to DTT 20mM or DA 2mM to estimate their responsiveness to reduction and oxidation, respectively. The graph represents the pooled results of three independent experiments. Columns and error bars represent the mean and the SD. For pair-wise comparison, statistical analysis was performed using unpaired t test with Welch's correction (A), or Mann-Whitney test (B), comparing each condition to sucrose treatment. ****p<0.0001. Only significant comparisons are shown. **(C)** ARE-GFP flies were exposed by feeding with sucrose 100mM, Paraquat 5mM, EtOH 2,5% or caffeine 2,5 mg/mL, then dissected after 3h, 6h or 16h. Images of single intestines were taken by confocal microscopy, then the fluorescence intensity was measured using the ImageJ software and normalized to sucrose 3h. Each point represents a single midgut. Statistical analysis was performed using lmer linear mixed model. Only significant comparisons are shown.

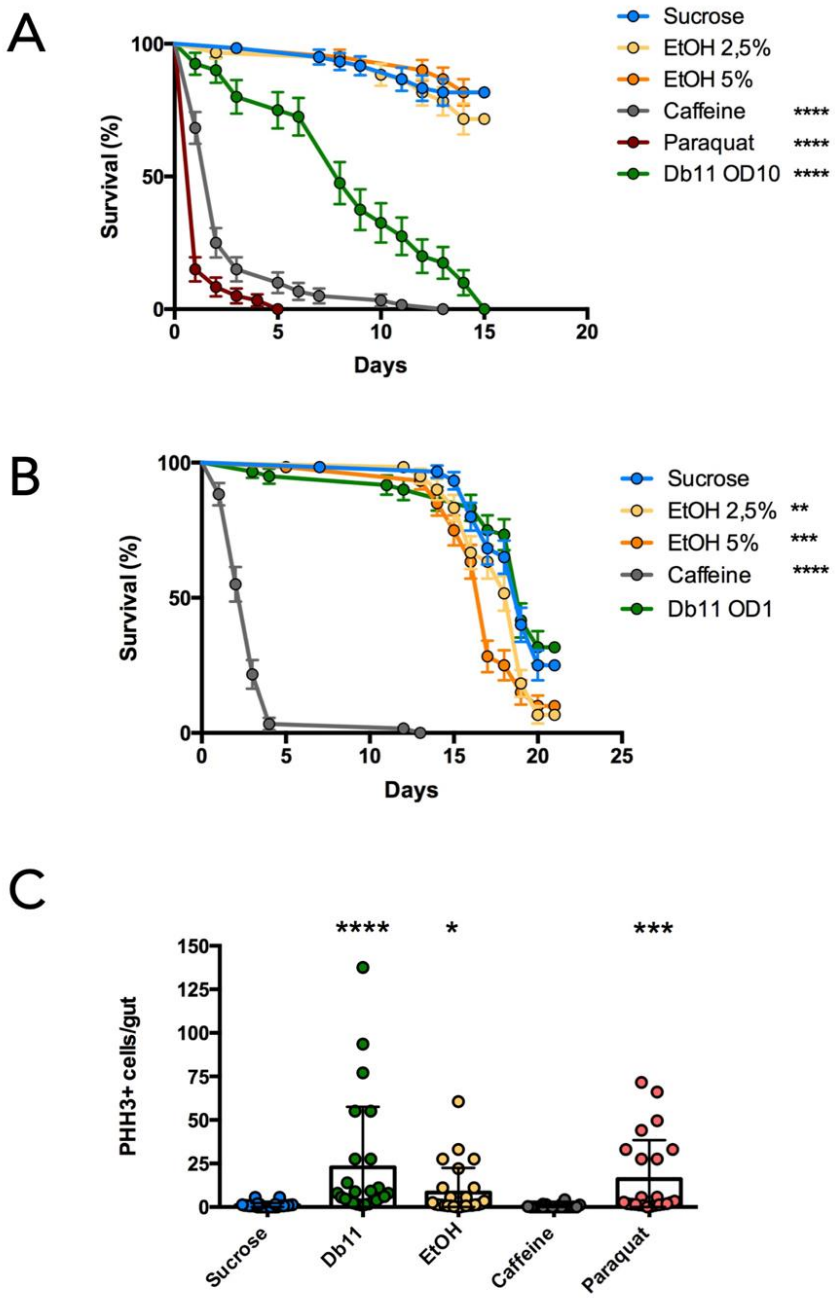


Figure 2.10. Ethanol and caffeine impact host survival and compensatory stem cells proliferation differently. (A) Survival curves of w^{A5001} flies exposed to xenobiotics and to Db11. **(B)** Same as (A), but this time ethanol was added every day and bacterial load was decreased to OD_{600}=1. Error bars represents the standard error. Statistics were performed using Logrank. **(C)** $PHH3^{+}$ cell count in midguts of w^{A5001} flies after 24h of exposure. The graph represents pooled results of three independent experiments. Each point represents an individual midgut. Error bars show mean with standard deviation (SD). Statistics were done using Kruskal-Wallis test for multiple comparisons, comparing each condition to sucrose treatment. *p<0,05, ***p<0,001, ****p<0,0001.

Cell death is not triggered in epithelial thinning, and actually the purge prevents it during a Db11 oral infection (Lee et al., 2016). Nevertheless, ISCs compensatory proliferation is induced in the steady state of the infection 1 day after Db11 ingestion (Lee et al., 2016). Then, we determined whether this mechanism is also induced after continuous xenobiotics treatment. As expected, we confirmed the results obtained with Db11 infection, and at the same time we found an important increase also after paraquat feeding (Figure 2.10C). We remarked an increase of phospho-histone3 (PHH3) positive cells in the midguts of flies which have ingested ethanol but not caffeine.

Taken together, these results suggest that caffeine is much more lethal to the insects, although it does not seem to induce critical damage to intestinal cells. On the contrary, *Drosophila* tolerates exposure to ethanol quite well, although this causes some harm to the midgut epithelium.

2.4 The Priming: protective effect against sustained exposure to hemolysin and xenobiotics

As described above, we failed in the attempt of inducing repeated cycles of purge feeding Db11 frequently. Since we see that alcohol and caffeine induce extrusions and consequent epithelial thinning, we hypothesized that those xenobiotics could equally prime enterocytes in order to prevent a second event of purge. To test that, flies were exposed to xenobiotics and then orally infected with bacteria the third day after the first challenge. Interestingly, midguts previously exposed to ethanol and caffeine were significantly thicker than naive ones (Figure 2.11A). As shown in pictures in Figure 2.11B, enterocytes thinning was very marked in non-primed flies but much less pronounced in primed ones, which kept to some extent their dome-like structures and relevant thickness. This effect was not caused by a reduced Db11 ingestion, as bacteria were well visible into the gut lumen. Notably, the prevention of a second cycle of purge is stronger if the first exposure is made with ethanol or caffeine compared to Db11 itself. Therefore, we conclude that xenobiotics may prime the enterocytes and prevent subsequent cytoplasmic purges from taking place in the case of two close stimuli.

To define how much time the enterocytes retain this imprint of the first purge, we decided to perform a second challenge farther from the first one. Then, we challenged flies with Db11 only after one-week past xenobiotics priming. In this case, differences in epithelial thinning were minor or even null (Figure 2.12A). Priming with ethanol still slightly improved the epithelial thickness, whereas an initial exposure to caffeine had no effect and double infections with Db11 even exacerbated the thinning. Hence, prevention of cytoplasmic purge after priming lasts between 3 and 7 days.

In order to establish whether a compensatory proliferation of ISCs is observed after the short six hours exposure used to prime flies with xenobiotics, we monitored PHH3 positive cells prior to the second *Serratia* infection (Figure 2.12B). In this case, no major difference was noted compared with the

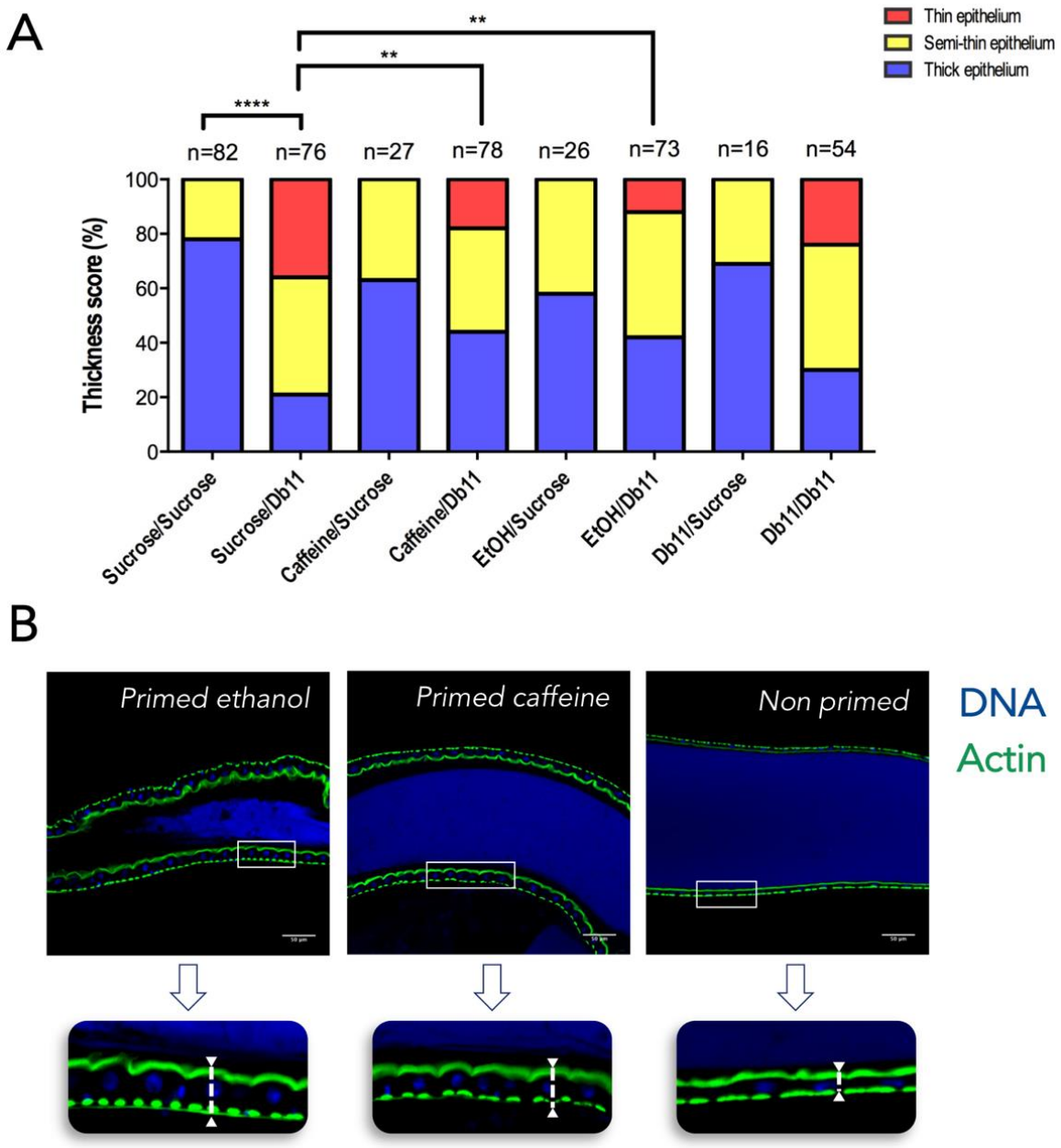


Figure 2.11. The priming: a first challenge with ethanol or caffeine prevents thinning during a second challenge with Db11. w^{A5001} flies were exposed to sucrose 100mM, Ethanol 2,5%, Caffeine 2,5mg/mL, or Db11 OD_{600}=10, in order to induce a first cytoplasmic purge. After this first challenge, flies were put back on regular medium. The third day after the first exposure, flies were fed with sucrose 100mM or Db11 OD_{600}=10 and then dissected 3h after infection. Intestines were then stained with phalloidin in order to visualize actin. **(A)** Intestines were scored according to their epithelial thickness: thick, semi-thin or thin. The graph represents pooled results of multiple experiments. Statistical tests were performed using χ^2 test. ****p<0.0001 (non-primed), **p=0.0047 (caffeine), **p=0.0013 (ethanol). Only significant comparisons are shown.
(B) Confocal pictures and zoom of infected R2 midgut region of flies which received a first challenge with ethanol, caffeine or sucrose (non-primed).

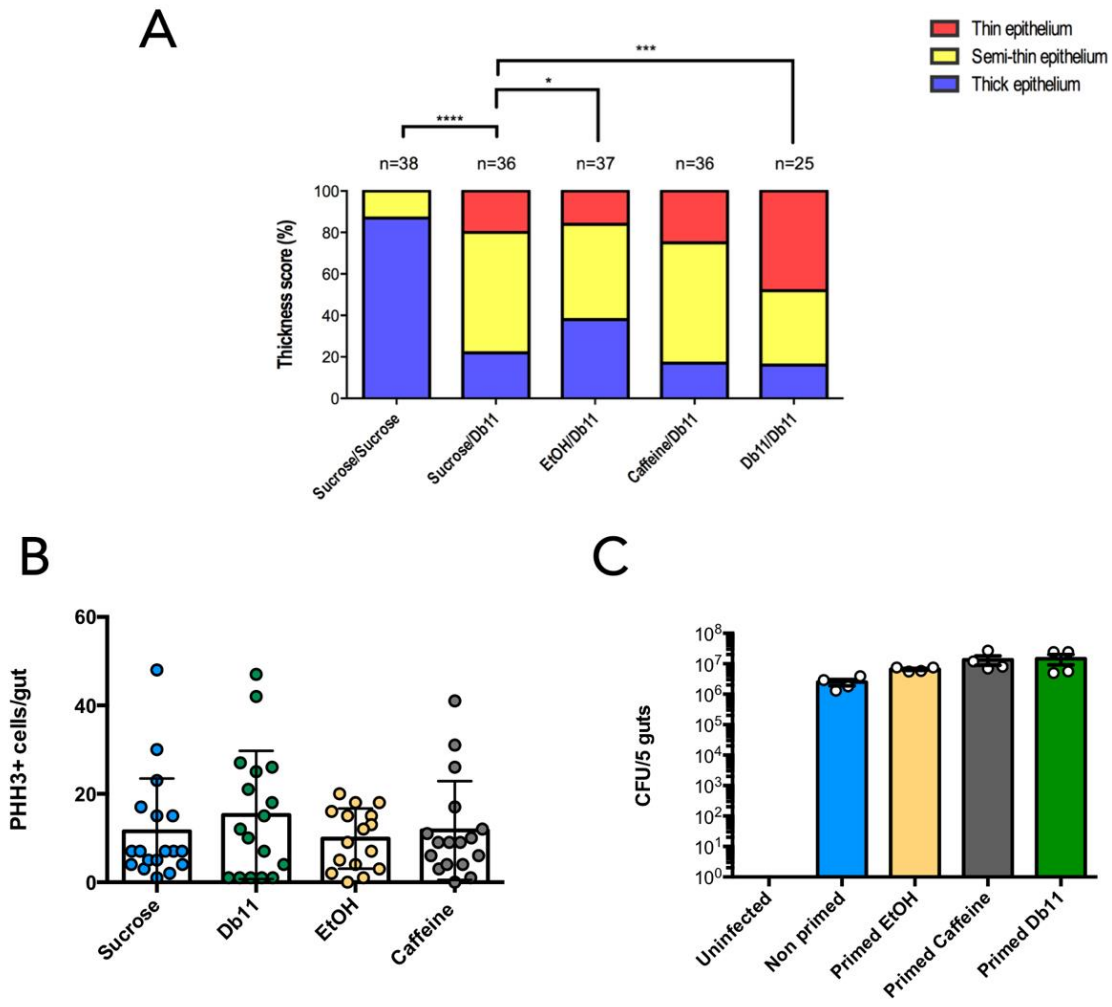


Figure 2.12. Priming protects flies from a second cycle of purge only for a few days after the first challenge. (A) w^{A5001} flies were exposed to sucrose 100mM, Ethanol 2,5%, Caffeine 2,5mg/mL, or Db11, in order to induce a first cytoplasmic purge. After this first challenge, flies were put back on regular medium. One week after the first exposure, flies were infected with Db11 OD_{600}=10 and then dissected 3h after infection. Intestines were stained with phalloidin in order to visualize actin and scored according to their epithelial thickness: thick, semi-thin or thin. The graph represents pooled results of four independent experiments. Statistical tests were performed using χ^2 test. ****p<0.0001, ***p=0.0001, *p=0.0475. Only significant comparisons are shown. **(B)** $PHH3^+$ cell count in midguts of w^{A5001} flies after 6h of exposure + 16h on regular food. Each point on the graph represents a single midgut. The graph represents pooled results of two experiments. Error bars show mean with standard deviation (SD). Statistics were done using Kruskal-Wallis test. Only significant comparisons are shown. **(C)** CFU count of bacteria in midguts of w^{A5001} flies 3h after the second infection with bacterial OD_{600}=10. The flies were primed two days before with Ethanol 2,5%, Caffeine 2,5mg/mL, or Db11 OD_{600}=10. Each point represents one biological replicate composed of 5 midguts. Error bars represents mean with SEM. The graph represents one out of two experiments yielding similar results.

uninfected flies, which suggest that limited exposure to xenobiotics did not harm much intestinal epithelium using the estimation of ISCs proliferation readout.

Finally, we considered the possibility that the first challenge may activate differential resistance mechanisms. Thus, we asked if the reduced thinning observed during the second challenge was not a consequence of a reduced bacterial load. Therefore, we measured the bacterial content in the midgut in infected flies that had previously been primed (Figure 2.12C). The result was very clear, showing that during the second challenge primed or non-primed flies carried the same number of bacteria in their midguts. Consequently, the reduced thinning in primed flies does not result from a reduction in bacterial titer.

2.5 Consequence of priming on host fitness

A crucial point was to determine whether the priming constitutes a beneficial or detrimental response for the host. If from one side the blocking of a resilience mechanism as the cytoplasmic purge may confer a disadvantage, on another side avoiding frequent cycles of purge may avoid the exhaustion of organism's energetic reserves, by constant depletion of enterocyte apical cytoplasm or alternatively by impairing their physiological functions. For that reason, flies were primed with alcohol, caffeine or Dbl1 and then survival tests started at the moment of the second infection (Figure 2.13). Unfortunately, those experiments did not yield clear cut results. Indeed, if in some cases we observed an increase in the survival rates of primed flies (Figure 2.13A), in others primed or non-primed animals died approximately at the same rate (Figure 2.13B), or we observed no difference between infected or non-infected conditions (Figure 2.13C). Consequently, we were not able to conclude whether priming is beneficial or not basing on those survival experiments. A better way to assess the physiological relevance of priming would be to analyze the phenotype of mutants in which priming is blocked.

2.6 Role of *CycJ* and *whe* genes in priming

One of the main points of the enterocyte purge and recovery article was that a *CycJ* deficiency leads to a lack of recovery, with an epithelium that remains thin, and that the ectopic expression of either *whe* or *lcs* genes can rescue this phenotype. Then, in our model, *CycJ*, by the induction of *whe* class of genes, would be required for the recovery program of enterocytes after the cytoplasmic extrusion. But, on the basis of results obtained with priming, we can actually propose another interpretation to the *CycJ* long-lasting thin epithelium. In fact, the flies fed on the bacterial solution continuously for 24h;

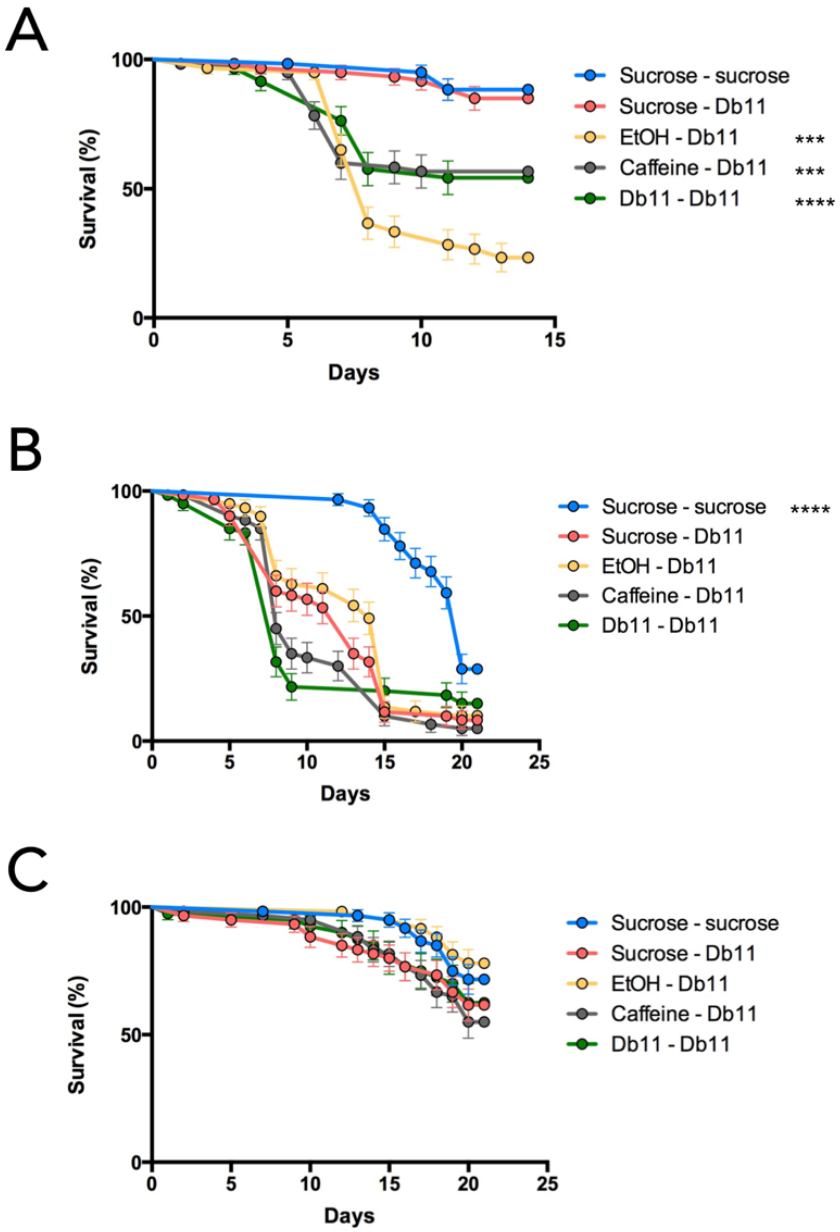


Figure 2.13. It is uncertain if priming is beneficial or not for the host. Survival curves of w^{A5001} flies that have been pre-exposed either to sucrose 100mM, Ethanol 2,5%, Caffeine 2,5mg/mL or Db11 OD_{600}=10. Survival test starts when the flies have been later infected with Db11 OD_{600}=1 or fed with sucrose 100mM, following the priming protocol (Figure 2.11). Each graph represents an independent experiment. Statistics were performed using Logrank, comparing each condition to non-primed infected flies (sucrose-Db11). ****p<0.0001, ***p=0.0004.

if the *CycJ* were necessary for priming, flies lacking this gene would undergo repeated cycles of purge or a continuous purge presenting always a thin epithelium, making indistinguishable a lack of priming from a lack of recovery. To distinguish between the two, flies were either fed for 12h continuously on Dbl1 or shifted on sucrose after the first three hours of infection (Figure 2.14A). We observed that in the case of the discontinuous infection, the midgut epithelium of $CycJ^{RNAi}$ flies was thick, therefore able to recover. This suggests that *CycJ* is a mediator of priming and not of recovery. Nevertheless, we remark that also control flies recovered more easily in the case of a discontinuous infection. Similarly, as *whe* or *lcs* expression rescued the recovery in *CycJ* mutants, we reasoned that the overexpression of those genes can mimic the first challenge, priming the midgut for a second exposure. To our surprise, *whe* and *lcs* overexpression presented different outcomes (Figure 2.14B). Indeed, the first provoked a substantial amelioration on the epithelium thickness, whereas the second had no effect, since we observed a thin epithelium after bacterial infection. Again, controls gave unexpected results: overexpression of GFP consistently decreased the thinning of epithelium, compared to the other reference conditions.

2.7 Cell turnover in the *Drosophila* intestine

It is reported that midgut epithelium is able to renew itself rapidly, within a few days (Nászai et al., 2015). We speculated that enterocytes that undergo a first purge are imprinted with a mark that prevents them from activating repeated extrusion cycles. Therefore, we set up an experiment to establish if priming duration and cell turnover are linked. We used *esgGal4Gal80^{ts}UAS-GFP* flies to induce fluorescent ISCs clones which will afterwards mature into enterocytes, with the goal to estimate their lifespan. We estimated the initial population of GFP positive cells, and then monitored their number in infected or non-infected condition for one week (Figure 2.15). We measured an important decrease of in the initial population around day 4 in both infected and non-infected conditions, although we still found a few GFP positive clones at day 7. Nevertheless, the drastic diminution at day 4 would coincide well with a priming protection period of three days but not seven days from the first exposure. The infection did not affect the turnover of the cells. Thus, this observation is compatible with the hypothesis of an imprint of enterocytes having been exposed to hemolysin or xenobiotics.

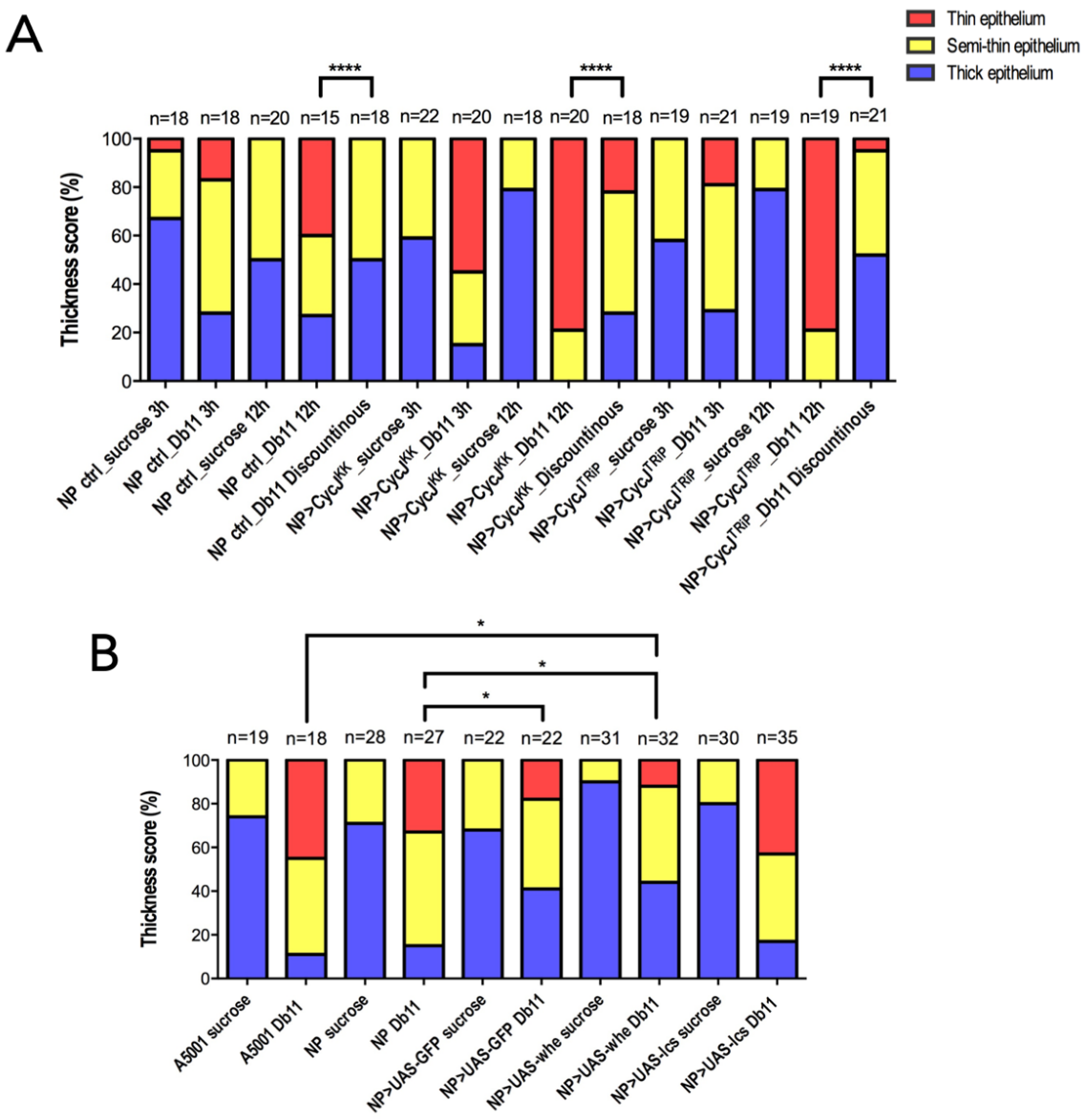


Figure 2.14. *whe* and *CycJ* may mediate the priming response. (A) *NpGal4Gal8o*^{ts} and *NpGal4Gal8o*^{ts} flies crossed with two different *CycJ* RNAi were fed with Db11 OD_{600}=10 for 3h, 12h, or for 3h then switched to sucrose for 12h (discontinuous), then dissected. Intestines stained with phalloidin in order to visualize actin and graded according to their epithelial thickness: thick, semi-thin or thin. The graph represents the pooled results of two independent experiments. Statistical tests were performed using χ^2 test. ****p<0,0001. Not all significant comparisons are shown. **(B)** w^{A5001}, *NpGal4Gal8o*^{ts} and *NpGal4Gal8o*^{ts} flies crossed with UAS-GFP, UAS-*whe* or UAS-*lcs* were infected with Db11 OD_{600}=10 and then dissected after 3h. Intestines were graded according to their epithelial thickness: thick, semi-thin or thin. The graph represents the pooled results of three independent experiments. Statistical tests were performed using χ^2 test. *NpGal4Gal8o*^{ts} VS *NpGal4Gal8o*^{ts}*>UAS-GFP:* *p=0,02; *NpGal4Gal8o*^{ts} VS *NpGal4Gal8o*^{ts}*>UAS-whe:* *p=0,02; w^{A5001} VS *NpGal4Gal8o*^{ts}*>UAS-whe:* *p=0,01. Only significant comparisons are shown.

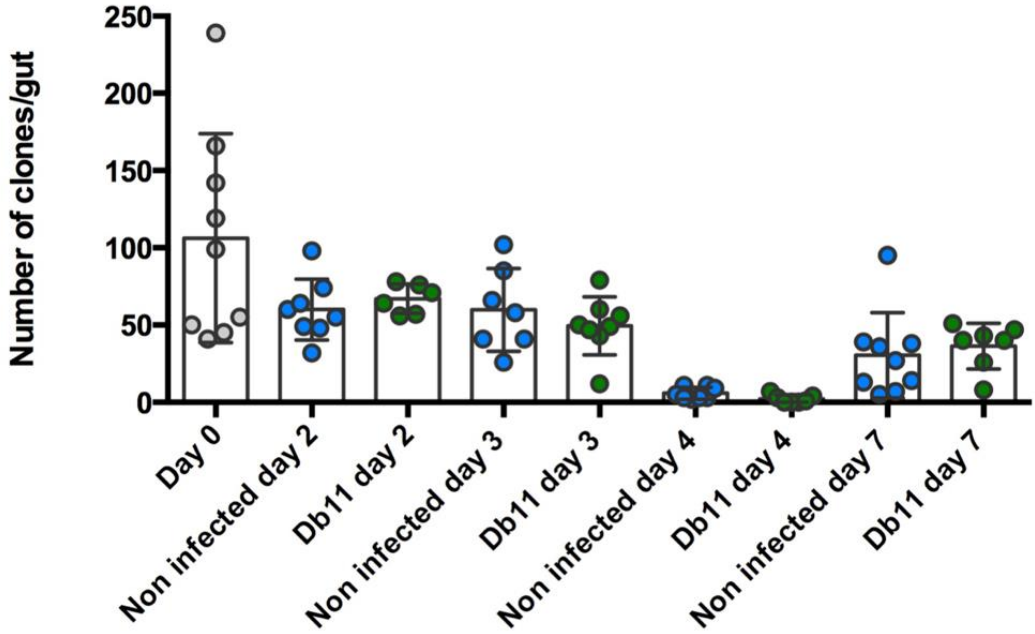


Figure 2.15. Enterocytes population undergoes a drastic renewal in four days. *esgGal4Gal80^{ts}UAS-GFP* flies were switched from 18°C to 29°C for three days in order to induce the formation of enterocytes clones expressing GFP. The flies were then switched at 18°C again for the rest of the experiment, and some intestines were dissected at day 0 to estimate the initial number of fluorescent clones. Next, the flies were divided into two batches, one infected and the other non-infected, and the midguts dissected at different days, to estimate the cell turnover. GFP positive cells within intestines were counted using the program ImageJ. Error bars indicate the mean with SD. The graph represents one out of two experiments yielding similar results.

3. Discussion

3.1 Birth of xenobiotic project

After several years of research, we have currently described multiple features of the cytoplasmic purge caused by *S. marcescens*. Beyond the conservation of this mechanism in mammals and humans, it is important to understand if this represents a specific response to pore-forming toxins or if it is a more general intestinal defense response. Long before my arrival in the laboratory, other researchers failed in the attempt to induce recurrent cycles of purge by repeated exposures to Dbl1. Moreover, comparing the molecular outcome of bacterial infection with publicly available databases, we found multiple compounds that equally induce the *CycJ*-dependent *whe* class of genes. Some of them were tested in the oral infection model, and especially caffeine and ethanol showed common characteristics to bacterial infection, namely the formation of megamitochondria and the alteration of the enterocyte's morphology. Additionally, a few years ago we changed the fly media recipe for a richer food containing five times more yeast and in which the antifungal agent was diluted in 2,5% ethanol instead of water. Suddenly after this change, we lost the epithelial thinning upon *S. marcescens* infection. Subsequent tests indicated that those two elements, yeast and especially ethanol, were the perturbators responsible of the phenotype's alteration.

Based on this background, the aim of this project was to better characterize the response to xenobiotics. During my PhD we mostly focused on caffeine and ethanol for the following reasons: i) their use has become part of human's lifestyle, and they are largely the most voluntarily ingested xenobiotics worldwide; ii) other compounds tested, such as paraquat and cadmium cause alteration of the intestinal epithelium but this occurs with a very late kinetics and is followed immediately after by death. Therefore, although paraquat and cadmium can cause a cytoplasmic purge, in their case this mechanism may not be sufficient to ensure fly's survival to the noxious effects brought by those compounds.

3.2 Caffeine, EtOH and the epithelial thinning

The choice of concentrations was key, since as we saw in the introduction, these two compounds have very different outcomes depending on the quantity taken. We used caffeine at 2,5 mg/mL (around 13mM) that is approximately the concentration found in a cup of expresso, and this was the concentration also used by others (Brown et al., 2014). EtOH was used at 2,5%, as it was the

concentration used for the fly food preparation, which caused an effect on the intestine. At these doses, our time-course analysis revealed that xenobiotics did cause a cytoplasmic purge (Figure 2.7). We were able to image groups of cells extruding starting from 30 minutes after xenobiotics exposure (Figure 2.8). After 1h of feeding, morphological alterations and loss of cell thickness were well visible, which means that cytoplasmic purge with caffeine and EtOH presents faster kinetics compared to Dbl1. This may be attributed to the cell's rapid absorption of ethanol and caffeine; conversely, activation of the Dbl1 virulence program implying hemolysin secretion may require more time. Besides, the epithelial thinning appeared not exactly the same: upon bacterial infection all the cell in the same region generally look similar; after xenobiotic feeding, we witnessed a more heterogenous degree of flattening by neighboring enterocytes. This could be due to the implementation of different programs within the cells or different communication between them, depending on whether the exposure is to hemolysin or to xenobiotics. Indeed, although a *CycJ/whe* response is induced in both cases, the different kinetics suggest that some variation is occurring. Although the heterogenous thinning observed with caffeine and EtOH may suggest that it is a cell-autonomous process, this hypothesis is unlikely because the extrusion occurred in gathered enterocyte's clusters (Figure 2.8), as in the case of Dbl1. Alternatively, the explanation might be purely mechanistic: bacteria exercise pressure on the peritrophic matrix and consequently on the cells all along the midgut, provoking a more homogenous thinning. This kind of physical constraint is absent with abiotic stresses such as xenobiotics. Nevertheless, we should remind that no stretching of the enterocytes is observed during Dbl1-induced purge (Lee et al., 2016). In parallel, EtOH treatment enhances membrane fluidity, affecting membrane composition and cell viability (Huffer et al., 2011; Tóth et al., 2014). These effects on membrane fluidity are in relation with ROS homeostasis (Sergent et al., 2005), which is involved also in EtOH-mediated actin cytoskeletal disruption (Loureiro et al., 2011). As mentioned before, alcohol and its metabolism also affects the epithelial barrier function by altering tight junctions and therefore increasing intestinal permeability (Dunagan et al., 2012; Elamin et al., 2013a, 2014b; Ying et al., 2014). Membrane fluidity, cytoskeleton and junctions reshaping are definitely factors that influence enterocyte morphology and therefore they may intervene in the phenotype that we observed. Nonetheless, a common point that brings together hemolysin, caffeine and ethanol is that all three can induce (with different mechanisms) a potassium efflux (Dopico et al., 2014; Garcia et al., 2019; Hertle, 2005; Zhang et al., 2018). Therefore, this should be studied more in detail, since it may represent an important factor in the initiation of the enterocyte purge.

3.3 ROS & xenobiotics: an ambiguous relationship

H_2O_2 signaling seems to be involved in the early steps of the cytoplasmic purge process triggered by *S. marcescens* (see chapter 1). However, in the case of caffeine and ethanol data interpretation is puzzling. Indeed, while all the three stimuli induced the aperture in the enterocytes with subsequent cytoplasm leak and cell thinning, mitochondrial H_2O_2 increase was observed after Db11 and caffeine but not EtOH feeding (Figure 1.15A & 2.9A). Instead, H_2O_2 levels seemed slightly enhanced in the cytoplasm after alcohol feeding, despite the difference was not statistically significant and the cytoplasmic-Orp1-roGFP2 probe presented a small dynamic range (Figure 2.9B). Therefore, these results do not allow us to generalize about the H_2O_2-mediated induction of the cytoplasmic purge. The effects of caffeine on ROS homeostasis is contradictory in the literature: despite the majority of the studies point out an antioxidant effect of trimethylxanthine (Khan et al., 2019; Nilnumkhum et al., 2019), there are also reports that describe it as a pro-oxidant (Li et al., 2019; Wang et al., 2019). On the other side EtOH is believed to have oxidant properties (Lu and Cederbaum, 2008; Nordmann et al., 1992), but this is mostly due to generation of ROS as a by-product of EtOH metabolism. Indeed, both compounds are metabolized by CYPs, which are a potential source of ROS (Hrycay and Bandiera, 2012). However, it is unlikely that these processes are relevant in our case of study, because we observed a thin epithelium as soon as 1h after ingestion, and metabolic dynamics likely take more time. Even so, we should verify whether knockdown of *Cyp2e1*/ADH and *Cyp12d1*/*Cyp1a2* affects xenobiotics-induced purge and H_2O_2 induction.

The lack of ARE-containing genes induction after xenobiotic exposure (Figure 2.9C) represents another layer of complexity. Indeed, despite we noted a slight progressive augmentation after EtOH treatment, feeding of the two xenobiotics did not significantly stimulate CncC activity. Therefore, this pointed out an additional difference compared to Db11 ingestion. Nevertheless, given the great diversity and broadness of the Nrf2 response, it is logical to think that not all genes regulated by this transcription factor are expressed at the same time and to the same extent. Nrf2 binding sites contain consensus motifs for other cofactors, which fine-tune the response, and other Cnc transcription factors also recognize ARE sequences (Tonelli et al., 2018). Hence, we cannot completely exclude that EtOH and caffeine activate an antioxidant transcriptional response through alternative pathways. Nonetheless, according to our data we cannot propose a general mechanism implying ROS as the inducer of cytoplasmic extrusions after xenobiotics exposure, despite caffeine displayed a mitochondrial H_2O_2 increase very similar to Db11 one in time and intensity.

3.4 Diversity in lifespan and gut damage

Ethanol and caffeine altered fly's survival very differently. The former was not harmful when fed at the concentration we used for our experiments on epithelial thinning: *Drosophila* withstand very well one single exposure to EtOH 2,5% or 5%. These quantities become marginally lethal only when they are administered chronically. Conversely, single acute exposure to caffeine drastically decreased the lifespan. This was expected since toxicity to insects was already reported (Ashihara et al., 2008; Nathanson, 1984), although the large difference in mortality rates compared to EtOH or even Db11 was appreciable. Albeit survival assays are essential to study organism's responses to a given treatment, they do not provide explanations for the causality of the death. Since all three agents (Db11, EtOH, caffeine) trigger the cytoplasmic purge but they produced three very different survival curves when ingested, we can speculate that the observed differences in mortality did not depend on this resilience mechanism. On one hand, we can hypothesize that after the enterocyte thinning and recovery, EtOH is efficiently detoxified by the host's metabolism, principally by ADH, having no relevant consequences on *Drosophila* lifespan. It would be interesting to test if ADH-deficient flies still undergo the cytoplasmic purge. On the other hand, caffeine's high toxicity to insects has been attributed to phosphodiesterases inhibition (Mustard, 2014; Nathanson, 1984). The exhaustion of intracellular Ca^{2+} stores and impact on nervous system through adenosine or dopamine receptor might be involved as well.

When we evaluated the midgut response, we observed that ethanol induced more compensatory proliferation of the ISCs than caffeine. This represent a good indirect method to estimate the damages occurring in the intestine. Accordingly, alcohol ingestion moderately injured enterocytes, most likely by the epithelium disruption mechanisms described above. We should verify this hypothesis by testing epithelium permeability through a SMURF assay and directly measure cell death upon EtOH feeding. Conversely, caffeine did not seem to cause damage to the intestinal epithelium. We can therefore imagine that it triggers the cytoplasmic purge (through H_2O_2 signaling in mitochondria) to protect the intestinal epithelium, but at the dose we used the inhibition of phosphodiesterases and/or the neurological effect are still lethal. Another possibility is that caffeine blocks ISCs proliferation. Firstly, we should test lower caffeine concentrations to find the sublethal dose for *Drosophila*, in order to verify whether in these conditions we still observe the cytoplasmic extrusion without inducing a strong mortality. Additionally, the use of high concentrations (mM) might selectively induce some kind of responses and mask the effects of caffeine exposure in the midgut. Secondly, we could measure cAMP and Ca^{2+} levels alongside caffeine survival assay.

Although our oral infection model with *Serratia* is well established nowadays, the causes that leads to fly's death are still not very clear. Host sensitivity is strictly depending on bacterial load. We

know that enterocyte purge and subsequent recovery is a protective mechanism that limits bacterial passage into the hemolymph and allows to get rid of damaged organelles and cellular components and possibly also the PFT. Even though this process does not involve cell death, we measured an increase of ISCs proliferation 1 day after Db11 ingestion (Lee et al., 2016 & Figure 2.10). We hypothesize that cytoplasmic purge is a first and rapid reaction to accidental exposure to toxin and xenobiotics, aimed at preserving the barrier integrity and reducing the vulnerability to damage. The subsequent epithelial renewal operated by ISCs proliferation may resolve completely the exposure-related stress and ensure a healthy and functional intestine.

3.5 The Priming: a first exposure to xenobiotics prevents Db11-induced thinning

Besides the fact that alcohol and caffeine induce a cytoplasmic purge per se, one very interesting feature is that they can prime the enterocytes to a subsequent exposure to *S. marcescens*. When infected with Db11 a few days after a xenobiotics challenge, the midgut epithelium was not undergoing a second cycle of purge. Despite that enterocytes were lightly less thick than under uninfected conditions, the overall morphology is maintained, with a relevant size and a dome-like structure. Surprisingly, this is not true if the two stimuli are inversed (first infection with *Serratia* and second challenge with xenobiotics), or in the case of double, sequential exposure to EtOH or caffeine (data not shown). Another unexpected observation was that a repeated infection with Db11 induce less priming: this is not in accordance with past results, where precisely the impossibility of inducing repeated cytoplasmic purges with Db11 led us to hypothesize the phenomenon of priming. However, this "resistance" to the purge was no longer present when a week elapsed between the first and second exposure. Moreover, we did not observe a reduction in the bacterial titer during the second challenge, ruling out the possibility that the lack of thinning is a consequence of a lighter microbial load. Therefore, our case presents differences with the examples of priming in *Drosophila* published previously (Christofi and Apidianakis, 2013; Pham et al., 2007). The term priming is even more appropriate here because: i) this event is not specific to a given pathogen, but it is extended to abiotic compounds and ii) it is not long lasting, as it disappears after a few days. We can identify these two aspects as the distinctive tracts between a "priming" and a "memory" response.

But what is the physiological meaning of the priming? If the cytoplasmic purge is protective and beneficial for the host, why inhibit it? It could be that the purge serves as a defense mechanism against occasional exposures but not to chronic threats. In fact, although the recycling of cells is energetically convenient compared to *de novo* synthesis, the activation of this program in a continuous way can be

metabolically very expensive. Thus, going through continuous cycles of purge may lead to the rapid exhaustion of organism's energetic reserves. This need to save energy may also be the basis for the lack of ISCs proliferation after a short exposure to Dbl1 or xenobiotics (Figure 2.12B). In addition, an intestinal epithelium that remains constantly flat and thin and in which enterocytes have lost multiple cellular components will most likely not be functional for digestion processes. Yet, we lack experimental evidences to support or refuse this assumption. Indeed, survival assays on primed flies gave no valuable information about the advantage or disadvantage of priming: only once we observed that primed flies were more sensitive to a second infection (Figure 2.13A), but this phenotype was not reproducible. At least, we can affirm that priming the flies did not confer resistance to a second infection. With due caution, this may be seen as a confirmation that the cytoplasmic purge is a beneficial response for the host. Key insights about the biological relevance of priming could be brought by the identification of a genetic mutant constitutively lacking a priming response; although we have some promising candidates (see paragraph below), at present we have not spotted with certainty such a gene. Then, as we know that cytoplasmic purge limits bacterial passage from gut to hemolymph, an alternative strategy should be to determine Dbl1 titer in hemolymph, expecting to observe more bacteria in flies that have been primed. If it is the case and the bacteria overcome the cellular immune response in the circulation, this might be at the basis of the increased mortality observed in primed flies in Figure 2.13A. Also, we should measure the $PHH3^+$ cells after the second challenge.

3.6 Which cellular/molecular mechanism regulates priming?

When exposed to EtOH or caffeine, flies are unable to go through a second purge during a Dbl1 oral infection in the next three days. Interestingly, this effect faded a week after the first exposure: primed flies could accomplish again a cytoplasmic extrusion when they fed *S. marcescens* seven days after xenobiotics (Figure 2.12A). Hence, we reasoned that there must be a transient imprint in the enterocytes that undertook a first purge which vanishes over the days.

CycJ was the first gene we identified to be important during the cytoplasmic purge process. The first evidence collected was that flies lacking this gene failed to recover the normal epithelial shape after the thinning. Therefore, the interpretation that was always given to this result is that *CycJ* is necessary for the recovery phase. However, after the discovery of the priming, we can propose another interpretation of this phenotype. In fact, our classical infection protocol does not allow to distinguish between recovery and priming, since the flies are constantly kept in contact with Dbl1. Thereby, a lack in the priming response would result in constant cycles of purge and an epithelium that looks always thin. To figure out the real function of *CycJ*, we came up with a discontinuous infection protocol: the purge is induced by feeding $CycJ^{RNAi}$ flies with Dbl1 only for 3h and then they were switched to sucrose.

In this manner, if *CycJ* is mediating the recovery, we should observe a thin epithelium in the case of a discontinuous infection. However, the interpretation was not so simple. First, we observed that the control line and the $CycJ^{TRiP}$ did not display a great thinning 3h post ingestion: this fact complicates the analysis of recovery at later time points. Indeed, we remarked that controls which were constantly on Db11 carried a consistent portion of thin and semi-thin epithelia at 12h p.i., while a full recovery was expected. This might indicate a time shift in the flies' feeding behavior. Nevertheless, we also observed that both $CycJ^{RNAi}$ had a much greater proportion of thin epithelia in the same condition. This can be seen as a confirmation that *CycJ* is mediating the restoration of the normal enterocyte shape (via priming or recovery). The discontinuous infection protocol definitely led to an improvement of the recovery phase, both in the RNAi lines and in the controls. The fact that $CycJ^{RNAi}$ could actually recover indicates that this gene is mediating the priming rather than the recovery after the thinning. Unfortunately, we cannot claim that with certainty, because the driver alone showed the same kind of behavior. To confirm this result we will have to repeat this experiment more times, possibly using a null mutant for *CycJ*. Additionally, we must verify that the priming mechanism is not working in *CycJ*-deficient flies.

CycJ lack of recovery is closely linked to *whe* genes induction: the ectopic expression of only one between *whe* or *lcs* rescued the phenotype of *CycJ* mutant (Lee et al., 2016). Therefore, we reasoned that overexpression of these peptides would mimic a priming: flies carrying an additional constitutively expressed copy of *whe* or *lcs* should be resistant to the cytoplasmic purge. Again, the analysis of this result was more complicated than we initially expected. First, GFP overexpression provoked an odd phenotype once more (see chapter 1): those flies did not undergo an epithelial thinning properly. Regrettably, this was the best control for our experiment, so this weakens considerably our conclusions. Secondly, for the first time we observed a differential role for *whe* and *lcs*, two genes until now we believed to have a redundant function. While *whe* overexpression appreciably ameliorates the thickness score, this is not true for *lcs*. Despite this result has been reproduced three times, it seems premature to draw conclusions on *whe* as a determinant of the priming, at least until the variation in our controls is settled. Moreover, it should be confirmed in an independent manner, e.g., measuring by qPCR an increase in *whe* expression in WT primed flies, or visualizing real-time and lineage expression using the G-TRACE system (Evans et al., 2009). In parallel, we should also perform the same experiments on the *lcs* gene, to establish whether it has a function on the priming or not.

The idea of a transient imprint may correspond well with epigenetic modifications of enterocytes, which have a short half-life. Unfortunately, preliminary experiments did not indicate the implication of chromatin modifiers in the priming process (data not shown). An alternative or complementary hypothesis to the *CycJ/whe* pathway involves the enterocytes turnover in the midgut. This tissue presents high self-renewal capacity, with the epithelium that is completely renewed within

1-2 weeks in steady-state conditions (Micchelli and Perrimon, 2006; Nászai et al., 2015; Ohlstein and Spradling, 2006). We reasoned that enterocytes that have experienced a first purge and received the imprint might be removed over the following days. These primed enterocytes would have memory of the first extrusion and would be resistant to a second purge, but once removed they would be replaced by naïve cells, able to trigger cytoplasmic purge again. This would explain why we did not observe epithelial thinning when the two stimuli are close (3 days) but not when they are farther (7 days). Estimation of enterocyte turnover in the midgut by clonal analysis, inducing marked cells with the esgGal4›UAS-GFP lineage (Buchon et al., 2009b) revealed a great decrease in the initial enterocyte pool at day 4. Although this result need confirmation by other methods, it provides a coherent explanation for the duration of the priming effect. We remarked that the infection did not increase the turnover, which is matching with the lack of increase in ISCs proliferation in the short-time exposure we used for priming. Of note, a factor that must be taken into account is that for technical needs the experiment was performed at 18°C, and the lower temperature certainly affects cellular processes such as division, differentiation and death, hence the need to find another independent method. Also, and unexpectedly, we detected more GFP$^+$ enterocytes at day 7 than at day 4. This could be due to a leaky residual activation of the promoter even at 18°C. Alternatively, as we constated a great variability of the fluorescent clones number at day 0, this might just be a matter of chance, that the flies examined at day 7 were those that initially had the most GFP$^+$ clones. Repeating the experiment more times will allow us to dispel this doubt.

3.7 Conclusions and future directions

In addition to being an important defense process during oral infection with the bacterium *Serratia marcescens*, our work has highlighted that the cytoplasmic purge is also active after ingestion of caffeine and ethanol. This represents a crucial point, because it demonstrates that this mechanism is not specific to the bacterial challenge but instead it is shared in the case of abiotic stressors. We showed that the cytoplasmic extrusion induced by Dbl1 is conserved in mammals (Lee et al., 2016), so it would be interesting to test if this is also true in the case of EtOH and caffeine. Since those compounds are frequently consumed by the majority of the world's population, this would be very relevant to human's health.

In order to get a deep understanding of the whole purge and recovery process, we have to elucidate the common points and the possible differences between bacterial and xenobiotics exposure. This could prove to be not an easy task, since despite all three agents cause the cytoplasmic purge and the priming, exposure to ethanol, caffeine or Dbl1 induces a series of responses specific to each

compound. The main features revealed in this work are summarized in Table D1. First of all, we measured a difference in the lethality of these compounds, which however is not correlated with the intestinal damage caused by them. For example, caffeine kills flies within a few days, but does not cause intestinal damage. Conversely, flies survived quite well after Db11 and EtOH feeding, but ISCs compensatory proliferation is induced. Also, while caffeine and Db11 shared a mitochondrial H_2O_2 increase, this is not the case for EtOH. This point in particular was very disappointing, as it would have allowed us to identify a common mechanism for the induction of the extrusions. However, the data we collected did not completely rule out this possibility.

An interesting question relies beyond these observations: why flies have evolved a common mechanism in response to such different stimuli? In the case of Db11 infection and caffeine exposure, we may speculate that flies perceive them as harmful regardless the dose, and thus activate a defense program to protect the midgut. Conversely, EtOH is an attractant for the insect, and it is well tolerated. We must however consider that flies encounter EtOH quite commonly in their natural life, and the daily intake can be very high: rotten fruits are particularly rich on it, and it can also derive from yeast fermentation in fly's midgut. This may be also the cause of why we do not observe a cytoplasmic purge in flies raised on a yeast-rich food: the EtOH produced by the microorganism may constantly prime the enterocytes, which becomes unresponsive to hemolysin attack. Another stimulating question is why the priming does not work in both directions? Flies recovered when fed continuously with EtOH and caffeine for 24h, but they were not primed against a second exposure of the same compounds. The elucidation of the molecular pathway regulating the priming and also its consequences, like the passage of bacteria into the hemolymph or ISCs compensatory proliferation after a second challenge might help us to address this question.

As previously mentioned, we have to keep in mind that a similarity shared by the three agents is a K^+ efflux. Interestingly, this is an activator of the inflammasome complex (He et al., 2016). Inflammasome activation principally activates caspase-1, which cleaves not only pro-inflammatory cytokines into their mature and active form but also the Gasdermin-D. This protein raises our interest because it causes large pores to the plasma membrane as we observe during the extrusion phase. Moreover, the pore formation does not necessarily lead to cell death: large protein complexes as ESCRT-III can be recruited to the membrane and repairs the pore avoiding inflammasome-induced pyroptosis (Rühl et al., 2018). Given that there is no inflammasome in *Drosophila*, another recent ramification of the cytoplasm purge project is currently investigating a possible similar mechanism with the implication of non-apoptotic caspases and cytoskeleton remodelers. In particular, recent studies reported that *Dronc*, a caspase which is required for the epithelial thinning, binds to actin cytoskeleton adaptors proteins and influences its dynamics (Kang et al., 2017; Orme et al., 2016).

Phenotype	Db11	Ethanol	Caffeine
Lifespan	Slightly affected	Not affected	Severly affected
Cytoplasmic purge	Yes. Thin epithelium at 3h p.i.	Yes. Thin epithelium at 1h p.i.	Yes. Thin epithelium at 1h p.i.
H_2O_2 induction	Yes, mitochondrial	No	Yes, mitochondrial
Priming against second Db11 challenge	Mild	Yes	Yes
ISCs proliferation 24h p.i.	High	Moderate	No

Table D1. Comparison and recapitulation of the effects of Db11, EtOH and caffeine exposure highlighted in this work.

Another important common point between EtOH, caffeine and Db11 is represented by the *whe* genes induction. The induction of these after the first event of purge may be the imprint at the basis of the transient resistance to consecutive rounds of extrusions in the following days. Thus, this will be an important axis to develop for the continuation of the project, in order to describe a full mechanism for the priming response. Alternatively, we should further explore the possibility of the existence of an epigenetic mark in the experienced enterocytes. The determination of a transcriptomic profile in primed midguts through an RNA sequencing would allow us to have a more complete view of what is happening in the cell after the cytoplasmic purge, confirming or not the hypothesis exposed just above and favoring the identification of new targets. Besides the molecular pathways, priming looks intimately linked to enterocytes turnover. The thinning extent depends on the presence of cells that previously underwent a purge event. We should study whether blocking or accelerating midgut's renewal dictate the duration of the priming: we hypothesize that slowing down the turnover would elongate priming duration, and by stimulating the replacement we would shorten it. A proposed model for the whole priming process is illustrated in Figure D2. Finally, one intriguing possibility would be that *CycJ* intervenes in the renewal process, since it is a cyclin showed to bind to Cdk1 (Althoff et al., 2009). We should test this hypothesis by crossing the $CycJ^{RNAi}$ line with an esg-Gal4 driver and evaluating the response to priming.

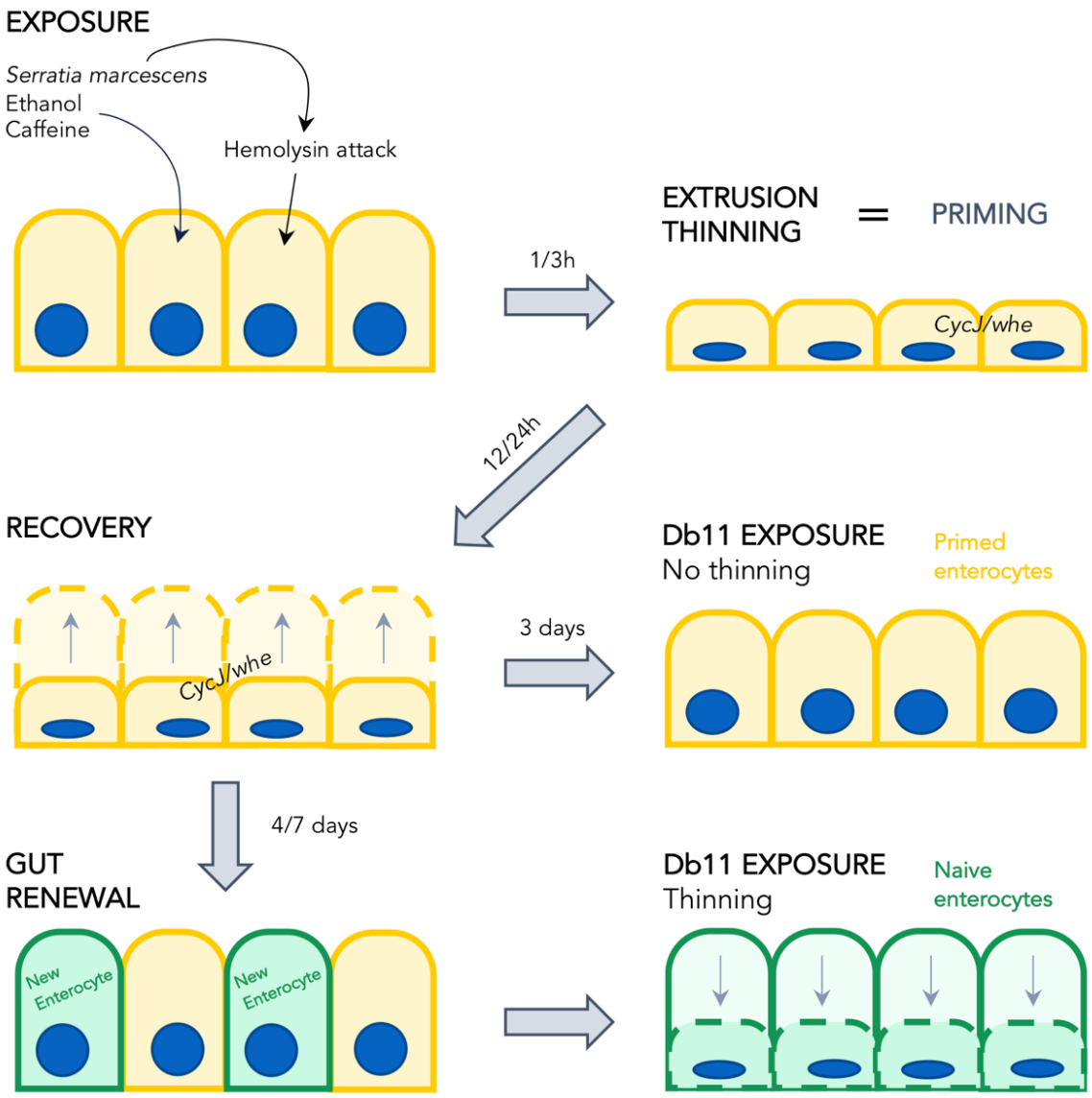


Figure D2. Time-lapse of the priming response after xenobiotics and *Serratia* exposure. From bottom left: flies feed on plants and rotten fruits, which are potential sources of caffeine, EtOH or bacteria such as *S. marcescens.* The PFT hemolysin of the latter causes cytoplasmic extrusion and consequent epithelial thinning within 3h; the same process is triggered by xenobiotics as soon as 1h after ingestion. Then, the epithelium progressively recovers its shape in 12/24h. In parallel, a *CycJ*-dependent *whe* induction is activated. The first cytoplasmic purge primed the enterocytes against a second exposure, likely by the expression of this gene. Indeed, if infected again with *S. marcescens* Db11 in the following three days, there is no thinning of the midgut epithelium. Meanwhile, the intestine self-renew itself over a few days: new "naive" enterocytes replace the "experienced" ones. When the majority of the cells have been changed, the intestinal epithelium is responsive to hemolysin again, triggering cytoplasmic extrusion and thinning.

1. General conclusion

Nosocomial infections represent a major threat in hospitals, affecting up to 15% of patients (Khan et al., 2017): the Gram-negative bacterium *S. marcescens* is one of the most frequent causes. Also, the vast majority of the world's population regularly consumes exogenous agents such as caffeine and ethanol. The study of the host's defense reactions to pathogens and xenobiotics is of great interest in our laboratory. It will contribute not only to improve the condition of infected patients, but also to understand physiological mechanisms in response to commonly consumed substances. While investigating the pathogenicity of *S. marcescens* in the intestine of *Drosophila melanogaster*, previous work in the laboratory identified a new resilience process, the cytoplasmic purge. The pore-forming toxin hemolysin secreted by the *S. marcescens* strain Db11 triggers an extrusion of cytoplasm and damaged organelles from the ECs into the gut lumen. Consequently, the intestinal epithelium becomes very thin and flat, but avoiding cell death it starts a progressive recovery process which restores the initial volume and shape within a day. In addition to being beneficial for the host, reducing the number of bacteria that disseminate through the digestive tract, this mechanism is also conserved in mammals (Lee et al., 2016). Finally, a molecular pathway involving uncharacterized genes has been described: the *CycJ*-dependent *whe* class of genes secretion is an important factor to reinstate the normal morphology of the midgut epithelium.

1.1 Contribution of this work

Nowadays, the enterocyte purge is a well-established model in our laboratory. Nevertheless, numerous questions remain to be addressed. During my PhD, a major axis has been dedicated to the investigation of how the cytoplasmic extrusion is triggered from the host point of view. I have shown that there is a very early ROS production in the midgut in the phase that precedes the leak of cytoplasm. This ROS induction has been measured with two independent methods: an estimation of total H_2O_2 content after infection by a biochemical technique and the evaluation of specific redox couples with the genetically encoded ratiometric reporter roGFP2. This evidence is further supported by other events linked to ROS biology: the lipid droplets and megamitochondria formation in the steps preceding the extrusion and the subsequent transcription of antioxidant genes after the cytoplasmic purge occurred. One important point was to determine the source of the ROS. Based on our data with the roGFP2 probe, the H_2O_2 increase arises mainly from mitochondria. Additionally, we could alleviate the degree of thinning using the mitochondrial ROS scavenger mitoTEMPO. Nevertheless, manipulation of fly genetics pointed out an implication of the DuOx system: DuOx knockdown or IRC overexpression prevent the purge process and result in reduced H_2O_2 induction after infection.

The cellular localization may constitute the bridge between the two sources. Indeed, both megamitochondria and DuOx are found in close association with the apical plasma membrane of enterocytes, at the sites where the extrusion events take place later. Therefore, this led us to hypothesize a working model where both systems are contributing to the cytoplasmic purge induction (Figure D1). Still, what we observed is a ROS induction: to be able to conclude that we are really observing a signaling event, we have to identify target proteins with potential H_2O_2 sensitive sites, and which are implicated in mechanisms relative to the purge, e.g., MAPK cascades or cytoskeleton modifications. When we tested a second RNAi line for DuOx ($DuOx^{RNAi}$ GD), this gave different outcomes both in survival and in thinning, therefore interpretation must be cautious. Precisely, highlighting the differences between the two $DuOx^{RNAi}$ strains, the one that purges and the one that does not, and also the data obtained with the UAS-IRC, would bring us an important insight about key elements that determine the activation of the extrusion program.

Alongside the role of ROS during the cytoplasmic purge, this project also led us to highlight important side effects of N-acetylcysteine. Albeit it is used worldwide as the most common antioxidant compound, NAC exercised a strong effect on bacteria, preventing their growth in culture and killing them inside the fly midgut. Furthermore, NAC reduces the lifespan of *Drosophila* even in the absence of infection. Taken together, these results suggest not only a prudent use of this compound during infection studies, but also an impulse to better understand and characterize its mode of action.

I have shown that the enterocyte purge and recovery is not a response specific to *S. marcescens* PFT hemolysin: this mechanism is also active upon ingestion of ethanol and caffeine. These two xenobiotics induce the epithelial thinning that reaches its peak already one hour after ingestion, subsequently starting the recovery process. Of note, the enterocyte morphology does not look exactly the same in the two cases. Although intestinal cells are always extruding in clusters, the flattening is more homogenous in the case of the bacterial infection and looks more irregular upon EtOH and caffeine feeding. In perspective, it would be interesting to study what are the divergence points between extrusion induced by bacteria or by xenobiotics.

Despite activating the same process, caffeine and ethanol also have some differences between them: feeding the former is very lethal but does not seem to induce damages to the intestinal epithelium, whereas EtOH is well tolerated by flies that yet increase ISCs compensatory proliferation, at least at the concentration we used. Finally, upon caffeine ingestion we observed a production of mitochondrial H_2O_2 similar to that caused by Dbl1, but it is not the same for EtOH. On the one hand this was disappointing, as those differences did not allow us to describe a common mechanism for the induction of enterocyte purge. On the other hand, this is stimulating because it could mean that this resilience mechanism can occur in various ways and therefore represents a broad defense tool rather than a specific response to a single molecule inducing a single pathway. As a matter of facts, the program for the purge might be triggered by two different mechanisms at least.

However, it is critical to remark that EtOH and caffeine are both triggering a priming process in the fly's midgut, preparing the enterocytes to a second challenge with Db11 by preventing repeated purge events. The intestinal epithelium remains insensitive to hemolysin for a few days, but one week after the first xenobiotics exposure it becomes able to extrude again. We suspect that at the basis of this phenotype there is a transient imprint: the best molecular candidates are *CycJ* and the associated *whe* gene, which until now we thought to be mediators of the recovery. Indeed, *CycJ* knockdown flies were able to recover if the Db11 exposure is not sustained, and overexpressing *whe* reduced the amount of thin guts, mimicking a priming event. Besides, the duration of priming appears to be linked to the enterocyte's lifespan. We measured a massive cell turnover at day four, which would coincide with the loss of the cells having received the imprint during the first exposure. Although more experiments are needed to fully elucidate the priming process, this work paved the path for the future investigations.

1.2 The variability of the epithelial thinning

The digestive tract is one of the main interfaces between the external and internal milieu, and as such many factors influence its physiology. Nutrition and microbiota are two major parameters to take into account, since even little changes may affect gut homeostasis in a considerable manner. The intestine is also at the center of a communication network with other organs, like the CNS and the fat body, regulating wide biological processes as behavior and metabolism. This high sensitivity to environmental variations surely constitutes a significant setback (but also a more realistic view) for *in vivo* studies on this organ.

Several aspects of the enterocyte purge and recovery are well established: nevertheless, we cannot help noticing variability of the thinning degree in our experiments. First of all, we must have a critic view of our scoring system. Indeed, it remains a semi-quantitative method that relies in part on the perception of the observer and therefore with a relevant subjective prospect. We tried to minimize this limitation by doing blind scores and/or performing a second check by another observer. However, to completely eliminate the bias we must setup an automated routine method that allows us to measure the thickness of the intestine with confidence and rapidity. Nevertheless, such analysis is not as easy as it seems: it already implicates taking multiple photos of each intestine used in the experiment, being very time-consuming. Moreover, we have to proportion the thickness of the enterocytes with the total width of the intestine, which varies from individual to individual. To further complicate the interpretation, it can happen that the two sides of the R2 region present different cellular morphologies, making multiple measurements necessary for each gut. Consequently, we must define a method which takes into consideration all these factors and in parallel ensure rapidity and reliability of execution.

Many factors influence the intestine and by consequence also our phenotype. Although rarely enough, some individuals or some strains display variability of epithelial thickness also after sucrose treatment. When in this control condition we observe a considerable amount of semi-thin epithelium the interpretation becomes complicated. Moreover, as we are in a model of oral infection, we cannot have total control over the quantity and the timing of feeding. The identification of the exact moment when the flies start to feed is of crucial importance to have precision in the time point. We addressed the issue with the addition of a food dye in the solutions, which partially solves the problem although not totally. These concerns appear to be linked somehow to the fly genotype: certain strains eat faster and more than others, and differences in the degree of thinning are also found. Since *Drosophila* has a very fast life cycle, the stocks have to be regularly isogenized to guarantee a stable and uniform genetic background. Another factor that may vary from individual to individual is the microbiota. The microbial community in the gut significantly influence many aspects of the host physiology, and some data suggest that also the extrusion extent convey the presence of certain bacteria. This aspect is also intertwined with the diet, which has a critical importance for any study on the intestine. The use of a rich or a poor diet regulates the host metabolism, shapes differentially the microbial community and has a deep impact on the thinning phenotype. During my PhD I found myself facing these disturbers several times, and their understanding will create a more straightforward system that will facilitate future studies.

1.3 The biological relevance of the cytoplasmic purge

In conclusion, we ask ourselves an important final question: what is the physiological meaning of the extrusion and recovery process? Why has *Drosophila* evolved such a mechanism and what advantage does it confer in nature? Our data led us to wonder whether it protects against occasional exposure or it represents a defense to chronic threats. The discovery of the priming, where a second exposure to *S. marcescens* after xenobiotics feeding does not cause an epithelial thinning, tells us that the purge does not happen repeatedly. Although we do not have definitive answers from survival studies in primed flies, we hypothesize that a recurrent or sustained cytoplasmic extrusion has deleterious consequences for the organism. This occurs for instance in the case of another resilience mechanism: in response to prolonged mitogenic signals repeated regenerative episodes lead to ISCs loss impairing gut homeostasis (Haller et al., 2017). Hence, the priming may serve to hinder frequent purges in order to avoid energetic exhaustion and disruption of intestinal functionality. Indeed, *CycJ*-deficient flies, which display a thin epithelium constantly upon infection likely because of repeated extrusion events, succumb readily after. Moreover, host defense can come with a remarkable cost (). In an evolutionary regard, *Drosophila* may have evolved this regulation as they encounter bacteria and EtOH very often in the wild. Then, humans conserved the mechanism to prevent deleterious consequences from food intoxication; in more

modern times, cytoplasmic purge and priming might be helpful to withstand frequent consumption of caffeine and ethanol.

Albeit the cytoplasmic purge and the subsequent recovery does not involve ISCs compensatory proliferation, we did observe an activation of cellular division later. Thus, it is logical to ask what the usefulness of this mechanism is if the epithelium is renewed the following day. We can think that enterocyte's cytoplasmic extrusion serves as a first line of defense upon acute exposure to toxin to preserve the integrity of the gastrointestinal barrier. The expulsion of cytoplasm and damaged organelles it is likely to protect the organ from toxins and virulence factors, alleviating the stress prior to cellular turnover. This hypothesis is supported by the fact that a short exposure to prime the flies does not induce ISCs proliferation the next day, likely because the extrusion is sufficient to endure the stress. Notably, the purge also limits the bacterial passage from the gut to the hemolymph avoiding a systemic infection. It would be interesting to know how many bacteria enter the circulation if the fly was previously primed.

S. marcescens hemolysin is not the only PFT triggering the cytoplasmic extrusion: the laboratory has previously reported that also *Pseudomonas entomophila* monalysin possesses this ability (Lee et al., 2016). Moreover, we showed here the conservation of this resilience process upon exposure to xenobiotics, caffeine and ethanol. Whether other bacteria owning PFTs and xenobiotics with a "*whe* genes profile" are also able to induce the intestinal thinning represents an appealing perspective. Expanding our view, we might ask whether this purge is relevant for intestinal inflammation, diarrheal diseases and stunting. In the year 2000, the World Health Organization (WHO) and United Nation Children's Fund (UNICEF) report on Water supply, Sanitation and Hygiene (WASH) estimated at 500 the number of children who died in Sub-Saharan region for these deficits (WHO & UNICEF, 2000). The poor WASH measures in these countries is at the basis of growth impairment and pathologies (Cumming and Cairncross, 2016; Hutton and Chase, 2016), which cannot be cured by nutrient supplementation. Therefore, research on contaminants (biotic or abiotic) and their effect in the digestive tract acquires primary relevance for human health.

2. Materials and methods

2.1 Fly husbandry and strains

Drosophila melanogaster flies were raised at 25°C with 60% humidity, 14h of day light on a semi-solid cornmeal medium. Composition: 6,4% (w/v) cornmeal (Moulin des Moines, France), 4,8% (w/v) granulated sugar (Erstein, France), 1,2% (w/v) yeast brewer's dry powder (VWR, Belgium), 0,5% (w/v) agar (Sobigel, France), 0,004% (w/v) 4-hydroxybenzoate sodium salt (Merck, Germany).

All experiments were performed on three to seven days old female flies, unless diversely specified. The WT reference strain was *white* (w^{A5001}). The tissue-specific driver NP1 (NpGal4Gal80^{ts}), also known as Myo31D, was used to guide genetic modifications specifically in the enterocytes (Cronin et al., 2009; Nehme et al., 2007). The Gal4Gal80^{ts} construction allowed the genetic manipulation in a tissue- and time-specific manner. For the experiments involving transgenic flies, this driver was used either alone or crossed with the GD RNAi control line from Vienna Drosophila RNAi Center (VDRC stock number #60.000) or the GFP overexpressing line (UAS-GFP). Crosses were performed at 18°C, using virgins from the driver line and males of the chosen genetic line. The progeny of these crosses was harvested and placed at 29°C with 70% humidity for 5 days for the degradation of Gal80^{ts} and the activation of Gal4 (Figure M1). The transgenic lines come either from the Vienna Drosophila RNAi Center (marked VDRC) or from the Bloomington Stock Center (marked Bl). For RNAi lines, the lines used are the following: UAS-DuOx^{RNAi} GD (VDRC #2593), UAS-IRC TRiP (Bloomington #57814), UAS-CycJ^{RNAi} KK (VDRC #10222), UAS-CycJ^{RNAi} TRiP (Bloomington #37521). For overexpression lines, the lines used are the following: UAS-GFP (Bloomington #1521), UAS-Cat (Bloomington #24621). UAS-DuOx^{RNAi} WJL and UAS-IRC are a generous courtesy of professor Won Jae Lee (Ha et al., 2005a, 2005b). The roGFP2 flies strains were a generous courtesy of Jörg Großhans (Albrecht et al., 2011). Genotypes are the following: cytoplasmic-Grx1 (P003, pUAST cyto-Grx1-roGFP2 II HV (line 13)/CyO; tubulinGal4/TM3Ser, strong expression); mitochondrial-Grx1 (P006, pUAST mito-roGFP2-Grx1 II HV (line 9)/CyO; tubulinGal4/TM3Ser, strong expression); cytoplasmic-Orp1 (P015, pCasPeR4-cyto-roGFP2-Orp1 II HV (line 1) strong expression); mitochondrial-Orp1 (P018, pCasPeR4-mito-roGFP2-Orp1 II HV (line 6) strong expression). CncC-ARE-GFP flies for ARE containing genes induction come from Sykiotis & Bohmann, 2008. For clonal analysis of enterocyte total number the line w; esgGal4tubGal80^{ts} UAS-GFP; UAS-flp Act>CD2>Gal4 (Jiang et al., 2009) was used. Lines overexpressing *what else* or *lacosta* (UAS-whe, UAS-lcs) were previously constructed in the laboratory (Lee et al., 2016).

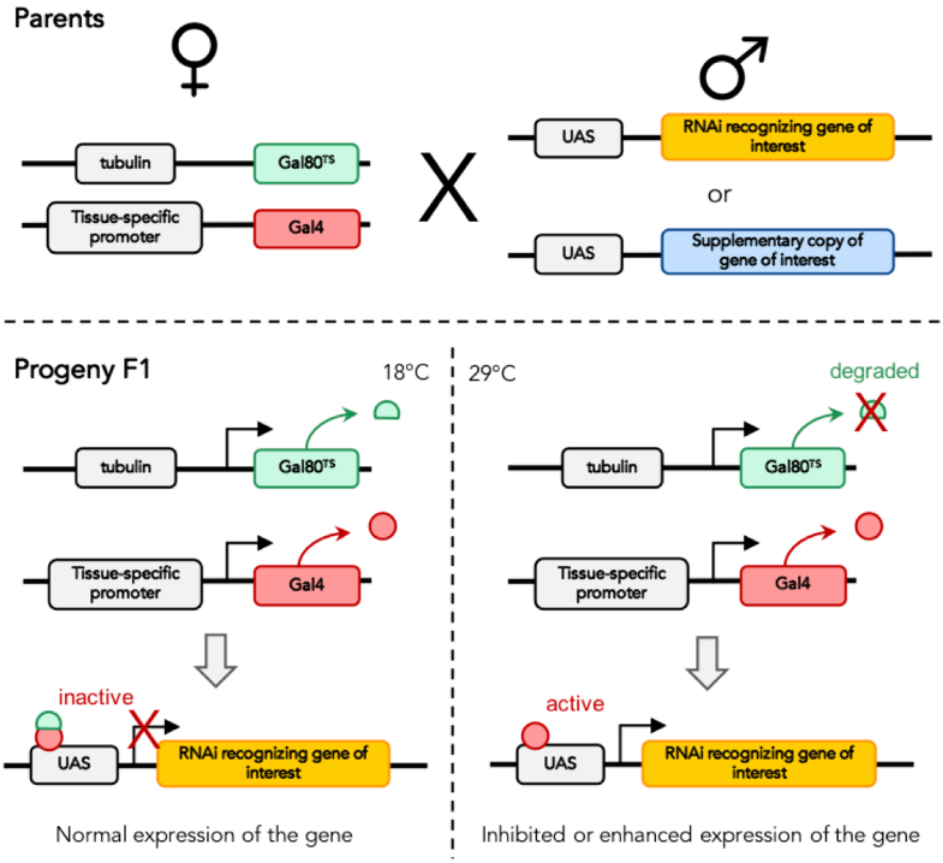


Figure M1. The UAS-Gal4Gal80^{ts} system. A fly line carries the yeast's Gal4 transcription factor under the control of a tissue-specific promoter and the Gal80^{ts}, which is a thermosensitive Gal4 inhibitor, under the control of the tubulin ubiquitous promoter. This line is crossed with another fly strain carrying interference RNA (RNAi) specific to a gene of interest or a supplementary copy of the gene of interest downstream a UAS (Upstream activating sequence) sequence. Crosses and development of the progeny is performed at 18°C, where the Gal80^{ts} inhibits the activity of Gal4. This avoids any influence during fly's development. When progeny F1 is switched to 29°C, Gal80^{ts} is degraded, thus the binding of Gal4 to UAS allows to express the RNAi or the supplementary copy, thus silencing or overexpressing the gene of interest.

2.2 Bacterial strain

Serratia marcescens Db11 strain (Flyg et al., 1980) was cultured in LB-agar plates with 100 µg/mL of streptomycin and incubated overnight at 37°C to obtain colonies. The plate is then kept at 4°C for around two weeks. One single bacterial colony is inoculated into liquid LB medium. These cultures were placed overnight at 37°C with agitation to induce bacterial growth.

2.3 Oral exposure

Except for survival assays, the bacterial infections were performed using a final bacterial OD_{600} (optical density measured at 600nm) of 10. A liquid culture was grown overnight (see above), and the resulting OD_{600} measured with a spectrophotometer. A part of this culture as needed was centrifuged at 4000rcf for 10minutes at 4°C. The pellet was resuspended in the appropriate volume to reach an OD_{600}=10 using a 50mM sucrose solution containing 10% of LB. EtOH solutions were prepared diluting pure EtOH in sucrose 50mM in order to obtain a final concentration of 2,5%. To obtain a 2,5mg/mL caffeine solution, 125mg of caffeine powder (Sigma-Aldrich, C0750) was diluted in 50mL of sucrose 50mM and placed 30 minutes in bain-marie at 50°C to favor the solubilization. Antioxidants (DTT: Euromedex EU0006-D; NAC: Sigma-Aldrich, A7250; Vit. C: Fluka/ Sigma-Aldrich 95210; mitoTEMPO: Sigma-Aldrich SML0737), H_2O_2 (Fisher Scientific H1750/15) and paraquat (Sigma-Aldrich 856177) solutions were prepared in a similar manner by diluting the powder or the stock solution in sucrose 50mM.

2mL of the solutions described above were added in a medium-size tube (3,5cm diameter) containing two absorbent pads (Millipore AP1003700). A maximum of 20 females flies were putted on a single tube to feed these solutions or sucrose 50mM only as a control. Time points used are specified in the single experiments.

2.4 Survivals

Survival assay were performed on three to seven days old flies, at 29°C with 70% humidity, in biological triplicates of 20 flies per vial. Bacterial OD_{600} was adjusted to 1, unless diversely specified. Caffeine final concentration was 2,5mg/mL. EtOH final concentrations were either 2,5% or 5%. NAC was used at 20mM. Sucrose 50mM was used as control. At day0, 2mL of each solution were added in each tube (see oral infection). Each day survivors were counted, and 200µL of sucrose 100mM was added to all the vials, unless diversely specified. Logrank test was used for statistics with the GraphPad software Prism6.

2.5 Fluorescent histochemical staining and observation

2.5.1 Dissection

Fly were anesthetized with CO_2. Midguts were dissected in PBS 1x and fixed for 30 minutes in 8% paraformaldehyde (Electron Microscopy Science, cat #15710). Tissues were then washed three times with PBS-TritonX-100 0,1% (PBT 0,1%), and then saturated at least 1h with BSA 2%.

2.5.2 Actin staining

Samples were incubated in 10µM Fluorescein Isothiocyanate (FITC) phalloidin (Sigma-Aldrich #5282) or Texas-Red phalloidin (Invitrogen™ #7471) for 1h30 at room temperature (RT) or overnight at 4°C. Samples were then washed three times with PBT 0,1%.

2.5.3 Propidium iodide staining

Flies were fed with a solution (sucrose 50mM+10% LB) containing GFP-expressing bacteria (OD_{600}=10) and 50µg/mL of propidium iodide for 3h at 29°C. Midguts were dissected and fixed as described above.

2.5.4 Phosphohistone3 (PHH3) staining

The anti-PHH3 antibody (Millipore ref 09-797) was diluted 1:500 in PBT 0,1% + BSA 2%. Samples were incubated in this solution 2h at room temperature or overnight at 4°C, then washed three times in PBT 0,1% and incubated with goat anti-rabbit FITC antibody (Abcam #6717) diluted 1:1000 in PBT 0,1% + BSA 2%. Samples were finally washed three times in PBT 0,1% prior to mounting.

2.5.5 Mounting & Microscopy

All samples were mounted on 8-wells diagnostic microscopy slides (Thermo Fisher Scientific) with Vectashield containing DAPI (Vector Laboratories) and then stored in the dark at 4°C. Samples were observed using a LSM780 confocal microscope (Zeiss) or an epifluorescence Axioscope2 microscope (Zeiss) as needed. Images were taken using a plan/apochromat 20x/0,8 dry objective. Raw files were treated and analyzed using the ImageJ/Fiji software if needed.

2.6 Bacterial titer in midguts

To measure bacterial CFU per gut (Db11), biological quadruplicates of 5 midguts were dissected in 100µL of sterile PBS 1x. Tissues were homogenized using a pestle motor mixer (Argos). Serial dilutions (10^{-1} to 10^{-6}) were applied to PBS volumes and 5µL of each dilution was plated on LB-agar plates containing 100 µg/mL of streptomycin. These plates were dried 10 minutes under bacterial hood and then incubated overnight at 37°C. The next day isolated colonies that grew on plates were counted. Bacterial CFU per gut was determined with respect to the dilution, the plated volume (5µL), the number of midguts (5) and the initial volume of PBS (100µL).

2.7 Fluorometric H_2O_2 quantification assay

H_2O_2 content in midguts was estimated using the fluorometric H_2O_2 quantification assay (Sigma-Aldrich, MAK165). Sample preparation: biological duplicates of 10 midguts per condition were dissected in sterile PBS 1x and readily putted in tubes on dry ice. 50µL of the kit's Assay Buffer were added and tissues were crushed with a Precellys homogenizer (Bertin Instruments). The rest of the protocol was performed following the manufacturer's instructions. This kit utilizes a peroxidase

substrate that generates a red fluorescent product after reaction with H_2O_2. This fluorescence (λ_{ex}=540/λ_{em}=590 nm) was measured using the Varioskan™ Lux microplate reader (Thermo Fisher).

2.8 Epithelial thickness score

To estimate the level of thinning and/or the recovery capacity, midguts were classified in three different categories: thick (a), semi-thin (b) or thin (c) (Figure M2). To determine overall epithelial thickness, two main parameters were evaluated: presence of the dome-like structures and cellular thickness. For this evaluation, only the R2 region of the midgut was considered. To make the assessment more objective, a blind score method or confirmation from a second observer is often applied.

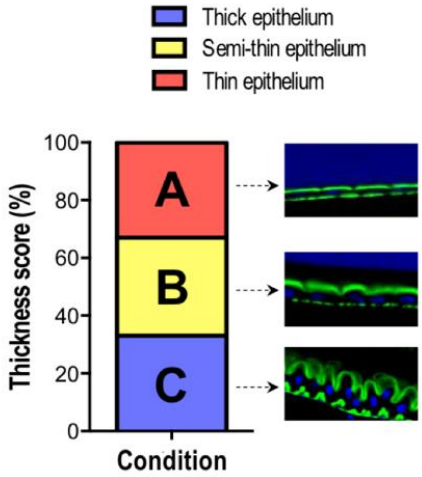


Figure M2. Estimation of epithelial thickness. To assess the level of the thinning and the capacity to recover, midguts were classified in three different categories. This thickness score is based on two main parameters: cellular volume and dome-like structure. If both are lacking or severely reduced, the midgut is classified as thin (A, red). If only one of the two is affected, the intestine is included in the semi-thin category (B, yellow). Finally, if enterocytes are thick and with the dome-like structure, the epithelium is considered thick (C, blue). χ^2 statistical test was performed.

2.9 roGFP2

Original and more detailed protocol: Barata & Dick, 2013.

2.9.1 Reference samples

To obtain fully oxidized and fully reduced samples, midguts were dissected in PBS 1x and then incubated 10 minutes in diamide (DA) or dithiothreitol (DTT) for oxidized and reduced references, respectively. After washing in PBS, these samples were placed 15 minutes in N-ethyl-maleimide (NEM) 20mM in order to preserve the redox state of the tissue. Midguts were quickly washed again in PBS and then incubated in PFA 4% for 20 minutes and mounted in Vectashield without DAPI.

2.9.2 Experimental samples

For dissection of experimental conditions, PBS was supplemented with NEM 20mM to preserve the redox state of the tissue. The midguts were further alkylated in NEM for 15 minutes after dissection. After washing in PBS, samples were fixed in PFA 4% for 20 minutes and mounted in Vectashield without DAPI. Prior to image acquisition, laser intensity for 405nm and 488nm excitation was adjusted using the fully oxidized and fully reduced samples, respectively. These settings are no longer modified for all the samples in the same experiment. For imaging, the probe was excited sequentially at 405nm and 488nm and emission was recorded at 500-530nm.

2.9.3 Image processing

Image treatment was performed adapting the protocol from (Kardash et al., 2011). Briefly, rolling ball procedure set to 50 pixels was used to subtract background and images were converted to 32-bit. The image intensity was thresholded with the default settings in greyscale and dark background. Values below the threshold were set to “not a number”. The plugin RatioPlus was used to generate ratio images by dividing the 405nm picture by the 488nm corresponding picture. The lookup table “fire” was used for final coloring. The intensities were finally normalized to the DTT control (fully reduced).

3. Annex

The Lipidic Purge

Since the discovery of the cytoplasmic purge, several ramifications arose from this project to understand in detail this mechanism of resilience. Nevertheless, this is not the only subject of interest in our laboratory. During my PhD, I spent almost two years on the study of what we though were another kind of purge: the lipidic purge.

This project began from a previous one conducted in collaboration with Delbac's team in Clermont-Ferrand. The microsporidium *Tubulinosema ratisbonensis*, a fungal intracellular pathogen, preferentially targets the fat body, a key metabolic organ in insects. When invaded by the parasite, fat body cells progressively lose their lipid reserves, scavenged by the parasite at the expense of the host (Franchet et al., 2019). Microsporidia contribute to the loss of honeybee colonies; they succumb even faster when also exposed to sub-lethal doses of pesticides, *e.g.*, the phenylpyrazole fipronil (Aufauvre et al., 2012; Vidau et al., 2011).

A previous member of the team showed that also *Drosophila* succumbs earlier to the double exposure, yet the parasite load decreases. This phenomenon was accounted by an exacerbated loss of lipids: both the parasite and the pesticide would compete for lipids and the host likely succumbs for the exhaustion of its energy reserves. The continuous ingestion of low doses of fipronil led to a constant formation of lipid droplets in the anterior midgut that were ultimately released in the gut lumen and feces. A detailed analysis with higher doses revealed that there was first the formation of numerous small lipid droplets, then a giant droplet formed in the apical region of enterocytes and was ultimately released into the lumen. There was no simultaneous loss of cytoplasm or any thinning of the epithelium, thus the lipidic purge is distinct from the cytoplasmic purge. Blocking the genes involved in droplets transport and fusion by RNAi was sufficient to block the lipidic purge.

Fipronil is known to inhibit the GABAa receptor Resistant to Dieldrin (Rdl) (Buckingham et al., 2005; Hosie et al., 1995; Remnant et al., 2014; Zhang et al., 2016); thereby, it activates neurons acting downstream of GABAergic neurons, since GABA is the main inhibitory neurotransmitter. Upon prolonged ingestion flies showed a behavioral phenotype, with strong seizures and uncontrolled movement of wings and legs. 24h after the ingestion of high doses of fipronil, only flies in which purge was blocked exhibited seizures and succumbed shortly thereafter. Chemically, fipronil molecule has hydrophobic properties. A fipronil fluorescent version is found into the lipid droplets of the fat body

and in enterocytes. He concluded that also the lipidic purge is protective for the fly, in this case from ingested fipronil. The crosstalk between the gut and the fat body would also have a crucial role for the phenotype, because the lipid level in these compartments determines whether the fly will be more sensitive on a low chronic or a high acute fipronil exposure. These data indicated that the lipidic purge protects the flies from the deleterious effects of an acute exposure to this pesticide but becomes deleterious upon prolonged exposure to low doses by depletion of lipid reserves. Interestingly, fipronil also induced a lipidic purge in honeybees and Caco-2 cells exposed to fipronil underwent a loss of lipid reserves. Injection of fipronil in the hemocoel induced the purge at least 3h faster than upon ingestion, likely because it has not to cross the midgut epithelium to reach the central nervous system. When Rdl was targeted by RNAi using a ubiquitous driver, the lipidic purge occurred constitutively, likely activating the neurons that are normally inhibited by *Rdl*-expressing cells. Blocking synaptic transmissions of these neurons, the lipidic purge induced by fipronil ingestion was prevented. In the proposed model, after the ingestion, the hydrophobic molecule fipronil crosses the gut and reach the Rdl receptor in the brain, acting as an inhibitor. *Rdl*-neurons inhibition triggers the activation of downstream neurons which innervate the intestine, inducing the formation, fusion and then extrusion of lipid droplets containing the pesticide.

All those data were collected until August 2017. Surprisingly, when I took the project in September those results could not be reproduced. I started to observe big lipid accumulation in enterocytes even in absence of fipronil (Figure A), not only in our wild type strain w^{A5001}. The lipid increase is particularly evident after exposure to sucrose, but also when flies are dissected directly from the food vials. Even if lethality and seizure are still present after pesticide exposure, for months I cannot see any purge in the intestine or the lipid loss in the fat body. Therefore, we thought about a food or water contamination with a compound capable of enhancing lipid accumulation in gut cells. Nonetheless, all the studies performed on food or water sources could not restore the previous phenotype. A list of all the test executed is exposed in Table A. Moreover, the phenotype with motor proteins for transport was not confirmed, and flies survived well after injection of high doses of fipronil. Another PhD student of the team, Gaétan Caravello, joined the project to solve the problem, and other tests were conducted on the chemical product itself: we ordered fipronil from different suppliers and we payed particular attention to the solution preparation (solvents, sucrose and solubilization), also checking their chemical profile by HPLC, without visible improvement. Then, we reasoned that the problem must reside in the flies. I bleached the flies with the goal to clear them from potential pathogens and microbes that can perturb lipid homeostasis, but the droplets amount did not change. In the end, one last hope came out when we tested different wild type strains for exposure to fipronil. Indeed, a w^{1118} strain that we receive some months before from Lemaitre's laboratory was not displaying

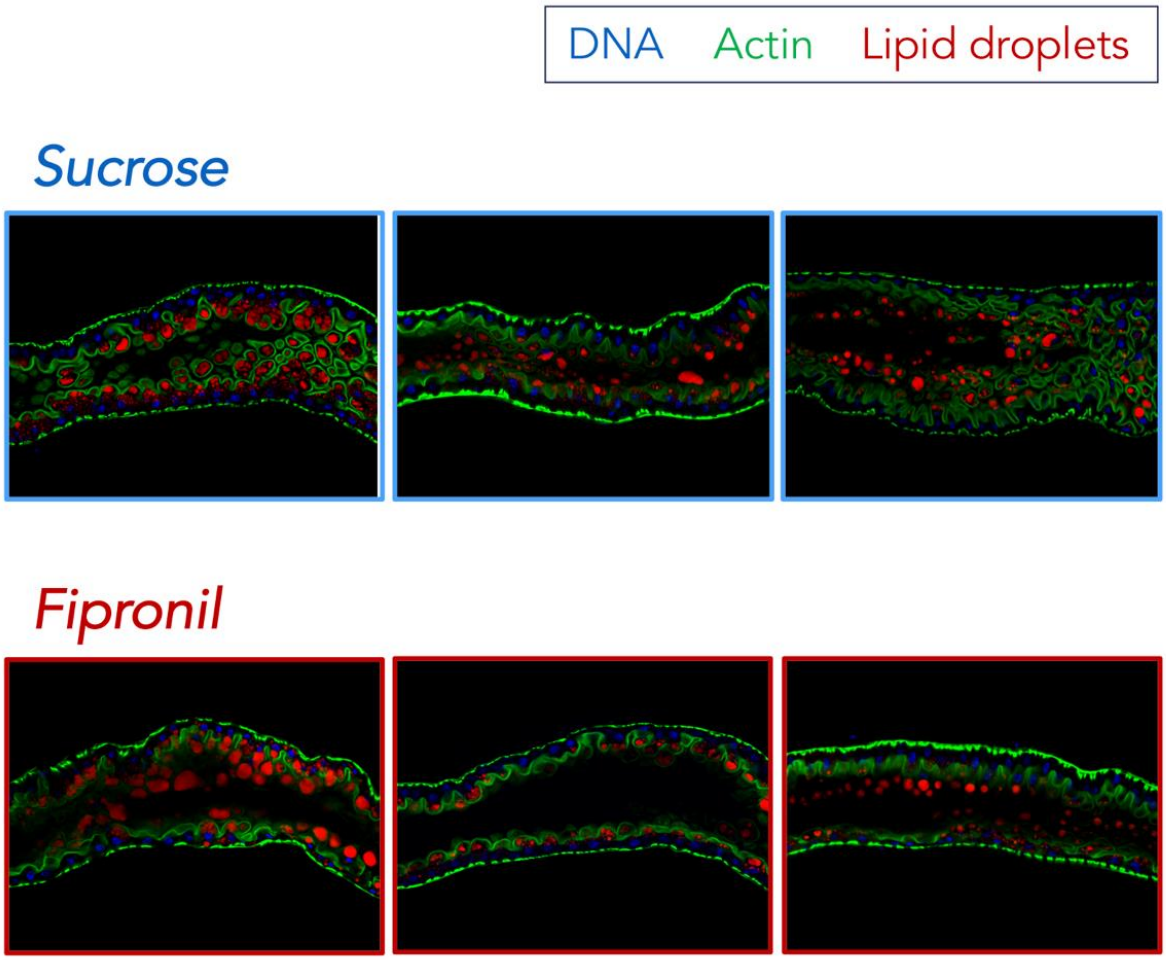


Figure A. Lipid droplets in the *Drosophila* midgut. Ethanol 2,5% or Fipronil 100ng/mL. Dissection started 5 hours after ingestion and then stained with phalloidin in order to visualize actin and Nile Red to visualize lipid droplets. Numerous lipid droplets are present: some of them are inside the plasma membrane of enterocytes, others seem to be extruded into the gut lumen.

exacerbated lipid accumulation in the intestine, neither on food nor in sucrose. Paradoxically, in this case we did not observe a lipidic purge upon fipronil exposure.

It is well known that the gut microbiota impacts host's metabolism. For instance, the presence of species of the genus *Acetobacter* strongly impact the lipid quantity in the digestive tract by producing the short chain fatty acid acetate (Kamareddine et al., 2018). We compared the microbiota of w^{A5001} and w^{1118}, looking for a differential presence of *Acetobacter*, or we tried administration of acetate to directly rescue the accumulation phenotype remarked in w^{A5001}. Yet, the generation of axenic flies or subsequent mono-association with interesting microbial species (*Acetobacter*, *Lactobacillus*) failed similarly to the other approaches.

In conclusion, what appeared to be a solid and appealing project almost ready for publication turned out to be an enigma. Excluding the lipid stores loss in the fat body upon fipronil feeding, I could not reproduce any of the precedent results. Not only we lost the phenotype within a few weeks, but all the experimental tests that followed did not give us an answer about the triggering cause. After much time spent in unsuccessful trials, I left the project in the hand of my colleague Gaétan. Actually, we currently suspect that fipronil exposure evokes some features of a starvation. Flies avoid fipronil or they eat less, activating pathways to provide energy from internal stores as autophagy and beta oxidation.

Food	Water	Exposure	Flies	Solutions
Poor/ Rich diet	Tap	Feeding pads	Bleached strains	Sucrose different batches
Yeast added/No yeast added	Osmosed	Food (top or mixed)	Axenics	Prepared from another person
Food vials from another laboratory (IGBMC, Ilkich)	MilliQ	Eppendorf cap	Monoassociations: Lactobacillus and Acetobacter	Solubility: HPLC
Starvation (3h or 14h)	Commercial brands (Cristaline, Volvic, La roche des écrins)	Injection	Antibiotics fed	Different providers of fipronil (pestanal, EP, traceCERT, Santa-Cruz)
Sucrose + amino acids supplementation (5h, 1day, 2days, 3days)			Feces transfer	Fipronil metabolites: fipronil sulfone
Water source			w^{A5001} from other teams	Sonication
Acetate supplementation			Other WT strains	Doses reponses
			Temperature	

Table A. List of tests performed in order to identify possible contaminants or variables interfering with the lipidic purge phenotype.

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Partie en Langue Française (10%)

1. But de l'etude

Il y a plus de dix ans, Nadine Nehme avec le reste de l'équipe a établi un modèle d'infection orale avec la bactérie *Serratia marcescens* (Nehme et al., 2007). Dans les années qui ont suivi, d'autres chercheurs dont Kwang-Zin Lee, Matthieu Lestradet, Richard Bou Aoun, Stephanie Limmer, Catherine Socha et Samuel Liegeois ont décrit un tout nouveau mécanisme de résilience dans l'intestin moyen de la mouche, la purge cytoplasmique (Lee et al., 2016). Leurs travaux ont permis non seulement d'identifier le rôle central de la toxine formant des pores hémolysine dans l'induction de l'extrusion cytoplasmique par les entérocytes, mais aussi de caractériser les gènes Cycline J et what else.

A mon arrivée au laboratoire, si d'une part il était bien établi que l'hémolysine de *Serratia* était un facteur nécessaire et suffisant pour déclencher l'ensemble du processus, le signal responsable de l'induction de l'extrusion cytoplasmique chez la mouche était encore inconnu. Dans le but d'identifier le facteur de l'hôte requis pour démarrer la purge cytoplasmique, nous avons concentré notre attention sur les événements qui précèdent l'amincissement épithélial. Comme détaillé dans l'article, les premiers événements d'extrusion ont lieu environ 1h après l'ingestion bactérienne. Avant cela, nous avons pu imager une accumulation transitoire de gouttelettes lipidiques et la formation de mégamitochondries. Comme indiqué dans la littérature, ces deux processus peuvent viser à atténuer le stress oxydatif au sein de la cellule (Bailey et al., 2015 ; Liu et al., 2015a ; Nagaraj et al., 2012 ; Shutt et al., 2012). De plus, dans notre expérience de séquençage d'ARN, nous avons trouvé l'induction de certains gènes antioxydants (peroxiredoxin2540, jafrac1, jafrac2, GST-D10, GST-E8) à 6h et 9h post-infection. Enfin, en cherchant dans les données génomiques disponibles sur FlyBase, nous avons remarqué que certains métaux (Cu, Zn, Cd) et le paraquat, agents oxydants bien connus, induisent également les gènes whe (ainsi que d'autres xénobiotiques comme l'éthanol et la caféine). Ces indices peuvent indiquer les ROS comme des molécules de signalisation dans les phases très précoces de la purge cytoplasmique. Des résultats antérieurs ont également indiqué que certains de ces xénobiotiques, notamment le Cd, le paraquat et la caféine, semblaient altérer la morphologie de l'intestin moyen avec la formation préalable de mégamitochondries. Cela peut suggérer que la purge des entérocytes est une réponse cellulaire courante à l'exposition à des toxines formant des pores ou à des xénobiotiques. Le cas de la caféine et de l'éthanol est particulièrement intrigant, car ce sont les xénobiotiques auxquels l'homme est (volontairement) le plus exposé. Par conséquent, nous avons également cherché à comprendre s'il existe

une interaction entre le défi bactérien et le défi xénobiotique, dans ce qui pourrait être une réponse « de priming » des entérocytes contre des expositions répétées à ces stimuli.
Pour cela, l'objet de ma thèse peut se résumer en trois questions principales :

Les ROS sont-ils impliqués dans l'induction de la purge cytoplasmique et quelle est leur dynamique ?
(Chapitre 1)

La purge cytoplasmique est-elle une réponse conservée face aux stimuli xénobiotiques tels que la caféine et l'éthanol ?
(Chapitre 2)

Y a-t-il une réponse de priming ou un priming croisé, qui prépare les entérocytes à un deuxième défi et empêche les extrusions continues de se produire ?
(Chapitre 2)

Enfin, dans une courte annexe à la fin du manuscrit, je résumerai les principaux points d'un projet parallèle sur lequel j'ai travaillé pendant ma thèse.

2. DISCUSSION

2.1 Naissance du projet xénobiotique

Après plusieurs années de recherche, nous avons actuellement décrit de multiples caractéristiques de la purge cytoplasmique provoquée par *S. marcescens*. Au-delà de la conservation de ce mécanisme chez les mammifères et l'homme, il est important de comprendre si cela représente une réponse spécifique aux toxines formants des pores ou s'il s'agit d'une réponse de défense intestinale plus générale. Bien avant mon arrivée dans le laboratoire, d'autres chercheurs ont échoué dans la tentative d'induire des cycles récurrents de purge par des expositions répétées au Db11. De plus, en comparant le résultat moléculaire de l'infection bactérienne avec des bases de données accessibles au public, nous avons trouvé de multiples composés qui induisent également la classe de gènes whe dépendants de la CycJ. Certains d'entre eux ont été testés dans le modèle d'infection orale, et en particulier la caféine et l'éthanol ont montré des caractéristiques communes à l'infection bactérienne, à savoir la formation de mégamitochondries et l'altération de la morphologie de l'entérocyte. De plus, il y a quelques années, nous avons changé la recette du milieu de culture des mouches pour un aliment plus riche contenant cinq fois plus de levures et dans lequel l'agent antifongique était dilué dans de l'éthanol à 2,5 % au lieu d'eau. Soudainement, après ce changement, nous avons perdu l'amincissement épithélial lors de l'infection par *S. marcescens*. Des tests ultérieurs ont indiqué que ces deux éléments, la levure et surtout l'éthanol, étaient les perturbateurs responsables de l'altération du phénotype.

Sur la base de ce contexte, le but de ce projet était de mieux caractériser la réponse aux xénobiotiques. Au cours de ma thèse, nous nous sommes principalement concentrés sur la caféine et l'éthanol pour les raisons suivantes : i) leur utilisation fait désormais partie du mode de vie de l'homme, et ils sont largement les xénobiotiques les plus volontairement ingérés dans le monde ; ii) d'autres composés testés, tels que le paraquat et le cadmium, provoquent une altération de l'épithélium intestinal, mais celle-ci se produit avec une cinétique très tardive et est suivie immédiatement par la mort. Par conséquent, bien que le paraquat et le cadmium puissent provoquer une purge cytoplasmique, dans leur cas ce mécanisme peut ne pas être suffisant pour assurer la survie de la mouche aux effets nocifs apportés par ces composés.

2.2 La caféine, l'EtOH et l'amincissement épithélial

Le choix des concentrations était essentiel, car comme nous l'avons vu dans l'introduction, ces deux composés ont des résultats très différents selon la quantité prise. Nous avons utilisé la caféine à 2,5 mg/mL (environ 13mM) qui est approximativement la concentration trouvée dans une tasse d'expresso, et c'est la concentration également utilisée par d'autres (Brown et al., 2014). L'EtOH a été utilisé à 2,5%,

car c'est la concentration utilisée pour la préparation de la nourriture de la mouche, qui a provoqué un effet sur l'intestin. À ces doses, notre analyse temporelle a révélé que les xénobiotiques provoquaient effectivement une purge cytoplasmique (figure 2.7). Nous avons pu visualiser des groupes de cellules en train d'extruder à partir de 30 minutes après l'exposition aux xénobiotiques (Figure 2.8). Après 1h d'alimentation, les altérations morphologiques et la perte d'épaisseur des cellules étaient bien visibles, ce qui signifie que la purge cytoplasmique avec la caféine et l'EtOH présente une cinétique plus rapide par rapport au Db11. Cela peut être attribué à l'absorption rapide de l'éthanol et de la caféine par la cellule ; à l'inverse, l'activation du programme de virulence de Db11 impliquant la sécrétion d'hémolysine peut nécessiter plus de temps. En outre, l'amincissement de l'épithélium n'est pas apparu exactement de la même manière : lors d'une infection bactérienne, toutes les cellules d'une même région ont généralement le même aspect ; après l'administration de xénobiotiques, nous avons constaté un degré plus hétérogène d'aplatissement des entérocytes voisins. Cela pourrait être dû à la mise en œuvre de différents programmes au sein des cellules ou à une communication différente entre elles, selon que l'exposition est à l'hémolysine ou aux xénobiotiques. En effet, bien qu'une réponse CycJ/whe soit induite dans les deux cas, les différentes cinétiques suggèrent qu'une certaine variation se produit. Bien que l'amincissement hétérogène observé avec la caféine et l'EtOH puisse suggérer qu'il s'agit d'un processus cellulaire autonome, cette hypothèse est peu probable car l'extrusion s'est produite dans les amas d'entérocytes réunis (Figure 2.8), comme dans le cas de Db11. Alternativement, l'explication pourrait être purement mécaniste : les bactéries exercent une pression sur la matrice péritrophique et par conséquent sur les cellules tout au long de l'intestin moyen, provoquant un amincissement plus homogène. Ce type de contrainte physique est absent lors de stress abiotiques tels que les xénobiotiques. Néanmoins, il faut rappeler qu'aucun étirement des entérocytes n'est observé lors de la purge induite par Db11 (Lee et al., 2016). Parallèlement, le traitement à l'EtOH augmente la fluidité membranaire, ce qui affecte la composition de la membrane et la viabilité des cellules (Huffer et al., 2011 ; Tóth et al., 2014). Ces effets sur la fluidité membranaire sont en relation avec l'homéostasie des ROS (Sergent et al., 2005), qui est également impliquée dans la perturbation du cytosquelette d'actine médiée par l'EtOH (Loureiro et al., 2011). Comme mentionné précédemment, l'alcool et son métabolisme affectent également la fonction de barrière épithéliale en altérant les jonctions serrées et en augmentant ainsi la perméabilité intestinale (Dunagan et al., 2012 ; Elamin et al., 2013a, 2014b ; Ying et al., 2014). La fluidité membranaire, le cytosquelette et le remodelage des jonctions sont certainement des facteurs qui influencent la morphologie des entérocytes et ils peuvent donc intervenir dans le phénotype que nous avons observé. Néanmoins, un point commun qui réunit l'hémolysine, la caféine et l'éthanol est que tous trois peuvent induire (avec des mécanismes différents) un efflux de potassium (Dopico et al., 2014 ; Garcia et al., 2019 ; Hertle, 2005 ; Zhang et al., 2018). Ce phénomène devrait donc être étudié plus en détail, car il pourrait représenter un facteur important dans l'initiation de la purge des entérocytes.

2.3 ROS et xénobiotiques : une relation ambiguë

La signalisation H_2O_2 semble être impliquée dans les premières étapes du processus de purge cytoplasmique déclenché par *S. marcescens* (voir chapitre 1). Cependant, dans le cas de la caféine et de l'éthanol, l'interprétation des données est déroutante. En effet, alors que les trois stimuli ont induit l'ouverture des entérocytes avec une fuite du cytoplasme et un amincissement des cellules qui s'ensuivent, une augmentation du H_2O_2 mitochondrial a été observée après l'administration de Db11 et de caféine mais pas d'EtOH (Figure 1.15A & 2.9A). En revanche, les niveaux de H_2O_2 semblaient légèrement augmenter dans le cytoplasme après l'ingestion d'alcool, bien que la différence ne soit pas statistiquement significative et que la sonde cytoplasmique-Orp1-roGFP2 ait présenté une faible gamme dynamique (figure 2.9B). Par conséquent, ces résultats ne nous permettent pas de généraliser l'induction de la purge cytoplasmique médiée par H2O2. Les effets de la caféine sur l'homéostasie des ROS sont contradictoires dans la littérature : bien que la majorité des études soulignent un effet antioxydant de la triméthylxanthine (Khan et al., 2019 ; Nilnumkhum et al., 2019), il existe également des rapports qui la décrivent comme un pro-oxydant (Li et al., 2019 ; Wang et al., 2019). De l'autre côté, l'EtOH aurait des propriétés oxydantes (Lu et Cederbaum, 2008 ; Nordmann et al., 1992), mais cela est principalement dû à la génération de ROS comme sous-produit du métabolisme de l'EtOH. En effet, les deux composés sont métabolisés par les CYP, qui sont une source potentielle de ROS (Hrycay et Bandiera, 2012). Cependant, il est peu probable que ces processus soient pertinents dans notre cas d'étude, car nous avons observé un épithélium mince dès 1h après l'ingestion, et la dynamique métabolique prend probablement plus de temps. Néanmoins, nous devrions vérifier si le knockdown de Cyp2e1/ADH et Cyp12d1/Cyp1a2 affecte la purge induite par les xénobiotiques et l'induction de H2O2.

L'absence d'induction des gènes contenant l'ARE après l'exposition aux xénobiotiques (Figure 2.9C) représente une autre couche de complexité. En effet, bien que nous ayons noté une légère augmentation progressive après le traitement à l'EtOH, l'alimentation des deux xénobiotiques n'a pas stimulé de manière significative l'activité du CncC. Par conséquent, cela a mis en évidence une différence supplémentaire par rapport à l'ingestion de Db11. Néanmoins, étant donné la grande diversité et l'étendue de la réponse Nrf2, il est logique de penser que tous les gènes régulés par ce facteur de transcription ne sont pas exprimés au même moment et dans la même mesure. Les sites de liaison de Nrf2 contiennent des motifs consensus pour d'autres cofacteurs, qui affinent la réponse, et d'autres facteurs de transcription Cnc reconnaissent également des séquences ARE (Tonelli et al., 2018). Par conséquent, nous ne pouvons pas exclure complètement que l'EtOH et la caféine activent une réponse transcriptionnelle antioxydante par des voies alternatives. Néanmoins, d'après nos données, nous ne pouvons pas proposer un mécanisme général impliquant les ROS comme inducteur des extrusions cytoplasmiques après une exposition aux xénobiotiques, bien que la caféine ait montré une augmentation mitochondriale de H2O2 très similaire à celle de Db11 en temps et en intensité.

2.4 Diversité de la durée de vie et des dommages intestinaux

L'éthanol et la caféine ont altéré très différemment la survie des mouches. Le premier n'était pas nocif lorsqu'il était administré à la concentration que nous avons utilisée pour nos expériences sur l'amincissement épithélial : les drosophiles supportent très bien une seule exposition à l'EtOH 2,5% ou 5%. Ces quantités ne deviennent marginalement létales que lorsqu'elles sont administrées de manière chronique. A l'inverse, une exposition unique et aiguë à la caféine réduit considérablement la durée de vie. Ceci était attendu puisque la toxicité pour les insectes a déjà été rapportée (Ashihara et al., 2008 ; Nathanson, 1984), bien que la grande différence dans les taux de mortalité par rapport à l'EtOH ou même au Db11 était appréciable. Bien que les tests de survie soient essentiels pour étudier les réponses des organismes à un traitement donné, ils ne permettent pas d'expliquer la causalité de la mort. Puisque les trois agents (Db11, EtOH, caféine) déclenchent la purge cytoplasmique mais qu'ils ont produit trois courbes de survie très différentes lorsqu'ils ont été ingérés, nous pouvons spéculer que les différences observées dans la mortalité ne dépendaient pas de ce mécanisme de résilience. D'une part, nous pouvons émettre l'hypothèse qu'après l'amincissement et le rétablissement des entérocytes, l'EtOH est efficacement détoxifié par le métabolisme de l'hôte, principalement par l'ADH, ce qui n'a aucune conséquence pertinente sur la durée de vie de la drosophile. Il serait intéressant de vérifier si les mouches déficientes en ADH subissent toujours la purge cytoplasmique. D'autre part, la forte toxicité de la caféine pour les insectes a été attribuée à l'inhibition des phosphodiestérases (Mustard, 2014 ; Nathanson, 1984). L'épuisement des réserves intracellulaires de Ca^{2+} et l'impact sur le système nerveux par le biais des récepteurs de l'adénosine ou de la dopamine pourraient également être impliqués.

Lorsque nous avons évalué la réponse de l'intestin moyen, nous avons observé que l'éthanol induisait une prolifération compensatoire des CSI (cellules souches intestinales) plus importante que la caféine. Ceci représente une bonne méthode indirecte pour estimer les dommages survenant dans l'intestin. Par conséquent, l'ingestion d'alcool a modérément endommagé les entérocytes, très probablement par les mécanismes de perturbation de l'épithélium décrits ci-dessus. Nous devrions vérifier cette hypothèse en testant la perméabilité de l'épithélium par un essai SMURF et en mesurant directement la mort cellulaire lors de l'ingestion d'EtOH. À l'inverse, la caféine n'a pas semblé causer de dommages à l'épithélium intestinal. Nous pouvons donc imaginer qu'elle déclenche la purge cytoplasmique (par le biais de la signalisation H_2O_2 dans les mitochondries) pour protéger l'épithélium intestinal, mais qu'à la dose que nous avons utilisée, l'inhibition des phosphodiestérases et/ou l'effet neurologique sont encore mortels. Une autre possibilité est que la caféine bloque la prolifération des CSI. Tout d'abord, nous devrions tester des concentrations plus faibles de caféine pour trouver la dose sub-létale pour la drosophile, afin de vérifier si dans ces conditions on observe toujours l'extrusion cytoplasmique sans induire une forte mortalité. De plus, l'utilisation de concentrations élevées (mM) pourrait induire sélectivement certains types de réponses et masquer les effets de l'exposition à la caféine dans l'intestin

moyen. Deuxièmement, nous avons pu mesurer les niveaux d'AMPc et de Ca2+ parallèlement au test de survie à la caféine.

Bien que notre modèle d'infection orale par *Serratia* soit bien établi de nos jours, les causes qui conduisent à la mort de la mouche ne sont toujours pas très claires. La sensibilité de l'hôte dépend strictement de la charge bactérienne. Nous savons que la purge des entérocytes et la récupération subséquente est un mécanisme de protection qui limite le passage des bactéries dans l'hémolymphe et permet de se débarrasser des organelles et des composants cellulaires endommagés et peut-être aussi du PFT. Même si ce processus n'implique pas la mort cellulaire, nous avons mesuré une augmentation de la prolifération des CSI 1 jour après l'ingestion de Db11 (Lee et al., 2016 & Figure 2.10). Nous émettons l'hypothèse que la purge cytoplasmique est une première réaction rapide à une exposition accidentelle à une toxine et à des xénobiotiques, visant à préserver l'intégrité de la barrière et à réduire la vulnérabilité aux dommages. Le renouvellement épithélial ultérieur opéré par la prolifération des CSI peut résoudre complètement le stress lié à l'exposition et garantir un intestin sain et fonctionnel.

2.5 Le priming : une première exposition aux xénobiotiques empêche l'amincissement induit par Db11

Outre le fait que l'alcool et la caféine induisent une purge cytoplasmique en soi, une caractéristique très intéressante est qu'ils peuvent préparer les entérocytes à une exposition ultérieure à *S. marcescens*. Lors de l'infection par Db11 quelques jours après une exposition aux xénobiotiques, l'épithélium de l'intestin moyen ne subissait pas un second cycle de purge. Bien que les entérocytes soient légèrement moins épais que dans des conditions non infectées, la morphologie globale est maintenue, avec une taille pertinente et une structure en forme de dôme. Étonnamment, ceci n'est pas vrai si les deux stimuli sont inversés (première infection avec *Serratia* et deuxième défi avec les xénobiotiques), ou dans le cas d'une double exposition séquentielle à l'EtOH ou à la caféine (données non montrées). Une autre observation inattendue a été qu'une infection répétée avec Db11 induit moins de priming : ceci n'est pas en accord avec les résultats antérieurs, où précisément l'impossibilité d'induire des purges cytoplasmiques répétées avec Db11 nous a conduit à faire l'hypothèse du phénomène de priming. Cependant, cette "résistance" à la purge n'était plus présente lorsqu'une semaine s'écoulait entre la première et la seconde exposition. De plus, nous n'avons pas observé de diminution du titre bactérien lors de la seconde provocation, écartant la possibilité que l'absence d'amincissement soit la conséquence d'une charge microbienne plus légère. Notre cas présente donc des différences avec les exemples de priming publiés précédemment chez la drosophile (Christofi et Apidianakis, 2013 ; Pham et al., 2007). Le terme priming est encore plus approprié ici car : i) cet événement n'est pas spécifique à un pathogène donné, mais il est étendu aux composés abiotiques et ii) il n'est pas durable, puisqu'il disparaît après quelques jours.

Nous pouvons identifier ces deux aspects comme les traits distinctifs entre une réponse "de priming" et une réponse "mémoire".

Mais quelle est la signification physiologique du priming ? Si la purge cytoplasmique est protectrice et bénéfique pour l'hôte, pourquoi l'inhiber ? Il se pourrait que la purge serve de mécanisme de défense contre des expositions occasionnelles mais pas contre des menaces chroniques. En effet, bien que le recyclage des cellules soit énergétiquement avantageux par rapport à la synthèse de novo, l'activation de ce programme de manière continue peut-être métaboliquement très coûteuse. Ainsi, le fait de passer par des cycles continus de purge peut conduire à l'épuisement rapide des réserves énergétiques de l'organisme. Ce besoin d'économiser l'énergie peut également être à l'origine de l'absence de prolifération des CSI après une courte exposition au Db11 ou à des xénobiotiques (figure 2.12B). En outre, un épithélium intestinal qui reste constamment plat et mince et dans lequel les entérocytes ont perdu de multiples composants cellulaires ne sera très probablement pas fonctionnel pour les processus de digestion. Pourtant, nous manquons de preuves expérimentales pour confirmer ou infirmer cette hypothèse. En effet, les essais de survie sur des mouches amorcées n'ont pas donné d'informations précieuses sur l'avantage ou le désavantage de l'priming : nous avons observé une seule fois que les mouches amorcées étaient plus sensibles à une seconde infection (Figure 2.13A), mais ce phénotype n'était pas reproductible. Au moins, nous pouvons affirmer que le priming des mouches n'a pas conféré de résistance à une seconde infection. Avec la prudence nécessaire, cela peut être considéré comme une confirmation que la purge cytoplasmique est une réponse bénéfique pour l'hôte. L'identification d'un mutant génétique dépourvu de manière constitutive d'une réponse de priming pourrait apporter des informations clés sur la pertinence biologique du priming ; bien que nous ayons quelques candidats prometteurs (voir paragraphe ci-dessous), nous n'avons pas encore repéré avec certitude un tel gène. Ensuite, comme nous savons que la purge cytoplasmique limite le passage des bactéries de l'intestin à l'hémolymphe, une stratégie alternative devrait être de déterminer le titre de Db11 dans l'hémolymphe, en s'attendant à observer plus de bactéries chez les mouches qui ont été amorcées. Si c'est le cas et que les bactéries surmontent la réponse immunitaire cellulaire dans la circulation, cela pourrait être à la base de la mortalité accrue observée chez les mouches amorcées dans la Figure 2.13A. Nous devrions également mesurer les cellules PHH3+ après la deuxième exposition.

2.6 Quel mécanisme cellulaire/moléculaire régit l'priming ?

Lorsqu'elles sont exposées à l'EtOH ou à la caféine, les mouches sont incapables de passer par une seconde purge lors d'une infection orale Db11 dans les trois jours suivants. Il est intéressant de noter que cet effet s'estompe une semaine après la première exposition : les mouches amorcées peuvent à nouveau accomplir une extrusion cytoplasmique lorsqu'elles nourrissent *S. marcescens* sept jours après l'administration des xénobiotiques (Figure 2.12A). Par conséquent, nous avons pensé qu'il devait y avoir

une empreinte transitoire dans les entérocytes qui ont entrepris une première purge qui disparaît au fil des jours.

CycJ a été le premier gène que nous avons identifié comme étant important au cours du processus de purge cytoplasmique. La première preuve recueillie a été que les mouches dépourvues de ce gène n'ont pas réussi à retrouver la forme normale de l'épithélium après la purge. Par conséquent, l'interprétation qui a toujours été donnée à ce résultat est que CycJ est nécessaire pour la phase de récupération. Cependant, après la découverte du priming, nous pouvons proposer une autre interprétation de ce phénotype. En effet, notre protocole d'infection classique ne permet pas de distinguer la phase de récupération de la phase de priming, puisque les mouches sont constamment maintenues en contact avec Db11. Ainsi, un manque dans la réponse de priming résulterait en des cycles constants de purge et un épithélium qui semble toujours mince. Pour découvrir la fonction réelle de CycJ, nous avons mis au point un protocole d'infection discontinue : la purge est induite en nourrissant les mouches CycJRNAi avec du Db11 uniquement pendant 3h, puis elles passent au saccharose. De cette manière, si CycJ est le médiateur de la récupération, nous devrions observer un épithélium mince dans le cas d'une infection discontinue. Cependant, l'interprétation n'était pas si simple. Tout d'abord, nous avons observé que la lignée témoin et la CycJTRiP ne présentaient pas un grand amincissement 3h après l'ingestion : ce fait complique l'analyse de la récupération à des points de temps ultérieurs. En effet, nous avons remarqué que les contrôles qui étaient constamment sur Db11 portaient une portion consistante d'épithéliums minces et semi-minces à 12h p.i., alors qu'une récupération complète était attendue. Cela pourrait indiquer un décalage temporel dans le comportement alimentaire des mouches. Néanmoins, nous avons également observé que les deux CycJRNAi avaient une proportion beaucoup plus importante d'épithéliums minces dans la même condition. Cela peut être considéré comme une confirmation que CycJ agit sur la restauration de la forme normale des entérocytes (via l'priming ou la récupération). Le protocole d'infection discontinue a définitivement conduit à une amélioration de la phase de récupération, à la fois dans les lignées ARNi et dans les contrôles. Le fait que CycJRNAi puisse effectivement récupérer indique que ce gène est le médiateur du priming plutôt que de la récupération après l'amincissement. Malheureusement, nous ne pouvons pas l'affirmer avec certitude, car le driver seul a montré le même type de comportement. Pour confirmer ce résultat, nous devrons répéter cette expérience plusieurs fois, en utilisant éventuellement un mutant nul pour CycJ. De plus, nous devons vérifier que le mécanisme d'priming ne fonctionne pas chez les mouches déficientes en CycJ.

Le manque de récupération de CycJ est étroitement lié à l'induction des gènes whe : l'expression ectopique d'un seul entre whe ou lcs a sauvé le phénotype du mutant CycJ (Lee et al., 2016). Par conséquent, nous avons raisonné que la surexpression de ces peptides mimerait un priming : les mouches portant une copie supplémentaire exprimée de manière constitutive de whe ou lcs devraient être résistantes à la purge cytoplasmique. Là encore, l'analyse de ce résultat s'est avérée plus compliquée que prévu. Tout d'abord, la surexpression de la GFP a provoqué un phénotype étrange une fois de plus

(voir chapitre 1) : ces mouches n'ont pas subi correctement un amincissement épithélial. Malheureusement, il s'agissait du meilleur contrôle pour notre expérience, ce qui affaiblit considérablement nos conclusions. Deuxièmement, pour la première fois, nous avons observé un rôle différentiel pour whe et lcs, deux gènes dont nous pensions jusqu'à présent qu'ils avaient une fonction redondante. Alors que la surexpression de whe améliore sensiblement le score d'épaisseur, ce n'est pas le cas pour lcs. Bien que ce résultat ait été reproduit trois fois, il semble prématuré de tirer des conclusions sur whe comme déterminant du priming, au moins jusqu'à ce que la variation dans nos contrôles soit réglée. De plus, il devrait être confirmé de manière indépendante, par exemple en mesurant par qPCR une augmentation de l'expression de la whe chez les mouches WT amorcées, ou en visualisant l'expression en temps réel et l'expression des lignées à l'aide du système G-TRACE (Evans et al., 2009). En parallèle, nous devrions également réaliser les mêmes expériences sur le gène lcs, afin d'établir s'il a une fonction sur le priming ou non.

L'idée d'une empreinte transitoire pourrait bien correspondre aux modifications épigénétiques des entérocytes, qui ont une courte demi-vie. Malheureusement, les expériences préliminaires n'ont pas indiqué l'implication des modificateurs de la chromatine dans le processus de priming (données non montrées). Une hypothèse alternative ou complémentaire à la voie CycJ/whe implique le renouvellement des entérocytes dans l'intestin moyen. Ce tissu présente une grande capacité d'auto-renouvellement, l'épithélium étant complètement renouvelé en 1 à 2 semaines dans des conditions stables (Micchelli et Perrimon, 2006 ; Nászai et al., 2015 ; Ohlstein et Spradling, 2006). Nous avons raisonné que les entérocytes qui ont subi une première purge et reçu l'empreinte pourraient être éliminés au cours des jours suivants. Ces entérocytes amorcés auraient la mémoire de la première extrusion et seraient résistants à une seconde purge, mais une fois retirés, ils seraient remplacés par des cellules naïves, capables de déclencher à nouveau la purge cytoplasmique. Ceci expliquerait pourquoi nous n'avons pas observé d'amincissement épithélial lorsque les deux stimuli sont proches (3 jours) mais pas lorsqu'ils sont plus éloignés (7 jours). L'estimation du renouvellement des entérocytes dans l'intestin moyen par analyse clonale, en induisant des cellules marquées avec la lignée esgGal4›UAS-GFP (Buchon et al., 2009b) a révélé une grande diminution du pool initial d'entérocytes au jour 4. Bien que ce résultat doive être confirmé par d'autres méthodes, il fournit une explication cohérente de la durée de l'effet d'priming. Nous avons remarqué que l'infection n'a pas augmenté le turnover, ce qui correspond à l'absence d'augmentation de la prolifération des ISCs dans la courte période d'exposition que nous avons utilisée pour le priming. Il convient de noter que, pour des raisons techniques, l'expérience a été réalisée à 18°C. Or, une température inférieure affecte certainement les processus cellulaires tels que la division, la différenciation et la mort, d'où la nécessité de trouver une autre méthode indépendante. De plus, et de manière inattendue, nous avons détecté plus d'entérocytes GFP+ au jour 7 qu'au jour 4. Cela pourrait être dû à une activation résiduelle du promoteur, même à 18°C. Alternativement, comme nous avons constaté une grande variabilité du nombre de clones fluorescents au jour 0, cela pourrait être simplement une question de chance, que les mouches examinées au jour 7

étaient celles qui avaient initialement le plus de clones GFP+. Répéter l'expérience plus de fois nous permettra de dissiper ce doute.

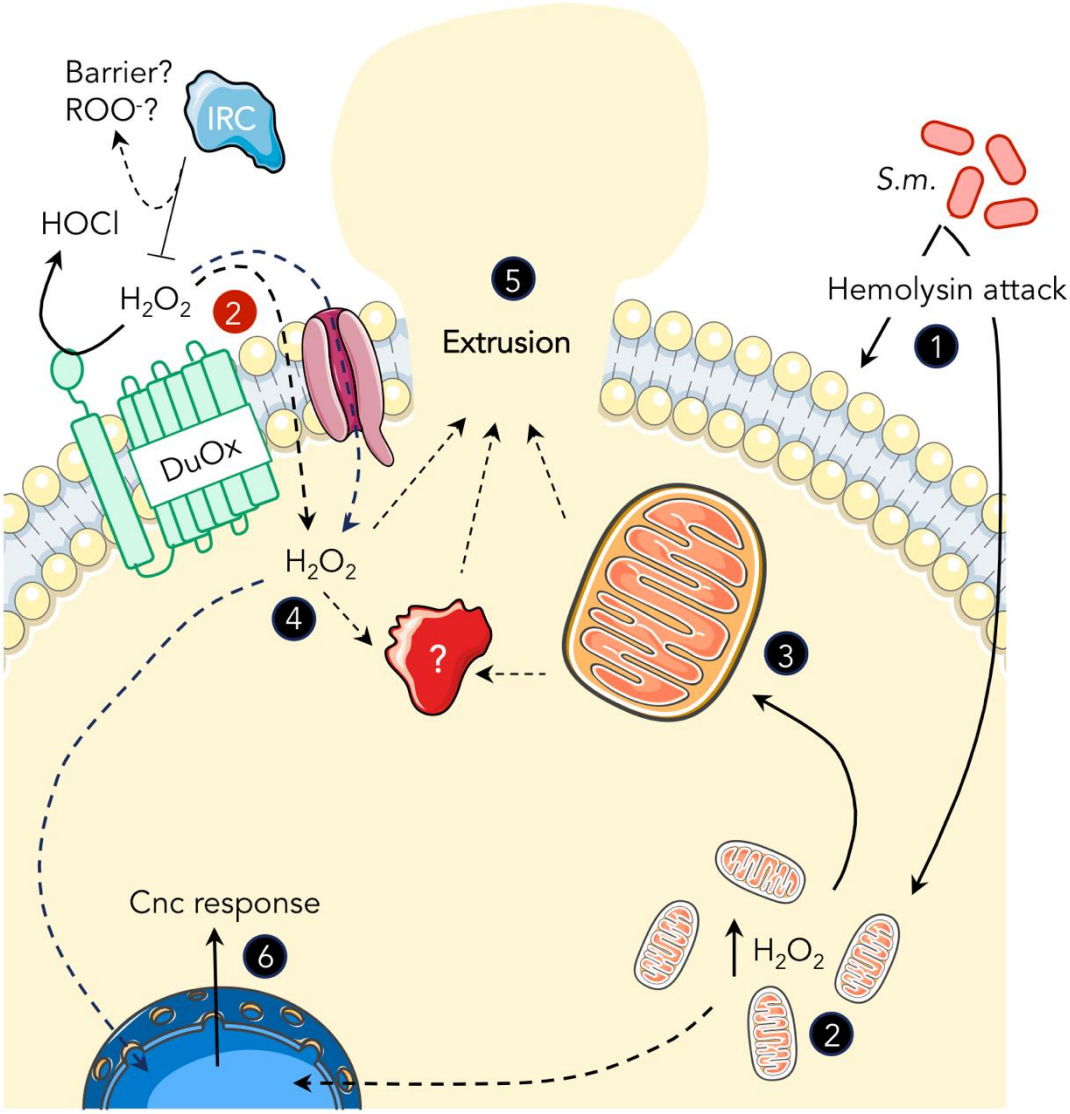

Figure D1. Hypothèse schématique de la dynamique des ROS pendant les premiers stades de l'infection par S. marcescens. Après l'ingestion de Db11, l'hémolysine, toxine formant des pores, attaque les entérocytes de l'hôte (1), formant de minuscules pores dans la membrane plasmique et atteignant éventuellement le cytoplasme et les mitochondries par les OMV (Bielaszewska et al., 2013). L'infection bactérienne provoque une augmentation rapide de H2O2 dans les mitochondries (2), qui s'accumule dans la partie apicale des entérocytes et fusionne en formant des mégamitochondries (3). Alternativement ou simultanément, la génération de ROS est induite par la Dual Oxidase DuOx (2). Le H2O2 produit par cette enzyme peut être transformé en HOCl ou entrer dans la cellule par diffusion ou via les aquaporines. La catalase immuno-régulée (IRC) piège le peroxyde d'hydrogène mais peut aussi produire des peroxyradicalaires (ROO-) ou renforcer la barrière du mucus intestinal. Le gradient de H2O2 formé sous la membrane (4) par l'une ou les deux voies peut induire une réorganisation du cytosquelette et de la membrane, directement ou indirectement, conduisant à la formation d'une ouverture et à une extrusion cytoplasmique ultérieure (5). Dernièrement, une réponse antioxydante est activée environ 6h après l'ingestion de Db11 (6) pour restaurer l'homéostasie cellulaire des ROS.

2.7 Conclusions et orientations futures

En plus d'être un processus de défense important pendant l'infection orale par la bactérie *Serratia marcescens*, notre travail a mis en évidence que la purge cytoplasmique est également active après l'ingestion de caféine et d'éthanol. Ceci représente un point crucial, car il démontre que ce mécanisme n'est pas spécifique au défi bactérien mais qu'il est au contraire partagé dans le cas de facteurs de stress abiotiques. Nous avons montré que l'extrusion cytoplasmique induite par Db11 est conservée chez les mammifères (Lee et al., 2016), il serait donc intéressant de tester si cela est également vrai dans le cas de l'EtOH et de la caféine. Comme ces composés sont fréquemment consommés par la majorité de la population mondiale, cela serait très pertinent pour la santé humaine.

Afin d'obtenir une compréhension approfondie de l'ensemble du processus de purge et de récupération, nous devons élucider les points communs et les différences possibles entre l'exposition aux bactéries et aux xénobiotiques. Cela pourrait s'avérer être une tâche difficile, car bien que les trois agents provoquent la purge cytoplasmique et le priming, l'exposition à l'éthanol, à la caféine ou au Db11 induit une série de réponses spécifiques à chaque composé. Les principales caractéristiques révélées dans ce travail sont résumées dans le tableau D1. Tout d'abord, nous avons mesuré une différence dans la létalité de ces composés, qui n'est cependant pas corrélée aux dommages intestinaux qu'ils provoquent. Par exemple, la caféine tue les mouches en quelques jours, mais ne provoque pas de dommages intestinaux. À l'inverse, les mouches survivent assez bien après l'administration de Db11 et d'EtOH, mais une prolifération compensatoire des CSI est induite. De plus, alors que la caféine et le Db11 partagent une augmentation mitochondriale de H_2O_2, ce n'est pas le cas de l'EtOH. Ce point en particulier était très décevant, car il nous aurait permis d'identifier un mécanisme commun pour l'induction des extrusions. Cependant, les données que nous avons recueillies n'ont pas permis d'exclure complètement cette possibilité.

Une question intéressante se pose au-delà de ces observations : pourquoi les mouches ont-elles évolué vers un mécanisme commun en réponse à des stimuli aussi différents ? Dans le cas de l'infection par Db11 et de l'exposition à la caféine, nous pouvons supposer que les mouches les perçoivent comme nuisibles quelle que soit la dose, et activent ainsi un programme de défense pour protéger l'intestin moyen. À l'inverse, l'EtOH est un attractif pour l'insecte, et il est bien toléré. Nous devons cependant considérer que les mouches rencontrent l'EtOH assez fréquemment dans leur vie naturelle, et que l'apport quotidien peut être très élevé : les fruits pourris en sont particulièrement riches, et il peut également provenir de la fermentation de levures dans l'intestin moyen de la mouche. Cela peut également expliquer pourquoi nous n'observons pas de purge cytoplasmique chez les mouches élevées avec une nourriture riche en levures : l'EtOH produit par le micro-organisme peut constamment amorcer les entérocytes, qui deviennent insensibles à l'attaque de l'hémolysine. Une autre question stimulante est de savoir pourquoi le priming ne fonctionne pas dans les deux sens. Les mouches se sont

rétablies après avoir été nourries en continu avec de l'EtOH et de la caféine pendant 24 heures, mais elles n'ont pas été amorcées contre une seconde exposition aux mêmes composés. L'élucidation de la voie moléculaire régulant le priming et de ses conséquences, comme le passage des bactéries dans l'hémolymphe ou la prolifération compensatoire des CSI après un second défi, pourrait nous aider à répondre à cette question.

Comme mentionné précédemment, nous devons garder à l'esprit qu'une similarité partagée par les trois agents est un efflux de K^+. Il est intéressant de noter qu'il s'agit d'un activateur du complexe de l'inflammasome (He et al., 2016). L'activation de l'inflammasome active principalement la caspase-1, qui clive non seulement les cytokines pro-inflammatoires dans leur forme mature et active mais aussi la Gasdermin-D. Cette protéine suscite notre intérêt car elle provoque de larges pores à la membrane plasmique comme nous l'observons lors de la phase d'extrusion. De plus, la formation du pore ne conduit pas nécessairement à la mort cellulaire : de grands complexes protéiques comme ESCRT-III peuvent être recrutés à la membrane et réparer le pore en évitant la pyroptose induite par l'inflammasome (Rühl et al., 2018). Étant donné qu'il n'y a pas d'inflammasome chez la drosophile, une autre ramification récente du projet de purge du cytoplasme étudie actuellement un possible mécanisme similaire avec l'implication de caspases non apoptotiques et de remodeleurs du cytosquelette. En particulier, des études récentes ont rapporté que Dronc, une caspase nécessaire à l'amincissement de l'épithélium, se lie aux protéines adaptatrices du cytosquelette d'actine et influence sa dynamique (Kang et al., 2017 ; Orme et al., 2016).

Phenotype	Db11	Ethanol	Caffeine
Lifespan	Slightly affected	Not affected	Severly affected
Cytoplasmic purge	Yes. Thin epithelium at 3h p.i.	Yes. Thin epithelium at 1h p.i.	Yes. Thin epithelium at 1h p.i.
H_2O_2 induction	Yes, mitochondrial	No	Yes, mitochondrial
Priming against second Db11 challenge	Mild	Yes	Yes
ISCs proliferation 24h p.i.	High	Moderate	No

Tableau D1. Comparaison et récapitulation des effets de l'exposition au Db11, à l'EtOH et à la caféine mis en évidence dans ce travail.

Un autre point commun important entre l'EtOH, la caféine et le Db11 est représenté par l'induction des gènes whe. L'induction de ces derniers après le premier événement de purge peut être l'empreinte à la base de la résistance transitoire aux séries d'extrusions consécutives dans les jours suivants. Il s'agira donc d'un axe important à développer pour la suite du projet, afin de décrire un mécanisme complet de la réponse de priming. Alternativement, nous devrions explorer davantage la possibilité de l'existence d'une marque épigénétique dans les entérocytes expérimentés. La détermination d'un profil transcriptomique dans les entérocytes amorcés par le biais d'un séquençage de l'ARN nous permettrait d'avoir une vision plus complète de ce qui se passe dans la cellule après la purge cytoplasmique, confirmant ou non l'hypothèse exposée ci-dessus et favorisant l'identification de nouvelles cibles. Outre les voies moléculaires, le priming semble intimement lié au renouvellement des entérocytes. L'ampleur de l'amincissement dépend de la présence de cellules qui ont précédemment subi un événement de purge. Nous devrions étudier si le blocage ou l'accélération du renouvellement de l'intestin moyen détermine la durée du priming : nous supposons que le ralentissement du renouvellement allongerait la durée du priming, et qu'en stimulant le remplacement, nous la raccourcirions. Un modèle proposé pour l'ensemble du processus de priming est illustré à la figure D2. Enfin, une possibilité intrigante serait que CycJ intervienne dans le processus de renouvellement, puisqu'il s'agit d'une cycline montrée pour se lier à Cdk1 (Althoff et al., 2009). Nous devrions tester cette hypothèse en croisant la lignée CycJRNAi avec un driver esg-Gal4 et en évaluant la réponse au priming.

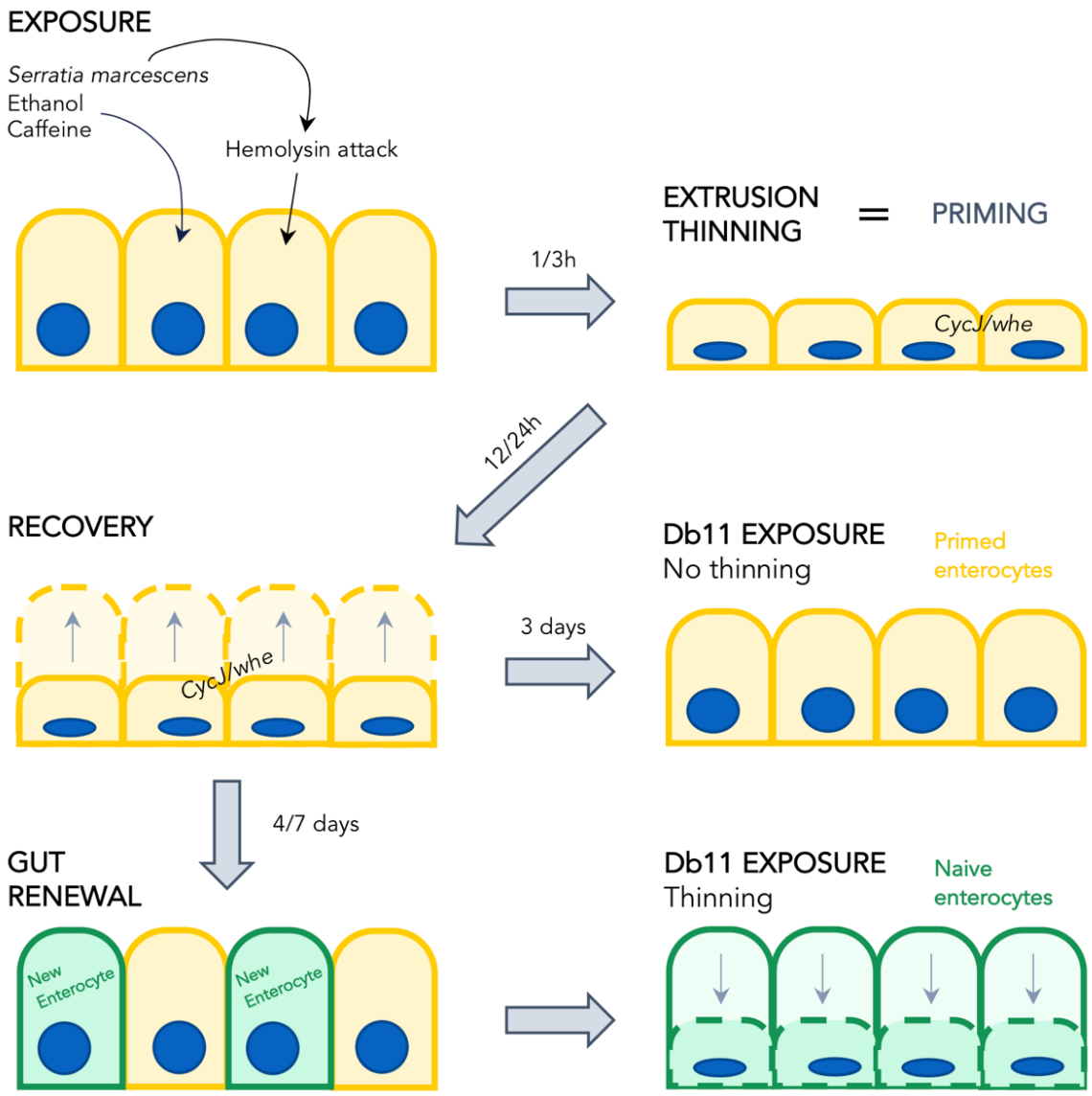

Figure D2. Time-lapse de la réponse d'amorçage après l'exposition aux xénobiotiques et à Serratia. En partant du bas à gauche : les mouches se nourrissent de plantes et de fruits pourris, qui sont des sources potentielles de caféine, d'EtOH ou de bactéries telles que S. marcescens. L'hémolysine PFT de cette dernière provoque une extrusion cytoplasmique et un amincissement épithélial conséquent dans les 3 heures ; le même processus est déclenché par les xénobiotiques dès 1 heure après l'ingestion. Ensuite, l'épithélium récupère progressivement sa forme en 12/24h. En parallèle, une induction de whe dépendante de la CycJ est activée. La première purge cytoplasmique a amorcé les entérocytes contre une seconde exposition, probablement par l'expression de ce gène. En effet, si l'on infecte à nouveau avec S. marcescens Db11 dans les trois jours suivants, il n'y a pas d'amincissement de l'épithélium de l'intestin moyen. Pendant ce temps, l'intestin s'auto-renouvelle en quelques jours : de nouveaux entérocytes "naïfs" remplacent les entérocytes "expérimentés". Lorsque la majorité des cellules ont été changées, l'épithélium intestinal réagit à nouveau à l'hémolysine, déclenchant une extrusion cytoplasmique et un amincissement.

3. CONCLUSION GÉNÉRALE

Les infections nosocomiales représentent une menace majeure dans les hôpitaux, affectant jusqu'à 15 % des patients (Khan et al., 2017) : la bactérie Gram négatif *S. marcescens* est l'une des causes les plus fréquentes. De plus, la grande majorité de la population mondiale consomme régulièrement des agents exogènes tels que la caféine et l'éthanol. L'étude des réactions de défense de l'hôte face aux pathogènes et aux xénobiotiques présente un grand intérêt pour notre laboratoire. Elle contribuera non seulement à améliorer l'état des patients infectés, mais aussi à comprendre les mécanismes physiologiques en réponse aux substances couramment consommées. En étudiant la pathogénicité de *S. marcescens* dans l'intestin de *Drosophila melanogaster*, des travaux antérieurs du laboratoire ont permis d'identifier un nouveau processus de résilience, la purge cytoplasmique. L'hémolysine, toxine formant des pores, sécrétée par la souche Db11 de *S. marcescens*, déclenche une extrusion du cytoplasme et des organites endommagés des entérocytes dans la lumière intestinale. En conséquence, l'épithélium intestinal devient très mince et plat, mais en évitant la mort cellulaire, il entame un processus de récupération progressive qui rétablit le volume et la forme initiaux en un jour. En plus d'être bénéfique pour l'hôte, en réduisant le nombre de bactéries qui se disséminent dans le tube digestif, ce mécanisme est également conservé chez les mammifères (Lee et al., 2016). Enfin, une voie moléculaire impliquant des gènes non caractérisés a été décrite : la sécrétion de gènes de la classe whe dépendante de CycJ est un facteur important pour rétablir la morphologie normale de l'épithélium de l'intestin moyen.

3.1 CONTRIBUTION DE CE TRAVAIL

La purge des entérocytes est aujourd'hui un modèle bien établi dans notre laboratoire. Néanmoins, de nombreuses questions restent à résoudre. Au cours de ma thèse, un axe majeur a été consacré à l'étude du déclenchement de l'extrusion cytoplasmique du point de vue de l'hôte. J'ai montré qu'il y a une production très précoce de ROS dans l'intestin moyen dans la phase qui précède la fuite du cytoplasme. Cette induction de ROS a été mesurée par deux méthodes indépendantes : une estimation du contenu total en H_2O_2 après l'infection par une technique biochimique et l'évaluation de couples redox spécifiques avec le rapporteur ratiométrique génétiquement codé roGFP2. Cette preuve est étayée par d'autres événements liés à la biologie des ROS : la formation de gouttelettes lipidiques et de mégamitochondries dans les étapes précédant l'extrusion et la transcription ultérieure de gènes antioxydants après la purge cytoplasmique.

Un point important était de déterminer la source des ROS. D'après nos données avec la sonde roGFP2, l'augmentation de H2O2 provient principalement des mitochondries. De plus, nous avons pu atténuer le degré d'éclaircissement en utilisant le piégeur de ROS mitochondrial mitoTEMPO. Néanmoins, la

manipulation de la génétique des mouches a mis en évidence une implication du système DuOx : le knockdown de DuOx ou la surexpression de l'IRC empêchent le processus de purge et entraînent une induction réduite de H2O2 après l'infection.

La localisation cellulaire peut constituer le pont entre les deux sources. En effet, les mégamitochondries et DuOx sont toutes deux étroitement associées à la membrane plasmique apicale des entérocytes, aux endroits où les événements d'extrusion ont lieu ultérieurement. Par conséquent, cela nous a conduit à émettre l'hypothèse d'un modèle de travail où les deux systèmes contribuent à l'induction de la purge cytoplasmique (Figure D1). Pourtant, ce que nous avons observé est une induction de ROS : pour pouvoir conclure que nous observons réellement un événement de signalisation, nous devons identifier les protéines cibles avec des sites potentiels sensibles au H_2O_2, et qui sont impliquées dans les mécanismes relatifs à la purge, par exemple, les cascades MAPK ou les modifications du cytosquelette. Lorsque nous avons testé une deuxième lignée d'ARNi pour DuOx (DuOxRNAi GD), celle-ci a donné des résultats différents tant au niveau de la survie que de l'éclaircissement, l'interprétation doit donc être prudente. Précisément, la mise en évidence des différences entre les deux souches DuOxRNAi, celle qui purge et celle qui ne purge pas, ainsi que les données obtenues avec l'UAS-IRC, nous apporterait un éclairage important sur les éléments clés qui déterminent l'activation du programme d'extrusion.

Outre le rôle des ROS pendant la purge cytoplasmique, ce projet nous a également conduit à mettre en évidence d'importants effets secondaires de la N-acétylcystéine. Bien qu'elle soit utilisée dans le monde entier comme le composé antioxydant le plus courant, la NAC a exercé un fort effet sur les bactéries, empêchant leur croissance en culture et les tuant à l'intérieur de l'intestin moyen de la mouche. En outre, la NAC réduit la durée de vie des drosophiles, même en l'absence d'infection. Pris ensemble, ces résultats suggèrent non seulement une utilisation prudente de ce composé lors des études d'infection, mais aussi une impulsion pour mieux comprendre et caractériser son mode d'action.

J'ai montré que la purge et la récupération des entérocytes n'est pas une réponse spécifique à l'hémolysine PFT de *S. marcescens* : ce mécanisme est également actif lors de l'ingestion d'éthanol et de caféine. Ces deux xénobiotiques induisent l'amincissement de l'épithélium qui atteint son pic déjà une heure après l'ingestion, ce qui déclenche ensuite le processus de récupération. Il convient de noter que la morphologie des entérocytes n'est pas exactement la même dans les deux cas. Bien que les cellules intestinales soient toujours extrudées en groupes, l'aplatissement est plus homogène dans le cas de l'infection bactérienne et semble plus irrégulier lors de l'ingestion d'EtOH et de caféine. En perspective, il serait intéressant d'étudier quels sont les points de divergence entre l'extrusion induite par les bactéries ou par les xénobiotiques.

Bien qu'ils activent le même processus, la caféine et l'éthanol présentent également quelques différences entre eux : l'ingestion de la première est très létale mais ne semble pas induire de dommages à l'épithélium intestinal, alors que l'EtOH est bien toléré par les mouches qui pourtant augmentent la prolifération compensatoire des CSI, du moins à la concentration que nous avons utilisée. Enfin, lors de l'ingestion de caféine, nous avons observé une production de H_2O_2 mitochondrial similaire à celle

provoquée par le Dbl1, mais il n'en est pas de même pour l'EtOH. D'une part, c'est décevant, car ces différences ne nous ont pas permis de décrire un mécanisme commun pour l'induction de la purge des entérocytes. D'autre part, cela est stimulant car cela pourrait signifier que ce mécanisme de résilience peut se produire de différentes manières et représente donc un outil de défense large plutôt qu'une réponse spécifique à une seule molécule induisant une seule voie. En fait, le programme de purge pourrait être déclenché par deux mécanismes différents au moins.

Cependant, il est essentiel de remarquer que l'EtOH et la caféine déclenchent tous deux un processus de priming dans l'intestin moyen de la mouche, préparant les entérocytes à un second défi avec Dbl1 en empêchant des événements de purge répétés. L'épithélium intestinal reste insensible à l'hémolysine pendant quelques jours, mais une semaine après la première exposition aux xénobiotiques, il redevient capable d'extruder. Nous soupçonnons qu'une empreinte transitoire est à la base de ce phénotype : les meilleurs candidats moléculaires sont CycJ et le gène whe associé, que nous pensions jusqu'à présent être des médiateurs de la récupération. En effet, les mouches knockdown CycJ sont capables de se rétablir si l'exposition au Dbl1 n'est pas prolongée, et la surexpression de whe réduit la quantité d'intestins minces, imitant un événement de priming. En outre, la durée de l'priming semble être liée à la durée de vie de l'entérocyte. Nous avons mesuré un renouvellement cellulaire massif au quatrième jour, ce qui coïnciderait avec la perte des cellules ayant reçu l'empreinte lors de la première exposition. Bien que d'autres expériences soient nécessaires pour élucider complètement le processus de priming, ce travail a ouvert la voie aux investigations futures.

3.2 LA PERTINENCE BIOLOGIQUE DE LA PURGE CYTOPLASMIQUE

En conclusion, nous nous posons une dernière question importante : quelle est la signification physiologique du processus d'extrusion et de récupération ? Pourquoi la drosophile a-t-elle évolué vers un tel mécanisme et quel avantage lui confère-t-il dans la nature ? Nos données nous ont amenés à nous demander s'il protège contre une exposition occasionnelle ou s'il représente une défense contre des menaces chroniques. La découverte du priming, où une seconde exposition à *S. marcescens* après l'alimentation en xénobiotiques ne provoque pas d'amincissement épithélial, nous indique que la purge ne se produit pas de manière répétée. Bien que nous n'ayons pas de réponses définitives à partir d'études de survie chez les mouches primées, nous émettons l'hypothèse qu'une extrusion cytoplasmique récurrente ou soutenue a des conséquences délétères pour l'organisme. Cela se produit par exemple dans le cas d'un autre mécanisme de résilience : en réponse à des signaux mitogènes prolongés, des épisodes régénératifs répétés conduisent à la perte de CSI, ce qui nuit à l'homéostasie intestinale (Haller et al., 2017). Par conséquent, le priming peut servir à empêcher les purges fréquentes afin d'éviter l'épuisement énergétique et la perturbation de la fonctionnalité intestinale. En effet, les mouches

déficientes en CycJ, qui présentent un épithélium mince en permanence lors de l'infection, probablement en raison d'événements d'extrusion répétés, succombent rapidement après. De plus, la défense de l'hôte peut avoir un coût remarquable. Du point de vue de l'évolution, les drosophiles ont pu développer cette régulation car elles rencontrent très souvent des bactéries et de l'EtOH dans la nature. Ensuite, les humains ont conservé ce mécanisme pour prévenir les conséquences délétères d'une intoxication alimentaire ; à une époque plus moderne, la purge et l'priming cytoplasmiques pourraient être utiles pour résister à la consommation fréquente de caféine et d'éthanol.

Bien que la purge cytoplasmique et le rétablissement qui s'ensuit n'impliquent pas de prolifération compensatoire des CSI, nous avons observé une activation de la division cellulaire ultérieure. Il est donc logique de se demander quelle est l'utilité de ce mécanisme si l'épithélium est renouvelé le lendemain. Nous pouvons penser que l'extrusion cytoplasmique des entérocytes sert de première ligne de défense lors d'une exposition aiguë à une toxine pour préserver l'intégrité de la barrière gastro-intestinale. L'expulsion du cytoplasme et des organites endommagés est susceptible de protéger la cellule des toxines et des facteurs de virulence, en atténuant le stress avant le renouvellement cellulaire. Cette hypothèse est soutenue par le fait qu'une courte exposition pour amorcer les mouches n'induit pas la prolifération des CSI le jour suivant, probablement parce que l'expulsion est suffisante pour supporter le stress. Notamment, la purge limite également le passage bactérien de l'intestin vers l'hémolymphe évitant ainsi une infection systémique. Il serait intéressant de savoir combien de bactéries entrent dans la circulation si la mouche a été préalablement amorcée.

L'hémolysine de *S. marcescens* n'est pas la seule PFT déclenchant l'extrusion cytoplasmique : le laboratoire a précédemment rapporté qu'également la monalysine de *Pseudomonas entomophila* possède cette capacité (Lee et al., 2016). De plus, nous avons montré ici la conservation de ce processus de résilience lors de l'exposition à des xénobiotiques, caféine et éthanol. La question de savoir si d'autres bactéries possédant des PFT et des xénobiotiques avec un "profil de gènes whe" sont également capables d'induire l'amincissement intestinal représente une perspective intéressante. En élargissant notre vision, nous pourrions nous demander si cette purge est pertinente pour l'inflammation intestinale, les maladies diarrhéiques et le retard de croissance. En 2000, le rapport de l'Organisation mondiale de la santé (OMS) et du Fonds des Nations unies pour l'enfance (UNICEF) sur l'approvisionnement en eau, l'assainissement et l'hygiène (WASH) a estimé à 500 le nombre d'enfants morts dans la région subsaharienne à cause de ces déficits (OMS & UNICEF, 2000). Les mauvaises mesures WASH dans ces pays sont à la base de troubles de la croissance et de pathologies (Cumming et Cairncross, 2016 ; Hutton et Chase, 2016), qui ne peuvent être soignés par une supplémentation en nutriments. Par conséquent, la recherche sur les contaminants (biotiques ou abiotiques) et leur effet dans le tube digestif acquiert une pertinence primordiale pour la santé humaine.

3.3 LA VARIABILITÉ DE L'AMINCISSEMENT ÉPITHÉLIAL

Le tube digestif est l'une des principales interfaces entre le milieu externe et interne, et à ce titre, de nombreux facteurs influencent sa physiologie. La nutrition et le microbiote sont deux paramètres majeurs à prendre en compte, car même de petits changements peuvent affecter l'homéostasie intestinale de manière considérable. L'intestin est également au centre d'un réseau de communication avec d'autres organes, comme le SNC et le corps adipeux, régulant de vastes processus biologiques comme le comportement et le métabolisme. Cette grande sensibilité aux variations environnementales constitue certainement un revers important (mais aussi une vision plus réaliste) pour les études in vivo sur cet organe.

Plusieurs aspects de la purge et de la récupération des entérocytes sont bien établis : néanmoins, nous ne pouvons pas empêcher de remarquer la variabilité du degré d'amincissement dans nos expériences. Tout d'abord, nous devons avoir une vision critique de notre système de notation. En effet, il reste une méthode semi-quantitative qui repose en partie sur la perception de l'observateur et donc avec une perspective subjective pertinente. Nous avons essayé de minimiser cette limite en effectuant des scores en aveugle et/ou en effectuant une seconde vérification par un autre observateur. Cependant, pour éliminer complètement ce biais, nous devons mettre en place une méthode de routine automatisée qui nous permette de mesurer l'épaisseur de l'intestin avec confiance et rapidité. Néanmoins, une telle analyse n'est pas aussi facile qu'il n'y paraît : elle implique déjà de prendre plusieurs photos de chaque intestin utilisé dans l'expérience, ce qui est très chronophage. De plus, il faut proportionner l'épaisseur des entérocytes à la largeur totale de l'intestin, qui varie d'un individu à l'autre. Pour compliquer encore l'interprétation, il peut arriver que les deux côtés de la région R2 présentent des morphologies cellulaires différentes, rendant nécessaires de multiples mesures pour chaque intestin. Il faut donc définir une méthode qui prenne en compte tous ces facteurs et assurer parallèlement la rapidité et la fiabilité d'exécution.

De nombreux facteurs influencent l'intestin et par conséquent aussi notre phénotype. Bien que cela soit assez rare, certains individus ou certaines souches présentent une variabilité de l'épaisseur épithéliale également après un traitement au saccharose. Lorsque dans cette condition de contrôle nous observons une quantité considérable d'épithélium semi-fini, l'interprétation devient compliquée. De plus, comme nous sommes dans un modèle d'infection orale, nous ne pouvons pas avoir un contrôle total sur la quantité et le moment de l'alimentation. L'identification du moment exact où les mouches commencent à se nourrir est d'une importance cruciale pour avoir la précision du point de temps. Nous avons abordé cette question en ajoutant un colorant alimentaire dans les solutions, ce qui résout partiellement le problème, mais pas totalement. Ces préoccupations semblent être liées d'une manière ou d'une autre au génotype de la mouche : certaines souches mangent plus vite et plus que d'autres, et des différences

dans le degré d'amaigrissement sont également constatées. Le cycle de vie de la drosophile étant très rapide, les stocks doivent être régulièrement isogénisés pour garantir un fond génétique stable et uniforme. Un autre facteur qui peut varier d'un individu à l'autre est le microbiote. La communauté microbienne de l'intestin influence de manière significative de nombreux aspects de la physiologie de l'hôte, et certaines données suggèrent que l'ampleur de l'extrusion dépend également de la présence de certaines bactéries. Cet aspect est également lié à l'alimentation, qui revêt une importance capitale pour toute étude sur l'intestin. L'adoption d'un régime riche ou pauvre régule le métabolisme de l'hôte, façonne différemment la communauté microbienne et a un impact profond sur le phénotype d'amincissement. Au cours de mon doctorat, je me suis retrouvé plusieurs fois face à ces perturbateurs, et leur compréhension permettra de créer un système plus simple qui facilitera les études futures.

4. MATÉRIAUX ET MÉTHODES

4.1 ÉLEVAGE DES MOUCHES ET SOUCHES

Les mouches *Drosophila melanogaster* ont été élevées à 25°C avec 60% d'humidité, 14h de lumière du jour sur un milieu semi-solide de farine de maïs. Composition : 6,4% (p/v) de farine de maïs (Moulin des Moines, France), 4,8% (p/v) de sucre cristallisé (Erstein, France), 1,2% (p/v) de levure de bière en poudre (VWR, Belgique), 0,5% (p/v) d'agar (Sobigel, France), 0,004% (p/v) de sel de sodium de 4-hydroxybenzoate (Merck, Allemagne).

Toutes les expériences ont été réalisées sur des mouches femelles âgées de trois à sept jours, sauf indication contraire. La souche de référence WT était blanche (wA5001). Le driver NP1 (NpGal4Gal80ts) spécifique au tissu, également connu sous le nom de Myo31D, a été utilisé pour guider les modifications génétiques spécifiquement dans les entérocytes (Cronin et al., 2009 ; Nehme et al., 2007). La construction de Gal4Gal80ts a permis la manipulation génétique d'une manière spécifique au tissu et au temps. Pour les expériences impliquant des mouches transgéniques, ce pilote a été utilisé seul ou croisé avec la lignée de contrôle GD RNAi du Vienna Drosophila RNAi Center (numéro de stock VDRC #60.000) ou la lignée surexprimant la GFP (UAS-GFP). Les croisements ont été effectués à 18°C, en utilisant des vierges de la lignée pilote et des mâles de la lignée génétique choisie. La progéniture de ces croisements a été récoltée et placée à 29°C avec 70% d'humidité pendant 5 jours pour la dégradation de Gal80ts et l'activation de Gal4 (Figure M1). Les lignées transgéniques proviennent soit du Vienna Drosophila RNAi Center (marqué VDRC), soit du Bloomington Stock Center (marqué Bl). Pour les lignées RNAi, les lignées utilisées sont les suivantes : UAS-DuOxRNAi GD (VDRC #2593), UAS-IRC TRiP (Bloomington #57814), UAS-CycJRNAi KK (VDRC #10222), UAS-CycJRNAi TRiP (Bloomington #37521). Pour les lignées de surexpression, les lignées utilisées sont les suivantes : UAS-

GFP (Bloomington #1521), UAS-Cat (Bloomington #24621). UAS-DuOxRNAi WJL et UAS-IRC sont une généreuse courtoisie du professeur Won Jae Lee (Ha et al., 2005a, 2005b). Les souches de mouches roGFP2 sont une généreuse courtoisie de Jörg Großhans (Albrecht et al., 2011). Les génotypes sont les suivants : cytoplasmique-Grx1 (P003, pUAST cyto-Grx1-roGFP2 II HV (ligne 13)/CyO ; tubulinGal4/TM3Ser, forte expression) ; mitochondrial-Grx1 (P006, pUAST mito-roGFP2-Grx1 II HV (ligne 9)/CyO ; tubulinGal4/TM3Ser, forte expression) ; Orp1 cytoplasmique (P015, pCasPeR4-cyto-roGFP2-Orp1 II HV (ligne 1) forte expression) ; Orp1 mitochondrial (P018, pCasPeR4-mito-roGFP2-Orp1 II HV (ligne 6) forte expression). Les mouches CncC-ARE-GFP pour l'induction des gènes contenant l'ARE proviennent de Sykiotis & Bohmann, 2008. Pour l'analyse clonale du nombre total d'entérocytes, la lignée w ; esgGal4tubGal80ts UAS-GFP ; UAS-flp Act>CD2>Gal4 (Jiang et al., 2009) a été utilisée. Les lignées surexprimant what else ou lacosta (UAS-whe, UAS-lcs) ont été précédemment construites au laboratoire (Lee et al., 2016).

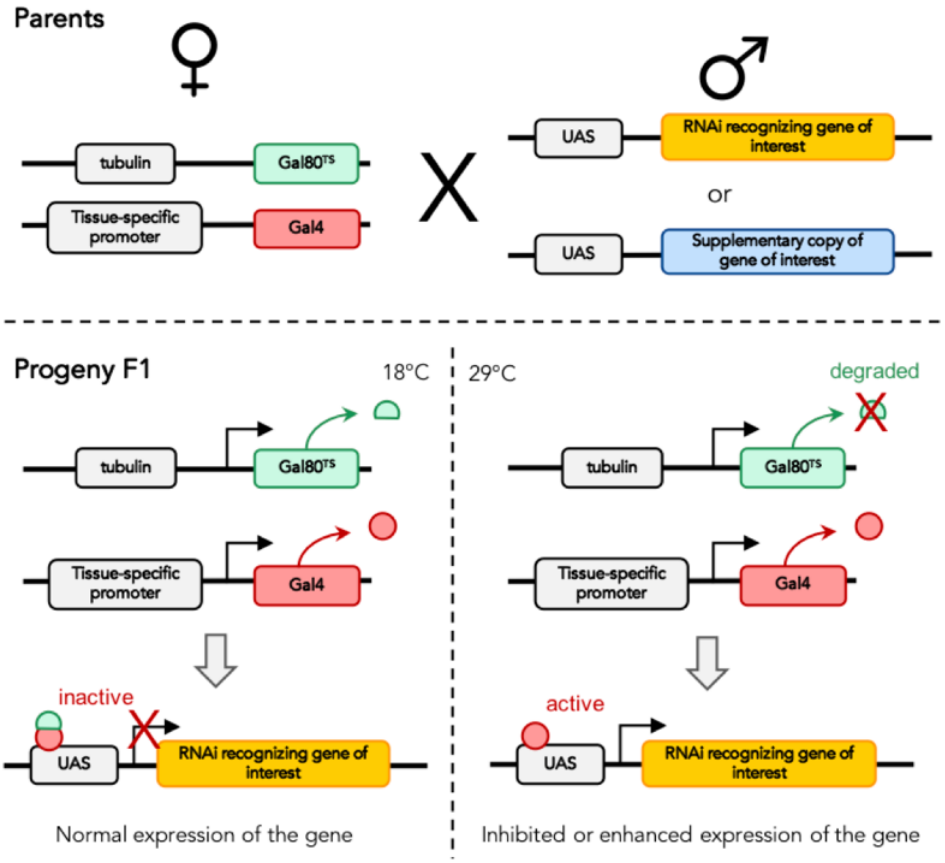

Figure M1. Le système UAS-Gal4Gal8ots. Une lignée de mouche porte le facteur de transcription Gal4 de la levure sous le contrôle d'un promoteur spécifique du tissu et le Gal8ots, qui est un inhibiteur thermosensible de Gal4, sous le contrôle du promoteur ubiquitaire de la tubuline. Cette lignée est croisée avec une autre souche de mouche portant un ARN interférent (ARNi) spécifique à un gène d'intérêt ou une copie supplémentaire du gène d'intérêt en aval d'une séquence UAS (Upstream activating sequence). Les croisements et le développement de la progéniture sont réalisés à 18°C, où le Gal8ots inhibe l'activité de Gal4. Cela permet d'éviter toute influence pendant le développement de la mouche. Lorsque la progéniture F1 passe à 29°C, Gal8ots est dégradé et la liaison de Gal4 à UAS permet d'exprimer l'ARNi ou la copie supplémentaire, réduisant ainsi au silence ou surexprimant le gène d'intérêt.

4.2 SOUCHE BACTÉRIENNE

La souche *Serratia marcescens* Db11 (Flyg et al., 1980) a été cultivée dans des plaques LB-agar avec 100 µg/mL de streptomycine et incubée pendant une nuit à 37°C pour obtenir des colonies. La plaque est ensuite conservée à 4°C pendant environ deux semaines. Une seule colonie bactérienne est inoculée dans du milieu LB liquide. Ces cultures sont placées pendant une nuit à 37°C avec agitation pour induire la croissance bactérienne.

4.3 EXPOSITION ORALE

A l'exception des tests de survie, les infections bactériennes ont été réalisées en utilisant une OD600 (densité optique mesurée à 600nm) bactérienne finale de 10. Une culture liquide a été cultivée pendant une nuit (voir ci-dessus), et l'OD600 résultante a été mesurée avec un spectrophotomètre. Une partie de cette culture, selon les besoins, a été centrifugée à 4000rcf pendant 10minutes à 4°C. Le culot a été remis en suspension dans le volume approprié pour atteindre une OD600=10 en utilisant une solution de saccharose 50mM contenant 10% de LB. Les solutions d'EtOH ont été préparées en diluant de l'EtOH pur dans du sucrose 50mM afin d'obtenir une concentration finale de 2,5%. Pour obtenir une solution de caféine à 2,5mg/mL, 125mg de poudre de caféine (Sigma-Aldrich, C0750) ont été dilués dans 50mL de sucrose 50mM et placés 30 minutes au bain-marie à 50°C pour favoriser la solubilisation. Les antioxydants (DTT : Euromedex EU0006-D ; NAC : Sigma-Aldrich, A7250 ; Vit. C : Fluka/ Sigma-Aldrich 95210 ; mitoTEMPO : Sigma-Aldrich SML0737), H2O2 (Fisher Scientific H1750/15) et paraquat (Sigma-Aldrich 856177) ont été préparés de manière similaire en diluant la poudre ou la solution mère dans du saccharose 50mM.

2mL des solutions décrites ci-dessus ont été ajoutés dans un tube de taille moyenne (3,5cm de diamètre) contenant deux tampons absorbants (Millipore AP1003700). Un maximum de 20 mouches femelles ont été placées sur un seul tube pour recevoir ces solutions ou du sucrose 50mM seulement comme contrôle. Les points de temps utilisés sont spécifiés dans les expériences individuelles.

4.4 TEST DE SURVIE

Les tests de survie ont été réalisés sur des mouches âgées de trois à sept jours, à 29°C avec une humidité de 70%, en triplicats biologiques de 20 mouches par flacon. La DO600 bactérienne a été ajustée à 1, sauf indication contraire. La concentration finale de caféine était de 2,5mg/mL. Les concentrations finales d'EtOH étaient de 2,5% ou 5%. La NAC a été utilisée à 20mM. Le saccharose 50mM a été utilisé comme contrôle. Au jour 0, 2mL de chaque solution ont été ajoutés dans chaque tube (voir infection orale). Chaque jour, les survivants ont été comptés, et 200µL de saccharose 100mM ont été ajoutés dans tous les flacons, sauf indication contraire. Le test de Logrank a été utilisé pour les statistiques avec le logiciel GraphPad Prism6.

4.5 COLORATION HISTOCHIMIQUE FLUORESCENTE ET OBSERVATION

4.5.1 Dissection

Les mouches ont été anesthésiées au CO2. Les viscères ont été disséqués dans du PBS 1x et fixés pendant 30 minutes dans du paraformaldéhyde à 8% (Electron Microscopy Science, cat #15710). Les tissus ont ensuite été lavés trois fois avec du PBS-TritonX-100 0,1% (PBT 0,1%), puis saturés pendant au moins 1h avec du BSA 2%.

4.5.2 Coloration de l'actine

Les échantillons ont été incubés dans de la phalloïdine isothiocyanate de fluorescéine (FITC) 10µM (Sigma-Aldrich #5282) ou de la phalloïdine Texas-Red (InvitrogenTM #7471) pendant 1h30 à température ambiante (RT) ou pendant une nuit à 4°C. Les échantillons ont ensuite été lavés trois fois avec du PBT 0,1%.

4.5.3 Coloration à l'iodure de propidium

Les mouches ont été nourries avec une solution (saccharose 50mM+10% LB) contenant des bactéries exprimant la GFP (OD600=10) et 50µg/mL d'iodure de propidium pendant 3h à 29°C. Les viscères ont été disséqués et fixés comme décrit ci-dessus.

4.5.4 Coloration de la Phosphohistone3 (PHH3)

L'anticorps anti-PHH3 (Millipore ref 09-797) a été dilué au 1:500 dans du PBT 0,1% + BSA 2%. Les échantillons ont été incubés dans cette solution pendant 2h à température ambiante ou une nuit à 4°C, puis lavés trois fois dans du PBT 0,1% et incubés avec un anticorps de chèvre anti-lapin FITC (Abcam #6717) dilué au 1:1000 dans du PBT 0,1% + BSA 2%. Les échantillons ont finalement été lavés trois fois dans du PBT 0,1% avant le montage.

4.5.5 Montage et microscopie

Tous les échantillons ont été montés sur des lames de microscopie diagnostique à 8 puits (Thermo Fisher Scientific) avec du Vectashield contenant du DAPI (Vector Laboratories), puis stockés dans

l'obscurité à 4°C. Les échantillons ont été observés à l'aide d'un microscope confocal LSM780 (Zeiss) ou d'un microscope à épifluorescence Axioscope2 (Zeiss) selon les besoins. Les images ont été prises à l'aide d'un objectif plan/apochromatique 20x/0,8 sec. Les fichiers bruts ont été traités et analysés à l'aide du logiciel ImageJ/Fiji si nécessaire.

4.6 TITRE BACTÉRIEN DANS L'INTESTIN

Pour mesurer les UFC bactériennes par intestin (Db11), des quadruplicats biologiques de 5 intestins moyens ont été disséqués dans 100µL de PBS 1x stérile. Les tissus ont été homogénéisés à l'aide d'un mélangeur à moteur à pilon (Argos). Des dilutions sérielles (10-1 à 10-6) ont été appliquées aux volumes de PBS et 5µL de chaque dilution ont été plaqués sur des plaques LB-agar contenant 100 µg/mL de streptomycine. Ces plaques ont été séchées 10 minutes sous hotte bactérienne puis incubées toute la nuit à 37°C. Le lendemain, les colonies isolées qui ont poussé sur les plaques ont été comptées. Les UFC bactériennes par boyau ont été déterminées par rapport à la dilution, au volume plaqué (5µL), au nombre de boyaux intermédiaires (5) et au volume initial de PBS (100µL).

4.7 TEST FLUOROMÉTRIQUE DE QUANTIFICATION DE L'H_2O_2

La teneur en H_2O_2 dans les viscères a été estimée à l'aide du test fluorométrique de quantification du H_2O_2 (Sigma-Aldrich, MAK165). Préparation des échantillons : des duplicatas biologiques de 10 intestins par condition ont été disséqués dans du PBS 1x stérile et facilement mis dans des tubes sur glace sèche. 50µL du tampon d'analyse du kit ont été ajoutés et les tissus ont été écrasés avec un homogénéisateur Precellys (Bertin Instruments). Le reste du protocole a été réalisé en suivant les instructions du fabricant. Ce kit utilise un substrat de peroxydase qui génère un produit fluorescent rouge après réaction avec H_2O_2. Cette fluorescence (λex=540/λem=590 nm) a été mesurée à l'aide du lecteur de microplaques VarioskanTM Lux (Thermo Fisher).

4.8 SCORE D'ÉPAISSEUR ÉPITHÉLIALE

Pour estimer le niveau d'amincissement et/ou la capacité de récupération, les viscères ont été classés en trois catégories différentes : épais (a), semi-fin (b) ou fin (c) (Figure M2). Pour déterminer l'épaisseur épithéliale globale, deux paramètres principaux ont été évalués : la présence de structures en forme de dôme et l'épaisseur cellulaire. Pour cette évaluation, seule la région R2 de l'intestin moyen a été prise en compte. Pour rendre l'évaluation plus objective, une méthode de notation en aveugle ou la confirmation d'un second observateur est souvent appliquée.

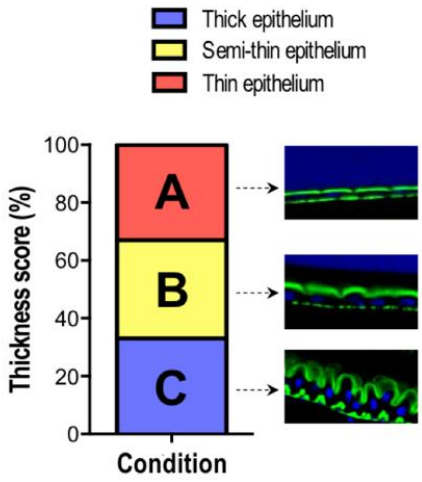

Figure M2. Estimation de l'épaisseur de l'épithélium. Afin d'évaluer le niveau d'amincissement et la capacité de récupération, les viscères ont été classés en trois catégories différentes. Ce score d'épaisseur est basé sur deux paramètres principaux : le volume cellulaire et la structure en dôme. Si les deux sont absents ou sévèrement réduits, l'intestin moyen est classé comme mince (A, rouge). Si un seul des deux est affecté, l'intestin est inclus dans la catégorie semi-mince (B, jaune). Enfin, si les entérocytes sont épais et présentent une structure en forme de dôme, l'épithélium est considéré comme épais (C, bleu). Le test statistique $\chi 2$ a été effectué.

4.9 ROGFP2

Protocole original et plus détaillé : Barata & Dick, 2013.

4.9.1 Echantillons de référence

Pour obtenir des échantillons entièrement oxydés et entièrement réduits, les viscères ont été disséqués dans du PBS 1x, puis incubés 10 minutes dans du diamide (DA) ou du dithiothreitol (DTT) pour des références oxydées et réduites, respectivement. Après lavage dans du PBS, ces échantillons ont été placés 15 minutes dans du N-ethyl-maleimide (NEM) 20mM afin de préserver l'état redox du tissu. Les viscères ont été rapidement lavés à nouveau dans du PBS puis incubés dans du PFA 4% pendant 20 minutes et montés dans du Vectashield sans DAPI.

4.9.2 Echantillons expérimentaux

Pour la dissection des conditions expérimentales, le PBS a été complété par du NEM 20mM pour préserver l'état redox du tissu. Les viscères ont été alkylés dans du NEM pendant 15 minutes après la dissection. Après lavage dans du PBS, les échantillons ont été fixés dans du PFA 4% pendant 20 minutes et montés dans du Vectashield sans DAPI. Avant l'acquisition des images, l'intensité du laser pour l'excitation à 405nm et 488nm a été ajustée en utilisant les échantillons complètement oxydés et complètement réduits, respectivement. Ces réglages ne sont plus modifiés pour tous les échantillons d'une même expérience. Pour l'imagerie, la sonde a été excitée séquentiellement à 405nm et 488nm et l'émission a été enregistrée à 500-530nm.

4.9.3 Traitement des images

Le traitement des images a été effectué en adaptant le protocole de (Kardash et al., 2011). Brièvement, la procédure de rolling ball réglée à 50 pixels a été utilisée pour soustraire le fond et les images ont été converties en 32 bits. Un seuil a été fixé pour l'intensité de l'image avec les paramètres par défaut en niveaux de gris et fond sombre. Les valeurs inférieures au seuil ont été définies comme "non chiffrées". Le plugin RatioPlus a été utilisé pour générer des images de rapport en divisant l'image 405nm par l'image correspondante 488nm. La table de consultation "fire" a été utilisée pour la coloration finale. Les intensités ont finalement été normalisées par rapport au contrôle DTT (complètement réduit).

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The cytoplasmic purge as a resilience response to pore-forming toxins and xenobiotics in the *Drosophila* intestinal epithelium

ROS signaling and protective effects against sustained exposures

Résumé en français

Tous les métazoaires rencontrent des pathogènes et des contaminants présents dans l'environnement. Les organismes ont développé des défenses pour lutter contre ces menaces : barrières physiques et système immunitaire limitent l'apparition de pathologies. Ces défenses sont complétées par la résilience, définie comme la capacité de supporter et de réparer les dommages, constituant un déterminant crucial pour la survie de l'hôte.

Nous étudions la résilience dans l'organisme modèle *Drosophila melanogaster*. Nous avions décrit un nouveau mécanisme de résilience dans l'intestin, la purge cytoplasmique. Toutefois, le mécanisme qui initie ce processus reste flou. Au cours de ma thèse j'ai étudié le signal requis pour déclencher l'extrusion, en me concentrant sur les ROS. En parallèle, j'ai étudié la conservation du processus avec d'autres composés. Ces travaux ont démontré que la purge des entérocytes pourrait aussi protéger contre certains xénobiotiques, comme l'alcool et la caféine. Enfin, j'ai décrit un mécanisme de *"priming"*, au cours duquel les entérocytes deviennent résistants à une seconde purge dans les jours suivant la première exposition .

Mots clés : *Drosophila*, intestin, *Serratia marcescens*, xenobiotics, purge cytoplasmique, espèces réactives de l'oxygène (ROS), priming

Summary in English

All metazoan encounter pathogens and contaminants presents in the environment. Organisms have developed defenses to fight against these threats: the physical barriers and the immune system limit the appearance of diseases and pathologies. These defenses are complemented by resilience, defined as the ability to withstand and repair damage, which is a crucial determinant for the survival of the host.

We study the resilience in the model organism *Drosophila melanogaster*. We had described a new resilience mechanism in the intestine, the cytoplasmic purge. However, the mechanism that initiates this process remains unclear. During my thesis I studied the signal required to trigger the extrusion, focusing on ROS. In parallel, I studied the conservation of the process with other compounds. This work has shown that the purge of enterocytes can also protect against certain xenobiotics, such as alcohol and caffeine. Finally, I have described a priming mechanism, in which enterocytes become resistant to a second purge in the following days of the first exposure.

Key words: *Drosophila*, intestine, *Serratia marcescens*, xenobiotics, cytoplasmic purge, reactive oxygen species (ROS), priming