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Chami Kim

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THÈSE DE DOCTORAT

Réponse immunitaire de la drosophile à la guêpe endoparasitoïde *Leptopilina boulardi*: caractérisation d'une réaction de résistance

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du grade de docteur en** Sciences de la Vie et de
la Santé
d'Université Côte d'Azur

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Soutenue le : 22 mars 2019

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Drosophila immune response to the
endoparasitoid wasp *Leptopilina boulardi*:
characterization of a resistance reaction

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Résumé

Drosophila melanogaster est un modèle majeur en biologie, notamment pour l'immunité et l'évolution.

L'immunité innée de la drosophile a été très étudiée dans le cadre de la réponse contre les bactéries et les champignons, mais on en sait moins sur la défense contre les guêpes endoparasitoïdes dont le développement à l'intérieur de l'insecte hôte entraîne sa mort. L'une des interactions les plus étudiées entre drosophiles et guêpes parasites implique *Leptopilina boulandi* qui pond des œufs à l'intérieur des larves hôtes et se développe à leurs dépens. Une fois que l'œuf parasitoïde a été reconnu comme un envahisseur étranger, la larve de *Drosophila* peut déclencher une réponse immunitaire qui mène à l'encapsulation : l'œuf est entouré de plusieurs couches d'hémocytes. La capsule ainsi formée est mélanisée et il y a formation d'espèces réactives de l'oxygène qui participe à la mort du parasitoïde. Alternativement, la réponse immunitaire peut être contournée grâce aux composants du venin injectés par la guêpe femelle en même temps que l'œuf.

Nous avons utilisé deux souches de *Drosophila*, résistante et sensible à *L. boulandi*, ne différant que par une région du chromosome 2R contenant un gène de résistance majeur. La résistance s'est révélée être monogénique, avec deux allèles, l'allèle de résistance étant dominant ($R/b+ > R/b-$). L'équipe avait précédemment identifié *edl* / *mae* (allèles R et S) en tant que gène candidat. *Mae* (modulateur de l'activité d'ETS) ou *edl* (ETS-domain lacking) a été décrit comme un médiateur de facteurs de transcription de la famille de l'ETS (E26 transformation-specific) chez la drosophile. *Mae* interagit avec les facteurs de transcription *via* son domaine SAM (Steril Alpha Domain), un domaine d'interaction protéine-protéine.

Les objectifs de ma thèse étaient de déchiffrer le rôle possible d'*edl* et d'identifier les événements moléculaires et cellulaires conduisant au succès ou à l'échec de l'encapsulation. J'ai utilisé diverses approches allant de la génétique des mouches à la cytométrie en flux.

L'implication de *edl* dans la résistance à *Drosophila* a été confirmée par surexpression et interférence d'expression de *edl*. La surexpression de l'allèle résistant dans un fond sensible conduit à un phénotype résistant. L'interférence de l'expression de l'allèle sensible entraîne une augmentation du taux d'encapsulation de parasitoïde. Au niveau cellulaire, une augmentation du nombre d'hémocytes après le parasitisme s'est produite plus tôt dans la souche résistante que dans la souche sensible. Il a également été observé que la glande lymphatique des larves résistantes éclate avant celle des larves sensibles. Au niveau moléculaire, des interactants potentiels de *edl* ont été

identifiés *in silico* et 2 ont été testés en utilisant la réduction de leur expression qui a conduit à l'observation d'une augmentation de l'encapsulation.

Dans l'ensemble, un acteur clé du mécanisme de résistance de la drosophile à la guêpe parasite a été identifié au cours de ce travail et permet d'ouvrir des pistes pour des travaux futurs sur le mécanisme de régulation de la réponse au niveau moléculaire.

Abstract

Drosophila melanogaster is a main model in biology, notably immunity and evolution.

Although the *Drosophila* innate immune processes to fight bacteria and fungi have largely been explored, less is known of the defence against endoparasitoid wasps whose successful development inside the insect host leads to its death. One of the most studied *Drosophila* – parasitoid wasp interaction involves *Leptopilina boulardi* that lays eggs inside host larvae and develop at their expense. Once the parasitoid egg has been recognized as a foreign invader, the *Drosophila* larva can mount a successful immune response, the encapsulation: the egg is surrounded by several layers of hemocytes and there is an increase of a specific types of hemocytes, the lamellocytes. The so-formed capsule is melanised and there is formation of reactive oxygen species, which kills the parasitoid. Alternatively, the immune response can be circumvented thanks to the venom components injected by the female wasp together with the egg.

Using two *Drosophila* strains, resistant and susceptible to *L. boulardi*, which differ only in a region of chromosome 2R containing a major resistance gene. The resistance was found to be monogenic, with two alleles, the resistance allele being dominant (Rlb+>Rlb). The team previously identified *edl/mae* (R and S alleles) as a candidate gene. *Mae* (Modulator of the Activity of ETS) or *edl* (ETS-domain lacking) was described as a mediator of specific transcription factors of the ETS (E26 transformation-specific) family in *Drosophila*. *Mae* interacts with transcription factors through a SAM (Steril Alpha Domain), a protein – protein interaction domain. *edl* is known to regulate *aop* and *pnt P2* transcription factors during the eye development and *aop* and *pnt P2* appear to have a role during haematopoiesis.

The objectives of my thesis were to decipher the possible role of *edl* and identify the molecular and cellular events leading to success or failure of encapsulation. I used various approaches from fly genetics to flow cytometry.

The involvement of *edl* in *Drosophila* resistance was confirmed by using overexpression and interference of *edl* expression. The overexpression of the resistant allele in a susceptible background leads to a resistant phenotype. The interference of the expression of the susceptible allele results in an increased rate of parasitoid encapsulation. At the cellular level, an increase in the number of hemocytes after parasitism occurred earlier in the resistant strain than in the susceptible strain. It was also observed that the hematopoietic lymph gland of the resistant larvae busted before the one of the susceptible larvae. At the molecular level, potential interactants of *edl* were identified in silico and 2 were tested using interference of their expression which led to observing an increase of encapsulation.

Overall, a key player in the resistance mechanism of the *Drosophila* to the parasitic wasp have been identify during this work and it lays the path for future work on regulation mechanism of the response at the molecular level.

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Table of abbreviations

Abbréviation	Signification	Abbréviation	Signification
ADGF	adenosine deaminase-related growth factor	MAPK	Mitogen-activated protein kinases
AdoR	Adenosine receptor	mRNA	messenger RNA
AGO	Argonaute	MZ	medullary zone
AMPs	antimicrobial peptides	NF- κ B	NF-kappa-B
aop	anterior open	PAMP	pathogen-associated molecular pattern
bsk	basket	PG	peptidoglycan
Crq	Croquemort	PGRP	Peptidoglycan Recognition protein
CZ	cortical zone	pi	piwi-interacting
DAMP	Damage-associated molecular pattern	pnt	pointed
DCV	<i>Drosophila C Virus</i>	PO	Phenoxidase
die	Diedel	PPO	prophenol-oxidases
Dif	Dorsal-related immunity factor	PRRs	pattern recognition receptors
DNA	Deoxyribonucleic acid	PSC	Posterior signalling center
Dome	Domless	PVF	platelet-derived growth factor/vascular endothelial growth factor-like factor
Dpp	decapentaplegic	R2D2	dsRNA binding co-factor of Dicer-2
DRK	Downstream of Receptor Kinase	RISC	RNA-induced silencing complex
dsRNA	double strand RNA	RNA	RiboNucleic acid
EBF	early B-cell factors enhancer binding factor	RNAi	RNA interference
edl	ETS binding lacking	ROS	Reactive Oxygen Species
EGFR	Epidermal Growth Factor Receptor	RTK	Receptor Tyrosine Kinase
ERKA	Extracellular-Regulated Kinase A	SAM	Sterile Alpha Motif
FGFR	Fibroblast Growth Factor Receptor	SINV	sindbis virus
FHV	Flock House Virus	siRNA	small interfering RNA
FOG	Friend of GATA	SOS	Son of Sevenless
gcm	missing glial cells	SPE	Spätzle-processing enzyme
GGBP3	Gram-negative bacteria binding protein 3	Spz	Spätzle
hh	hedgehog	srp	serpent
hop	hopscoth	Stat	Signal Transducer and Activator of Transcription
IKK	I κ B kinase	TEL	Translocation Ets leukemia
IL-6	Interleukine 6	TLRs	Toll-like Receptors
IIS	Insulin/IGF	TNF	tumor necrosis factor
imd	immunodeficiency	TOR	Target of Rapamycin
IMPDH	inosine monophosphate dehydrogenase	tsr	twinstar
Jak	Janus Kinase	upd	unpaired
JNK	Januse kinase	VSR	viral suppressors of RNAi
KO	knock-out	Wnt	wingless
LG	Lymph Gland	zir	Zizimin-related
lz	Lozenge		

I. Introduction

Types of interactions between organisms

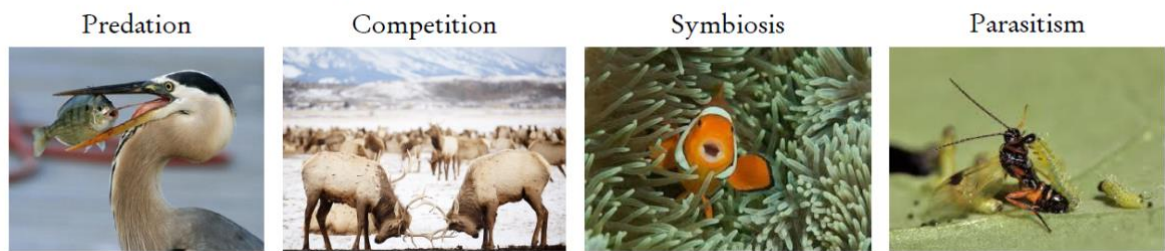


Figure 1: Examples of interaction between organisms. Predation: a heron caught a fish. Competition: 2 stags fighting. Symbiosis: a clownfish living in symbiosis with a sea anemone. Parasitism: a caterpillar parasitized by *Cotesia glomerate*. Pictures from National Geographic.

Every living organism interacts with other organism(s) during its life time. These species interactions form the basis for many ecosystem properties and processes such as nutrient cycling and food webs. They can be classified in several categories including predation, competition, symbiosis and parasitism (Figure 1). Predation might be the most obvious direct interaction people think about when asked about species interactions: the predator kills and eats the prey. Competition for the acquisition of a resource (food, reproductive partner) can be direct or indirect and it significantly influences the structuring of communities. Symbiosis refers to a long-term association between 2 different organisms. Parasitism is a form of symbiosis where one individual will thrive and survive at the expense of one or several hosts. Parasites can live in (endoparasite) or on the host (ectoparasite). The interactions' characterization as a function of the impact on the partner "host" fitness is not as clear as described above. It is admitted that environmental conditions or equilibrium breaks in the interaction can displace an interaction on a scale ranging from mutualism to parasitism or pathogenicity. For instance, commensal intestinal bacteria may behave as pathogens under certain conditions. Finally, we must also highlight the long-term impact of mutualism and parasitism on the evolution of past and present species through selection pressures, interactions of genomes and horizontal gene transfer.

Overall, these interactions may have positive, negative or neutral effects on either specie's ability to survive and reproduce and are widespread causes of natural selection and *in fine* of species evolution.

Insects are the most successful group of all animals. The origin of insects was dated to the early Ordovician ~479 millions years ago and ~345 millions years ago for major extant lineages (Misof *et al.*, 2014). They are the most successful group of all animals, considering either the number of individuals (1019 living simultaneously) (McGavin, 2010) or the number of species (55% of the species biodiversity, 85% of the animal biodiversity). Entomologists estimate the actual number of living insect species could be as high as 5 to 10 millions. The nutrition by consumption of other insects (entomophagy) could be quite old in this taxon and it is found in all the main insect orders. Entomophagous insects are broadly divided into two classes: the parasites and the predators. At least 87 families, in 50 different insects orders contain parasitic species, while those which are predatory are present in 167 families from 14 orders. Allowing for duplications, at least 224 families in 15 orders have adopted entomophagous habit. Between 10 and 20% of known insect species are parasitoids, of which a quarter are *Diptera* or *Coleoptera* and three quarters are *Hymenopteran* wasps.

The parasitoid lifestyle is considered intermediate between parasite and predator. Indeed, a parasitoid develops in interaction with and at the expense of a host (parasite) but consumes the

tissues of the host which usually leads to its death (predator). Thus, parasitoid insects are mainly species with a unique reproductive strategy: they lay eggs in or on other insects (egg, larva, pupa or adult), their larvae develop as parasites while adults have a free life. Therefore, food sources usually differ between larva and adult. A distinction can be made between gregarious parasitoids that can lay up to hundreds of eggs in a host, and solitary parasitoids for which even if several eggs are laid in a host, a single parasitoid will emerge. During oviposition, some parasitoid females kill or paralyze their host which is immediately consumed by their larvae (idiobiont parasitoids), while others allow the host to continue its development to ensure the proper development of the parasitoid offspring (koinobiont parasitoids). Koinobiont parasitoids, that parasitize host stages with a developed immune system (larval or adult hosts), must then be able to evade or suppress host defences, as well as manipulate host physiology to ensure their survival. This requires a delicate balance between the parasite and the host, the parasite allowing the host to be kept alive while bypassing its immunity and diverting its nutrients. Another alternative to the interaction is the triggering of a successful immune response by the host. The egg/larva of the parasitoid is then "inactivated" and/or killed, which usually allows the survival of the host. These two situations leading to "a single winner" are often described as "resistance" of the host (ability of the host to survive parasitism) and "virulence" (ability of the parasitoid to succeed in parasitism). However, the notion of resistance or virulence in the genetic sense is based on the existence of a variation and therefore presupposes the presence of resistant/susceptible or virulent/avirulent individuals in the population.

Like other insects, *Drosophila* species (*Dipteran*) can be parasitized at larval or nymphal stages by many species of parasitoids, including more than 40 *Hymenopteran* species, which constitute an important factor in the field population regulation (Carton *et al.*, 1986). A large number of laboratory investigations on the biology, behaviour, ecology, genetics, physiology have been performed on these parasitoids, mainly on koinobiont larval parasitoids of the genus *Leptopilina* (Eucoilidae) and *Asobara* (Braconidae) (Bouletreau, 1986; Carton *et al.*, 1986; Carton & Nappi, 1997, 2001; Kraaijeveld *et al.*, 1998; Van Dijken & Van Alphen, 1998; Fauvergue *et al.*, 1999; Eijs & Van Alphen, 1999; Eslin & Prévost, 2000; Fellowes & Godfray, 2000; Fleury *et al.*, 2000; Vavre *et al.*, 2000; Ellers *et al.*, 2002). In all these studies, *Drosophila* parasitoids have proven to be suitable biological models because of the extensive knowledge available on *Drosophila* biology. It is thus not surprising that this model is also one of the most advanced for the study of the immune interaction between host and parasitoid and the genetic bases of host resistance and wasp virulence.

In the rest of the introduction, I will focus mainly on the model of interaction between *Drosophila* and its larval parasitoids. I will first summarize the knowledge on the immune system of *Drosophila*

with a focus on the cellular aspects of immunity, essential for the response to parasitoids. I will then present data on the interactions between *Drosophila* and parasitoid wasps. Finally, I will detail our knowledge of the genetic resistance of *D. melanogaster* to these wasps and outline the issues and objectives of my PhD work.

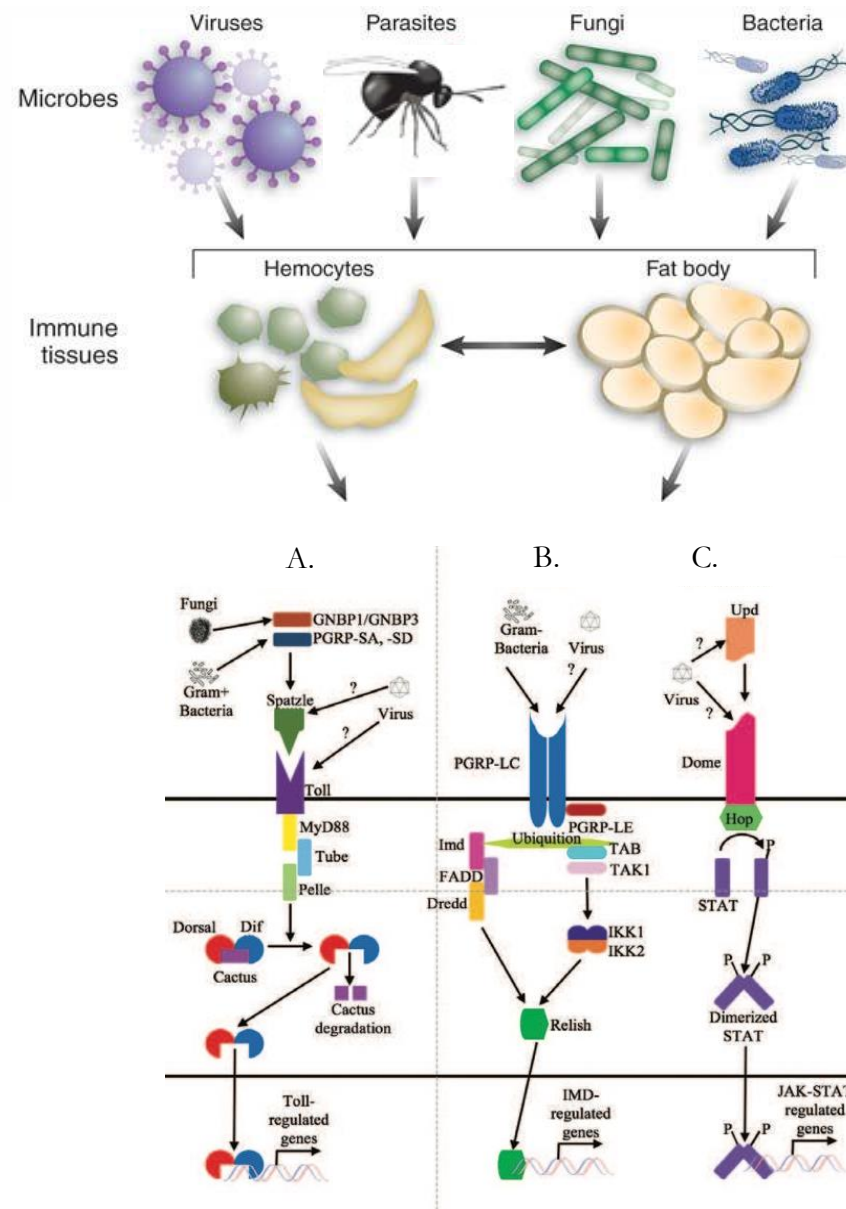


Figure 2: Schematics of the activation of immune defences in *Drosophila* adapted from (Lopez *et al.*, 2018) and (Tanji & Ip, 2005). A: Toll pathway, B: imd pathway, C: Jak-Stat pathway

A. Immunity in *Drosophila melanogaster*

Since the discovery of the “white” mutation and its positioning on the X chromosome by Thomas Hunt Morgan in 1910, *D. melanogaster* has been a central model organism for studies in genetics, population genetics and other aspects of biology, including immunology. Indeed, the conservation of basic signalling pathways and key transcription factors controlling the development and functions of blood cells from *Drosophila* to human, makes *D. melanogaster* a simplified and interesting model to decipher the fundamental mechanisms governing hematopoietic system formation and homeostasis. Therefore, *Drosophila* is a study model for diseases associated with these mechanisms, such as leukaemia. Like other insects, the fruit fly lacks the mechanisms of adaptive immunity and relies solely on its innate immunity to defend against pathogens and parasites.

Innate immune defences are central mediators of the metazoan immune system and are essential to the health and the success of complex organisms (Hoffmann, 2003). The discovery of antimicrobial peptide responses 40 years ago has established *D. melanogaster* as a leading system to investigate fundamental components of the innate immune defences: The Nobel Prize in physiology or medicine has been awarded to Jules Hoffmann and Bruce A. Beutler for their discoveries concerning the activation of innate immunity in 2011.

During its life cycle, *D. melanogaster* flies feed, lay eggs and develop on decaying environments, notably fruits. Therefore, they are more exposed to pathogens than other organisms. This explains that *Drosophila* produces efficient humoral and cellular mediators to fight against the different pathogenic infections as well as parasitoids wasps. Its immune defences are based on the existence of cellular components, different types of hemocytes, and the production of antimicrobial peptides (AMPs) secreted mainly by fat and hemocytes. At the molecular level, innate immunity has been shown to involve several different pathways, including the Toll-Dorsal (Toll) pathway, the immunodeficiency (*imd*) pathway, the Janus Kinase (Jak)/Signal Transducer and Activator of Transcription (Stat) pathway, autophagy, and RiboNucleic Acid interference (RNAi) interference (Figure 2)(Mussabekova *et al.*, 2017). The Toll and *imd* pathways contribute mainly to the antibacterial and antifungal defences even if the Toll pathway may also be an actor of the anti-parasite response (Lemaitre & Hoffmann, 2007; Valanne *et al.*, 2011; Lamiable *et al.*, 2016).

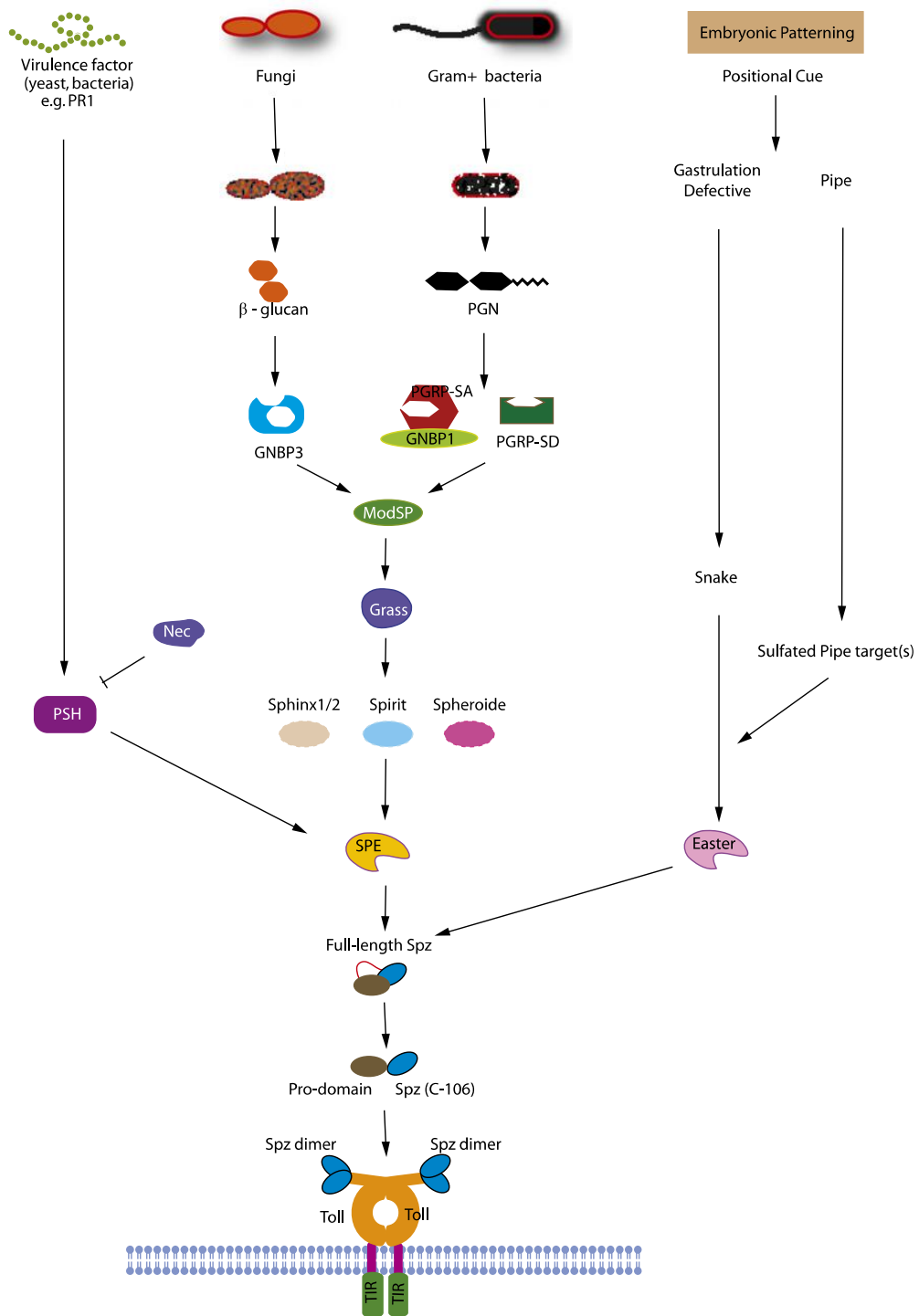


Figure 3: Toll pathway from Valane, Wang and R met, 2011

1. Antibacterial and Antifungal defences

a) The Toll pathway

The first immune receptors, the Toll receptors, have been discovered in *Drosophila* (see Vogel, 2012 for an historical review). Yet, the Toll pathway was first known for its role in the establishment of dorso-ventral polarity in the embryo, thanks to a series of genetic screens for genes involved in early *Drosophila* embryonic development (Nüsslein-Volhard & Wieschaus, 1980). What is interesting with the Toll pathway is that there is crossing over between the discoveries in humans and *Drosophila*. The identification of Toll as an activator of the immune response happened in 1995 (Rosetto *et al.*, 1995). Human Toll was found soon after that and it was followed by the discoveries Toll-like Receptors (TLRs) in both *Drosophila* and mammals, and it was shown that the Toll pathway is conserved across phyla.

In *Drosophila* innate immunity and embryonic patterning, the Toll signalling pathway (Figure 3) is activated by the endogenous protein ligand Spätzle (Spz) (Valanne *et al.*, 2011). Spz is secreted as an inactive precursor that during embryogenesis is processed into an active form by the serine protease Easter. During infection, pattern recognition receptors (PRRs) initiate the immune responses when they encounter damage-associated molecular pattern molecules (DAMPs) and pathogen-associated molecular pattern molecules (PAMPs). PAMPs such as microbial Lysine-type peptidoglycan recognized by Peptidoglycan Recognition Protein (PGRP) or β -glucans recognized by Gram-negative bacteria binding protein 3 (GNBP3) activate proteolytic cascades and signal-transduction pathways. Insect PGRPs are classified as short or long: short PGRPs have signal peptides and can be extracellular proteins, whereas long PGRPs can be intracellular, extracellular, and transmembrane proteins. This PGRP-PNG interaction initiates a proteolytic cascade in which Spätzle is processed by a specific serine protease, Spätzle-processing enzyme (SPE). Among, the *Drosophila* PGRPs, only PGRP-SA and PGRP-SD are involved in the activation of the Toll pathway. The Toll receptor activation allows the degradation of the NF-kappa-B (NF- κ B) inhibitor Cactus and the liberation of transcription factors Dorsal-related immunity factor (Dif) and Dorsal, the *Drosophila* NF- κ B homolog. Upon translocation to the nucleus, Dorsal binds to the kappa B-related nucleotide sequences of the antifungal peptide Drosomycin and different antibacterial peptides such as Cecropins, Attacin, and insect Defensin. In larvae and adult, Dorsal is expressed in the fat body, and both its expression level and nuclear localization are enhanced upon microbial challenge.

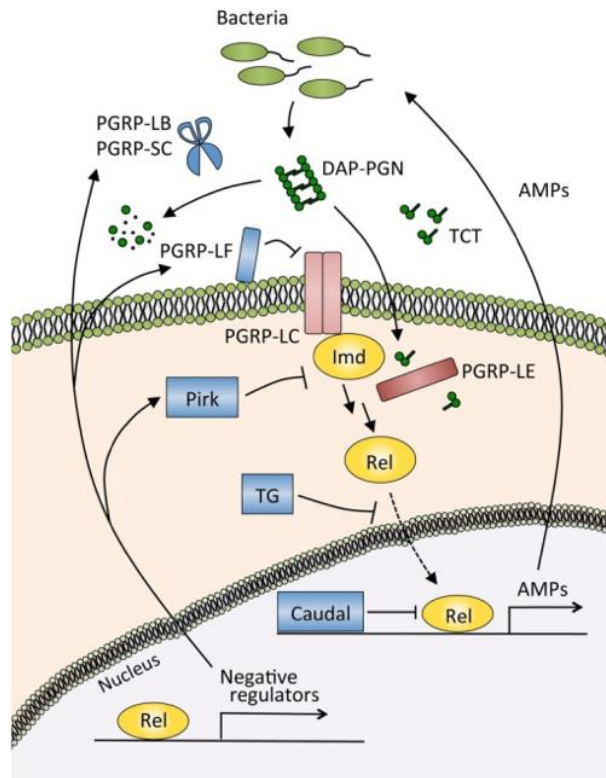


Figure 4: Representation of the Imd pathway From Kuraishi *et al.*, (2013)

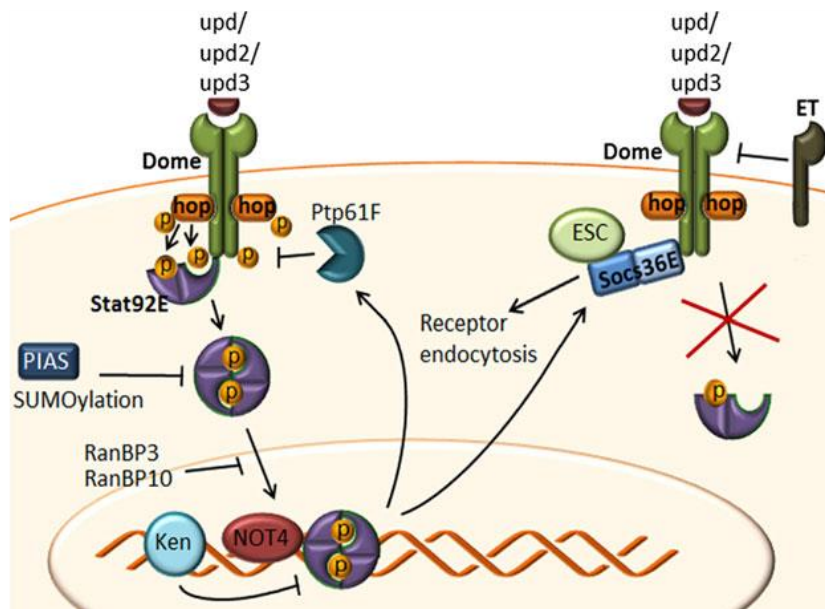


Figure 5: JAK/STAT pathway from Myllymäki and Rämetsä, 2014

The gene signalling pathway between *Spätzle* and *Cactus*, which includes *Toll*, *Tube*, or *Pelle*, constitute the Toll signalling “cassette” also required for the antifungal response in adult flies.

b) The Imd pathway

Another evolutionarily conserved signalling cascade regulates *Drosophila* immunity, the imd pathway (Figure 4), which activates a third NF- κ B gene, *Relish* (Kuraishi *et al.*, 2013; Myllymaki *et al.*, 2014). Activation of Relish requires phosphorylation and cleavage of the inhibitory C-terminal part (I- κ B like), probably by the caspase 8-homolog Dredd, thus exposing a novel ubiquitin binding site. This leads to activation of the Tab2/Tak1 complex, which in turn phosphorylate the *Drosophila* I κ B kinase (IKK) complex. The phosphorylation of Relish by the IKK complex led to its cleavage and the translocation of its N-terminal part (Rel-68) into the nucleus where it activates the transcription of genes coding for AMPs (Diptericin and Cecropin). IMD, a death domain protein, is part with Dredd of the signalling complex recruited after binding of peptidoglycan (PG) to the receptor. Several PGRPs are involved in the imd pathway activation, the main one being the transmembrane protein PGRP-LC that binds bacterial PG (Tanji & Ip, 2005). PGRP-LE in its short-secreted form binds PG in the hemolymph and presents it to PGRP-LC. The cytoplasmic PGRP-LE can also interact with IMD, independently of PGRP-LC, to activate autophagy, whereas the transmembrane form can activate a prophenoloxidase cascade together with PGRP-LC. PGRP-LE is the only intracellular pathogen receptor identified in *Drosophila*. The imd pathway is required for expression of most *Drosophila* AMPs and flies with defects in the pathway die from bacterial infection while resisting fungal infection (Ben-Ami *et al.*, 2009). Interestingly, the pathway initiates the systemic response in the fat body but is also locally activated in various tissues, trachea, brain and gut epithelia and mucosal surfaces.

c) The JAK-STAT pathway

The JAK/STAT pathway (Figure 5) was first discovered and studied in vertebrates. In human, this pathway plays a role in the differentiation of T and B lymphocytes, the control of inflammatory and wound repair in response to interferons, interleukins, other cytokines, and growth factors.

In *Drosophila*, a conserved JAK/STAT signalling pathway controls segmentation in embryos, as well as blood cell development and many other processes (eye development, gut renewal ...) (Morin-Poulard *et al.*, 2013; Zeidler & Bausek, 2013).

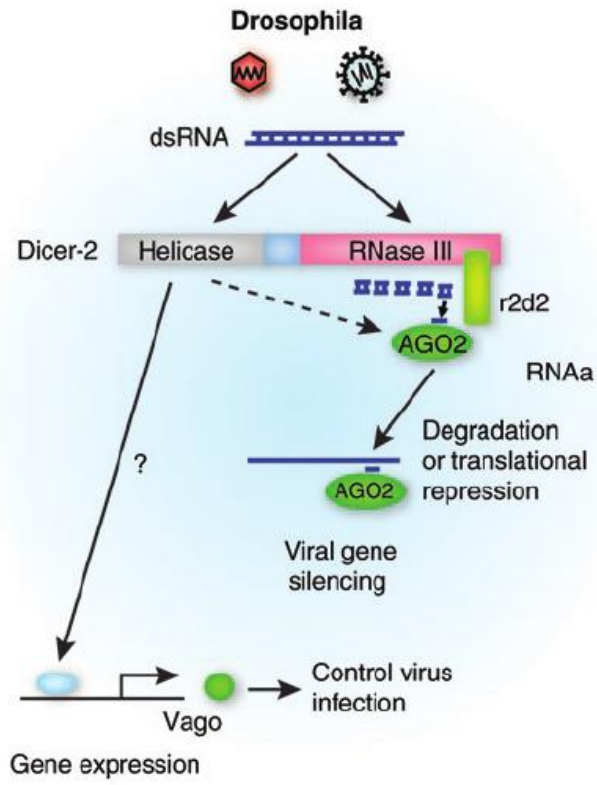


Figure 6: RNAi machinery in *Drosophila* from Mussabeokva, Daeffler and Imler, 2017

The *Drosophila* known ligands in the JAK/STAT pathway consist of three cytokine-like proteins (closely related to vertebrate leptins) called unpaired (upd), upd2 and upd3. The expressions of these 3 ligands are induced in response to tissue damage: upd3 in adult hemocytes after a bacterial infection and upd2 and upd3 in response to viral infection (Myllymäki & Rämet, 2014). Extracellular upds bind to the cytokine receptor Domeless (Dome), which shares similarities with the mammalian IL-6 receptor family. Dome transmits the signal through the only *Drosophila* JAK kinase hopscotch (hop) which phosphorylate Stat92E (Stat92E/Marelle) which then forms a dimer and translocate to the nucleus where it binds to the promoters of the target genes.

A role for JAK-STAT signalling in cellular immunity was suggested by studies of the hop^{Tum-1} mutant. This mutation leads to an increased number of plasmatocytes and the massive differentiation of lamellocytes ready to encapsulate “self” tissue, leading to the formation of black masses/melanotic pseudo-tumours (Hanratty & Dearolf, 1993; Luo *et al.*, 1995). Plasmatocytes that adhere to injured tissue upon the detection of basement membrane disruption, start a systemic response by producing cytokine (mainly Upd3) that eventually result in hemocyte proliferation (Agaïsse *et al.*, 2003). Activation of JAK-STAT signalling in hemocytes is thus required for their increased proliferation in response to both tumours and wound (Asha *et al.*, 2003; Sorrentino *et al.*, 2004). It is interesting to note that upd3 is secreted by the posterior signalling center (PSC) of the larval lymph gland (LG) to maintain the prohemocytes of the medullary zone (MZ) in an undifferentiated state (Makki *et al.*, 2010). In case of parasitism, the JAK/ STAT pathway is switch off to allow lamellocytes differentiation (Makki *et al.*, 2010).

2. Antiviral immunity

Like many organisms, *Drosophila* can be infected by viruses and is a good model to study the mechanisms of antiviral defence (Kemp & Imler, 2009). The viruses described as able to infect *Drosophila* are RNA viruses and the best characterized *Drosophila* immune reactions are for the Sigma virus, the *Drosophila* C Virus (DCV) and the Flock House Virus (FHV) (Lopez *et al.*, 2018). Antiviral immunity appears to be mediated by two general mechanisms: RNAi inhibition of viral RNAs, such as the piwi-interacting pathway and an induced response calling on the expression of specific antiviral proteins. As in mammals, the induced response involves the activity of several signalling pathways, among which the JAK/STAT pathway (described in I.A.1.c).

RNAi consists of the formation of an RNA-induced silencing complex (RISC, Figure 6). In the case of a virus infection, the viral mRNA is processed by Dicer-2, a RNase, into a 21 nucleotides double strands siRNA (small interfering RNA). Dicer-2 is joined by R2D2 (a dsRNA binding co-

factor of Dicer-2), a homodimer of TAR11 and an Argonaute (AGO) protein. The mature RISC complex contains a single strand siRNA and is the guide strand to allow specific degradation of the target RNA by AGO2. This process is essential for the *Drosophila* survival as the virus need the *Drosophila* machinery for multiplication. Indeed, loading of the siRNA duplex onto AGO2 to form a pre-RISC complex cannot occur solely in the presence of the Dicer-2/R2D2 complex. Three chaperone proteins, Hsc70, Hsp90 and Hop, are essential for pre-RISC formation, whereas two others (Droj2 and p23) further improve the efficiency of AGO2- RISC assembly. Many insect viruses, including those of *Drosophila*, encode viral suppressors of RNAi (VSRs). Some of them bind to long viral dsRNAs and prevent binding of Dcr-2. For example, the FHV encodes for B2 binds to dsRNA and inhibits the loading of the 21 nucleotides double strands siRNA onto AGO2 (Mussabekova *et al.*, 2017)(Figure 6).

The Toll pathway may play a role in the antiviral response *via* the AMPs production, silencing of dipterin (Relish-dependent) and attacin (STAT-dependent) resulted in increased of Sindbis virus (SINV) viral load (Lester & Li, 2014). Interestingly, while the Toll pathway is activated in the fat body upon oral infection or direct hemolymph injection of DCV, only the oral infection route gave a phenotype. This suggests that the antiviral action of the Toll pathway targets a specific step of the viral cycle of the oral infection route, which is bypassed when the virus is directly injected in the body cavity.

Genome-wide profiling upon DCV infection identified upregulated genes that contain STAT-binding sites in their promoter like the virus-induced RNA1 (*vir-1*). In the JAK/STAT signalling pathway, Hop and Dome activity is required for the induction of *vir-1* in response to DCV infection and hop mutant flies express low levels of *vir-1*, have high viral titers and succumb rapidly to DCV infection. Altogether, these data suggest a model in which DCV infected cells produce a cytokine that activates the JAK-STAT pathway and the immune defence in non-infected cells (Morin-Poulard *et al.*, 2013).

Other components of viral particles may be sensed by the fly immune system. For example, *Diedel* (*die*) is an immunomodulatory cytokine, which down-regulates the imd pathway, is strongly upregulated by the enveloped viruses SINV, but not by the non-enveloped viruses DCV. *Die* mutant flies have reduced viability and succumb more rapidly than controls when infected by the RNA Sindbis virus. Induction of *die* does not involve Relish, but *Dif* which is activated by a non-canonical Toll pathway. *Die*, encodes a circulating 12 kDa protein, and belongs to a larger family of proteins encoded by insect DNA viruses and present in the venom of parasitic wasps (Lamiabile *et al.*, 2016).

In summary, gene expression profiling studies and experiments with mutant flies point to the involvement of the Toll, imd, and JAK/STAT pathways in the control of viral infections. Apoptosis is also a major response to viral infection, initially characterized in *Lepidopteran* insects in the context of DNA viruses. Programmed cell death can stop the infection before viral replication is completed. Additionally, apoptosis may promote clearance of infected cells by phagocytes, thus preventing dissemination. Clearly, both the presence of hemocytes and active phagocytosis are required to control some virus in infected flies (Nainu *et al.*, 2015).

3. Cellular immunity

We have seen that the penetration of a pathogen into the *Drosophila* hemolymph triggers the activation of signalling pathways, especially in the fat body and hemocytes. In healthy larvae, the circulating hemocytes are mainly plasmatocytes, the remainder being crystal cells and a very small number of prohemocytes and lamellocytes. The number of circulating hemocytes in the larval stage increases from a few hundred at the beginning of the first larval stage to about 6000-7000 cells at the end of the third larval stage (Lanot *et al.*, 2001).

a) Hemocyte types

Prohemocytes have been described in the embryonic head mesoderm, in embryo-derived larval hemocytes, in the larval lymph gland and in adult hematopoietic hubs (Williams, 2007; Makki *et al.*, 2010; Gold & Brückner, 2014; Ghosh *et al.*, 2015). They are small cells (4-6 μm in diameter) with a high nuclear/cytoplasmic ratio. So far, there is no known specific marker universally labelling *Drosophila* prohemocytes.

Plasmatocytes are the most abundant type of hemocytes in healthy larvae hemolymph, making up to 95% of the circulating hemocytes. These spherical shaped cells of about 8-10 μm in diameter are characterized by the presence in their cytoplasm of numerous lysosomes, phagosomes (phagocytosis vacuoles) and resorption bodies, which reflect their phagocytic activity. The phagocytic ability of plasmatocytes depends on the expression of scavenger and pattern recognition receptors on their surfaces such as Croquemort (Crq) for recognition of apoptotic cells and members of the Nimrod family, Eater and NimC1, for Gram-positive and Gram-negative bacteria.

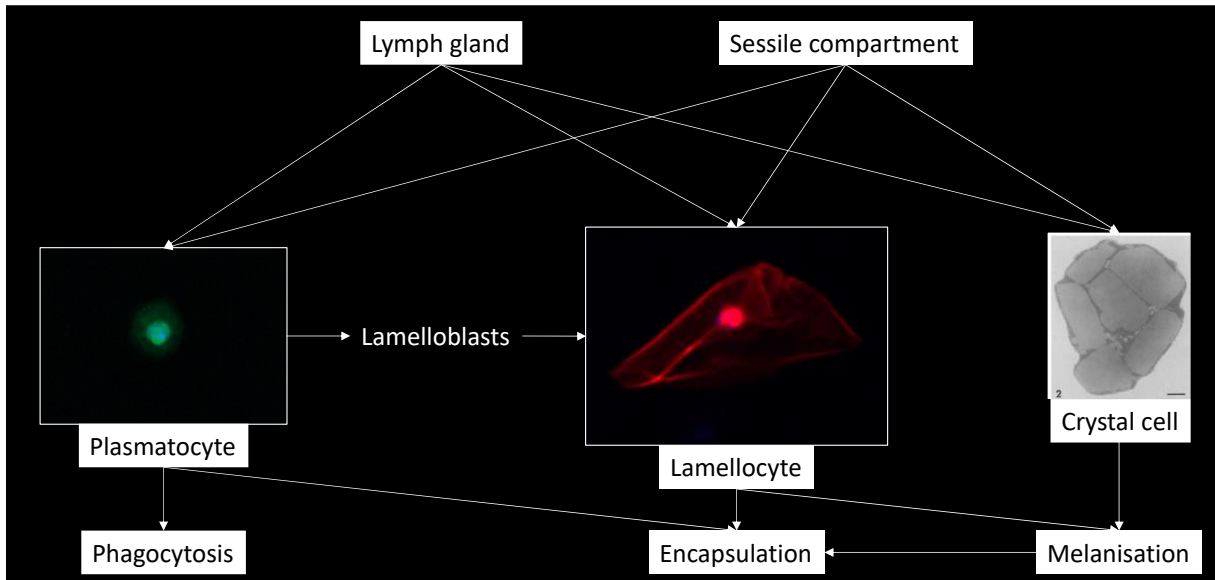


Figure 7: Origin and function of hemocytes in *Drosophila melanogaster* (in green: plasmatocytes expressing eater-GFP; in red: lamellocytes expressing msnCherry (personal pictures, Axioplan Z1, x400); electronic microscopy picture of crystal cell from (Rizki *et al.*, 1980)

They form the primary layer of cells during a response to eliminate bacteria (nodulation (Gandhe *et al.*, 2007; Dubovskiy *et al.*, 2016)) or to form a cellular "capsule" around parasitic agents such as nematodes or the egg of a parasitoid too large to be phagocyted (Rizki, 1968; Carton *et al.*, 2008; Bajgar *et al.*, 2015; Dubovskiy *et al.*, 2016). They are involved in wound healing and also synthesize extracellular matrix proteins (Martinek *et al.*, 2008) and antimicrobial peptides (Wang *et al.*, 2014). Plasmatocytes have important functions in the innate cellular immune response, but also in tissue remodelling and homeostasis during embryogenesis. While plasmatocytes are usually considered as a single entity, populations expressing different subsets of markers have been identified (Jung *et al.*, 2005; Honti *et al.*, 2014). Moreover, two plasmatocytes subpopulations with distinct functions in the adult immune response have been identified (Clark *et al.*, 2011).

Crystal cells are non-adhesive round cells of the same size as plasmatocytes and represent 5% of the cells of the hemolymph of healthy larvae (Lemaitre & Hoffmann, 2007). They are named according to the presence of large paracrystalline inclusions in their cytoplasm, which contain prophenol-oxidases (PPO1 and PPO2) which are the inactive form of the enzymes catalysing the melanisation reaction. Upon injury, activation of the JNK pathway and the TNF homolog Eiger leads to crystal cells rupture and release of PPO zymogens into the hemolymph (Bidla *et al.*, 2007). These cells play a major role in clotting, wound healing and antimicrobial defences. The transcription factor Lozenge, expressed only during the development of this cell type (Muratoglu *et al.*, 2007), constitute a specific marker to identify them.

Lamellocytes are described as large flat (40µm diameter) with adherent cells. Although they are rare in the hemolymph of non-parasitized larvae, their number largely increases at the pre-pupal stage (Holz *et al.*, 2003). They are not observed in the embryo nor the adult stages. During the larval stage, the production and differentiation of lamellocytes can be induced following injury or intrusion of a large foreign body such as a parasitoid egg (Rizki & Rizki, 1992). Lamellocytes form the successive outer layers of the capsule formed around foreign bodies by attaching to the plasmatocyte cells and participate in its melanisation since they contain one specific pro-phenoloxidase (PPO3) (Irving *et al.*, 2005; Dudzic *et al.*, 2015). The lamellocytes origin upon parasitism is still under debate: they can be derived from sessile or circulating plasmatocytes that form intermediate lamelloblasts during the trans-differentiation of into lamellocytes (Honti *et al.*, 2010; Stofanko *et al.*, 2010; Anderl *et al.*, 2016) or release as mature lamellocytes from an accelerated proliferation and bursting of the lymph gland (Lanot *et al.*, 2001). Lamellocytes present several surface markers such as L1/Attila (Kurucz *et al.*, 2007), PS4 (Crozatier *et al.*, 2004) or the integrin myospheroid (Xavier & Williams, 2011).

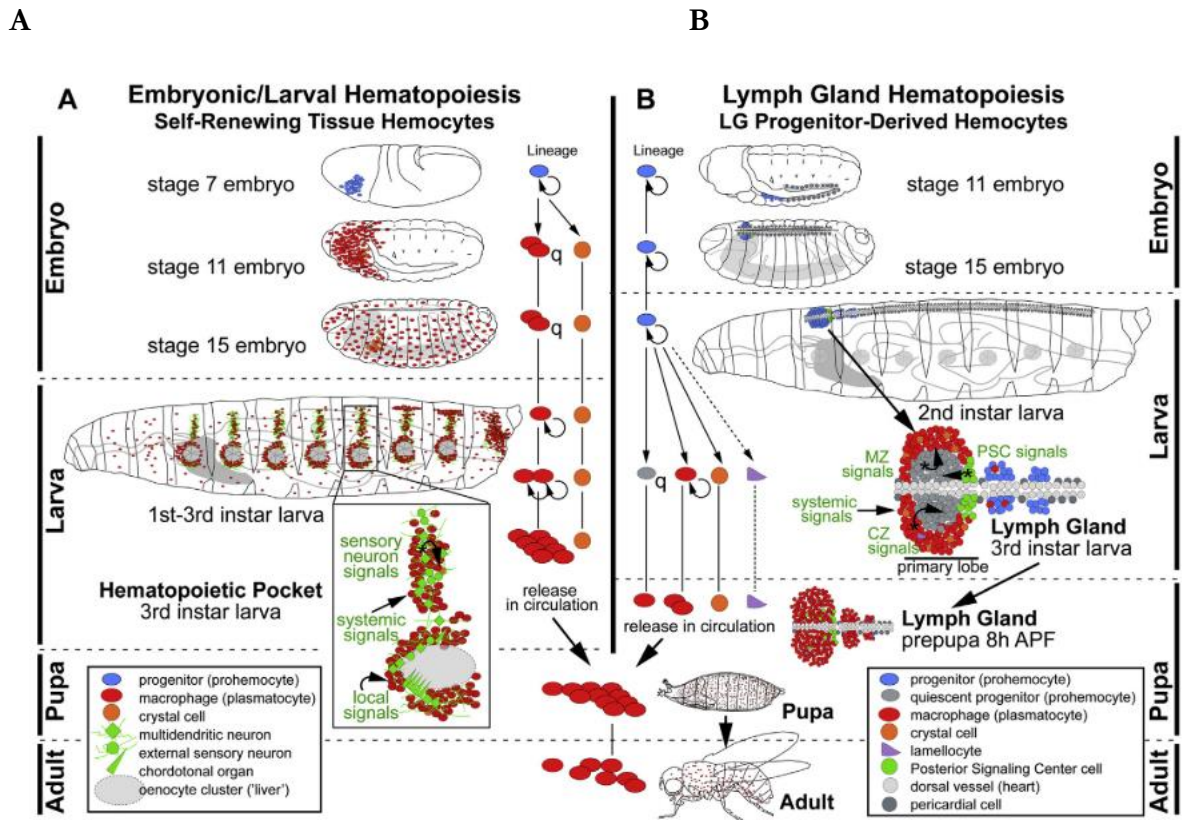


Figure 8: Ontogenesis of blood cell lines and regulation of hematopoiesis in *Drosophila*. (see text for explanation; from (Gold & Brückner, 2014)).

b) Hematopoiesis

The ability to perform genetic manipulations and the conservation of genes and pathways involved with those in mammalian hematopoiesis make *D. melanogaster* hematopoiesis a powerful model to study the complex biological process involved in the balance between the quiescence of hematopoietic stem cells and their differentiation into lineages. There are many reviews on *Drosophila* hematopoiesis (Krzemien *et al.*, 2010; Makhijani & Brückner, 2012; Gold & Brückner, 2014, 2016; Ramond *et al.*, 2015; Hillyer, 2016; El Chamy *et al.*, 2017; Yu *et al.*, 2017) and new findings are published almost every week. The following text will not provide an exhaustive overview of all the work in this field but will highlight the main features of *Drosophila* hematopoiesis.

Until recently in *Drosophila*, hemocytes were thought to be produced in two successive waves, one during embryogenesis and the other in a specific organ, the lymph gland, during larval stages, with differentiated adult hemocytes derived from embryonic and larval hemocytes (Gold & Brückner, 2014). However, recent studies suggest much higher plasticity, with hematopoietic sites distributed along the larvae and also present in adult flies (Gold & Brückner, 2014; Ghosh *et al.*, 2015).

The Figure 8 from Gold and Bruckner (Gold & Brückner, 2014) summarizes the different stages of embryonic and larval hematopoiesis. In the embryo (panel A), the prohemocytes emerging from the head mesoderm will differentiate into plasmatocytes and crystal cells. At embryonic stage 7, prohemocytic progenitors (in blue) are in the mesoderm of the head. After four cycles of division, these progenitors stop proliferating and differentiate into 600 to 700 plasma cells (in red) and a small number of crystal cells (in orange) that remain grouped around the proventriculus. At stage 11, the differentiated plasmatocytes begin to migrate to the antero-posterior end of the embryo and uniformly colonized the embryo at stage 15, then all cells remain quiescent until the end of embryogenesis. At larval transition, plasmatocytes and embryonic crystal cells persist. Plasmatocytes colonize hematopoietic niches at each larval segment which also contain peripheral sensory neuron clusters (in green) to form the hematopoietic pockets of the sessile compartment. The plasmatocyte Eater protein, a transmembrane receptor of the Nimrod family, is necessary to allow their recruitment as well as that of crystal cells to sessile compartments (Bretscher *et al.*, 2015). Signalling molecule produced by the sensory neurons, such as Activin-b, a TGF-b family ligand, induced the adhesion and proliferation of hemocytes in hematopoietic pockets suggesting strongly that environmental sensory stimuli and neural activity provide another regulatory level (Makhijani *et al.*, 2017).

The second wave of hematopoiesis occurs during the larval stages, where two hematopoietic compartments are present: the sessile compartment and the lymph gland (Figure 8; panel B). Lymph gland prohemocytes derive from precursors of the embryo cardiogenic mesoderm (in blue). At that stage, the lymph gland precursors form a single pair of lobes that are localized along the dorsal vessel. This single pair of lobes are designated as the primary lobes (the anterior lobes). At the end of the first larval instar, additional pairs of posterior lobes also emerge along the dorsal vessel, the secondary or posterior lobes. So, in third instar larvae, lymph gland is composed of a large pair of primary anterior lobes organized into three domains: the cortical zone (CZ), the medullary zone (MZ) and the posterior signalling center (PSC), followed by several small pairs of posterior lobes, each separated by a pair of pericardial cells. The posterior lobes are mainly composed of prohemocytes.

In normal condition, the differentiation of lymph gland hemocytes is detected starting from the third larval stage; it develops in a spatiotemporally organized manner. During the third larval stage, the primary lobes of the CZ dilate through proliferation and differentiation of hemocytes into plasmatocytes, a small number of crystal cells and occasionally a few lamellocytes. Then the progenitors present in the MZ become quiescent. As development progresses, almost all hemocytes in the lymph gland differentiate, and 8 hours after puparium formation, all cells in the lymph gland have been released into the circulation (Grigorian *et al.*, 2011). The proliferation and differentiation of hemocytes is controlled by a wide range of signals from the lymphatic gland and systemic sources, such as neurotransmitters and growth factors from the brain, and levels of nutritional compound.

During the larval life, hemocytes in the sessile compartments proliferate and differentiate, so these sites are functional haematopoietic sites (Markus *et al.*, 2009; Makhijani *et al.*, 2011; Leitão & Sucena, 2015). Plasmatocytes proliferate (self-renewal) and also transdifferentiate in crystal cells through a Notch signalling-dependent process (Leitão & Sucena, 2015). They first lose the expression of the differentiation factor Nimrod C1 as well as their phagocytic activity, to acquire the expression of the Lz marker (Leitão & Sucena, 2015). The hemocytes from the sessile compartment can move laterally between the integument and the muscle layer and reach the hemolymph. During normal larval development, sessile hemocytes contribute only gradually to the pool of circulating plasmatocytes and are only released at the beginning of metamorphosis. However, simple stimuli such as light brush strokes are sufficient to induce this release (Makhijani *et al.*, 2011) indicating that the fate of these sessile hemocytes also depends upon systemic and/or local signals. Immune challenge, such as *Hymenopteran* wasp oviposition can cause also their premature mobilization and induce their trans-differentiation into lamellocytes.

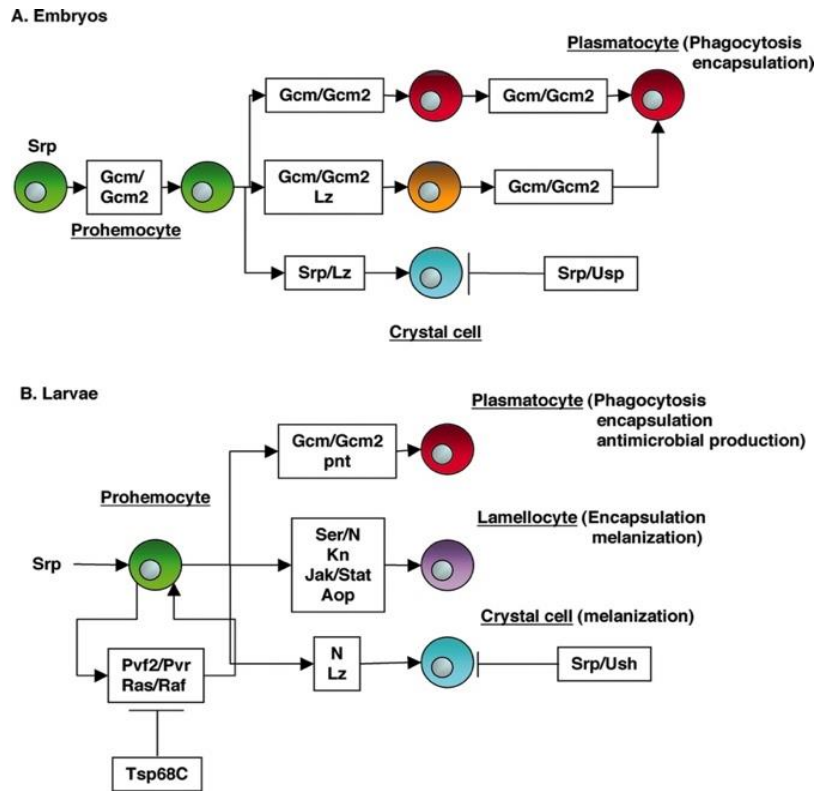


Figure 9: Hematopoiesis regulators and hemocyte functions in *D. melanogaster* from (Williams, 2007). A) At the embryonic level, progenitors in the procephalic mesoderm differentiate into two types of hemocytes (plasmatocytes and crystal cells). B) The lymphatic gland contains many progenitors that differentiate into three types of hemocytes (plasmatocytes, crystal cells and lamellocytes). Many factors and signalling pathways regulating the engagement of these hematopoietic lines in these two compartments have been identified (see text).

c) Factors controlling hematopoiesis

Hematopoietic progenitor maintenance, hemocyte differentiation and the overall homeostasis of the hematopoietic system are finely tuned by intrinsic factors and by environmental stimuli. During embryogenesis, the transcription factor GATA Serpent (*srp*) (Evans *et al.*, 2014; Spahn *et al.*, 2014; Shlyakhover *et al.*, 2018) (Figure 9) is required for the specification of the hemocellular primordium in the head mesoderm at the early embryonic stage and, later, for gene expression during hemocyte maturation. Similarly, the first cells of the lymphatic gland, begin to express *Srp* well before other differentiation or maturation markers can be detected. The activity of *Srp* is modulated by the recruitment of Friend Of GATA (FOG), U-Shaped and Lozenge (*Lz*) co-factors (Figure 9). *Srp* is necessary for the formation of plasmatocytes and crystal cells populations (Petersen *et al.*, 1999; Waltzer *et al.*, 2003; Muratoglu *et al.*, 2007; Shlyakhover *et al.*, 2018). The transcription factors "missing glial cells" *Gcm* and *Gcm2* are expressed in all prohemocytes and their inhibition in some cells leads to the expression of the transcription factor *Lz* which induces them to become crystal cells. The continuous expression of *Gcm*/*Gcm2* leads others to transform into plasmatocytes (Waltzer *et al.*, 2002). The binding of the Serrate ligand to the Notch receptor is critical in regulating *Lz* expression and in specifying crystal cell precursors (Lebestky *et al.*, 2003).

In the lymph gland PSC, high levels of Reactive Oxygen Species (ROS) (Owusu-Ansah & Banerjee, 2009), activation of the Wingless signalling pathway (Sinenko *et al.*, 2009) and expression of the EBF transcription factor Collier (Benmimoun *et al.*, 2015; Oyallon *et al.*, 2016) are required for the maintenance of a pool of pluripotent hematopoietic progenitors. The PSC also secretes diffusible signals such as hedgehog (*Hh*) and the platelet-derived growth factor/vascular endothelial growth factor-like factor (PVF1) to activate different pathways in the lymph gland compartments (Mondal *et al.*, 2011; Tokusumi *et al.*, 2012, 2018; Letourneau *et al.*, 2016). *Hh* acts directly on the progenitors of the MZ to maintain them from their pluripotent state. PVF1 acts on differentiating hemocytes, stimulating the secretion of adenosine deaminase-related growth factor-A (ADGF-A), which leads to the inactivation of the adenosine/adenosine receptor (*AdoR*) signalling pathway in MZ cells by modulating the extracellular adenosine level (Lazzaro, 2015). This double control allows the balance between progenitors and differentiated cells to be maintained. Other regulatory pathways are important for PSC cell proliferation such as the decapentaplegic (*Dpp*) and the wingless (*Wnt*) pathway. A balance between the signalling levels *Wnt* and *Dpp* determines the number of PSC cells. The Insulin/IGF (*IIS*) and Target of Rapamycin (*TOR*) signalling pathways, components of the nutrient detection system, also appear to be important for triggering progenitor proliferation (Benmimoun *et al.*, 2012; Shim *et al.*, 2015).

In addition, prohemocyte fate is controlled by local signals from the neighbouring heart tube and through the regulation of PSC morphology (Morin-Poulard *et al.*, 2016). In L3 larvae, PSC cells act to maintain JAK/STAT signalling activity in prohemocytes, thus preserving their multipotency necessary for these cells to adopt a lamellocyte fate in response to parasitism. When pathways such as JAK/STAT or Toll are constitutively activated, for example by expressing active forms of receptors in hemocytes, there are few sessile hemocytes and the majority of blood cells are circulating (Luo *et al.*, 1995; Benmimoun *et al.*, 2012; Shim *et al.*, 2015). The Ras/Raf/MAPK/Toll and Notch signalling pathways are also involved in the proliferation and differentiation of hemocytes (Asha *et al.*, 2003; Valanne *et al.*, 2011; Ferguson & Martinez-Agosto, 2014; Schmid *et al.*, 2014; Reimels & Pflieger, 2015; Hao & Jin, 2017).

Immune cells produced during the embryonic and larval stages are found in adults and, recently, progenitor cells have been shown to form groups of subcuticular cells (hematopoietic nodes) that can differentiate into crystal and plasmocytic cells (for review (Ramond *et al.*, 2015)).

d) Melanisation

Melanisation covers complex enzymatic and spontaneous reactions that lead to the formation and deposit of black melanin that forms the scar during wound healing and terminates the encapsulation of a large invader. Phenoloxidase (PO) catalyses the first reaction steps by forming indole groups from tyrosine which are then polymerized to form melanin. Enzymatic and spontaneous reactions in turn produce a set of intermediate products such as quinones, diphenols, superoxide, hydrogen peroxide and reactive nitrogen intermediates (Nappi & Christensen, 2005). These reactive species produced during the reaction contributes also to the killing of the aggressors (Nappi & Christensen, 2005; Hillyer, 2016). PO is synthesized intracellularly as an inactive pro-enzyme form, the prophenoloxidase (PPO). The *Drosophila* genome encodes for three PPOs: PPO1 and PPO2 that are produced in crystal cells (Binggeli *et al.*, 2014) and PPO3 specifically expressed in lamellocytes (Irving *et al.*, 2005; Dudzic *et al.*, 2015). The activation of phenoloxidases is normally tightly controlled to limit the reaction at the lesion site and prevent systemic melanisation. PPO1 and PPO2 require a proteolytic cleavage of their pro-domain to be activated (Chen *et al.*, 2012). The cleavage of PPO1 is mediated by a clip-domain protease serine called Hayan (Nam *et al.*, 2012). Hayan also exists as an inactive zymogen which is itself stimulated by a stepwise process involving other serine proteases, that activities are controlled by serine protease inhibitors (Tang *et al.*, 2006).

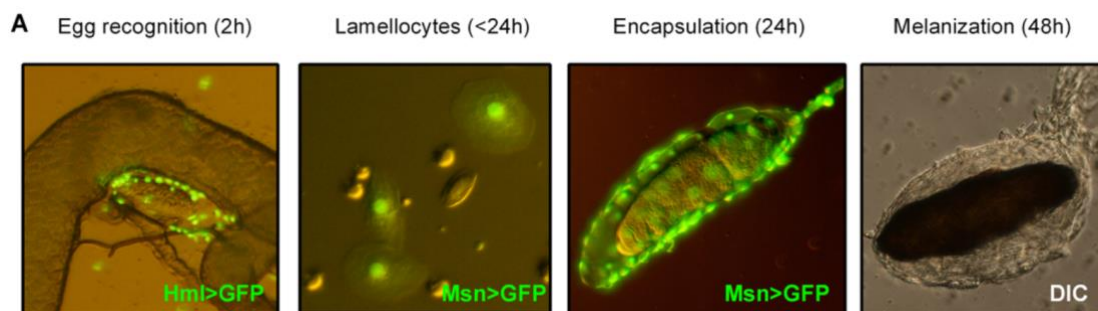


Figure 10: Formation of the multilayer cellular capsule around a parasitoid egg (from Bajgar et al, 2015). The wasp egg is recognized by plasmatocytes (green, Hml>GFP) within 2 h. Lamellocytes, labeled by the Msn>GFP marker appear in circulation less than 24h post-parasitism and are detected on the parasitoid egg 24h after parasitism. Melanisation is observed 48h after the parasitoid egg injection.

The activation of this proteases cascade in response to microbial and fungal attacks can be attributed to PGLP-LC and PGRP-LE expressed by hemocytes and fat body cells (Schmidt *et al.*, 2009). PPO3 in contrast is capable of self-activation without proteolysis (Chen *et al.*, 2012) but it is present only under parasitic conditions and involved in the melanisation of the cell capsule formed around the invader, suggesting a limited diffusion and a short period of activity.

e) Encapsulation

Encapsulation is an important defence mechanism for insects against parasites and other large foreign bodies. It makes it possible to limit the immune response around the intruder and destroy it while avoiding a possible negative effect of the systemic response on the host itself. Encapsulation refers to multiple hemocytes binding to larger invaders, like protozoans, nematodes and parasitoids (eggs and larvae), that cannot be phagocytized by a single cell. The binding of multiple hemocytes to aggregations of bacteria, fungi and protozoans is also sometimes called nodulation (Dubovskiy *et al.*, 2016).

The encapsulation of the parasitoid wasp *Leptopilina bouleardi* egg by the larvae of *D. yakuba* and *D. melanogaster* was described by Russo *et al.*, (1996). It involves three key steps with coordinated actions of both plasmatocytes and lamellocytes. Oviposition by *Leptopilina* wasps occurs mainly in second instar larvae. After perforation of the cuticle and basement membrane by the ovipositor, the egg is usually deposited at the abdominal level by the wasp where it floats freely in the hemocoel. Within minutes, it will be recognized as "non-self" by the larva's immune system, which will trigger the various mechanisms leading to encapsulation. The breaching of the basement membrane and the subjacent epithelium releases factors that certainly play a role in the triggering of the response. The egg is recognized by circulating plasmatocytes which form the first basal cellular layer around the parasitoid egg chorion (Figure 10). This recognition also leads to the increase of the number of circulating hemocytes, mainly plasmatocytes and lamellocytes. These hemocytes may be liberated from an accelerated differentiation and bursting of the lymph gland (Lanot *et al.*, 2001) or mobilized from the sessile compartments and by transdifferentiation of plasmatocytes in lamellocytes (Markus *et al.*, 2009; Honti *et al.*, 2010; Anderl *et al.*, 2016). Indeed, a novel population of infection-induced cells, named lamelloblasts, derived from plasmatocytes, appears in the circulation few hours after parasitism. Lamelloblasts proliferate vigorously and develop into lamellocytes (Anderl *et al.*, 2016). Plasmatocytes transdifferentiation into lamellocyte-like cells was also detected directly on the wasp egg (Anderl *et al.*, 2016). Different signalling pathways are important for lamellocytes proliferation upon parasitism (Zettervall *et al.*, 2004). The

transdifferentiation of plasmatocytes into lamellocytes seems to implicate the Charlatan (Chl) protein, a transcription factor that interacts with CoREST and it is also induced following activation of the JAK / STAT pathway (Stofanko *et al.*, 2010). In accordance with this, genes involved in Toll, Jak-Stat and PO pathways are upregulated 12 hours after *L. bouleardi* wasp infection (Schlenke *et al.*, 2007). In addition, mutations in the Toll and Jak-Stat pathways affect hemocyte counts, lamellocyte differentiation, and the rate of encapsulation of parasitoid eggs (Sorrentino *et al.*, 2004). As the Toll pathway is implicated in both PO (Ligoxygakis *et al.*, 2002) and JAK/STAT (Lagueux *et al.*, 2000) pathways, it might be a central regulator of the response to the parasitism. *Edin* (elevated during infection) expression is induced after parasitism in the fat body and is required for the encapsulation response through mobilization of sessile hemocytes leading to an increase in the number of plasmatocytes (Vanha-Aho *et al.*, 2015). The use of *Drosophila* mutant for the Rac1 GTPase also showed the role of this protein and the involvement of the Jun N-terminal Kinase Basket (Bsk), as well as the stabilization of actin filaments in the recruitment of the population of sessile hemocytes (Williams *et al.*, 2006). This stabilization of actin is necessary for Rac1-induced hemocyte activation by lowering expression of *Cofilin* (encoded by the *twinstar* (*tsr*) gene). Elimination of Bsk by RNAi in hemocytes suppresses the Rac1-induced sessile hemocyte release and the induction of Rac 1 in lamellocytes. Rac1 may thus act on Bsk activity and stable actin formation for cellular immune activation, leading to the release of sessile hemocytes and increasing their number in circulation. The first visible event in encapsulation is the deposition of a dense layer of unknown material on the chorion of the egg six hours after infection. This layer could consist of extracellular matrix deposited by the first plasma cells. Indeed, in mutant *Drosophila* larvae without Laminin A, an essential component of the extracellular matrix, plasmatocytes are not able to adhere to eggs and there is no encapsulation (Howell *et al.*, 2012). The following plasmatocytes spread on the surface of this layer by emitting filopodia that come into contact, the cells forming tight junctions between them to surround the egg (Williams, 2009). The migration, spreading and formation of tight junctions between plasmatocytes involve the Rho GTPase Rac2 (Williams *et al.*, 2005) and certainly the Rho nucleotide exchange factor protein (RhoGEF) Zizimin-related (*Zir*) that interacts with Rac2 and CDC42 (*Zir* KO gives a phenotype very similar to the Rac2 mutant). Then, lamellocytes and some crystal cells form several layers of cells joined by septate junctions that surround the egg in a close capsule. In Rac2 mutants, plasmatocytes and lamellocytes attach to the egg but fail to spread on its surface to form a complete capsule due to the inability to create tight junctions, and the capsule does not melanise. The *raspberry* gene (coding for inosine monophosphate dehydrogenase (IMPDH)) is also important for the role of plasmatocytes, notably the formation of filopodia (Kari *et al.*, 2016).

After 24h the parasitoid egg is already embedded and at 48h the capsule is melanised (Figure 10). PPO3 is the most important PPO contributing to this melanisation, certainly released upon the lysis of lamellocytes proximal to the egg (Russo *et al.*, 1996; Dudzic *et al.*, 2015). Interestingly, PPO2 is also necessary to achieve the melanisation of the capsule (Dudzic *et al.*, 2015).

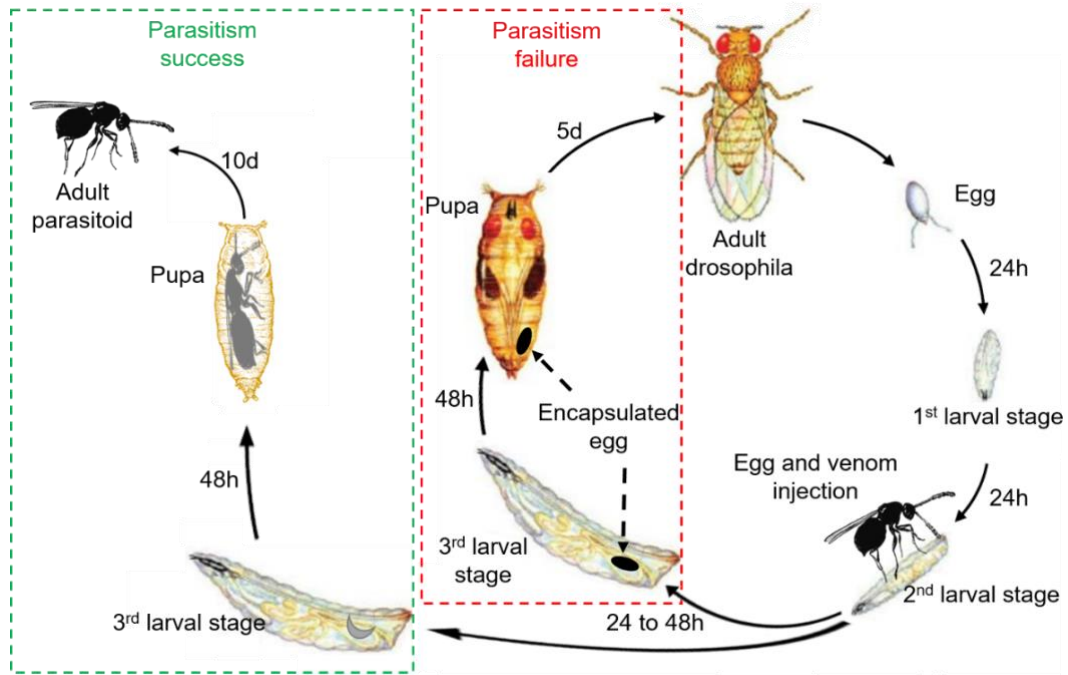


Figure 11: *D. melanogaster* and *L. boulardi* life cycle (adapted from Carolina Biological Supply Company)

B. Genetic interaction between *Drosophila melanogaster* and *Leptopilina boulardi*

In the previous chapter, the different immune weapons used by the fly in defence against the wasp egg have been described. When defence is successful (then the fly resistant) parasitoid egg is encapsulated and the fly can achieve its life cycle (Figure 11). However, in most cases the parasitoid is able to circumvent the host defences, the parasitoid larva hatches from the egg, consumes the host tissues and develops. After pupation, an adult parasitoid emerges from the pupal case instead of a fly (Figure 11).

The reproductive success of parasitoids is closely linked to their behavioural and physiological adaptations towards the host. These adaptations can be observed at different stages of the parasitism: location of the host's habitat, the host it-self, the acceptance the host and the ability to develop in the host. This ability to develop depends on the host's immune defence (resistance) and the parasite's ability to circumvent it (virulence), which are genetically determined and can be influenced by the environment. Interactions between *Drosophila* and parasitoid wasps are among the few examples in which the existence of intraspecific variations in parasitic success has been demonstrated.

As the parasitoid development is dependent of its host, the dynamics of host and parasitoid populations are interdependent: parasitoid populations evolve according to the host populations, which are impacted at a variable level according to the rate of parasite pressure. The first studies carried on a *L. boulardi* population from North Africa (Tunisia, Carton *et al.*, 1986) caught on opuntia fruits showed parasitic success on two sympatric species of fruit flies, *D. melanogaster* and *D. simulans*. However, these *L. boulardi* preferred to parasitize *D. melanogaster* than *D. simulans* in a non-choice situation (90% versus 60%), the difference being even more pronounced when the parasite has the choice between the two hosts (90% versus 50%). In addition, in about 10% of cases, *D. simulans* showed an effective immune response against the egg of *L. boulardi*. This result has been confirmed by other studies and extended to other *Drosophila* species (Carton & Kitano, 1981). Over the years, studies have demonstrated variation in both the virulence of *L. boulardi* and the resistance of *D. melanogaster* host. It was also shown that *L. boulardi* strains choice between larvae of *D. yakuba* and *D. melanogaster* hosts (probing and laying behaviour) positively correlated with the level of parasitism success on these hosts (Dubuffet *et al.*, 2006). This shows an ability of parasitoids to choose a preferential host in relation to the ability to succeed in this host.

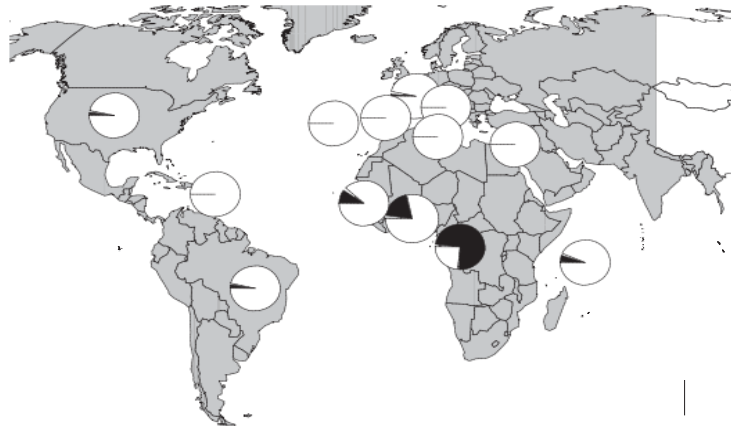


Figure 12: Geographical distribution of the encapsulation rate of *L. bouvardi* populations in sympatric populations of *D. melanogaster*. The encapsulation rate (%) is represented by the black part of the pie chart. (From (Dupas *et al.*, 2003))

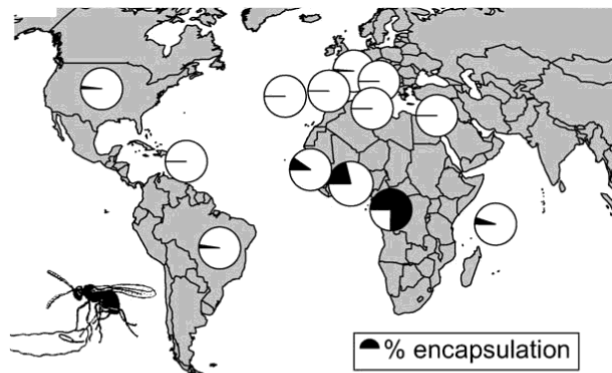


Figure 13: Distribution of *Drosophila* resistance phenotype. The resistance level is estimated from the encapsulation rate (%), represented by the black part of the pie chart. (from (Dupas *et al.*, 2003))

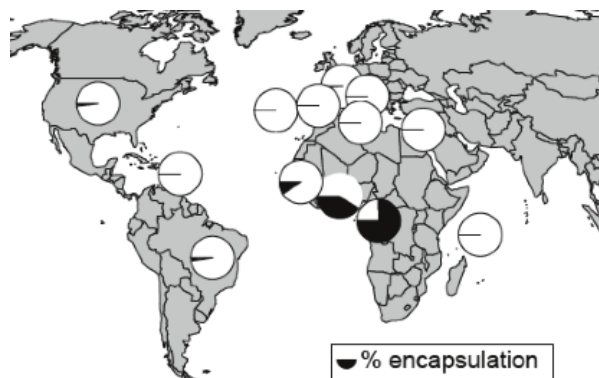


Figure 14: Geographic distribution of virulence in *L. bouvardi* populations. The virulence level is estimated from $(1 - \text{the encapsulation rate } (\%))$, represented by the black part of the pie chart. (from (Dupas *et al.*, 2003))

1. Resistance-virulence in *D. melanogaster*-*L. bouleardi* populations

L. bouleardi is a parasitoid species whose repartition is mainly Mediterranean and tropical. Different populations have been sampled throughout its geographical range and tested in encapsulation assays with sympatric populations of the main host species *D. melanogaster* (Dupas & Boscaro, 1999; Dubuffet *et al.*, 2009; Dupas *et al.*, 2013). Variation of encapsulation rates were evidenced with consistently low encapsulation levels in Europe, Asia and America but higher ones for some places in tropical Africa, particularly in Congo (Figure 12). The Congolese populations of *D. melanogaster* and *L. bouleardi* were at the base of Dr Carton's work on the interaction between these two species and more specifically of obtaining the *L. bouleardi* line G486 (ISy), and the *D. melanogaster* strains resistant/susceptible to this line. To understand the sympatric encapsulation rates, it is necessary to disentangle the effects associated with the parasitoid and with the host. This requires reference lines or strains whose choice is thus critical for those wishing to reveal the genetic variations in resistance and virulence. The term "reference line" should be restricted to the lines that allow the detection of such genetic variation in natural populations of the antagonistic species. The term "resistance" will refer to the encapsulation rate of a parasitoid reference line measured in a host population or line, and the term "virulence" as one minus the encapsulation rate of a parasitoid population or line by a host reference strain.

Using the reference lines, the geographic variation of *D. melanogaster* resistance to *L. bouleardi* was evaluated (Figure 13), as well as the geographic variation of *L. bouleardi* (resistant population of *D. melanogaster*) (Figure 14). Data showed that 80% of the variation in sympatric encapsulation rates was explained by the variation in *L. bouleardi* virulence (Dupas *et al.*, 2003). The addition of host resistance to the regression did not increase the variance explained since most parasitoid populations except in tropical Africa were highly virulent on *D. melanogaster* (Figure 14). It is interesting to note that a consistent resistance rate to the Congolese line of *L. bouleardi* was observed in almost all populations of *D. melanogaster* (except in Sweden and Sao Tome, Figure 13) although this type of *L. bouleardi* seems to be present only in tropical Africa. Interestingly, the lower virulence against *D. melanogaster* in this "Congo" line was mirrored by an increase in virulence against another host species, *D. yakuba* (only present in tropical areas) on which other *L. bouleardi* populations are largely unsuccessful (Dubuffet *et al.*, 2008).

It was further established that *L. bouleardi* virulence against *D. melanogaster* and *D. yakuba*, was genetically "determined" by two unrelated "loci" referred to as ISm ("immune suppression" of *D. melanogaster*) and ISy ("immune suppression of *D. yakuba*). However, the existence of a major locus of virulence and the characterization of various "virulence" factors that not necessarily localize in the same region of the genome are data that still need to be reconciled.

Since the outcome of the host-parasitoid interaction is always fatal to one of the antagonists, suggesting a very intense reciprocal selection, it thus represents a great scope for the study of coevolution (Godfray, 1994; Kraaijeveld *et al.*, 1998). Coevolution between pairs of antagonistic species is generally considered an endless "arms race" between attack and defence traits to counteract the adaptive responses of the other species. This continuous arm race between coevolving antagonists is referred to as the "Red Queen" hypothesis (Van Valen, 1973; Lively, 2010). Negative frequency-dependent reciprocal selection is a main force in the maintenance of genetic variation in natural populations (Judson, 1995), and possibly even in the maintenance of sexual reproduction (Jaenike, 1978; Lively, 2010). Negative frequency-dependence requires genetic variation for resistance in hosts and virulence in parasites, as well as costs for resistance and infectivity and/or a high genetic specificity of the host-parasite interaction (Hamilton *et al.*, 1990; Agrawal & Lively, 2002). Due to its importance in the dynamics of coevolving systems, evidences for genotype-specificity have been sought in various host-parasite interactions, from which many studies reported significant host genotype-by-parasite genotype interactions (Carius *et al.*, 2001; Lambrechts *et al.*, 2006; Salvaudon *et al.*, 2007). A significant genotype-by-genotype interaction between *D. melanogaster* and its parasitoid *L. boulandi* has been also described (Dubuffet *et al.*, 2007), while other host-parasitoid systems showed no clear evidence for such specificity (Kraaijeveld *et al.*, 2001a; Sandrock *et al.*, 2010).

Different not mutually exclusive scenarios were considered by Dupas *et al.*, (2009) to explain the *Drosophila-L. boulandi* pattern of interaction. i) the coevolutionary dynamics can advantage a host with a rare genotype: if parasitoids cannot parasitize all genotypes of a host species, those able to succeed on the most common represented host genotype will be selected, which will benefit the rare genotype. A maintained high level of polymorphism of virulence and resistance would thus be expected. ii) some virulence and resistance strategies might be effective against all genotypes of the antagonist species, meaning that adaptation of both the host and the parasite will be required. iii) the parasitoid may not adapt but change host species: the hosts that provides the highest selective value to the parasitoid would be parasitized more, for instance host species that do not have effective defences. If the new host adapts and becomes resistant, the parasitoid will change host again.

To test these scenarios, phylogenetic comparative methods were applied to virulence traits as 1-the encapsulation rate of 13 parasitoid strains of *Leptopilina* spp. in interaction with five host strains of species of the melanogaster subgroup of *Drosophila* (Dupas *et al.*, 2013). Strain-strain reciprocal specificity was observed in several host-parasitoid pairs of species. Results interpreted *Leptopilina* virulence as a threshold trait above an underlying continuity, so called liability. When

liability is far from the threshold, virulence is fixed. When liability is close to the threshold, virulence varies specifically, and reciprocal adaptations can take place. The data show that fixed virulence can be an ancestral trait for an entire group, thus preventing coevolution with host species. When virulence varies, variation mechanisms match specifically with host species or strains. The evolution of non-specific interactions would therefore be linked to phylogenetic constraints on coevolution that allow or not host-specific variation to be expressed. This, in combination with geographic variation in community composition, host choice mechanisms and mating systems linked to the host, may have favoured speciation and diversification in some clade interactions only. This hypothesis that coevolution stops in some clades and restarts in others led to a scenario of escape and radiation coevolution (Ehrlich & Raven, 1964) in this host–parasitoid system.

In the interaction between *D. melanogaster* and *L. boulardi*, coevolution does not seem to predominantly operate. Indeed, the vast majority of *L. boulardi* populations are virulent against all host populations. An exception would be tropical Africa since there is variation in resistance against sympatric parasitoids themselves variable in virulence.

2. Fly resistance

Wild *Drosophila* populations are a mixture of sensitive and resistant flies. Experimental evolution studies have been done to select resistant lines artificially and evaluate how quickly the *Drosophila* could adapt to an environment containing parasitoids (Kraaijeveld & Godfray, 1997; Kraaijeveld *et al.*, 2001b; Wertheim *et al.*, 2011; Jalvingh *et al.*, 2014). In response to an increased parasitoid pressure, the fly population evolves toward higher levels of resistance. A microarray survey of control and selected lines showed a complex transcriptomic response with a large number of genes differentially expressed (Wertheim *et al.*, 2011). Some of these genes were associated with immunity. Genome-wide gene expression study have also been done on different *Drosophila* species in order to compare the response to parasitism at the expression level (Salazar-Jaramillo *et al.*, 2017). *D. melanogaster* and *D. simulans* have the closest expression profiles and *D. sechellia* have to most different one (Salazar-Jaramillo *et al.*, 2017) which is understandable as it was not resistant to the parasitoids used (Salazar-Jaramillo *et al.*, 2014). *D. yakuba* presents a different set of genes differentially expressed compare to *D. melanogaster* and *D. simulans* (Salazar-Jaramillo *et al.*, 2017). One of the drawbacks of these studies is the use of different lines of parasitoids and flies, sometimes poorly characterized for their virulence and resistance or having an intermediate phenotype for these traits. It should be noted, however, that several genes expressed in response

to parasitism in *D. melanogaster* and involved in immunity were localized on chromosome 2R as the major resistance we are studying.

From a physiological point of view, the factor most often proposed to influence/explain the host resistance to parasitoids is the number of hemocytes. This number was increased for instance in *Drosophila* lines selected for increased resistance to the braconid wasp *A. tabida* (Kraaijeveld *et al.*, 2001b). The number of hemocytes was also shown to affect the resistance potential of non-selected *Drosophila* hosts against *A. tabida* (Eslin & Prévost, 1998).

3. Parasitoid virulence factors

To counteract the *Drosophila* defence mechanisms, parasitoid wasp species have developed different strategies. Passive strategies may include masking/mimicry of surface features that prevents recognition of the egg or larva by the host's immune system or laying the egg in host tissues inaccessible for immune cells (Vass & Nappi, 2000; Prévost *et al.*, 2005). Some *Asobara* species use this immunoevasive strategy by hiding their egg into the host tissues (Prévost *et al.*, 2005). Other species use active strategies that involve the specific modification and/or destruction of some of the immune components such as inhibition/reduction of melanization and/or alteration of the cellular response, by affecting the hemocyte-producing organ (hematopoietic lymph gland) and/or directly the circulating hemocytes. The *Leptopilina* genus (currently 32 known species, classified into three main groups: *Longipes*, *Heterotoma* and the *Boulardi*) used active strategies to suppress the host immune response based on the virulence factors present in the venom they inject along with the egg (Carton *et al.*, 1986; Carton & Nappi, 1997). Comparative studies from different animals indicate that venoms are usually complex mixtures of proteins and non-proteinaceous compounds whose production can be metabolically costly (Casewell *et al.*, 2013). In *Leptopilina* species venom is composed of soluble protein and contains small vesicles (from 150-300 µm) of specific shapes similar to virus and then were named Virus-like particles (VLPs) (Rizki & Rizki, 1990; Russo *et al.*, 1996). These vesicles have been the subject of numerous studies, they are mainly composed of proteins from the wasp and presence of nucleic acid has not been found to date (Gueguen *et al.*, 2011; Gatti *et al.*, 2012; Heavner *et al.*, 2017). Moreover, these vesicles are assembled by a still unknown mechanism in the lumen of female venom gland, a very different mechanism than this of virus formation (Morales *et al.*, 2005; Gueguen *et al.*, 2011). Thus, they are not viral particles and the term venosomes may be more appropriated. The mature venosomes are stored in the large venom reservoir linked to the venom gland (Dupas *et al.*, 1996). In *L. heterotoma*, it has been shown that purified reservoir venosomes induce the destruction of lamellocytes *in vitro*

and *in vivo* by modifying the cytoskeleton of these cells before inducing lysis (Rizki & Rizki, 1984, 1990, 1994). Similarly, when *L. boulandi* venom from a virulent ISm strain is injected, the lamellocytes shape change from discoidal to bipolar but no apparent lysis occurred. The shape modification is likely sufficient to prevent their ability to adhere to the egg and therefore to form capsules (Russo *et al.*, 2001; Labrosse *et al.*, 2003; Dubuffet *et al.*, 2008; Poirié *et al.*, 2009; Colinet *et al.*, 2010). Furthermore, microinjection of venom extracted from the reservoir of ISm strain of *L. boulandi* has been shown to be sufficient to protect eggs from the ISy strain by blocking the encapsulation reaction (Dubuffet *et al.*, 2009). The injection of the ISy venom has no effect on the host lamellocytes (Colinet *et al.*, 2009). Interestingly, electron microscopy studies of the reservoir of the ISy showed much less venosomes than in the ISm one (Dupas *et al.*, 1996; Wan *et al.*, submitted).

4. Proteomic of the *L. boulandi* ISm and ISy venom

A combined transcriptomic study of the venom gland coupled with a proteomic study of the venom of the ISm and ISy strain identified 65 and 49 major venom proteins, respectively. A significant number of venom proteins were unique to either ISm or ISy, and only 50% of the shared venom proteins were abundant in both strains (Colinet *et al.*, 2013).

Some proteins from the venom have also been purified and tested to study their role in parasitism. For example, LbSPNy a serine protease inhibitor of the serpin superfamily was previously characterized as a main venom virulence factor in ISy females' venom (Colinet *et al.*, 2009). LbSPNy inhibits the activation of the phenoloxidase cascade in its *D. yakuba* host (Dubuffet *et al.*, 2008; Colinet *et al.*, 2013). *L. boulandi* ISm encodes also a serpin (LbSPNm) similar in sequence to LbSPNy. LbSPNm is abundant in the ISm venom, however, amino acid sequence differences observed at key residues in the reactive center loop suggested a different protease targeting and thus a distinct biological activity (Colinet *et al.*, 2013).

A purified RhoGAP domain-containing protein, LbGAP, secreted in the ISm venom showed a protective effect against the encapsulation of the ISy egg (Labrosse *et al.*, 2005a,b). LbGAP was further immunolocalized as many spots in *D. melanogaster* lamellocytes with a modified shape after parasitism or venom microinjection (Colinet *et al.*, 2007). It is suggested that LbGAP, induces changes in the lamellocytes morphology by interacting with, and inactivating the two Rho GTPases, Rac1 and Rac2 (Colinet *et al.*, 2007), both essential for successful encapsulation of *Leptopilina* eggs (Williams *et al.*, 2005, 2006).

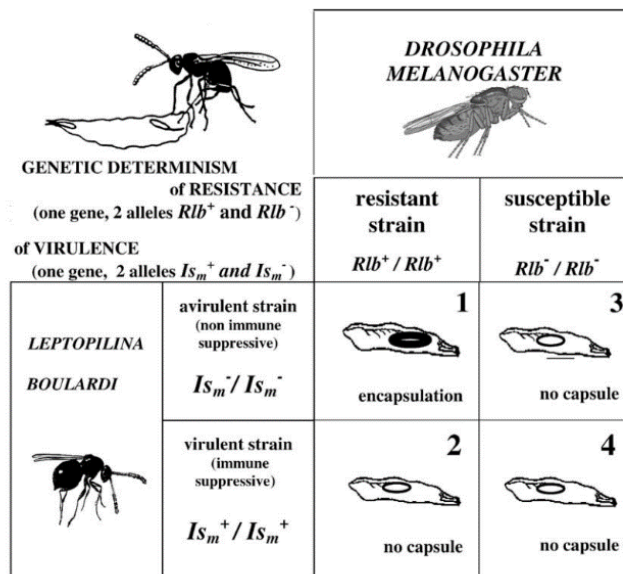


Figure 15: Matching interactions in the *D. melanogaster* – *L. bouvardi* reference system (Dupas, Carton, and Poirié 2003). The host alleles Rlb^+ and Rlb^- correspond to the resistant and susceptible phenotypes, respectively, and the alleles Ism^+ and Ism^- (Isy) are responsible for virulence and avirulence of the parasitoid. When host has a resistance allele and parasitoid an avirulence allele (case 1), the parasitoid egg is encapsulated. In the three other situations (cases 2, 3 and 4), the parasitoid develops successfully.

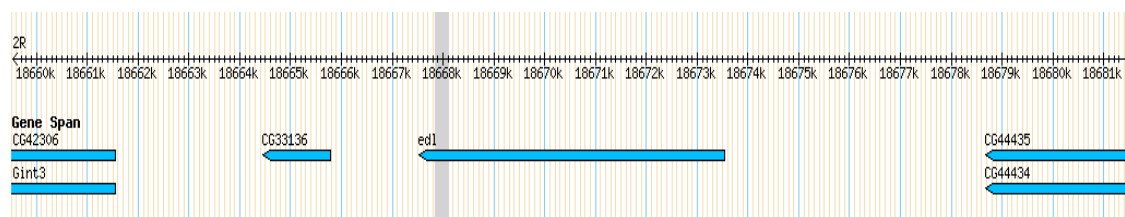


Figure 16: Region of chromosome 2R showing the position and distance between CG33136 and *edl*. 1Kb between blue lines (Flybase)

LbGAPy was not detected in ISy venom confirming previous results showing that the quantitative difference in LbGAP explains the intraspecific variation of virulence between the ISm and ISy strains. In each of the ISm and ISy venom gland transcriptomic libraries, 8 unisquences coding for other RhoGAP domain-containing proteins and sharing 40-60% of similarity with LbGAP were found. Most of these supplementary RhoGAPs were predicted to be secreted and accordingly identified in venom by proteomics. However, all contained mutations in the arginine key residue (R74) required for the catalytic GAP activity and involved in the LbGAP interaction with Rac GTPases (Colinet *et al.*, 2007). In addition, some of these RhoGAPs had one or more mutations at sites involved in binding to Rac GTPases (Colinet *et al.*, 2007). The role played by these mutated RhoGAP domain-containing proteins in the parasitism remains to be explored. These suggest that the venom evolutionary processes between the strains proceed by the same molecular mechanisms largely described in other venomous animals, i.e. the recruitment of venom proteins from cellular proteins with the acquisition of novel functions and the role of duplications in the emergence of multigenic families of virulence factors (Poirié *et al.*, 2014)

5. Origin of the *D. melanogaster* and *L. bouleardi* strains

From studies of populations from various geographical areas, substantial variation for both resistance and virulence was shown in this model. Parasitoid lines that are used in the laboratory were obtained from a population of Tunisia (Nasrallah) for G431 (named ISm) and from a population of Brazzaville (Congo) for G486 (named ISy). The parasitoid ISm line represents the pattern observed in most Mediterranean places: it is highly virulent in *D. melanogaster*, whichever the host strain, but is completely unable to escape encapsulation in any *D. yakuba* strain (Dupas & Boscaro, 1999). Its success is thus species dependant. By contrast, the parasitoid ISy can succeed in both *D. melanogaster* and *D. yakuba* but its success depends on the genotype of the host (susceptible vs. resistant; (Dupas *et al.*, 2003; Dubuffet *et al.*, 2007)) and is thus host-genotype specific.

Naturally occurring resistance variation between populations has been well described in *D. melanogaster* against *L. bouleardi* and *A. tabida* (Kraaijeveld & Van Alphen, 1995; Dupas *et al.*, 2003) and in *D. yakuba* against *L. bouleardi* (Dubuffet *et al.*, 2007). In Congo, encapsulation rates of the reference line ISy by *D. melanogaster* ranged from 12% to 90% (Carton & Boulétreau, 1985; Carton *et al.*, 1992). To analyse the genetics of resistance to parasitoid wasps in *D. melanogaster*, *Drosophila* isofemale lines with similar encapsulation rates were mixed to form a resistant and a susceptible population.

At the end, the selected inbred resistant (R) and susceptible (S) strains obtained thus originate from the same population as the ISy parasitoid (Brazzaville, Congo) (Carton *et al.*, 1992). Resistance to *A. tabida* was analysed using the same resistant strain that also proved to be resistant to *A. tabida*, and Canton S as a susceptible strain (Benassi *et al.*, 1998).

Variation in resistance was mostly expected to be multigenic due to the high number of genes involved in the encapsulation process. Yet, in both *D. melanogaster* and *D. yakuba*, resistance to parasitoids was explained by a single diallelic locus, with the resistant phenotype showing complete dominance over the susceptible one. In *D. melanogaster*, the loci were named *Rlb* (resistance to *L. boulandi*, Figure 15) and *Rat* (resistance to *A. tabida*; Benassi *et al.*, 1998; Carton *et al.*, 1992), and *Rlby* in *D. yakuba* (resistance to *L. boulandi*; Dubuffet *et al.*, 2007). The use of isofemale lines might have favoured the recovery of simple genetic systems, but an independent study also concluded on a simple genetic basis of *D. melanogaster* resistance to *A. tabida*. Using recombination experiments, *Rlb* and *Rat* were shown to be located on chromosome 2, 35 cM apart, and thus different genes (Poirié *et al.*, 2000).

C. The *Rlb* gene

The *D. melanogaster* resistant and susceptible strains described above (created from a *D. melanogaster* population of Brazzaville (Carton *et al.*, 1992)) were used to produce resistant and susceptible strains with a similar genetic background using chromosome transfer. For this, the chromosome 2 of the resistant strain, shown to be involved in the resistant phenotype, was transferred into the susceptible strain background. This was followed by several backcrosses to get as close as possible to the susceptible background on chromosome 2. The created strains (1088 or SY and 1089 or YR), respectively resistant and susceptible to *L. boulandi* ISy parasitoid differed only by a region on chromosome 2R that contains the “resistance gene”.

The team started working on locating the *Rlb* gene using these strains in collaboration with Dr. Carton. *Rlb* was first localised on chromosome 2R at a genetic location of ca. 2-86.7 using markers and genetic experiments (Poirié *et al.*, 2000). Its localization was then further restricted to a 300 kb region, in 55E2-E6; F3, using different strains bearing deletions (Hita *et al.*, 1999). Indeed, despite dominance of the *Rlb*⁺ allele, F1 larvae bearing a deletion in front of the *Rlb*-containing region exhibit a decreased encapsulation rate, probably due to transvection effects. The *Rlb*-containing region was then restricted to 100 kb by specifying the molecular limits of the deletions using *in situ* hybridization on chromosomes and Southern blotting experiments with cosmid probes. Finally, male recombination experiments were performed to tentatively localize *Rlb* to the

right or to the left of a P-element inserted in this region (the BL-10633 stock used was obtained in a genomic insertion screen experiment; (Török *et al.*, 1993)). Results showed that *Rlb* was very close to the P-element used for the experiments, leading to characterization of two possible candidates, the *mae/edl* gene and CG33136 of unknown function (Hita *et al.*, 1999) (Figure 16).

CG33136 codes for a putative transmembrane protein with an unknown function. It has homologues mainly in *Drosophila* species. In *D. melanogaster* its expression starts at the L3 stage and peaks in the adult where it is highly expressed in the testis and at a low level in the fat body (flybase.org/reports/FBgn0053136).

1. Discovery of *edl* as the Rbl gene

From male recombination experiments, *mae/edl* hereafter referred to as *edl* was the more likely candidate for Rlb. The insertion of the P{lacW} element in the 10633 insert line was mapped into the 5' UTR of the *edl* transcription unit, 800 bp upstream of the initiation codon (Baker *et al.*, 2001). Besides, the *edl* protein contains an ETS-specific pointed domain (SAM domain) and acts as a signalling intermediate that directly links the RTK/Ras/MAPK signalling pathway to its downstream transcription factor targets (Baker *et al.*, 2001).

Edl mediates MAPK phosphorylation of the ETS transcription factors Yan/Aop and pointed P2. Aop is involved in cell choice between cell proliferation and differentiation following RTK signalling (Rogge *et al.*, 1995). Besides, ectopic expression of a constitutively active allele of the *edl* target *aop* stimulate both proliferation of hemocytes and formation of lamellocytes in *Drosophila* larvae (Zettervall *et al.*, 2004). Differences between *edl* resistant and susceptible alleles, their expression or their regulation could thus possibly lead to differences in the timing of hemocytes proliferation/differentiation in response to parasitism.

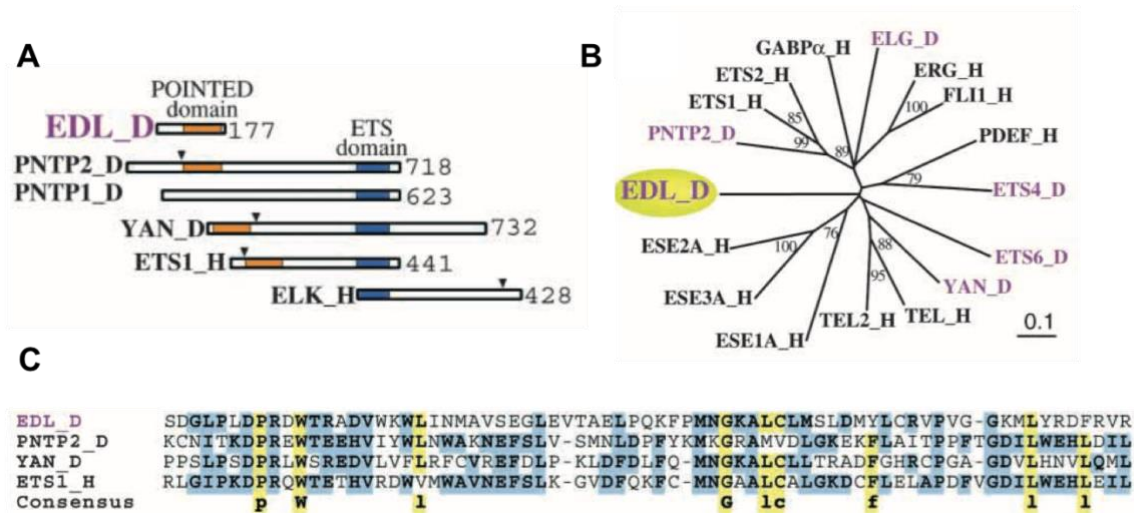


Figure 17: The structure and comparison of edl with other Ets-proteins. A: Line diagram showing the structures of edl and examples of other Ets proteins (D, *Drosophila*; H, Human). The ETS domain is shown in blue, Pointed domain in orange. Essential MAPK phosphorylation sites are shown by triangles. Numbers on the right indicate the amino acid length B: a phylogenetic tree of the PNT domain. Sequences of all Ets proteins containing PNT domain from *Drosophila* (five sequences) and human (eleven sequences) are aligned with edl using Clustal W. Bootstrap value more than forty (based on 100 replicates) are shown. Notice that all of the *Drosophila* members belong to different branches. C: An alignment of the Pointed domain. Amino acids that are conserved in all or most of the proteins (15-16 out of 17) analysed in Human are shaded yellow, and are shown with capital or lower-case letters, respectively. Other amino acids that match those of edl are shaded blue. (from Yamada, 2003)

2. Edl structure and functions

a) Structure

Edl was first described in 2001 (Baker *et al.*, 2001) as a regulator of the function of the *Drosophila* Ets transcription factors family. *Edl* codes for the 177 amino acids protein “ETS-domain lacking” (EDL), a protein with an Ets-specific Pointed domain (PNT) but not an ETS DNA-binding domain (Figure 17). This highly conserved PNT domain is found within a subset of the Ets transcription factors, including mammalian Ets-1, Ets-2, Erg, Fli-1, GABPalpha, and Tel, as well as *Drosophila* Pnt P2 (ETS-like protein pointed P2) and aop (anterior open, yan, Ets DNA-binding protein, pokkuri)(Figure 17).

The PNT domain is structurally related to the larger group of Sterile Alpha Motif (SAM) domains. SAM domains are known to exhibit diverse protein-protein interaction modes. They can form multiple self-association architectures and also bind to various non-SAM domain-containing proteins. SAM domains are also found in Polycomb-group proteins and in MAPK kinase kinases that are components of the MAPK cascade (Ponting, 1995). The SAM domain is also implicated in lipids and RNA binding (Barrera *et al.*, 2003; Denay *et al.*, 2017).

b) Fonctions

In *Drosophila*, work from multiple laboratories has defined MAPK, aop, pnt P2 and edl as a critical transcriptional effector circuit in the Receptor Tyrosine Kinase (RTK)/Ras/mitogen-activated protein kinase (MAPK) pathway. These four core components, are interconnected *via* multiple levels of transcriptional regulation, protein-protein interactions, and post-translational modifications. In-depth investigation of a small number of direct transcriptional targets identified from genetic studies has led to the suggestion that aop functions as a short-range passive repressor that competes with the ETS family activator Pointed (PNTs) for access to GGA(A/T) ETS consensus-binding motifs (Klämbt, 1993; Scholz *et al.*, 1993; O’Neill *et al.*, 1994). Genome-wide chromatin occupancy profile showed also that aop binds at developmentally important genes clusters packing multiple kilobases of DNA, a pattern that is conserved between *D. melanogaster* and *D. virilism*. These aop-bound regions forming large repressive domains. Competition between aop and pnt is also regulated by MAPK activation, which attenuates aop-mediated repression while stimulating pnt-mediated activation (Gabay *et al.*, 1996). Edl and aop interact *via* their SAM domain and this interaction affects the phosphorylation of aop (Baker *et al.*, 2001). Edl has also the capacity to bind to Pnt P2, but not Pnt P1 (Baker *et al.*, 2001) and inhibits the activation of the transcription by this protein (Yamada, 2003).

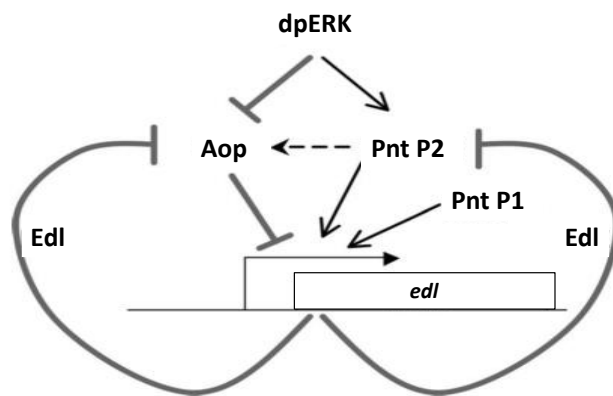


Figure 18: Feedback loop regulation of *edl* transcription by *yan/aop* and pointed proteins. EGF signalling leads to *edl/mae*-mediated phosphorylation and down-regulation of *aop*, resulting in activation of targets by *pnt P2*. As *edl* itself is a target of *pnt P2* and *aop* and negatively regulates their function, activation of *pnt P2* would result in induction of *edl* and subsequent down-regulation of the pathway by inhibition of *pnt P2* to limit the duration of the signal (adapted from Vivekanand, Tootle, and Rebay 2004)

If *edl* can regulate the transcriptional roles by binding to *aop* and *pnt* P2, both transcription factors can in turn regulate the expression of *edl* (Figure 18). *Aop* is an inhibitor of *edl* expression and Pointed proteins (*pnt* P1 and *pnt* P2) are enhancers of *edl* expression (Vivekanand *et al.*, 2004).

One of the most complete description of the role of *edl* in this regulatory switch has been made during the eye development (Yamada, 2003; Vivekanand *et al.*, 2004). In the absence of MAPK activation, un-phosphorylated *aop* polymerises and inhibits the transcription of target genes and *pnt* can bind to the ETS binding sites of those genes. Upon activation of the MAPK pathway, the basal level of *edl* binding to *aop* allows for the phosphorylation of *aop* by ERKA (Extracellular-Regulated Kinase A). *Aop* phosphorylation enhances its interaction with the nuclear exportin Crm1, thereby allowing Crm1 to export monomeric *aop* from the nucleus to the cytoplasm (Song *et al.*, 2005). The *aop* decrease allows for the increase in *edl* expression and then of its protein that interacts with *aop* inhibiting the formation of *aop* polymers. Indeed, the *edl*-SAM domain associates specifically with the surface on the *aop*-SAM domain required for *aop*-SAM polymerization (Qiao *et al.*, 2004). In terms of binding affinity, the *edl*-SAM/*aop*-SAM domains interaction is approximately 1000 times stronger than *aop*-SAM self-association. Mutations on *edl* that specifically disrupt its SAM domain-dependent interactions with *aop* disable the derepression function of *edl in vivo*. Thus *edl*-*aop* interaction serves to regulate *aop* nuclear export by both inhibiting export of the *aop* monomer in the absence of signalling and facilitating *aop* depolymerization (Song *et al.*, 2005).

At the same time, ERKA activate *pnt* P2 by phosphorylation which enhances the activation of the transcription of its target genes including *edl*. *edl* expression increases and since there is less of *aop* and more of activated *pnt*, *edl* binds to *pnt* blocking the ERKA binding and consequently inhibits *pnt* P2 phosphorylation. This will produce a negative feed-back loop inducing a decrease of transcriptional activity. This forward and feed-back loops seems a general mechanism implicated in cell proliferation and differentiation in a variety of tissues (Dubois *et al.*, 2016; Schwarz *et al.*, 2018).

3. EGF pathway and timing of lamellocytes liberation after parasitism

Many receptor tyrosine kinases (RTKs), including the epidermal growth factor receptor (EGFR) and fibroblast growth factor receptor (FGFR), signal through the Ras/ MAPK pathway (Nishida & Gotoh, 1993; Lusk *et al.*, 2017). These receptors have important developmental functions and are also misregulated in a variety of cancers.

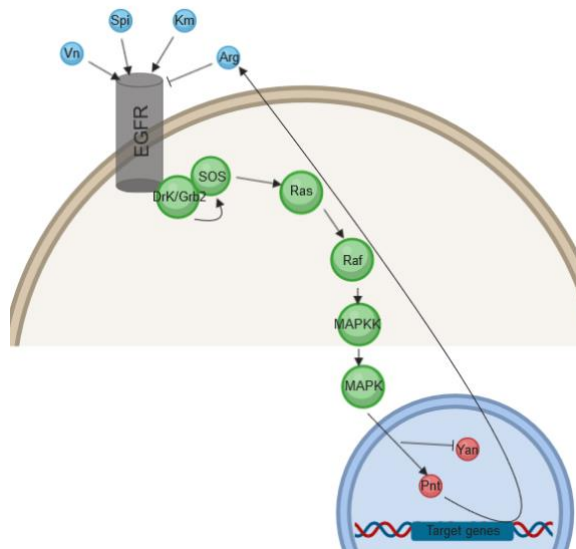


Figure 19: Diagrammatic representation of the EGF signaling pathway. EGFR ligands activated (vn, spi, krn) the pathway. Drk binds through its SH2 domain to the phosphorylated tyrosines on EGFR and in turn binds SOS which triggers activation of Ras. Ras activates Raf which phosphorylates MAPKK which phosphorylates MAPK which can phosphorylate pnt or yan which in turn lead to transcriptional responses, including the transcription of Arg which will down regulate the EGF pathway (adapted from Lusk *et al.*, 2017).

The *Drosophila* homolog of EGFR is a single-pass transmembrane receptor tyrosine kinase (RTK) that transduces signal from a series of different ligands, which can interact with the receptor through different molecular mechanisms (Klein *et al.*, 2008) (Figure 19). These ligands include gurken (grk), spitz (spi), keren (krn) that show homology to TGF- α and vein (*vn*), homologous to neuregulin (Paul *et al.*, 2013; Steinhauer *et al.*, 2013; Austin *et al.*, 2014). In addition to these four extracellular ligands, Argos (arg) serves as a ligand antagonist by inhibiting EGF signal transduction (Klein *et al.*, 2008).

Upon ligand binding, EGFR forms a dimer and trans-phosphorylates. DRK (Downstream of Receptor Kinase; the *Drosophila* homolog of mammalian *Grb2*) binds to the phosphorylated EGFR and is recruited at the plasma membrane. In turn, DRK binds Son of Sevenless (SOS), the guanine exchange factor, which triggers activation of RAS by promoting GTP binding. RAS activates RAF which phosphorylates MAPK kinases which phosphorylate rolled, a MAPK. Among many targets, activated rolled phosphorylates the transcription factors such as pnt and aop which in turn lead to transcriptional responses. In essence, the two transcription factors are in opposition with Pointed as the activator of EGF pathway target genes and aop as a repressor with edl balancing their function as seen before.

The first study that suggested a role of the MAPK pathway in *Drosophila* cellular immune response was done by Zettervall *et al.* (2004). To study the molecular mechanisms of this response, these authors have overexpressed different genes in the hemocytes, using the GAL4-upstream activating sequence system and a hemocyte-specific *Hemese-Gal4* driver. They showed that surexpression of receptor tyrosine kinases, such as Egfr, Pvr, and Alk caused a drastic increase in the number of circulating hemocytes. An increase was also observed with the downstream signaling components Ras85D and pointed, supporting the notion that the Ras–MAPK pathway regulates hemocyte numbers. Pvr and Alk, also increased the lamellocyte production but not Egfr. The surexpression of an aop mutant protein (Aop^{ACT}), with all possible MAPK phosphorylation sites mutated to acts as a constitutive repressor of Pointed, gives a massive lamellocyte response and a strong stimulation of hemocyte production instead of the antiproliferative effect expected. To explain this the authors suggested that different MAPKs may regulate a delicate balance between proliferation and lamellocyte activation by targeting different phosphorylation sites on aop and pnt.

Later, *Egfr* gene function in hemocytes in wild-type flies was suggested by Sinenko *et al.* (2012). They showed that just after wasp parasitism an increase of ROS levels occurred in the PSC cells of the lymph gland, leading to the secretion of Spi, one ligand of the EGFR signaling pathway.

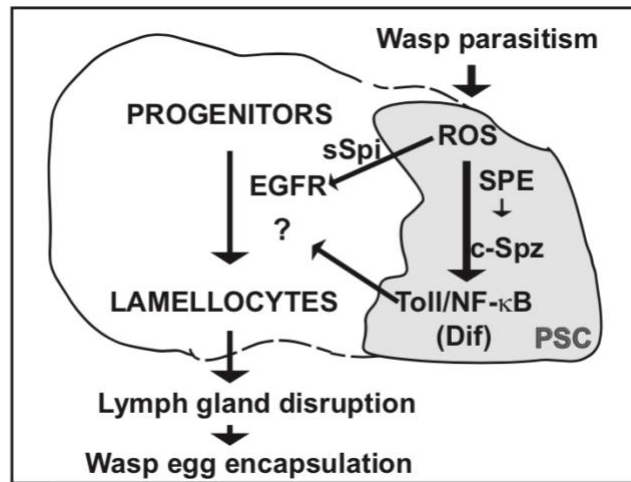


Figure 20: Proposed gene regulatory network that controls lymph gland rupture upon wasp parasitism. The PSC is drawn in grey. Wasp parasitism increases ROS in PSC cells that activate Toll/NF- κ B and Spitz secretion (sSpi). Toll/ NF- κ B activation in PSC cells requires SPE in the same cells for Spätzle processing (c-Spz). sSpi non cell-autonomously activates the EGFR pathway in lymph gland progenitors. Both EGFR and Toll/NF- κ B activation are required for lymph gland lamellocyte differentiation, lymph gland disruption and wasp egg encapsulation. From (Louradour et al., 2017)

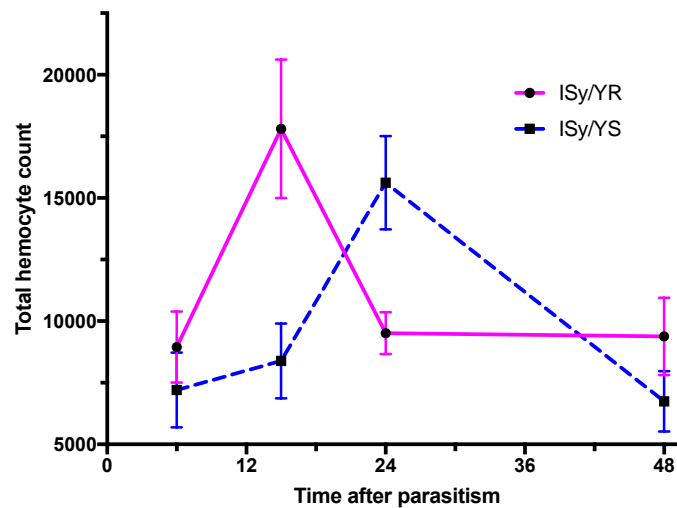


Figure 21: Total numbers of hemocytes in single parasitized larvae, 6 h, 15 h, 24 h and 48 h after parasitism by the avirulent ISy strain. YR, resistant strain; YS, susceptible strain. Results are means of number of hemocytes per mm^3 counted from 10 larvae (mean \pm S.D.). (from Russo et al. (2001)).

The secretion of Spi into the hemolymph activates EGFR/Erk signaling in circulating hemocytes and triggers their differentiation into lamellocytes (Sinenko *et al.*, 2012).

However, a direct role of EGFR activation in lymph gland progenitors was not rule out. More recently this was demonstrated that the co-activation of EGFR signaling and Toll/NF- κ B by ROS levels in the PSC controls the lymph gland hematopoiesis under parasitism (Louradour *et al.*, 2017). Toll/NF- κ B signaling in PSC cells, while not required for lamellocyte differentiation, controls the timing of wasp-induced lymph gland dispersal and the release of lymph gland lamellocytes into circulation. The EGFR pathway activation in lymph gland progenitors is also required for on time lymph gland dispersal. The phosphorylation of ERK, undetectable in the lymph gland under normal conditions, increased in lymph gland progenitors 6h post-parasitism, indicating an EGFR activation. Furthermore, decreasing Spi expression in the PSC or down-regulating the EGFR pathway by expressing a dominant-negative form of the EGFR receptor in lymph gland progenitor cells, delays the lymph gland dispersal post-parasitism.

From all these data, the hypothesis is that *edl* and its interactors *aop* and *pnt* are implicated in the EGFR pathway activation in *D. melanogaster* hemocyte progenitors is required for their multiplication, differentiation and the on-time lymph gland dispersal (Figure 20).

Interestingly one of the phenotypic difference described between the Resistant (YR) and Susceptible (YS) fly strains is a different timing for the hemocyte increase in the hemolymph after parasitism by the Isy avirulent parasitoids (Russo *et al.*, 2001) (Figure 21): the number of hemocytes in the R strain peaks at 15 h post-infestation and while it is 12h later for the YS strain. It is possible that this earlier “proliferation response” in the YR strain plays an important role for the encapsulation success.

At least, it is of note that TEL (translocation–Ets–leukemia or ETV6), the human *aop* ortholog, is required specifically for hematopoiesis within the bone marrow and its frequently found rearranged by chromosomal translocation in different human leukemias and cancers (Wang *et al.*, 1998; Seth & Watson, 2005; Telford *et al.*, 2016) In zebrafish, it has been shown that the TEL/ETV6 ortholog as several distinct roles for in embryonic hematopoiesis: *etv6* knockdown resulted in reduced levels of progenitor cells, erythrocytes and macrophages (Rasighaemi *et al.*, 2015).

D. Objectives

The *Drosophila/Leptopilina* is one of the best models to study intraspecific variation of both host resistance and parasite virulence, with well-characterized strains available in the laboratory. *Drosophila* resistance is only observed against *L. boulardi* wasps of the ISy type (originating from Brazzaville, Congo) and it naturally occurs from high to medium frequencies in most natural populations. While *edl* was the best *Rlb* candidate, its role in the fruit fly resistance remained to be demonstrated and the differences between the *Rbl+* and *Rbl-* alleles to be elucidated.

As an extension of these previous works, the aims of my PhD were:

- i) To explain the phenotypic differences observed between the YS and YR strains i.e. the difference in encapsulation of the parasitoid egg and an earlier peak of hemocytes in circulation in the resistant strain compare to the susceptible one (Russo et al, 2001). I have further analyzed the timing of the hemocyte production difference by creating a susceptible and a resistant reporter lines to follow the lamellocytes as they are produced in case of parasitism using flow cytometry. And to understand the origin of the increase of the circulating hemocytes and the lamellocytes, I looked at the lymph gland bursting by dissection at different time points after parasitism in the YS and YR lines.
- ii) To demonstrate the role played by *edl* in the resistance to *L. boulardi* and investigate the pathway in which it is involved by using genetic experiments including different UAS-Gal4 and RNAi lines. I have also started a bioinformatic analysis of the different potential interactants in order to understand the role of *edl* in the resistance reaction of the *Drosophila* to the parasitoid.

II. Materials and methods

Table 1: List of the *Drosophila* strains used (name, genotype, function, identification and use during the PhD)

Line	Genotype	Phenotype	Source
Nasrallah	WT, <i>edl</i> susceptible allele	Susceptible to <i>isy</i>	ESIM
Sefra	WT, <i>edl</i> susceptible allele	Mix population of resistant and susceptible <i>Drosophila</i>	ESIM
YR	WT, <i>edl</i> resistant allele	Resistant to <i>isy</i>	ESIM
YS	WT, <i>edl</i> susceptible allele	Susceptible to <i>isy</i>	ESIM
UAS - <i>edl</i> F	Insertion of the UAS- <i>edl</i> susceptible allele in W118	<i>edl</i> susceptible allele coding sequence under the control of UAS promoter	Dr Yamada
UAS - <i>edl</i> X	Insertion of the UAS- <i>edl</i> susceptible allele in W118	<i>edl</i> susceptible allele coding sequence under the control of UAS promoter	Dr Yamada
UAS - Mae 34 how	Insertion of the UAS- <i>edl</i> resistant allele in W118	<i>edl</i> resistant allele coding sequence under the control of UAS promoter	ESIM
UAS - Mae 8 how	Insertion of the UAS- <i>edl</i> resistant allele in W118	<i>edl</i> resistant allele coding sequence under the control of UAS promoter	ESIM
pHsp		Heat-stock promoter, expression of Gal4 is activated after a heat shock of 1h at 37°C	ESIM
pHml	w ¹¹¹⁸ , P{Hml-GAL4.G}5-6	Expression of Gal4 in the hemocytes	BL6395
pCg	w ¹¹¹⁸ ; P{Cg-GAL4.A}2	Expression of Gal4 in the hemocytes, the fatbody and the lymph gland	BL7011
pCol85	pCol85, UAS Dicer, UASmDC8GFP/Cyo;Dfd-GFP	Expression of Gal4 in the PSC	Labo Crozatier
PG125	pg125, UAS Dicer 2, UASmD8GFP/Cyo;Dfd-GFP	Expression of Gal4 in the medullary zone	Labo Crozatier
e33c		Expression of Gal4 in the lymph gland	Labo Crozatier
BL Ets97D RNAi	Y1 v1; P{TRIP.JF01805}attP2	Expresses dsRNA for RNAi of <i>Ets97D</i> under UAS control	BL23795
BL I(3)mbt RNAi	Y1 v1; P{TRIP.JF02913}attP2	Expresses dsRNA for RNAi of <i>I(3)mbt</i> under UAS control	BL28076
BL <i>edl</i> RNAi	Y1 sc [*] v1; P{TRIP.HMC04869}attP40	Expresses dsRNA for RNAi of <i>edl</i> under UAS control	BL57552
VDRc pnt P2 RNAi	Insertion in chromosome 2	Expresses dsRNA for RNAi of <i>pnt P2</i> under UAS control	VDRc771
VDRc <i>edl</i> RNAi	Insertion in chromosome 3	Expresses dsRNA for RNAi of <i>edl</i> under UAS control	VDRc51818
Eater-GFP	Eater-GFP;;	Expression of GFP under the control of eater promoter in the plasmatocytes	Lemaitre
msnCherry	;;MSNF9MO-mCherry	Expression of mCherry under the control of msnf9mo promoter in the lamellocytes	Lemaitre
BL 26759 aop RNAi	Y[1]v[1]; P{V{+7.7}v{+1.8}=TRIP.JF02323}attP2/TM3_Sb[1]	Expresses dsRNA for RNAi of <i>aop</i> under UAS control	BL26759
BL 35404 aop RNAi	Y[1]sc[*]v[1]; P{V{+7.7}v{+1.8}=TRIP.GI00324}attP2	Expresses dsRNA for RNAi of <i>aop</i> under UAS control	BL35404
BL pnt RNAi	Y[1]v[1]; P{V{+7.7}v{+1.8}=TRIP.JF02227}attP2	Expresses dsRNA for RNAi of <i>pnt P2</i> under UAS control	BL31936
UAS-CD8;;GFP	UAS-CD8;;GFP	Expresses GFP under UAS control at the membrane	labo Gallet
YW;-Sp/Cyo;Sk/Tm6B	YW;-Sp/Cyo;Sk/Tm6B	Balancer line	labo Gallet
YW hep1 yFM7 wa	YW hep1 yFM7 wa	Balancer line	labo Gallet

A. *D. melanogaster* strains and stocks, *L. bouhardi* lines, rearing conditions

The strains YR (Gif n°1088; homozygous for the *Rlb*⁺ allele) and SY (Gif n°1089; homozygous for the *Rlb*⁻ allele), respectively resistant and susceptible to the ISy strain of *L. bouhardi*, were kindly provided by Dr Yves Carton as part of an extended collaboration. They only differ by a region of chromosome 2R containing the *Rlb* gene (see introduction part; Carton *et al.*, 1992; Hita *et al.*, 1999). The Sefra strain was founded in 2013 from a population collected in the county of La Drôme part of the French Rhône Valley. All the other strains and stocks were either kindly donated from other laboratories or teams or ordered from *Drosophila* stock centres. These strains are listed in a dedicated table (Table 1).

All *Drosophila* flies were reared on a yeast rich medium (10% corn flour, 10% baker's yeast, 6% Moldex) at 25°C, with a 12 hours day/night cycle.

pcol-Gal4 (Krzemien *et al.*, 2007); e33c-Gal4 (Harrison *et al.* 1995) and PG125dome-gal4 (Jung *et al.*, 2005) were gifts from Dr M. Crozatier (Toulouse). UAS-mCD8-gfp / YW; Sp/CyO; Ski/Tm6B / and YW hep1 yFM7 wa were kindly provided by Dr A. Gallet (ISA); UAS-edl F and UAS-edl X were gifts of Dr T. Yamada (Japan), and eater-GFP and msnCherry of Dr B. Lemaitre (Switzerland). The UAS-mae 34how and UAS-mae 8how were made following the description in the annexe.

The other *Drosophila* strains were obtained from the Bloomington (BL) stock centre: pHml-Gal4 BL 6395), pCg-Gal4 (BL 7011), UAS-*mae* RNAi (BL57552), UAS-*pnt* RNAi (BL31936), UAS-*aop* RNAi (BL26759), UAS-*aop* RNAi (BL35404), or the Vienna *Drosophila* Ressource Center (VDRC): UAS-*mae* RNAi (51818), UAS-*pnt* RNAi (7171).

The *L. bouhardi* ISy line (Gif n°486) was kindly provided by Dr Yves Carton. It was obtained from an isofemale line selected from a population collected in Brazzaville (Congo) (“avirulent” strain) (Dupas *et al.*, 1998). Parasitoids were maintained on the susceptible *Drosophila* strain Nasrallah (originating from Tunisia) at 25°C with a 12 hours day/night cycle. Emerged adults were collected and kept on agar (1.5% agar, 10% sugar, 0.4% Nipagine) at 20°C with a 12 hours day/night cycle.

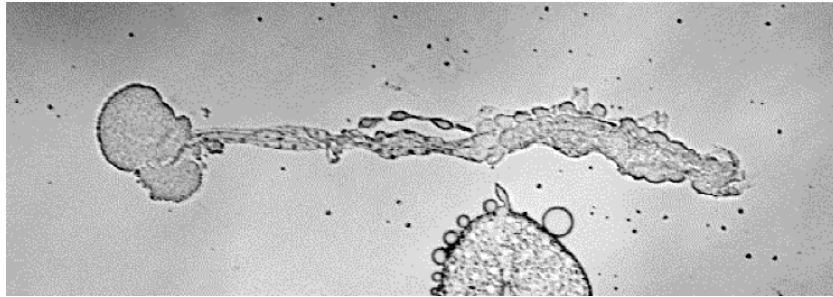


Figure 22: Picture of a dissected “intact” lymph gland: The two anterior lobes of the gland are present with clear sharp edge. Any lymph gland not resembling this gland was classified as “bursting”. (DIC, obj 200x Zeiss Axioplan Z1)

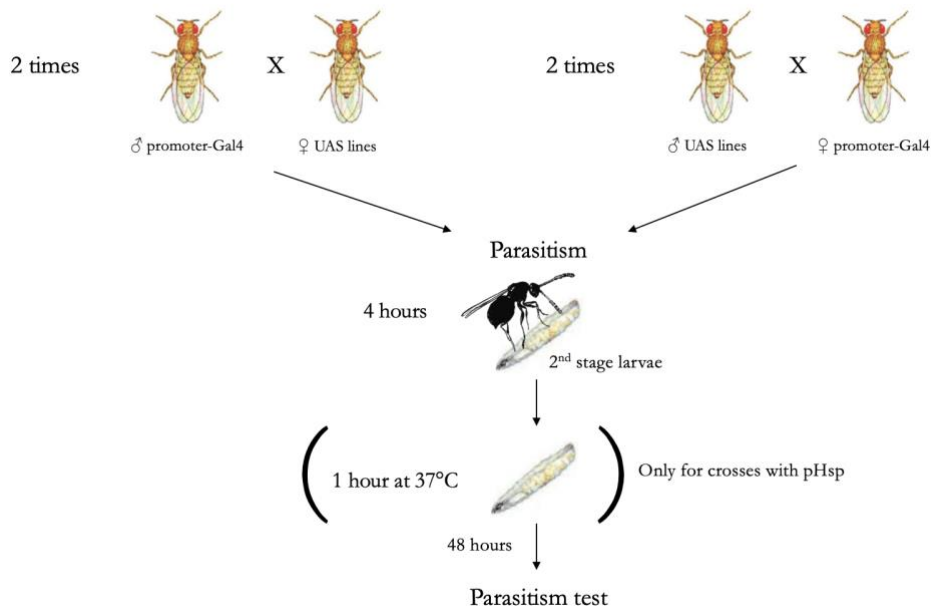


Figure 23: Crossover schemes for RNA interference experiments. The interfering RNAs for the gene of interest are produced according to the driver promoter-Gal4 line used. Parasitism is done 3 days after egg laying.

B. Larval encapsulation assays

For encapsulation tests, pools of 20 *D. melanogaster* second instar larvae (L2) were transferred on dishes (\emptyset 2.5cm) containing yeast-rich *Drosophila* medium and submitted to parasitism during 4h by two 1 to 14 days old *L. boulandi* ISy females. Larvae were then allowed to grow at 25°C for 48h (28°C for all experiments with RNAi lines, which allows to induce expression more effectively). Larvae were then dissected with forceps under a binocular microscope (x20) and the number of larvae containing an encapsulated egg/larva or a free parasitoid larva was recorded. Only monoparasitized host larvae were considered. In all experiments, the encapsulation rate (ER), an approximation for parasitism failure was calculated by dividing the number of host larvae containing an encapsulated egg/larva by the total number of monoparasitized larvae. When UAS/Gal4 lines were used, controls were (UAS line x Nasrallah) or (Gal4 line x Nasrallah).

C. Observation of the timing of the lymph glands bursting

Pools of 20 second instar YR and YS larvae were submitted to parasitism for 2h at 25°C by ISy females. Larvae were then kept at 25°C before dissection 8-10, 10-12, 13-15, 22-24, 26-28 or 28-30h post-parasitism (interval caused by the parasitism, there can be 2h between the first time the parasitoid parasitize and the last one). Lymph glands were observed (binocular 40x) on unfixed larvae and classified into two groups: “intact” when anterior lobes were present and their edge regular (Figure 22) and “bursting” when the anterior lobes were absent or rudimentary.

The number of larvae in each group was recorded for each period after parasitism. The percentage of lymph gland dispersion was then calculated by dividing the total number of larvae with absent or disrupted anterior lobes by the total number of monoparasitized larvae.

D. Genetic approaches, crossover schemes

1. RNA interference experiments

For the RNAi experiments, crosses and tests were carried out at 28°C as described in the Figure 23 below. For the crosses involving the heat shock inducible pHsp promoter, the larvae were transferred at 37°C for one hour just after parasitism to activate the promoter. The crosses were carried out twice in both directions: between a male of a promoter-Gal4 strain and a female of a UAS strain for the gene of interest and between a male of a UAS strain for the gene of interest and a female of a promoter-Gal4 strain. The offspring was then tested for the level of encapsulation as described II.B. The crosses have been achieved in 3 independent replicates.

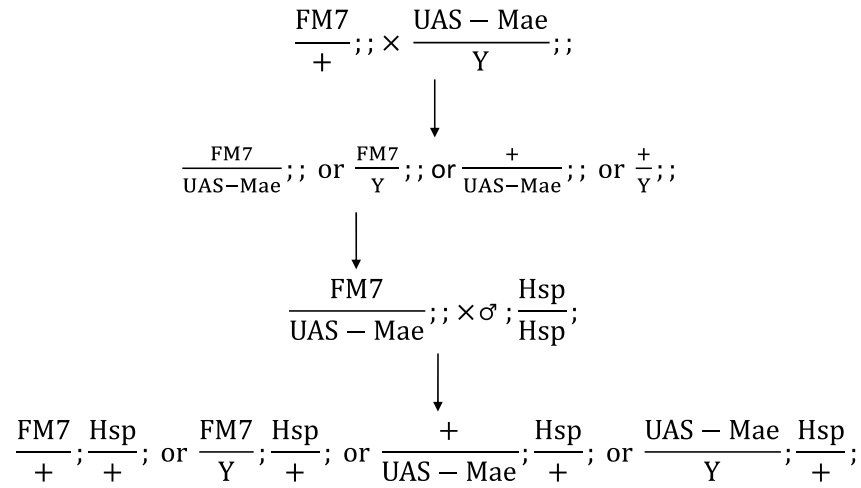


Figure 24: Cross-over scheme to test the localization of the insertion on chromosome 1

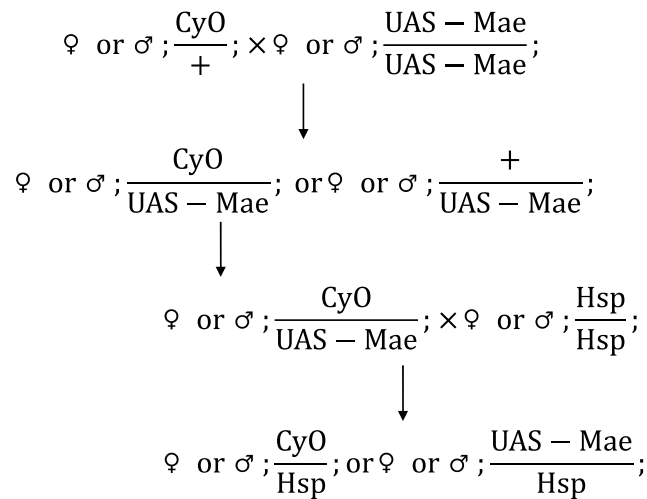


Figure 25: Crossover scheme to test localization of the insertion on chromosome 2

2. Localisation of the UAS-*mae* insertion in transgenic strains

To confirm the role of *mae* in *D. melanogaster* resistance, we have done rescue experiments. For that, we used two home-made transgenic strains homozygous for the *mae* resistant allele, UAS-*mae* 34how and UAS-*mae* 8how. A first step was to identify the chromosome carrying the UAS-*mae* insert in each of these strains. To do this, UAS-*mae* 34how and UAS-*mae* 8how were crossed with two different strains: YW; Sp/CyO; Ski/Tm6B which carries balancers of the chromosomes 2 and 3, and YW hep1 yFM7 wa which carries a balancer of chromosome 1. The crossover schemes are shown below.

a) Test of localization of the insertion on chromosome 1

Males of the two transgenic strains 8 and 34 were crossed with females carrying the FM7 balancer. F1 virgin females were then crossed with males of a Hsp-Gal4 driver line, 48h after the egg laying the L2 larvae were parasitized by 2 Isy, the larval progeny was heat-shocked at 37°C for 1 hour to induce expression and thereafter left to develop until emergence at 25°C with a 12 hours day/night cycle. Results of parasitism were observed at the *Drosophila* adult stage, at which individuals not carrying the FM7 balancer (selection of individual with normal eye shape) can be identified. 8 days after parasitism, as all the *Drosophila* have emerged from the pupa, the numbers of adult *Drosophila* flies containing a capsule (resistant) and pupae containing a parasitoid (susceptible) were observed and counted.

b) Test of localization of the insertion on chromosome 2

UAS-*mae* 8 and 34 flies were crossed with fly from a strain carrying the CyO balancer, YW; SP/CyO; Ski/Tm6B. [Cy] (curly wings phenotype) F1 offspring were then crossed with flies of a Hsp-Gal4 driver strain. L2 larvae from this cross were parasitized by 2 Isy for 4h and then heat-shocked at 37°C for 1 hour. They pursued their development at 25°C until they reached the adult stage at which the [Cy] phenotype is visible. Non [Cy] adult resistant and susceptible flies were estimated as described in II.D.2.a).

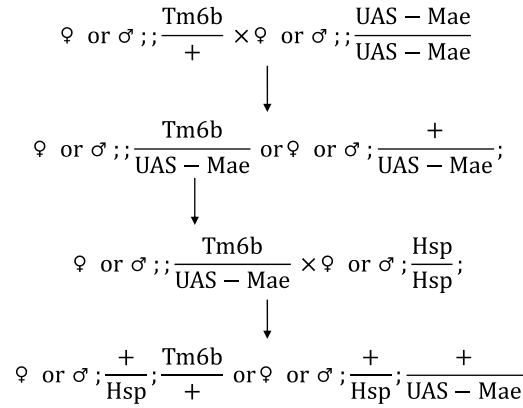


Figure 26: Crossover scheme to test the localization of the insertion on chromosome 3

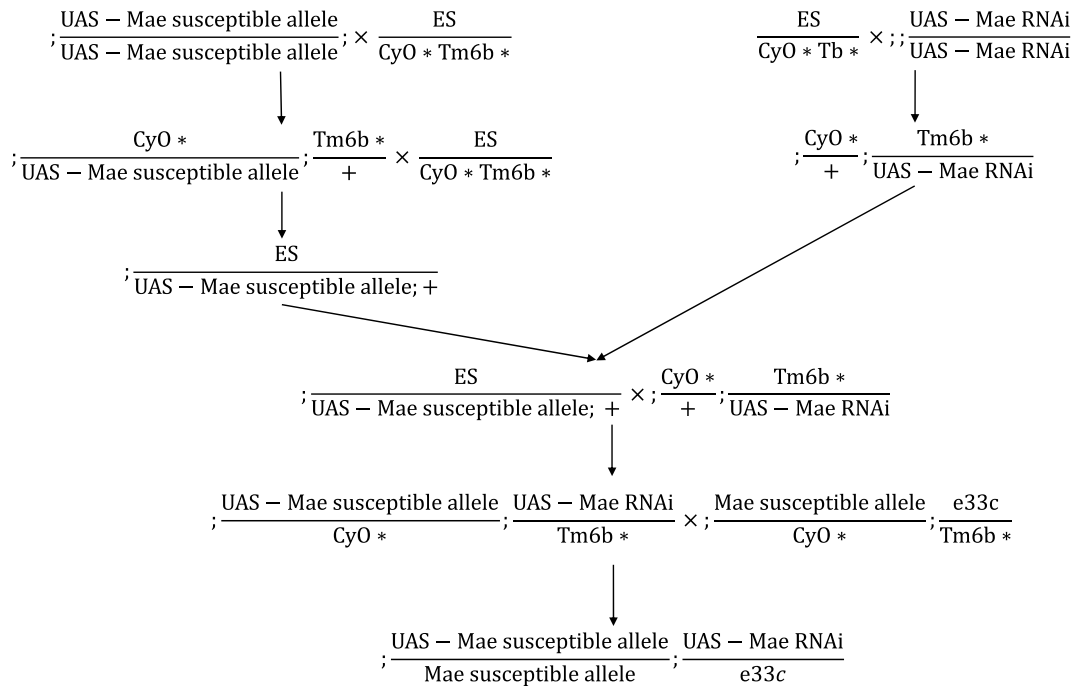


Figure 27: Crossover scheme for the rescue experiment

c) Test of localization of the insertion on chromosome 3

The hypothesis of the location of the insertion on chromosome 3 was tested by crossing flies of the transgenic UAS-*mae* resistant allele lines with flies of a strain carrying a Tb balancer, YW; SP/CyO; Ski/Tm6B. F1 [Tb] (tubby larvae and pupa phenotype) offspring were then crossed with flies of the Hsp-Gal4 driver strain and larval progeny was parasitized by 2 Isy for 4h and was maintained at 37 ° C for 1 hour before further development at 25 °C. The [Tb] phenotype being visible at the larval stage, the parasitism outcome was assessed at this stage on non [Tb] larva as stated in II.B.

3. Rescue experiment

To further confirm the results obtained with the UAS-*mae* 8how and 34how lines and the *mae* RNA interference experiments, a rescue experiment was performed since false positives caused by off-target effects happened, a careful validation of RNAi-induced phenotypes is required (Echeverri & Perrimon, 2006). The crossover scheme is described below. ES/(CyO* Tb*) (from Dr Crozatier's lab) allows keep the chromosomes 2 and 3 bearing the balancer genes linked together. The final offspring were tested for their resistant/susceptible phenotype as described in section II.B.

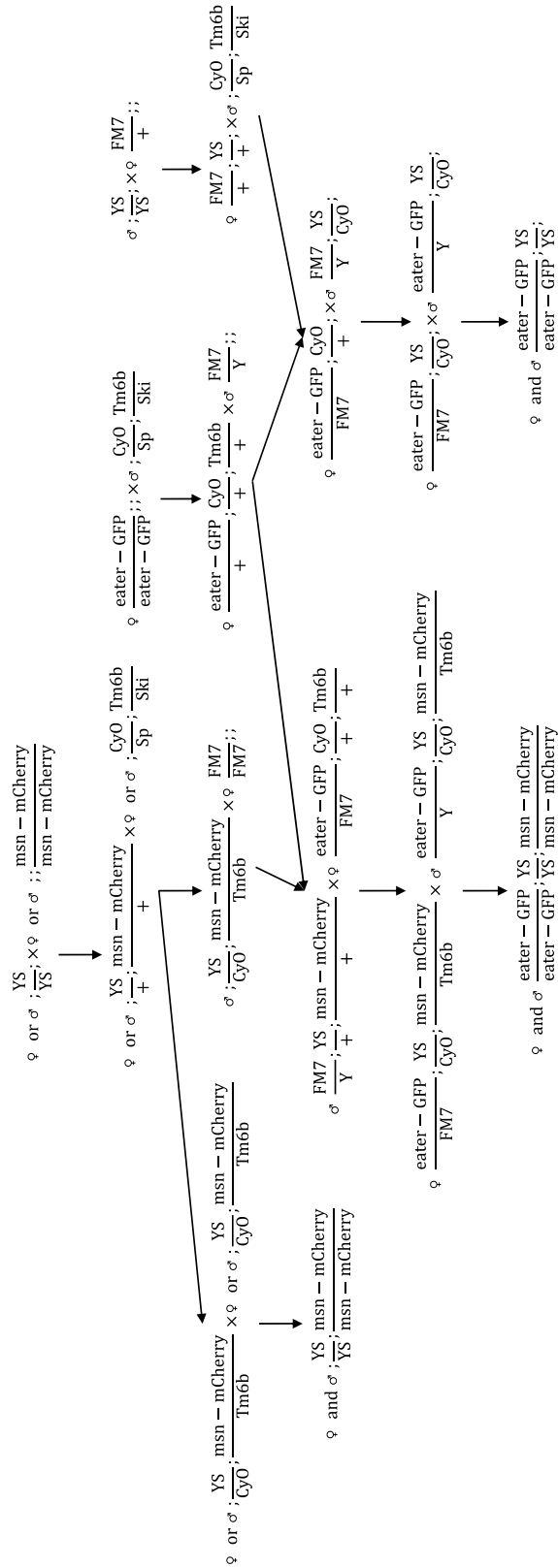


Figure 28: Crossover scheme to create resistant and susceptible strains with a double staining of plasmatocytes and lamellocytes (the scheme was used for both YS and YR). Presence of GFP, cherry and alleles of *edl* were checked using PCR.

4. Creation of new *Drosophila* lines

In order to study more easily the hemocyte response during parasitism we decided to make reporter strains with either, strains carrying insertions of *eater*-GFP (to stain the plasmatocytes) or *msnCherry* (to stain the lamellocytes) (Anderl *et al.*, 2016) were crossed with the YR and YS strains as shown below.

Direct observation of the hemocytes under fluorescent microscope indicated that the lamellocytes' cytoplasm of both created lines is stained in red due to the presence of *msnCherry*.

E. Immunolabeling of hemocytes

To observe the hemocytes, groups of 5 *D. melanogaster* second instar larvae (L2) were submitted to parasitism during 4h by two *L. boullardi* ISy females and dissected 24h later. The 5 larvae were washed in a drop of water and the posterior end of the larvae was gently teased with fine forceps under a binocular and hemolymph allowed to flow in a 30 μ L PBS drop. The drop containing the larval hemolymph was then transferred at the center of a circular glass coverslip (20 mm diameter) placed in a 24-well cell culture plate. The hemocytes were left for adhesion to the coverslip for 1h at RT. Then, the coverslip was gently washed with 500 μ L of PBS and the cells were fixed by a 15 minutes incubation with a 4% paraformaldehyde solution (4% PFA in 0.1 M phosphate buffer, pH 7.4). The coverslip was allowed to dry, washed 3 times 5 min with PBS, and blocked with 500 μ L PBS-3% BSA-0.1% TritonX100 for 30 min. After a PBS washing, the cells were then incubated with a 1/100 dilution of primary antibody for 45 minutes, followed by 3 PBS washes and the incubation with the secondary antibody (1/1000 dilution; 45 min). The coverslip was washed three times with PBS and a final quick wash with milliQ water, and mounted on a glass slide with an anti-fading medium containing DAPI (Interchim). A mouse monoclonal antibody was used: an anti-myospheroid (CF.6G11 from the DSHB) was used for the labelling of the lamellocytes. Secondary antibodies were 488-Alexa (Sigma) and m-cherry anti-mouse (GeneCopoeia) used. The observations were made using the fluorescent mode of the AxioImager Z1 (Zeiss).

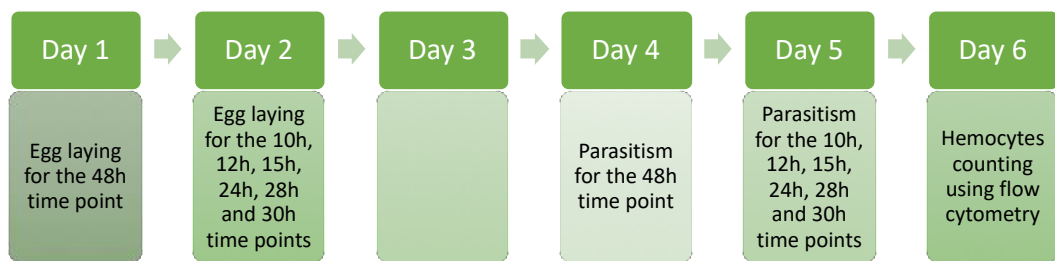


Figure 29: Representation of the sample preparation timeline for the hemocytes counting experiment

F. Flow cytometry procedures.

Hemocytes counting was performed by flow cytometry using the resistant and susceptible created *Drosophila* lines bearing fluorescent lamellocytes (II.D.4). Following egg laying on the planned days (Figure 29), 20 eggs were collected in dishes filled with fly medium. Second instar larvae were then submitted to parasitism for 2 hours at 25°C by 2 ISy *L. bouhardi* females and thereafter allowed to develop for 8-10, 10-12, 13-15, 22-24, 26-28, 28-30 or 46-48 hours (interval caused by the parasitism, there can be 2h between the first time the parasitoid parasitize and the last one) at 25°C before being dissected and the hemolymph collected in 50 µL of PBS. The experimental protocol was made so the analysis of the hemolymph from all the different times after parasitism were done the same day. This experiment was done in 3 independent replicates.

Multidimensional analyses were performed on a BD LSRFortessa™ cell analyzer (BD Bioscience) equipped with four lasers (488, 575, 640 and 405nm) with four PMT (PhotoMultiplier Tube) in the 488nm laser, 4 PMT in the 575nm, 3 PMT in the 640nm and 6 PMT in the 450nm laser. Quantification of the total collected hemocytes was performed directly (without fixation) after cell collection and staining for 10 minutes with CF®488A-WGA (1:10,000; Ref 29022-1, Biotium; observation at 490/515 nm) to label all hemocytes (Rizki & Rizki, 1984; Tirouvanziam *et al.*, 2004a). Counting beads (AccuCheck Counting Beads, Life technologies) were added just before injection to the cells suspension to calibrate the flow cytometer. Every hemocytes were stained by WGA and detected using the FICT-A channel and lamellocytes were mCherry positive and detected using the PECF594-A channel.

G. Molecular approaches (DNA, RNA extraction, PCR and qPCR)

Whole *Drosophila* DNA was extracted using home-made solutions (Buffer 1:1% SDS, 50mM Tris HCL (pH8), 25mM NaCl and 25mM EDTA (pH8); and 3M potassium acetate (pH8)).

DNA extraction from one *Drosophila* leg was performed using the prepGEM Insect Kit (ZyGEM) following the manufactory protocol.

RNA was extracted from the fat body of *Drosophila* larvae using RNazol RT from Sigma following the manufactory protocol.

PCR was done using the GoTaq 2 DNA polymerase and the primers' sequences provided in the following table (Table 2) on an Eppendorf Mastercycler PCR machine (40 cycles).

Table 2: Primers used and their characteristics (in green: PCR primers, in white: qPCR primers)

Name	Fwd/Rev	Sequence	Size	Tm (°C)	Amplicon size	Objectif
Mae.MAEIII2.fw	Fwd	GCAAGTGGAAATCGAGCTA	18	54	212 bp	Amplification of the portion of mae containing the SNP
Mae.SNP2.rev	Rev	TGGATGTAGATGAGATGGC	19	54		
Mae.Exon1.fw	Fwd	AATCAGTTGAGAACGTGGA	19	54	602 bp	Sequencing of mae exon 1
Mae.Exon1.rev	Rev	AACCGACTTACCTTGATCG	19	54		
Mae.EcoR1.2.fw	Fwd	AGAATTCAAATGCAAGTGGAAATCGAGCTA	29	64	654bp	For mae protein production in ordre to purify the antibody
Mae.Xho1.2.rev	Rev	GCTCGAGAAATGACGAGCAGAATAAGG	28	65		
cherry.fw	Fwd	GAGTTCATGCGCTTCAAGGTG	21	60	602bp	Amplification of mCherry sequence
cherry.rev	Rev	ATGGTGTAGTCCTCGTTGTGG	21	60		
gfp.fw	Fwd	GACGTAACGGCCACAAGTT	20	59	554bp	Amplification of GFP sequence
gfp.rev	Rev	GACTGGGTGCTCAGGTAGTG	20	59		
Mae.qPCR.fw	Fwd	CAGACGCCATCTGCATCATC	22	60,9	132 bp	To follow mae expression
Mae.qPCR.rev	Rev	GCGCTCATCAGTCGATTGTAT	22	60,8		
actin.qPCR.fw	Fwd	GCGTCGGTCAATTCAATCTT	20	60	138 bp	Reference gene - To check the presence of DNA contamination in the RNA purification
actin.qPCR.rev	Rev	AAGCTGCAACCTCTTCGTCA	20	60		
robl.qPCR.fw	Fwd	AGCGGTAGTGCTGCCGTGT	20	64	149 bp	Reference gene
robl.qPCR.rev	Rev	CCAGCTGGATTTGACCGGA	20	62,8		
Rap2l.qPCR.fw	Fwd	ACTCCGTGCATTACGTGCG	20	61,6	212 bp	Reference gene
Rap2l.qPCR.rev	Rev	CCGACCCGAGCACAACT	20	62,9		

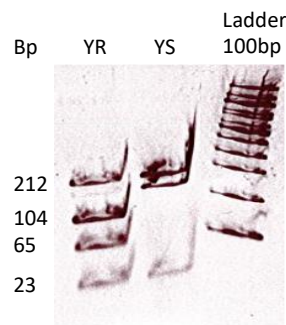


Figure 30: Acrylamide gel (12%) of DNA from YS (containing the susceptible allele of edl) and YR (containing the resistant allele of edl) lines treated with Mae III Endonuclease. YR is DNA from YR larvae and YS is DNA from YS larvae. Ctr is the water negative control. On the YR lane, 4 bands were detected (212, 104, 65 and 43bp) while on YS lane only 3 bands were visible (212, 169 and 43bp). The 104bp and 65bp YR bands correspond to the YS 169 bp band.

qPCR was performed using the Takyon No ROX SYBR MasterMix blue dTTP (Eurogentec) on an Agilent AriaMx, Real-Time PCR system following the takyon kit guidelines. cDNAs were produced using using Invitrogen SuperScript kit. RT qPCR data analysis were carried out using RqPCRBase, a R-package working on R computing environnement for analysis of qPCR data (Hilliou & Tran, 2013). 2 reference genes were chosen, *robl* and *rap2l* as their expression in the fatbody is described as low to moderately high and *edl* expression is described as low to moderate on Flybase (Ling & Salvaterra, 2011).

Primers and their characteristics are described in the Table 2. The sequencing was performed externally (Genewiz).

For identification of the *edl* allele, in the every experiment except the creation of reporter lines, PCR was performed on whole *Drosophila* DNA using the Mae.MAEIII2.fw and Mae.SNP2.rev primers. Digestion of the amplicon with a 1/5 dilution of the MAEIII restriction enzyme in 20 μ L at 1h at 55°C (Sigma). The products of the digestion were migrated on a 10% acrylamide gel at 50V for 2h. Revelation was done using BET bath (Figure 30).

H. Cloning and transformation

PCR amplicons of the resistant and susceptible alleles of *edl* were inserted in PCRII vectors using the TOPO TA Cloning Kit from Invitrogen. *EcoR1* and *Xho1* (NEB) were then used to transfer the insertion from the PCRII vectors to pGEX-4T-2 vectors for protein production. Plasmid extractions were performed using the GenElute Plasmid Miniprep kit (Sigma) followed by a separation on a 1,2% agarose gel. DNA gel purifications were done with the MinElute PCR purification kit (Quiagen) and the ligations using the T4 DNA ligase (Promega). The competent cells were the TOP10 electrocompetent cells (Invitrogen). Selection of the transformed bacteria was performed using classical LB-ampicillin medium.

I. Bioinformatics

Sequences analysis was performed using Geneious v9. The *edl* *D. melanogaster* sequence (only one mRNA sequence coding for one protein) was obtained from FlyBase (flybase.org). The amino acid sequences of the SAM domains of the various SAM domain-containing *D. melanogaster* proteins were obtained from Uniprot (uniprot.org). Multiple alignments were performed on Geneious® using multiple alignment functions MUSCLE (Edgar, 2004) and 150 iterations. Nucleotide

sequences were aligned using the pairwise alignment function with the Geneious global alignment with the free end gaps and the 51% similarity matrix.

J. Statistics

RStudio v1.1.456 was used for all statistical analysis.

For parasitism tests, the encapsulation rate was tested using generalized linear mixed models (GLMM) with the control as a fixed effect, the replicates as a random effect and a binomial error distribution.

For the lymph gland bursting tests, a generalized linear mixed model (GLMM) was used with the parasitism status of the larvae as a fixed effect, the replicates as a random effect and a binomial error distribution. In both cases, models were fitted with the `bglmer` function implemented in the “`blme`” R package (Dorie, 2015).

For the flow cytometry, data analysis was performed using the flow cytometer software (BD Bioscience). The proportion of lamellocytes were compared using the `prop.test` function in R. `prop.test` can be used to test the null hypothesis that the proportions are the same in several groups (probabilities of success), or that they are equal to given values (`prop.test(x=c(L1,L2),n=c(T1,T2))`). The p -values have been corrected by multiplying them by the number of points (7) during multiple comparisons. The mean of the quantities of hemocytes were compared using Mann-Whitney (`wilcox.test`).

III. Results

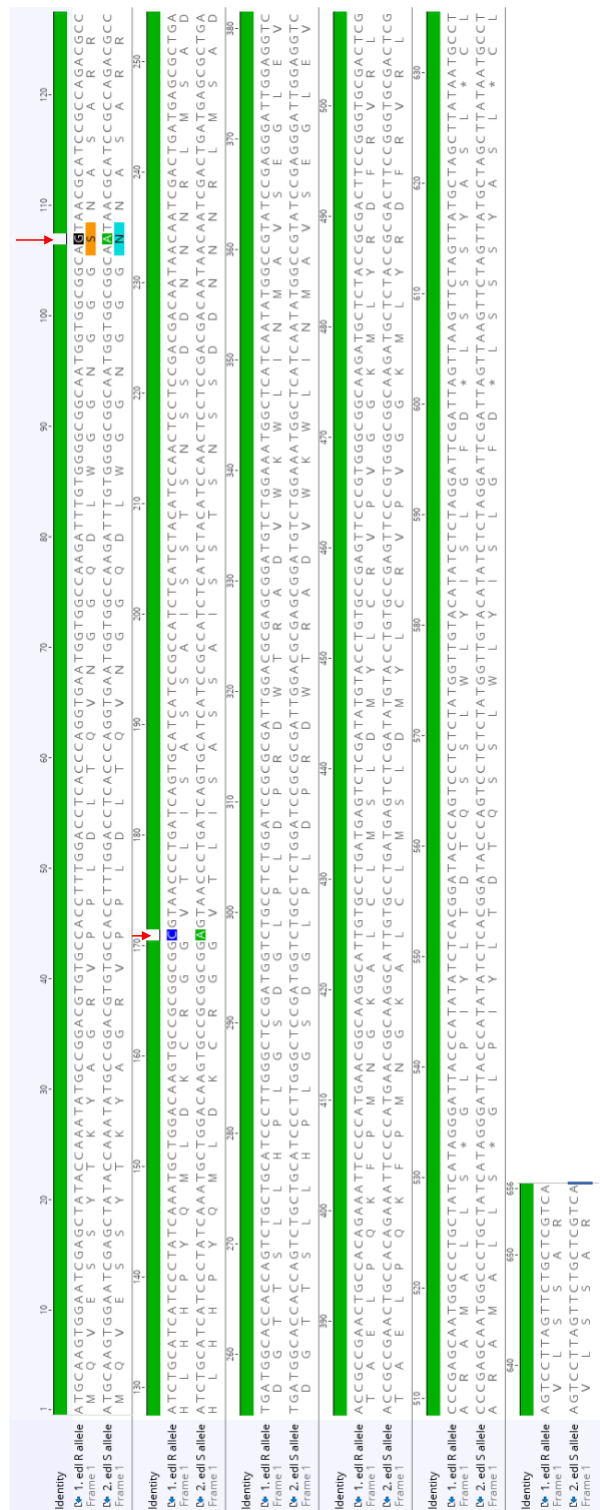


Figure 31: Alignment of the sequences of the CDS of 2 alleles of edl. Edl R is the allele Rlb+ (resistant) and edl S is the allele Rlb- (susceptible). Frame 1 is the translation of the genomic sequences into amino acids. The alignment was performed using Geneious software and the geneious alignment option with 51% similarity. Only the differences are highlighted: for nucleotide: guanine in black, adenine in green, cytosine in royal blue; for amino acid: serine in orange, asparagine in teal.

A. New tools for the characterization of the resistance/susceptible phenotype

As they are the basis of my work, I assessed the resistance level of the YR (resistant) and YS (susceptible) strains that were maintained in the laboratory for several years. The *L. boulandi* Isy strain of parasitoid was the one used to assess the resistant/susceptibility status of those 2 *Drosophila* lines. I found that the encapsulation rate of the YR line was only 40% (21/53 capsules out of 3 replicates) while for the YS one only 20% (48/59 capsules out of 3 replicates). Thus, the YR line much less resistant than at the time it was created (Carton *et al.*, 1992). This change is probably due to human error so for the project I needed to reselect resistant and susceptible lines.

In Hita *et al.*, (2006), a 100kb locus on chromosome 2 was linked to the resistance in the YR and YS lines. Two genes are present in this locus: CG33136 and *edl*. *Ecl* appeared to be the best candidate at the time and some work had previously been done on *edl* in the lab (unpublished). They sequenced the CDS (Coding DNA Sequence) of *edl* in the YS and YR lines and found 2 Single Nucleotide Polymorphism (SNP). They correspond to a guanine on the resistant allele and to an adenine on the susceptible allele at the 107th position on the CDS of *edl* and a cytosine on *edlR* and an adenine on *edlS* at the 171th position on the CDS (Figure 31). The SNP translates into a change in amino acid: the resistant allele has a serine at position 36 whereas the susceptible allele has an asparagine.

These two amino acids are not substitutable since they have different chemical and physical properties. This change occurred in the non-SAM domain region of *edl* then it may not be involved in the function of this domain and interaction with other SAM-domain proteins. Since serine are phosphorylatable by kinases, we tested *in silico* all possible sites used by all kinases in the GPS server (<http://gps.biocuckoo.org/wsresult.php> (Xue et al, 2005)) and the NetPhos 3.1 Server (<http://www.cbs.dtu.dk/services/NetPhos/> (Blom et al, 2004)). Both software predicted a large number of potential phosphorylation sites on *edl* by different kinases, including the S(36) on the *edlR* protein, but indeed absent on the *edlS* one. However, at this site the score was low and only above the threshold for the kinase *cdc2* involved in cell cycle progression

Most importantly, the presence of the first SNP (G/A) introduce a supplementary restriction site for the endonuclease *MaeIII* (/GTNAC) in the *edlR* sequence leading to the production of 4 fragments from the *edlR* amplification instead of three for *edlS* one. This allowed to differentiate the resistant and susceptible flies on acrylamide gel. This feature was thus used to select the flies carrying the different alleles by DNA extraction.

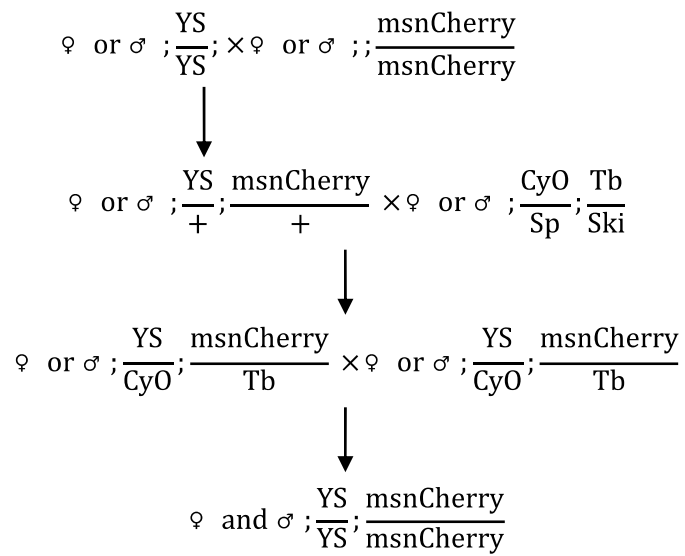


Figure 32: Cross-over scheme to create resistant and susceptible strains with a staining of lamellocytes (the scheme was used for both YS and YR).

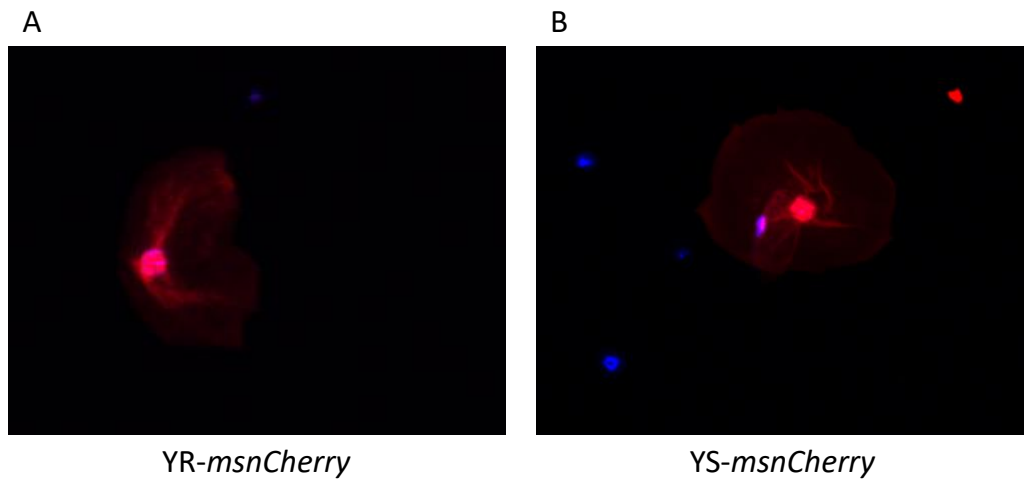


Figure 33: Pictures of lamellocytes from the YR-*msnCherry* (A) and YS-*msnCherry* (B) taken using fluorescent microscopy (*mCherry* from insertion in the lines under *msn* promotor and DAPI for the nucleus, X400)

1. Creation of the new resistance/susceptible lines

To create new lines of resistant fly, the YR larvae were first submitted to parasitism and after 48h larvae with visible capsule were selected to continue their development. At emergence, a virgin female and a male, both with a visible capsule, were allowed to mate and the female laid eggs for a week. Afterwards, DNA was extracted from the parents and tested to check the *edl* allele type using *MaeIII* test and then the *edl* amplicon of the *Drosophila* presenting a resistant digestion profile was sent for sequencing to check the homozygous/heterozygous status of *edl*. The chromatogram provided together with the consensus sequence after sequencing allows use to identify whether only nucleotide or more are detected for the same position.

For the susceptible line, a female and a male were allowed to mate and the female laid eggs for a week, DNA was extracted from the parents and tested to check for their *edl* alleles. Only the descendants of the couple where both parents had susceptible alleles were kept, every generation part of the progeny was tested by encapsulation test to check the susceptibility.

After 10 generations and the DNA from 150 *Drosophila* was extracted, 2 selected lines per genotype were pooled together to give the YR and YS lines having respectively 89% and 20% of resistance used in the rest of the project.

2. Creation of the new resistance/susceptible lines with fluorescent hemocytes

A phenotypic difference described in Russo *et al.*, (2001) between the YR and YS lines was an earlier increase in the number of circulating hemocytes in the YR line. But the time course of this cell number increase was not analysed in details in the first hours following the parasitism. Moreover difference in lamellocytes production occurring after parasitism was clearly reported (Russo *et al.*, 2001; Carton *et al.*, 2005). Recently, the use of flow cytometry to count of the different *Drosophila* hemocyte types has been developed and shown its efficiency (Anderl *et al.*, 2016; Gyoergy *et al.*, 2018). We thus decide to use this method to analyse more clearly the hemocyte response phenotype of the YR and YS lines upon *Isy* parasitism.

First, I had to develop YR and YS lines bearing fluorescent plasmatocytes and lamellocytes in order to create a double reporter line for each *Drosophila* strain. To do so I took example from the work developed by Anderl *et al.*, (2016) to observe the variation of hemocytes counts post-parasitism and to differentiate the fate of several hemocytes types. In their work, they took advantage of the enhancer-reporter constructs *eater-GFP*, which is specific for plasmatocytes, and *MSNF9MO-mCherry* (called *msnCherry* in this manuscript), which is specific for lamellocytes.

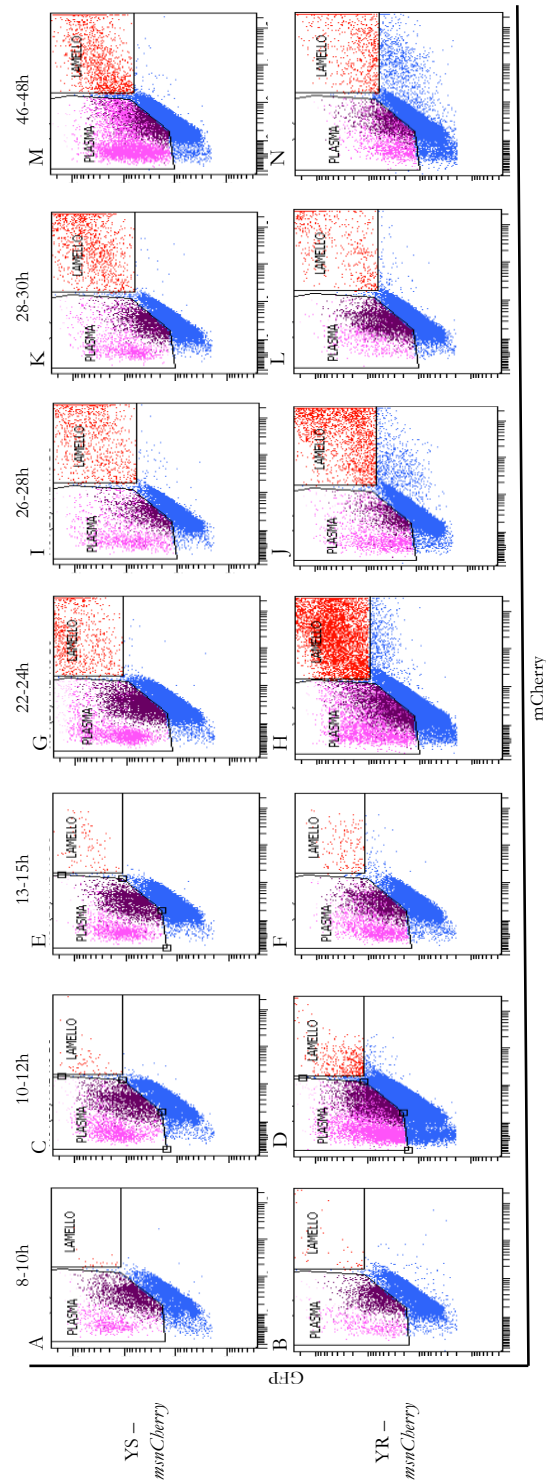


Figure 34: Flow cytometry plots at representative time points after parasitism. YS-*msnCherry* and YR-*msnCherry* larvae were infected by G486. The time points were chosen to follow the variation in the hemocytes populations after parasitism. GFP stains the hemocytes, mCherry is produced by lamellocytes. Blue dots: not taken into account for the countings (e.g. debris, dead cells). Pink and purple dots: GFP+ mCherry- cells, gated “plasma” for plasmatocytes. Red dots: GFP+ mCherry+, gated “lamello” for lamellocytes

Unfortunately, as the eggs from the crosses between the eater-GFP and FM7 lines did not hatch, the double reporter lines could not be obtained. I did not have the time to investigate the reason of the incompatibility between these lines. So, only lines containing the *msnCherry* insertion to follow the lamellocytes could be obtained Figure 32. Therefore, we now have in our possession a resistant (84% of encapsulation, 21/25 larvae with capsule in 3 replicates, YR-*msnCherry*) and a susceptible (20% of encapsulation, 2/10 larvae with capsule in 3 replicates, YS-*msnCherry*) line carrying the *msnCherry* insertion. The reporter lines were checked using fluorescent microscopy (Figure 33), larvae from the 2 lines were parasitized to induce lamellocytes differentiation and hemocytes were collected. The lamellocytes in the new YR-*msnCherry* and YS-*msnCherry* expressed mCherry in their cytoplasm (respectively Figure 33A and B).

B. Different timing-dependant cellular response between YS-*msnCherry* and YR-*msnCherry* after parasitism

As the egg recognition happens less than 2h in the parasitism process (Bajgar *et al.*, 2015) and that plasmatocytes and lamellocytes are attached to the egg at 24h, the production of hemocytes in the resistant and susceptible reporter lines was evaluated by flow cytometry in the first 24h after parasitism. Seven different time points were chosen: 8-10h, 10-12h, 13-15h, 22-24h, 26-28h, 28-30h and 46-48h after parasitism (3 replicates).

Since only lamellocytes were labelled *in vivo* by expression of *msnCherry*, we used WGA-GFP to label all hemocytes (Tirouvanziam *et al.*, 2004a). WGA is a carbohydrate-binding lectin, that we used to obtain the total number of circulating cells in our samples. The subtraction between the number of *msnCherry* and GFP lamellocytes and the GFP cells give us the number of plasmatocytes. Since crystal cells represent only a small number of cells, this cell category was not separated from plasmatocytes. Moreover, crystal cells are fragile cells that generally lysed rapidly after hemolymph collection. The data from this experiment are represented below in the figure format and the detail counts are available in the Table 13 in the Annexe.

Figure 34 showed one example of the results obtained by flow cytometry. At 8-10h after parasitism (Figure 34A and B), for YS and YR the main population of cells are labelled only with WGA-GFP and a small number of cells representing the lamellocyte population doubly labelled with GFP and mCherry red fluorescence. At 10-12h (Figure 34C and D), for both YS-*msnCherry* and YR-*msnCherry* lamellocytes is increasing but more in YR-*msnCherry* than YS-*msnCherry*. The situation was almost the same at 13-15h (Figure 34E and F).

8-10 10-12 13-15 22-24 26-28 28-30 46-48
(h)

Figure 35: Total hemocytes count post-parasitism evaluated by flow cytometry. Columns represent the mean total number of hemocytes per larva at the indicated time (in hours) after parasitism for the YR-*msnCherry* (dots) and YS-*msnCherry* (grey). The bar indicates the S.D. Each of the YR-*msnCherry* replicate is represented by a blue circle and YS-*msnCherry* by a purple square.

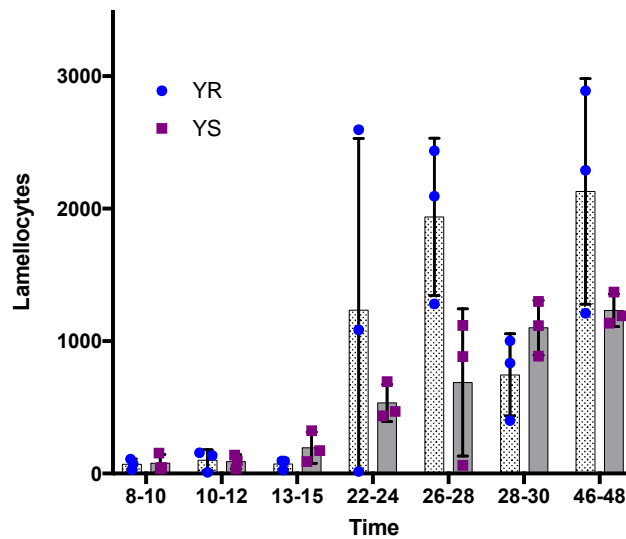


Figure 36: Lamelloocytes count on the resistant and the susceptible lines after parasitism. WGA-GFP+/*msnCherry*+ cells (considered as lamelloocytes) were extracted from the cells counted by flow cytometry. Columns represent the mean number of lamelloocytes per larva at the indicated time (in hours) after parasitism for the YR-*msnCherry* (dots) and YS-*msnCherry* (grey). The bar indicates the S.D. Each of the YR-*msnCherry* replicate is represented by a blue circle and YS-*msnCherry* by a purple square.

At the 22-24h (Figure 34G and H), an increase happened in the lamellocytes population which was stronger in YR-*msnCherry* compared to YS-*msnCherry*. The number of plasmatocytes seems to follow also the same pattern between the YR-*msnCherry* and YS-*msnCherry* lines. Thereafter, the number of lamellocytes looked constant in YS-*msnCherry* while it decreased in YR-*msnCherry* to reach a lower level at 28-30h (Figure 34K and L). The number of plasmatocytes seems also to decrease.

Figure 35 showed the mean of the cell populations (WGA+ cell population) and the value for each of the three replicates analysed by flow cytometry at each time. While some large scattering between the three replicates were apparent at some time points, the average total number of hemocytes per larva showed no significant difference at any of the time points between the resistant and susceptible lines.

Nevertheless, a sharp increase in the total number of hemocytes is observed for both *Drosophila* lines at 48h post-parasitism (Figure 35). We expected to see a difference in the number of hemocytes between the 2 lines around 15h post-parasitism as reported in Russo *et al.*, (2001). While fluctuation occurs around this time they were not significant, the large variations between replicate may explain that they were underevaluated. The difference may also due to the fact that two very different technics were used to estimate the number of cells: in Russo *et al.*, (2001), the number of cells was evaluated using hemocytometer which are more prone to human error, whereas the flow cytometry limits the human error to the preparation of the sample and the gating on the data collected but not the method of data collection.

From the cytometry data, we also extracted at the different time post-parasitism the WGA+/Cherry+ cell numbers, which represent the lamellocyte population (Figure 36). During the first 15h, the number of lamellocytes is low and no change was observed in both *Drosophila* lines. At 22-24h after parasitism, for both lines an increase occurred that seems stronger in the YR-*msnCherry* line although one of the replicates was very low (only 16 lamellocytes were counted). The difference in the lamellocytes number between the two lines even more visible at 26-28h where the YR-*msnCherry* have 2.8 times more lamellocytes than the YS-*msnCherry* line. Surprisingly the lamellocytes number dropped in the YR-*msnCherry* at 28-30h, returning to the same level than in the YS-*msnCherry* line, before increasing again at 46-48h. I do not have an explanation for this drop and more replicates and further work should be done to confirm and explain it. Altogether, the general trend in this experiment is a gradual increase in the number of lamellocytes in YS with a more chaotic variation in YR.

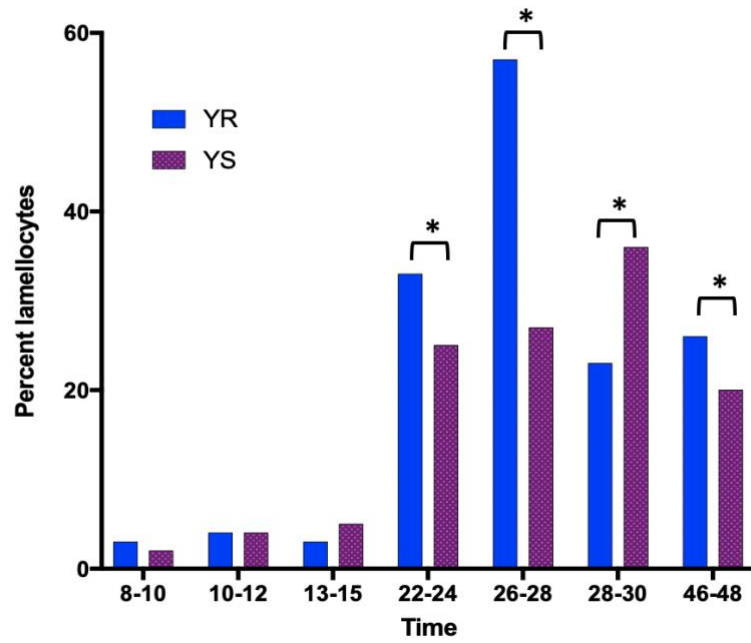


Figure 37: Lamellocytes proportion in hemolymph after parasitism. The number of lamellocytes (WGA+/msnCherry+) was divided by the total number of hemocytes (WGA+) to give the lamellocytes proportion in YR-msnCherry (blue) and YS-msnCherry (purple) hemolymph: Statistical comparison of the resistant strain to the susceptible strain: $p > 10^{-10}$.

For all these experiments, we collected the hemolymph from various number of larvae (depending on the survival, Table 13) and the quantity of hemolymph collected may varied from one sample to another. In order to attenuate this risk, we analysed the proportion of the lamellocytes (WGA+/mCherry+) among the total number of cells counted (WGA+) at each time after parasitism.

The lamellocytes proportions (Figure 37) obtained in YR-*msnCherry* and YS-*msnCherry* hemolymph at 10, 12 and 15h after parasitism is low (less than 5%) and showed no significative difference. Between 15h to 24h, the proportion of lamellocytes increased by 5-fold in the YS-*msnCherry* to reach 25% and 10-folds for the YR-*msnCherry* line (33%) and continue to increase in this line to reach 55% of the circulating cells between 26-28h. Afterwards, in the resistant line, the proportion of lamellocytes decreases by more than half and stay at this level until 48h. In YS-*msnCherry* it increased slightly at 28-30h to reach its maximum (36%), before going down at 48h.

The decrease in proportion at 30h can be explain by the drop in the number of lamellocytes observed in Figure 36, as less lamellocytes but about the same number in hemocytes leads to a decrease in the proportion of lamellocytes in the whole population. The one at 48h post-parasitism by the overall increase in the number of hemocytes (Figure 35), probably an increase in the number of circulating plasmatocytes.

In this experiment, we have observed an increase in lamellocytes proportion 24h post-parasitism in both the YR-*msnCherry* and YS-*msnCherry* lines, there are more lamellocytes when the *Drosophila* is resistant and the lamellocytes count increases more sharply in the resistant line when it looks steadier in the susceptible one. All those elements indicate that there is a difference in the cellular response to the parasitism between the YR and YS lines at the lamellocytes level.

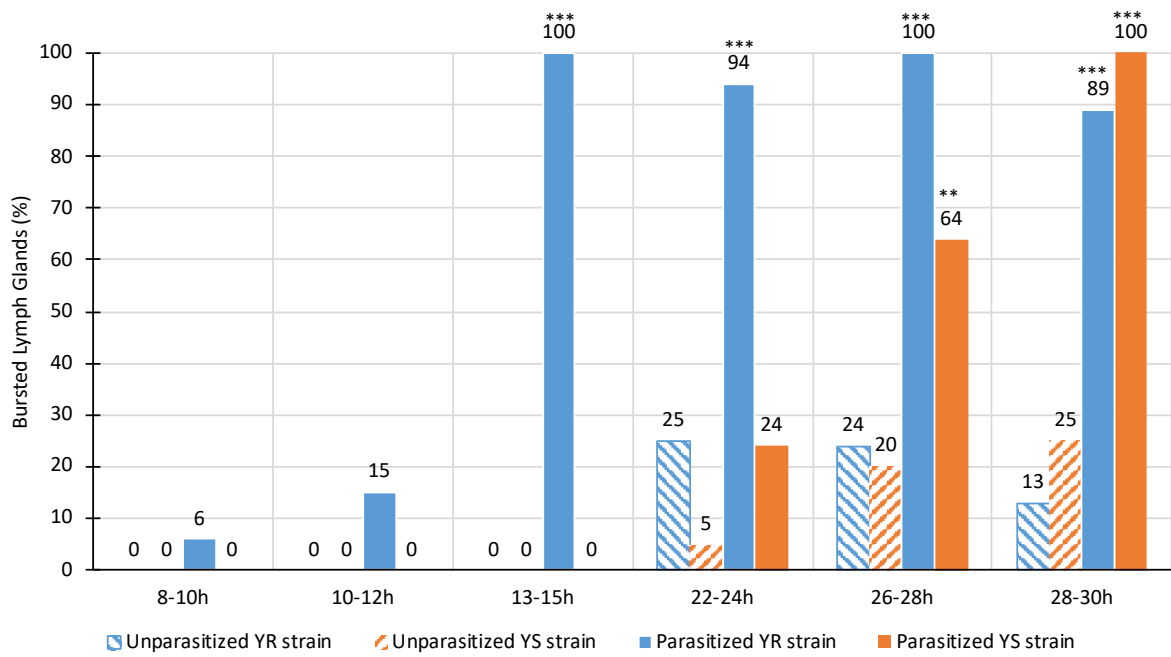


Figure 38: State of the lymph gland in control and in parasitized conditions. The percentage of bursting lymph gland was evaluated with time in parasitized and control larvae for the YR strain (control condition: hatched blue; parasitized condition: plain blue) and YS strain (unparasitized condition: hatched orange; parasitized condition: plain orange). For both fly lines, a replicate of stage 2 larvae was split in two at zero time, one was used as a control the other was parasitized. The unparasitized condition was compared to the parasitized condition using glmer on 3 replicates for each condition (p-value: *: <0,05; **: <0,01; ***: <0,005). Details of the number of dissected larvae in Table 2 in Annexe.

C. Timing of the lymph gland bursting in parasitized YR and YS lines

Lamellocytes can originate from the transdifferentiation of circulating plasmatocytes (Honti *et al.*, 2010; Stofanko *et al.*, 2010; Anderl *et al.*, 2016), the sessile compartment (Markus *et al.*, 2009) and the bursting of the lymph gland (Lanot *et al.*, 2001). To investigate further the origin of the lamellocytes peaks that was observed between 22-24h after parasitism, we have established the kinetic of the lymph gland bursting profile under normal developmental condition and *Isy* parasitism for both YR and YS lines. The lymph glands were observed by microscopy after dissection of the larvae, which allowed also to characterize their parasitism state.

At 8-10h post-parasitism, only on the YR parasitized larvae some individuals (1 on the 17 observed) have a bursted lymph gland (Figure 38). For the other conditions, all larvae had an intact lymph gland. At 10-12h post-parasitism, bursted lymph gland were only observed in the YR parasitized larvae (3 on the 20 observed). At 13-15h post-parasitism, 100% of the lymph gland of the resistant parasitized larvae were bursted (a similar situation to all following time) while all lymph gland observed in the YS parasitized or the unparasitized control larvae in both lines were intact. At 22-24h, 25% of the YR control larvae have bursted lymph gland but only 5% in the YS control, and the level for the parasitized YS reached 24%. At 26-28h, the YR control larvae remained the same (24%) while in the YS bursting increased to 20%. In parasitized condition, the level of bursted lymph gland for the susceptible line reached 64%. At the 28-30h post-parasitism, in control conditions, the percentage of burst lymph gland remained almost the same for YS and YR, and almost all lymph gland had bursted. In control condition, the lymph gland of all larvae will burst at the end of the 3rd larval stage, before or at pupation. The lymph glands of YR and YS start to burst 22-24h after parasitism but only in small proportion (25% and 5%). It could be due to the dissection, the manipulation of the larvae, or natural earlier bursting in some larvae. We thus observed that in the YR line, parasitism induces an accelerated bursting of the lymph gland and that after 13-15h, all have released their hemocytes (Figure 38). While, in YS, no reaction is observed in the first 13-15h post-parasitism, the bursting was observed starting at 22-24h and showed a gradual increasing until all lymph glands were bursted at 28-30h. This bursting kinetic difference is certainly a key element of the resistant phenotype: the faster release of hemocytes allows for a rapid encapsulation of the parasitoid egg that is needed to be done by 22-24h.

It will be interesting to interpret the earlier release of hemocytes from the lymph gland in YR in the context of the timing of formation of the capsule around parasitoid eggs in YR and YS (few capsules formed) fly larvae, which has not yet been described.

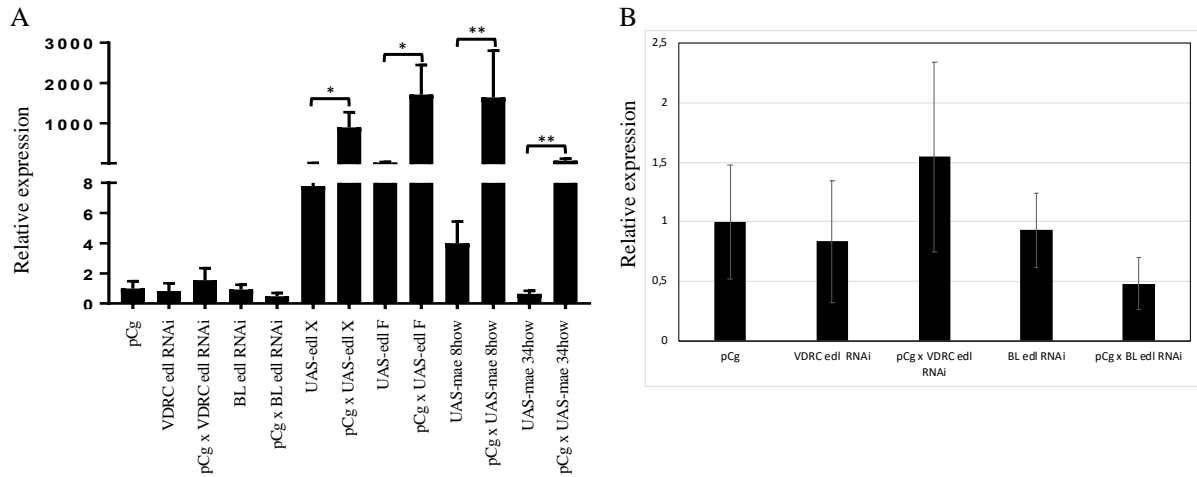


Figure 39: Measure Levels of relative gene expression in *Drosophila* larvae. A: relative expression of *edl* in the fat body of larvae from the following lines: pCg, VDRC *edl* RNAi, BL *edl* RNAi, UAS-*edl*X, UAS-*edl*F, UAS-*mae*8how, UAS-*mae*34how and from the F1 crosses between pCg and the RNAi lines and pCg and the UAS lines. B: relative expression of *edl* in the fat body of larvae from the following lines: pCg, VDRC *edl* RNAi, BL *edl* RNAi and from the F1 crosses between pCg and the RNAi lines. *edl* expression was normalized to 2 reference genes. Values are means \pm SE of three biological replicates, each composed of three technical replicates. Asterisks indicate statistical differences at $P < 0.05$ *, $P < 0.01$ **, and $P < 0.001$ ***, according to the Student's t-test.

D. Role of *edl* in the resistance phenotype

The two previous phenotypic studies indicated clearly a difference in the timing of the immune response between the YS and YR lines mainly due to a differential increase in lamellocytes (Russo *et al.*, 2001). This is compatible with a major role of the *edl* gene in resistance/sensitivity as suggested by: i) the genetic studies, ii) the results with the transgenic strains for the *edl* R allele, and iii) the *edl* role in cell proliferation/differentiation through regulation of two other transcription factors, *pnt* P2 and *aop* (Vivekanand *et al.*, 2004), we tested the effect of expression of *edl* transgene in different tissues, of RNAi lines for *edl* and predicted or known *edl* interactants, on the resistance against *L. bouhardi* ISy. We took advantage of one of the many tools available in *Drosophila*, the UAS-Gal4 system. It allows to drive or inhibit the expression of a gene in a spatial or temporal manner by crossing a strain carrying a specific promoter upstream of the *Gal4* encoding-gene with a strain carrying the UAS (Upstream Activation Sequence) promoter upstream of a gene of interest or RNAi sequence. Therefore, the Gal4 will be transcribed following the specific promoter expression pattern and the binding of Gal4 to the UAS promoter will activate the gene or RNAi expression (Neckameyer & Argue, 2013).

The Table 1 described all the different *drosophila* lines used for this approach. UAS-RNAi lines are available for *edl*, *pointed* and *aop* at the Bloomington Drosophila Stock Center and the Vienna Drosophila Resource Center. We also have transgenic lines in the lab containing the resistant allele coding sequence, UAS-*mae* 8how and UAS-*mae* 34how, and the susceptible allele under an UAS promoter (UAS-*edl*F and UAS-*edl*X, from Dr. Yamada). For this project, we selected 5 promoter strains: *pHml* that drive the expression in hemocytes, *pCg* in hemocytes, the fat body and the lymph gland, and *e33c*, *pg125*, *pCol* for the expression of the UAS-genes/RNAi in different parts of the lymph gland (the whole lymph, the medullary zone and the PSC, respectively).

First, to control that the UAS-lines worked, the level of expression of *edl* in the overexpressing and RNA interference lines was evaluated by qPCR. We crossed them with the *pCg-GAL4* line in order to have the expression of the transgenes in the fat body to facilitate the RNA extraction as the fat body is very abundant in the L2 and L3 larvae. In the overexpressing lines, the transgene expression is 115 times higher for the UAS-*edl*X line, 67 times higher for the UAS-*edl*F, 410 times higher for the UAS-*mae* 8how and 117 times higher for the UAS-*mae* 34how line than from the same line not crossed (Figure 39A). The expression of *edl* is low in the *pCg-GAL4* and the *edl*-RNAi lines (Figure 39B) in agreement with the level of expression normal conditions reported in the data base (Flybase). Due to this low expression, results showed a large variability, and level expression after the induction of the *edl*-RNAi in crosses were not significantly different from the control lines.

Table 3: Representation of the encapsulation rate of the promotor-Gal4 and UAS-sequence of interest lines before any cross. Encapsulation rate: in blue: between 0% and 30%; in grey: between 31% and 60%; in orange: between 61% and 100%.

	Function of <i>Drosophila</i> lines	Encapsulation rate
Nasrallah	Control	2%
pCg	Expression of Gal 4 in hemocytes, fatbody and lymph gland	4%
pCol	Expression of Gal 4 in the PSC	10%
e33c	Expression of Gal 4 in the lymph gland	85%
pHml	Expression of Gal 4 in hemocytes	0%
pg125	Expression of Gal 4 in the medullary zone	4%
UAS edl F	UAS - susceptible allele of <i>edl</i>	5%
UAS edl X	UAS - susceptible allele of <i>edl</i>	0%
UAS Mae 34 how	UAS - resistant allele of <i>edl</i>	35%
UAS Mae 8 how	UAS - resistant allele of <i>edl</i>	26%
BL edl RNAi	UAS - RNAi of <i>edl</i>	3%
VDRC edl RNAi	UAS - RNAi of <i>edl</i>	0%
VDRC Pnt P2 RNAi	UAS - RNAi of <i>pnt P2</i>	0%
BL pnt RNAi	UAS - RNAi of <i>pnt P2</i>	0%
BL 26759 aop RNAi	UAS - RNAi of <i>aop</i>	12%
BL 35404 aop RNAi	UAS - RNAi of <i>aop</i>	0%

Table 4: Representation of the encapsulation rate of the descendants of the crosses for the overexpression of *edl*. Encapsulation rate: in blue: between 0% and 30%; in grey: between 31% and 60%; in orange: between 61% and 100%. The encapsulation rate in the cross with Nasrallah is compared to the cross of interest using glmer on 3 replicates (p-value: *: <0,05; **: <0,01; ***: <0,005).

	Nasrallah	UAS edl F	UAS edl X	UAS Mae 34 how	UAS Mae 8 how
Nasrallah	2%	12%	14%	23%	28%
pCg	11%	4% *	13%	40% **	57% *
pCol	15%	0%	21%	62% ***	40% ***
e33c	39%	33%	21%	45%	34%
pHml	11%	4%	14%	64% ***	56% ***
pg125	13%	0%	0%	15%	17%

A rescue experiment was performed to check if the susceptible phenotype from the BL *edl* RNAi could be rescued. The insertion of the UAS-*mae* 34how line could be located on chromosome 3. The resistance level of descendants of the cross presented in Figure 27 had an encapsulation rate raised to 48% (34/70 larvae with capsules, 3 replicates). Which does not allow us to conclude on the subject of the rescue. Other crosses will have to be performed. Unfortunately, the insertion of the UAS-*mae* 8how could not be localised due to the death of the larvae. We used the crosses between the chosen UAS and Gal4 lines with the Nasrallah susceptible strain as a control to ensure the difference observed is not only due to a crossing effect.

For each used line, I also checked the resistant (encapsulation)/susceptible (no encapsulation) status (Table 3). Except for UAS-Mae 34how and e33c lines, the parasitism outcome was success suggesting susceptible fly lines. For UAS-Mae 34how (carrying the resistant allele sequence of *edl*), parasitism failure was intermediate at 35%, while for the e33c-GAL4 line a strong resistance phenotype of the larvae was observed (85%). There is thus a variation in the response to parasitism in the different lines.

1. Expression of the *edl* resistant allele in hemocytes confers the resistance phenotype

After induction of the expression of the susceptible allele of *edl* (UAS-*edl*F and UAS-*edl*X) with any GAL4-promotor, parasitism success was the major outcome of the Isy parasitism (Table 4). Therefore, the overexpression of the susceptible allele in the hemocytes, the lymph gland and the fat body did not change the susceptible phenotype of the parent lines. The crosses with e33c-GAL4 reduces the parasitism failure to the same level than the Nasrallah cross used as control.

When the expression of the resistant allele of *edl* (UAS-*mae* 8how and UAS-*mae* 34how) is induced in the hemocytes/fat body/lymph gland, the hemocytes, or the PSC (cross with pCg-GAL4, pHml-GAL4 and pCol-GAL4), a significant increase in the encapsulation rate occurred in the F1 (Table 4). When it was only in the medullary zone (cross with pg125-GAL4), the susceptible phenotype did not change (Table 4).

The overexpression of the resistant *edl* allele in hemocytes clearly conferred the expression of a resistance phenotype, while the overexpression in the whole lymph gland or its medullary zone is not sufficient as the pCg-Gal4 line induced an expression in the hemocytes, the fatbody and the lymph gland, the effect of an expression using this promotor is the combination of the effect of the expression in the 3 tissues.

Table 5: Encapsulation rate of the F1 from the crosses for the RNAi of edl. The crosses between the chosen UAS-RNAi and Gal4 driver lines with the Nasrallah susceptible strain were used as a control to ensure that the difference observed is not only due a crossing effect (Nasrallah column and line). Encapsulation rate: in blue: between 0% and 30%; in grey: between 31% and 60%; in orange: between 61% and 100%. The encapsulation rate of the cross of interest is compared with it cross with Nasrallah to using glmer on 3 replicates (p-value: *: <0,05; **: <0,01; ***: <0,005).

	Nasrallah	BL edl RNAi	VDRC edl RNAi
Nasrallah	2%	0%	21%
pCg	11%	28%	41% **
pCol	15%	20%	24%
e33c	39%	23%	38%
pHml	11%	37% ***	62% ***
pg125	13%	17%	30% **

Table 6: Encapsulation rate of the F1 from the crosses for the RNAi of pointed and aop. The crosses between the chosen UAS-RNAi and Gal4 driver lines with the Nasrallah susceptible strain were used as a control to ensure that the difference observed is not only due a crossing effect (Nasrallah column and line). Encapsulation rate: in blue: between 0% and 30%; in grey: between 31% and 60%; in orange: between 61% and 100%. The encapsulation rate of the cross of interest is compared with it cross with Nasrallah to using glmer on 3 replicates (p-value: *: <0,05; **: <0,01; ***: <0,005).

	Nasrallah	VDRC Pnt P2 RNAi	BL pnt RNAi	BL 26759 aop RNAi	BL 35404 aop RNAi
Nasrallah	2%	30%	50%	40%	61%
pCg	11%	-	57%	75% ***	41% **
pCol	15%	54%	32%	16%	14%
e33c	39%	68%	57%	55%	66% ***
pHml	11%	86% ***	59% *	40%	44% ***
pg125	13%	40%	34%	12% **	63% ***

To confirm the role of *edl* and better understand whether the classical molecular mechanism in which *edl* binding with either the ETS transcription factors *pnt* or *aop* fine-tunes target genes expression is also at work (Vivekanand *et al.*, 2004), I used different RNAi-lines induced with the same set of promoter-GAL4 lines.

2. Effect of the repression of *edl*, *aop* and *pointed*

RNAi is a cellular mechanism that uses RNA-guided degradation of messenger RNA transcripts. It is an important tool to identify and characterize gene function (Perrimon *et al.*, 2012; Heigwer *et al.*, 2018). When the promoter-Gal 4 is crossed to a the UAS^{-RNAi} lines, the expression of a specific hairpin structure is induced in a tissue specific manner and the silencing is done on the target gene in the tissue of interest. All the UAS^{-RNAi} lines I used (see Table 3) exhibited a susceptible phenotype toward the *L. boularidi* Isy (Table 1), when crossed with Nasrallah the *Edl*^{-RNAi} lines showed a susceptible phenotype (Table 5), however the two *Yan*^{-RNAi} and the *Pointed*^{-RNAi} lines showed an increase in resistance (Table 6).

After induction of the transgene expression by crossing the Bl-*edl*-RNAi line with the driver Gal4 lines, only the cross with the pHml-Gal4, which drives the expression in hemocytes, induced an increase in the encapsulation ability (Table 5). With the VDRC-*edl*-RNAi line, an increase in the encapsulation ability was observed when the RNAi was expressed in the medullary zone (pg125), the hemocytes (pHml), the lymph gland and the fat body (pCg) (Table 5). Induction of BL-*pnt*-RNAi expression by all driver Gal4 induced a moderate level of resistance (Table 6) including with the Nasrallah line. Thus, the only cross showing a statistically significant increase was the cross with the hemocyte driver pHml-Gal4 where the resistance was about 86% in average. The situation was different with VDRC-*Pnt*-P2 RNAi: when crossed with the Nasrallah line no significative effect was observed, and when crossed with pCg (hemocytes, lymph gland and fat body) no egg hatched. While the crosses with e33c-Gal4 and pHml-Gal4 increase the resistance only with this last it was statistically significative. Thus, for both RNAi lines only induction in hemocytes induced a significative increase in the encapsulation ability.

The crosses of the *UAS-Aop*^{RNAi(BL26759)} and *UAS-Aop*^{RNAi(BL35404)} lines with the driver-Gal4 and also the Nasrallah lines showed different outcome in parasitism. Cross of *UAS-Aop*^{RNAi(BL26759)} with Nasrallah increase moderately the resistance compare to the non-crossed line while this effect was more intense with *UAS-Aop*^{RNAi(BL35404)} (Table 3). When compared to the Nasrallah cross situation only the *UAS-Aop*^{RNAi(BL26759)} x pCg-Gal4 (hemocytes, lymph gland and fat body expression) showed a statistically significative increase in resistance while the cross with the pg125-Gal4

(expression in the medullary zone) induced a significant decrease (Table 6). For the *UAS-Aop^{RNAi(BL35404)}* crosses, a statistical decrease of the encapsulation ability was observed with pCg-Gal4 and pHml-Gal4, while a statistical increase in resistance was found with e33c-Gal4 and pg125-Gal4 (Table 6). Thus, the silencing of the two *Aop^{RNAi}* seems to have different and sometimes opposite effects in the different tissues.

The RNAi of *aop* and *pointed* conferred a resistance phenotype when done in specific tissues (hemocytes for *pointed* and different ones of *aop*), therefore, it was interesting to look at the other potential interactants of *edl* as its main function is to interact with transcription factors.

E. Potential interactants of *edl*

Edl, *pnt* and *aop* play important roles in *Drosophila* eye development (Vivekanand *et al.*, 2004) where *pnt* and *aop* act as nuclear effectors of the Ras/MAPK pathway (Lusk *et al.*, 2017) and provide an excellent example of the interplay between active and repressive functions of ETS proteins. The DNA binding domain, called the ETS-domain, is the common characteristic shared by all proteins that belong to this family of transcription factors. *Edl* has no ETS domain but interacts directly with *Pnt P2* and *Yan* through a domain termed the *Pointed (PNT)* domain (Klämbt, 1993). The PNT domain is approximately 80 residues long (Figure 40) and has a similar organization as the sterile alpha motif (SAM) domain that is involved in protein-protein or protein-RNA interactions (Qiao & Bowie, 2005). In about one-third of ETS proteins (ETS, ETV, ERG families plus GABP α and SPDEF), a functional PNT domain is present.

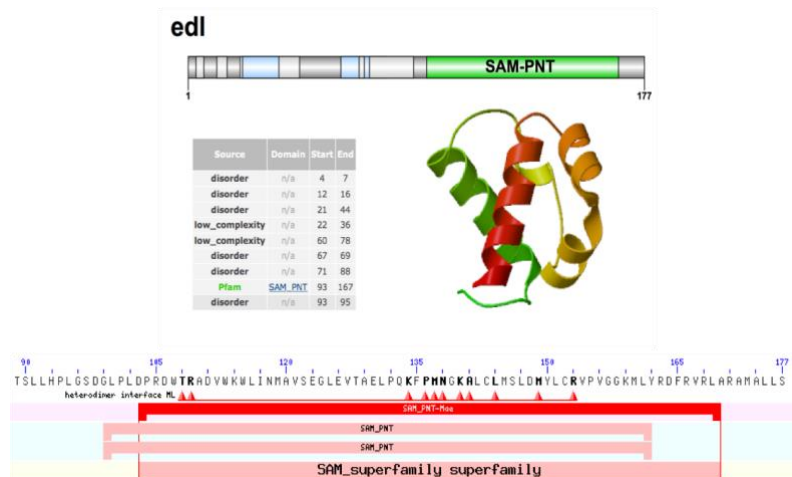


Figure 40: *Edl* structural feature. The *EDL* protein (top) has disordered (grey) and low complexity zones (blue), and only one clear *SAM-PNT* domain (green) shown in 3D. The lower panel shows the sequence of the *Edl* *SAM-PNT* domain with the important amino acids involved in interaction in bold.

1. Bioinformatic comparison of orthologs between *Drosophila* species

edl is conserved in evolution and thus is present in the genome of other *Drosophila* species. However, the level of variability of this gene within the *Drosophila* species has not been evaluated, and particularly of its SAM-PNT domain (Figure 40). I did a BLAST (Basic Local Alignment Search Tool) with the CDS sequence and the protein sequence of the *D. melanogaster edl* gene (BLASTn in Flybase and BLASTP in NCBI) to find the orthologous genes/proteins in other drosophila species. On Flybase, 24 hits were obtained some with annotated sequences and some with scaffolds, e-values varies from $1.8E^{-157}$ to $10E^{-28}$, and these sequences represented 20 different species (Table 7). The NCBI protein BLAST on *Drosophila* phylum returned 26 sequences each one from a different species that matched on their full length with the *D. melanogaster edl* sequence (Table 8) suggesting that one *edl* ortholog exist for these species. These 26 sequences ranged from 64 to 85 % of identity and e-value from $2E^{-70}$ to $8E^{-130}$. However, 66 sequences (with species redundancies) matched only on the C-terminal part on the SAM-PNT domain. The protein sequences alignment of the 27 species evidenced a very high level of amino acids conservation of the SAM domain (Figure 41), suggesting a strong evolutive pressure to maintain its structure unchanged in *Drosophila*. Conservation of genes across species indicates that this gene could be important for the organism (King Jordan *et al.*, 2002), therefore, the conservation of the protein sequence of *edl* could point out to the important of this protein for *Drosophila* development.

Since the SAM-PNT domains of proteins may interact together, the large number of proteins matches in *Drosophila* database observed with the *edl* SAM-PNT domain suggested that other interactants than *aop* and *pnt* P2 may bind to *edl*. I thus started a bioinformatic search for the most probable of these interactants.

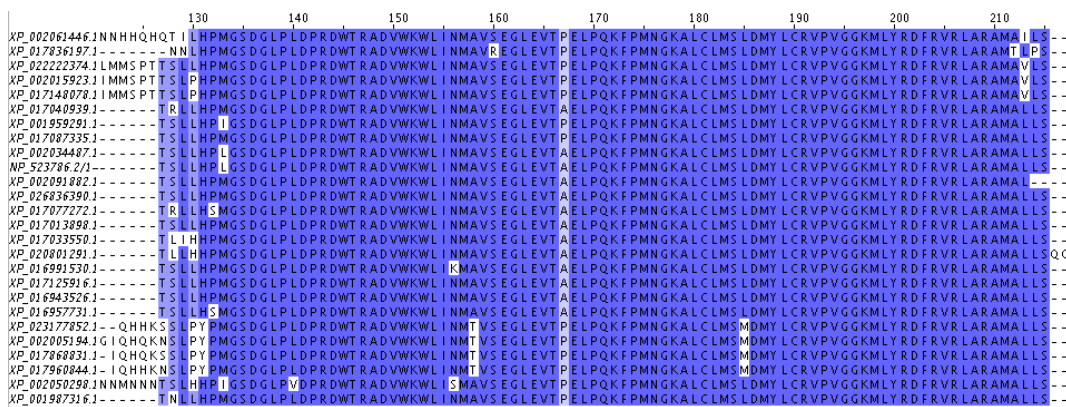


Figure 41: Muscle alignment of the 26 proteins with a SAM-PNT domain of the different *Drosophila* species. The sequence from position 130 to 210 is shown: Background dark blue, identical amino acid; light blue, substitution; white, different.

Table 7: Orthologues of *edl* in *Drosophila* species: the genomes of the different *Drosophila* species do not have the same quality, for some species only the scaffolds are available and have been represented here. The species for which a gene name is available are highlighted in yellow.

<i>Drosophila</i> species	Matching gene or scaffold
<i>Drosophila ananassae</i>	GF12797
<i>Drosophila biarmipes</i>	scf718000302291
<i>Drosophila bipectinata</i>	scf7180000395751
<i>Drosophila elegans</i>	scf7180000491228
<i>Drosophila erecta</i>	GG20954
<i>Drosophila eugracilis</i>	scf71800004096663
<i>Drosophila ficusphila</i>	scf7180000453811
<i>Drosophila grimshawi</i>	GH21853
<i>Drosophila miranda</i>	positions on chromosome 3
<i>Drosophila mojavenensis</i>	GI20354
<i>Drosophila persimilis</i>	GL11322
<i>Drosophila pseudoobscura</i>	GA13479
<i>Drosophila rhopaloa</i>	scf7180000766409
<i>Drosophila sechellia</i>	GM19886
<i>Drosophila simulans</i>	GD25369
<i>Drosophila suzukii</i>	scaffold2_1100000004510
<i>Drosophila virilis</i>	GJ22079
<i>Drosophila willistoni</i>	GK20716
<i>Drosophila yakuba</i>	GE13893
<i>Drosophila kikkawai</i>	scf718000302682

Table 8: List of proteins obtained by an edl blast on NCBI *Drosophilidae* (BLAST parameter were set as default values)

<i>Description</i>	<i>Species</i>	<i>Score</i>	<i>Evalue</i>	<i>ident</i>	<i>Accession n°</i>
<i>ETS-domain lacking</i>	<i>Drosophila melanogaster</i>	364	8,00E-130	1	NP_523786.2
<i>PREDICTED: uncharacterized protein LOC108112037</i>	<i>Drosophila eugracilis</i>	309	7,00E-108	0,88	XP_017077272.1
<i>uncharacterized protein LOC6547220</i>	<i>Drosophila erecta</i>	303	2,00E-105	0,89	XP_026836390.1
<i>PREDICTED: uncharacterized protein LOC108145186</i>	<i>Drosophila elegans</i>	302	4,00E-105	0,86	XP_017125916.1
<i>GM19886</i>	<i>Drosophila sechellia</i>	300	3,00E-104	0,95	XP_002034487.1
<i>PREDICTED: uncharacterized protein LOC108053401</i>	<i>Drosophila rhopaloa</i>	299	7,00E-104	0,89	XP_016991530.1
<i>PREDICTED: uncharacterized protein LOC108019927</i>	<i>Drosophila sukukii</i>	298	1,00E-103	0,86	XP_016943526.1
<i>uncharacterized protein Dana_GF12797</i>	<i>Drosophila ananassae</i>	296	6,00E-103	0,82	XP_001959291.1
<i>PREDICTED: uncharacterized protein LOC108118906</i>	<i>Drosophila bipectinata</i>	296	8,00E-103	0,82	XP_017087335.1
<i>uncharacterized protein Dyak_GE13893</i>	<i>Drosophila yakuba</i>	293	1,00E-101	0,88	XP_002091882.1
<i>PREDICTED: uncharacterized protein LOC108029749</i>	<i>Drosophila biarmipes</i>	293	2,00E-101	0,85	XP_016957731.1
<i>PREDICTED: uncharacterized protein LOC108082594</i>	<i>Drosophila kikkawai</i>	290	3,00E-100	0,85	XP_017033550.1
<i>uncharacterized protein LOC110178486</i>	<i>Drosophila serrata</i>	290	3,00E-100	0,82	XP_020801291.1
<i>PREDICTED: uncharacterized protein LOC108068686</i>	<i>Drosophila takahashii</i>	288	1,00E-99	0,8	XP_017013898.1
<i>PREDICTED: uncharacterized protein LOC108087866</i>	<i>Drosophila ficusphila</i>	272	2,00E-93	0,79	XP_017040939.1
<i>uncharacterized protein Dvir_GJ22079</i>	<i>Drosophila virilis</i>	255	2,00E-86	0,72	XP_002050298.1
<i>PREDICTED: ETS homologous factor</i>	<i>Drosophila busckii</i>	252	1,00E-85	0,73	XP_017836197.1
<i>GH21853</i>	<i>Drosophila grimshawi</i>	249	2,00E-84	0,73	XP_001987316.1
<i>PREDICTED: uncharacterized protein LOC108159364</i>	<i>Drosophila miranda</i>	249	5,00E-84	0,72	XP_017148078.1
<i>uncharacterized protein LOC6591154</i>	<i>Drosophila persimilis</i>	248	8,00E-84	0,72	XP_002015923.1
<i>ETS homologous factor</i>	<i>Drosophila obscura</i>	247	2,00E-83	0,7	XP_022222374.1
<i>Hypothetical predicted protein</i>	<i>Drosophila guanche</i>	246	8,00E-83	0,69	SPP73495.1
<i>uncharacterized protein LOC6638239</i>	<i>Drosophila willistoni</i>	238	1,00E-79	0,67	XP_002061446.1
<i>uncharacterized protein LOC111604157</i>	<i>Drosophila hydei</i>	229	1,00E-76	0,66	XP_023177852.1
<i>PREDICTED: protein YIPF1 homolog</i>	<i>Drosophila arizonae</i>	216	3,00E-71	0,67	XP_017868831.1
<i>PREDICTED: bifunctional serine/threonine-protein kinase/NEDD4-like E3 ubiquitin-protein ligase</i>	<i>Drosophila navojoa</i>	216	6,00E-71	0,66	XP_017960844.1
<i>uncharacterized protein Dmoj_G120354, isoform A</i>	<i>Drosophila mojavensis</i>	214	2,00E-70	0,64	XP_002005194.1

Table 9: List of all the genes coding for SAM domain IPR013761 containing proteins. The IPR003118 subfamily is listed first, then IPR001660 and last IPR013761.

	Subtype	Name
Pointed domain	IPR003118	aop
	IPR003118	l(3)mbt
	IPR003118	pnt
	IPR003118	Ets97D
	IPR003118	Ets98B
	IPR003118	Ets21C
	IPR003118	edl
Sterile alpha motif domain	IPR001660	Bicaudal C
	IPR001660	gnu (giant nuclei)
	IPR001660	Sex comb on midleg
	IPR001660	Polyhomeotic distal
	IPR001660	Polyhomeotic-proximal chromatin protein
	IPR001660	Protein matrimony
	IPR001660	D-spinophilin, core domains
	IPR001660	Protein Smaug
	IPR001660	Connector enhancer of KSR protein CNK
	IPR001660	CG2662
	IPR001660	Eph receptor tyrosine kinase
	IPR001660	Tankyrase
	IPR001660	CG4238
	IPR001660	CG15625
	IPR001660	CG9098
	IPR001660	CG13996
	IPR001660	Samuel or Moses
	IPR001660	Sfmbt
	IPR001660	CG16812
	IPR001660	Sans
	IPR001660	Caskin
	IPR001660	Liprin-γ
	IPR001660	Liprin-
	IPR001660	CG17625
	IPR001660	CG4393
	IPR001660	Stromal interaction molecule homolog
	IPR001660	Liprin-α
	IPR001660	Protein aveugle
	IPR001660	Shal K[+] channel interacting protein
	IPR001660	Sphingomyelin synthase-related 1
	IPR001660	Diacylglycerol kinase
	IPR001660	Ect4 (Ectoderm-expressed 4)
IPR001660	CG34377	
SAM domain	N.A.	Gasz
	N.A.	Gemini
	N.A.	Crossveinless c
	N.A.	CG42672

2. Bioinformatic search for new edl potential interactants

First, I search for all *D. melanogaster* proteins containing an InterPro tag from the SAM domain family (<https://www.ebi.ac.uk/interpro/>). The main identification number corresponding to the SAM domain family is IPR013761 and this family is subdivided into 5 subgroups:

- Sterile alpha motif domain (IPR001660)
- Pointed domain (IPR003118)
- Ste50, sterile alpha motif (IPR015316)
- Transcription factor, GA-binding, alpha subunit (IPR016312)
- Caskin1/2, SAM repeat 1 (IPR035497)

131 *Drosophila* proteins were in the SAM domain family list (IPR013761): 15 within the IPR003118, 97 in the IPR001660 and 19 in the IPR013761. Out of these 131 proteins, I could identify 44 genes (listed in Table 9): 4 within IPR013761, 33 with IPR001660 and 7 with IPR003118.

Since edl, aop and pnt P2 have a Pointed domain type (IPR003118), I hypothesized that potential interactants of edl belong also to this group. Indeed, one of the features of the SAM domain proteins is that, in addition to interact with other SAM domain proteins, they oligomerize by auto association through the SAM domain (Isono *et al.*, 2013; Denay *et al.*, 2017). Thus, more the protein domain will be similar to the edl/aop/pointed SAM domain, highest is the chance that it associates with them.

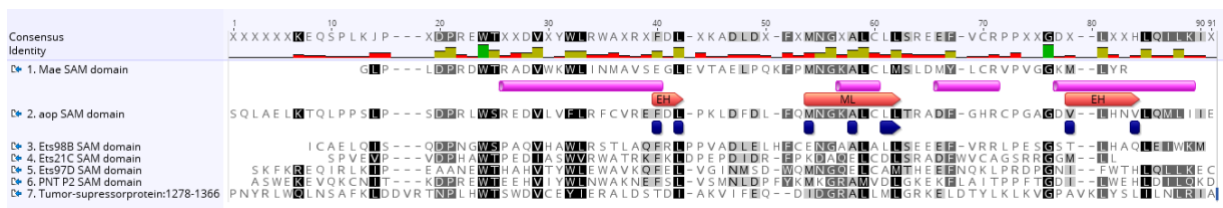


Figure 42: Alignment of the SAM domain IPR003118: The positions indicated in blue are the ones described as the most important amino acids for the interaction between aop and edl (Qiao *et al*, 2004). ML surface of the edl SAM domain are represented in pink and EH surface of the aop SAM domain in red.

Table 10: Summary of the amino acids positions important for SAM-SAM domain interaction (in red non-conservative substitution; in green conservative substitution). When amino acid/amino acid, the first amino acid is compared to edl and the second to aop. Position 82 is absent in EDL due to a shorter sequence. The amino acids in red are different to the one in aop SAM domain sequence.

AA position	Edl	Aop	Pnt P2	Ets98B	Ets21C	Ets97D	l(3)mbt
40	E	F	F	F	F	F	T
42	L	L	L	L	L	L	I/I
54	M	M	M	E	K	M	I
58	A	A	A	A	E	E	A
61	L	L	D	L	D	A/A	M
62	M	L	L/L	L/L	L/L	M	L/L
78	M	V	I/I	T	M	I/I	A/A
82	-	V	H	Q	-	H	L

In the IPR003118 subtype there are 2 proteins which only have a SAM domain (*l(3)mbt* (*CG5954*) and *edl* itself) and 5 with both a SAM and an ETS domain (*Ets21C*, *Ets98B*, *Ets97D*, *aop* and *pnt*). All these genes are transcription factors: for example, *l(3)mbt* (or lethal(3) malignant brain tumour) is a transcription factor implicated in cell proliferation (Meier et al, 2012) and the ETS domain proteins are known to bind with DNA domain (Sharrocks et al, 1997). Alignment of the SAM domains from these proteins was thus done to identify whether they retain the main amino acid regions implicated in the *edl* binding (Figure 42). It has been shown that the ML surface of the *edl* SAM domain binds to the EH surface of the *aop* SAM domain, the 8 aa at these positions are indicated by blue boxes in Figure 42 (Qiao et al, 2004). As we can see, 4 out of the 7 amino acids (due the shorter sequence of *edl*) in the interaction surface are identical between *edl* and *aop* SAM domains while only 4 were identical with *PNT-P2*. However, 6 amino acids (including one conservative substitution (CS)) were similar between *aop* and *pnt-P2*. For *ets98B*, 4 amino acids (+ 1CS) were identical with *edl* and 5 (+ 1CS) out of the 8 are identical with *aop*, while for *ets21C*, only 3 with *edl* and 4 with *aop*. For *ets97D*, 4 with *edl* (+ 1CS) and 3 with *aop* (+ 2CS). For *l(3)mbt*, 1 is identical to *edl* (+ 1CS) and 2 for *aop* with 3CS. (Table 10)

Those most conserved positions between the ETS transcription factors are the phenylalanine F40 (6 out of 7 factors), the leucine L42 (6/7 with 1CS), the leucine L62 (5/7) and the Alanine A58 (5/7) and then the methionine M54 (4/7) (Figure 42).

From these data, *Ets98B* and *Ets97D* seemed most interesting candidates to test their interaction with *edl in vivo*. From this analysis, *Ets79D* and *l(3)mbt* have been chosen to be tested in RNAi tests. *Ets97D* is an ETS transcription factor like *aop* and *pnt*. *L(3)mbt* is interesting due to its implication in cell proliferation (Wisnar et al, 1995).

Table 11: Representation of the encapsulation rate of the descendants of the crosses for the RNAi of l(3)mbt and ets97D as explained in (cf mat et met). The encapsulation rate was tested before crosses (- column and line). Encapsulation rate: in blue: between 0% and 30%; in grey: between 31% and 60%; in orange: between 61% and 100%. The encapsulation rate of the uncrossed line is compared to the cross of interest using glmer on 3 replicates (p-value: *: <0,05; **: <0,01; ***: <0,005).

	-	Nasrallah	BL L(3)mbt RNAi	BL Ets97D RNAi
-	-	2%	11%	8%
pCg	4%	11%	35% ***	32% ***
pCol	10%	15%	45% ***	42% **
e33c	85%	39%	38%	72%
pHml	0%	11%	67% ***	30% **
pg125	4%	13%	32% *	14%



Figure 43: The 2 peptides chosen for the antibody production

3. Ets97D and l(3)mbt *in vivo* RNAi tests

I have thus tested the effect of the expression of the UAS-Ets97D^{RNAi} and UAS-l(3)mbt^{RNAi} using the resistant/susceptible assay used before. The status of these mutant lines were tested before crossing and both were found susceptible (Table 11).

We induced the expression of the RNAi by crossing with the same driver-Gal4 lines previously used. Induction of the *l(3)mbt* RNAi expressed in the hemocytes, the whole lymph gland and the PSC increases in the encapsulation ability of the larvae (Table 11). The *Ets97D* RNAi as an effect when expressed in the hemocytes and the PSC. These results show that inhibition of both transcription factor may interfere with *edl* and then the resistance/susceptible phenotype. However, their action may be indirect, since the SAM domains shows large homology, expression of a RNAi against one of this protein domain produce an off target on the others. A direct interaction between these SAM-domain proteins and *edl* needs to be proved.

It shows that *l(3)mbt* expression inhibition allows more encapsulation when done in both hemocytes and the lymph gland whereas the effect on the ability to encapsulate is stronger when the inhibition of the expression of *Ets97D* is activated in the PSC.

4. Preliminary tools creation for testing physical interactions with *edl*

To find out which SAM domain proteins could physically interact with *edl* we wanted to use the co-immunoprecipitation method to catch *edl* and its interactants after overexpression in *Drosophila* S2 cells. This needs two things: to have a S2 line that overexpress *edl* and, since we did not want to modify the *edl* binding properties by adding a tag, an *edl* specific antibody.

To obtain an *edl* specific antibody we have synthesized 2 peptides that were not in the *edl* SAM domain sequence (to avoid cross reactions, Figure 43) that were used to immunized rabbits from which we have now the serums. In order to test/purify the antibody, we need to produce the *edl* protein.

To this end, I wanted to insert the CDS of *edl* in a pGEX expression vector. The pGEX vector allows the production of GST-tagged proteins under the control of the *tac* promoter, which is induced by the lactose analog isopropyl β -D-thiogalactoside (IPTG). pGEX vectors are also engineered with an internal *lacIq* gene which is a repressor protein that binds to the operator region of the *tac* promoter, preventing expression until induction by IPTG. The chosen pGEX vector allowed us to remove the GST tag using thrombin. After several attempts, I finally managed to insert the CDS of *edl* into the pGEX vector using a pGEX which previously contained an insert

(thanks to Dr Colinet) as my main challenge was to know if the restriction enzymes cleaved their restriction sites or not.

However, I did not test the protein production from this vector. Nevertheless, I believe it is of the utmost importance to validate physical interactions between edl and its interactants. It is facilitated by the fact that known interactants of edl are existing and can be controls to those experiments.

IV. Discussion

In this work, the two *D. melanogaster* lines used in this work derived from a same natural population caught in Congo more than 30 years ago and were characterized by their phenotypic response against the *L. bouhardi* ISy (Carton *et al.*, 1989). The larvae of the resistant line encapsulate and kills the parasitoid egg while the parasitoid egg succeeds to develop in the susceptible line larvae, killing the fly host. These lines are of interest since such model of intraspecific variation in resistance is scarce, and moreover the *Drosophila* model now provides all the tools necessary to decipher the molecular mechanism underlying this phenotype.

The phenotypic differences between these lines is due to a major gene, named *Rlb*, and the *Rlb*+ resistant allele is dominant (Hita *et al.*, 1999, 2006). The YR (*Rlb*+) and YS (*Rlb*-) lines have the same genetic background (that of the susceptible line) and differ only at by a 100 kb region of chromosome 2R containing the *Rlb* gene (Hita *et al.*, 2006). At that time, they could restrict the size of the region containing *Rlb* and hypothesis a main candidate for this gene that was further reduced to one candidate gene, *edl* (Hita *et al.*, 2006). *edl* encodes for a protein known to be involved in the switch of transcription factors which regulate the genes expression in response to different pathways activation (Vivekanand *et al.*, 2004; Schwarz *et al.*, 2018).

A. Comparison of the resistant and susceptible lines at the phenotypical level

1. No difference observed in the timing of the hemocyte response

It is known that the resistant and susceptible lines presented different quantities of hemocytes after parasitism (Russo *et al.*, 2001). As stated in the results part of the manuscript, we did not observe a difference of the hemocytes count between the resistant and susceptible lines (Figure 35) contrary to what was observed in Russo *et al.*, (2001) where the number of hemocytes increased 15h after infection by the parasitoid in the R strain and at 24h for the S strain (Figure 21). The differences between the 2 studies can be explained by the fact that 2 different technics were used to estimate the number of cells: Russo and colleagues used a Thomas hemocytometer counting chamber to evaluate the number of cells in the diluted hemolymph, while we used flow cytometry to limit the human factor. Both technics have their advantages and disadvantages. Although counting directly visualizes the details of cell types and their morphology, it is time consuming and requires many repetitions to reduce counting variability. Flow cytometry makes it possible to count a very large number of cells and to analyze numerous samples over a short period. However, it requires cell labelling. I have tried to create YS and YR lines expressing a fluorescent marker (eater-GFP and msnCherry) in two hemocyte types to facilitate the counting. However, only resistant and susceptible reporter lines allowing the labelling of lamellocytes could be produced. We thus use

WGA-GFP a lectin that has been shown to bind *Drosophila* hemocytes. WGA recognizes N-acetylglucosamine (GlcNAc) residues in protein-linked glycans. This lectin was used by Rizki & Rizki, (1984) to follow the fate of lamellocytes after *L. heterotoma* parasitism. They observed different labelling depending on the stage of lysis of these cells that is induced by this parasitoid venom, but not reported to occur with the ISy line of *L. boulandi*. Mortimer et al, reported that the labelling on hemocytes may vary with parasitism, but a clear labelling was observed on cells between 0 to 24 h (Mortimer *et al.*, 2012). Recently, WGA-GFP has been used in cytometry and reported to label almost all circulating hemocytes (Tirouvanziam *et al.*, 2004b), which is confirmed by our observations of hemocytes by fluorescent microscopy reports. Thus, we are quite confident that we have not missed the peak observed by Russo *et al.*, (2001) due to a technical problem, but we have no clear explanation yet for this discrepancy. One drawback of the cytometry study is the low number of repeats and the large variation observed. This variation both in quantity of cells and kinetics from one replicate to another and also within the same replicate may be due to a non-synchronization between the larvae. Indeed, although every precaution has been taken to have larvae uniform in age and size, reared under the same conditions, as well parasitoids in good shape, it is difficult to control everything with a living organism. One important variable is the interest of the female parasitoid for its hosts that could be influenced by multiple biotic and abiotic factors in the laboratory. For example, parasitism can be immediate, occurring a few minutes upon host finding or at any time during the 2h of contact, already inducing a time lag. Very few is known of the parasite synchronization with the host physiology and this may depend on the quality of the host and the wasp egg, two parameters that are difficult to manage. At last, the wasp/host encounter can stress the host more or less depending on the rapidity of the oviposition (multiple failed ovipositions, ovipositor insertion to search for unparasitized larvae, etc) and also the venom quantity injected that can be variable from one larva to another (unpublished laboratory results). All of these may induce variations in the immune response. Supplementary repeats are needed to strengthen these data, but they are already informative, particularly on the differential variation in lamellocytes proportion I have observed between the YR and YS strains.

2. Variations in the number and proportion of lamellocytes between resistant and susceptible lines

As one of the most notable hemocyte type implicated in the encapsulation of the parasitoid egg is lamellocytes (Rizki & Rizki, 1992), I have shown that the variations in the lamellocytes count greatly differ between the resistant and susceptible lines, and the way the increase happens is also different. There are twice as many lamellocytes in the resistant line compared to the susceptible

line 24h post-parasitism (Figure 36). Between 20 et 28h post-parasitism, the lamellocytes count increases more sharply in the resistant line, to represent up to 58% of the circulating hemocytes, while it looks steadier in the susceptible one and can represent 39% of the circulating hemocytes at its maximum (Figure 37). Thus, there is clearly a difference between the YS and YR strains in the induction of lamellocytes after parasitism. It seems that YS reacts later or more slowly at the cellular level, which may lead to less lamellocytes in circulation at the appropriate time and explain its less capacity to encapsulate this parasitoid. As the main feature of the resistance is the formation of a melanised multicellular capsule around the parasitoid egg (Carton & Boulétreau, 1985; Bajgar *et al.*, 2015) and lamellocytes are produced in case of parasitism or wounding, several hypothesis can be made. First, more lamellocytes in the hemolymph of the resistant *Drosophila* larvae could lead to an encapsulation of the parasitoid egg and therefore a resistant phenotype. Second, a faster release of lamellocytes could also lead to the creation of the capsule around the egg and not later when the parasitoid larvae has hatched from it. Third, the combination of the 2 previous hypothesis, an earlier faster release of lamellocytes in the hemolymph of *Drosophila* larvae can also be an option to trigger the capsule formation.

3. Induction of lamellocytes in relation with the early LG bursting in YR

Those newly differentiated lamellocytes could originated from several sources: transdifferentiated plasmatocytes (Honti *et al.*, 2010; Stofanko *et al.*, 2010), the lymph gland (Lanot *et al.*, 2001; Sorrentino *et al.*, 2002; Crozatier *et al.*, 2004) and the sessile compartment (Markus *et al.*, 2009; Honti *et al.*, 2010; Stofanko *et al.*, 2010; Anderl *et al.*, 2016). Therefore looking at the lymph gland was the next logical step as the sessile compartment is more difficult to study, even if technics and tools are available to observe it (Anderl *et al.*, 2016). Also there have been many studies on the implication of the lymph gland in the response against parasitoid infection (Sorrentino *et al.*, 2002; Crozatier *et al.*, 2004; Krzemień *et al.*, 2007; Sinenko *et al.*, 2012; Benmimoun *et al.*, 2015; Oyallon *et al.*, 2016). Most of these studies suggest that the cellular response is maximal in larvae 48 h after wasp egg-laying as the bursting of the lymph gland occurred around this time. However, they were done with different *D. melanogaster* strains, most often bearing mutation(s), or in the Hop^{tum-1} background, thus making it difficult to associate and generalize lymph gland bursting and encapsulation response.

Our experiments show that the lymph gland of resistant larvae releases hemocytes at least 9h earlier than the ones of susceptible larvae (Figure 38) which could lead to the resistant phenotype: a quicker bursting of the lymph gland leads to a faster release of mature hemocytes, which is an

advantage for the encapsulation of the parasitoid egg. All the lymph glands of the resistant line burst at the same time, whereas the ones of the susceptible line progressively burst (Figure 38). In YS, the first larvae with bursted lymph gland were observed at 24h and their number increased progressively to reach 100% at 30h (late L3). The lymph gland burst in YR larvae was not translated immediately into an increase in the total circulating hemocytes suggesting either that the number of cells released is low and does not affect the total count of cells in circulation or that their labelling is different from that of the mature plasmatocytes and lamellocytes retained by the gating on the flow cytometer. Lamellocytes are known to exhibit strong adhesive properties and could aggregate. They could also stick to the glass and plastic surfaces used for hemolymph collection and treatments of hemolymph, two effects that may preclude accurate counting of individual cells and lead to undervaluation of their numbers. However, the fact that we were able to observe their increase at 24h and 48h in both lines plead against these artifacts, but a double count (manual and cytometry) on the same samples will nevertheless have to be done to confirm it.

Since this lymph gland cells release occurs few hours before the lamellocyte number increases, it is tempting to suggest that pro-hemocytes differentiated in the lymph gland and were released. However, since the total number of hemocytes did not change at the time of the lamellocytes number increase, and we have observed an increase in the lamellocytes/plasmatocytes ratio, this could be in agreement with a trans-differentiation of circulating plasmatocytes. A final answer will require different approaches such as microscopic observations of the hemolymph and the lymph gland using different types of hemocytes markers.

Nevertheless, we have to bear in mind the fact that there is a 9h gap between the 15h and 24h post-parasitism time points in our experiment. A supplementary time point around 18-20h could be interesting as it could be possible to observe a delay in the lamellocytes increase in the susceptible line compared to the resistant line which would correspond to what is observed in the lymph gland experiment. Also, we observed a drop in the number of lamellocytes at 28-30h post-parasitism (Figure 36), when retranscribed in the lamellocytes proportion in the hemolymph (Figure 37), we observed a diminution of the proportion at 28-30h and 46-48h. The drop in the number of lamellocytes observed in the YR strain between 28-30h and 46-48h post-parasitism may be partly related to the capsule formation that requires a certain number of lamellocytes directly for its formation but perhaps also indirectly for melanisation. Once the *Drosophila* larva has enough hemocytes to form the capsule, it can be hypothesized it do not need to keep an important pool of lamellocytes in its hemolymph as it is costly for the organism (Kraaijeveld *et al.*, 2001b; Lazzaro, 2015; McGonigle *et al.*, 2017), as infection-induced proliferation of blood cells leads to a diversion of carbon away from somatic growth and development, and sugars being directed to the

hematopoietic organ for cell proliferation (Lazzaro, 2015) and dangerous for the organism since these cells can form pseudo-tumors by encapsulating normal tissues when their concentration is too high (Gateff, 1994).

Once that the resistant and susceptible reporter lines for lamellocytes have been created, it was interesting to follow up and to try to insert the eater-GFP reporter insertion into those lines. We knew that 2 reporter lines have been created by the past (Anderl *et al.*, 2016) and when I observed the hemocytes for the cross between msn-mCherry and eater-GFP lines, I saw mCherry lamellocytes and GFP plasmatocytes. If resistant and susceptible reporter lines for lamellocytes and plasmatocytes end up being created, it gives access to the observation of the sessile compartment in whole larvae and examine the hemocytes differentiation in the lymph gland.

Table 12: Recapitulative table of the effect of edl alleles overexpression and RNAi. Upward arrow indicates an increase; number of arrows indicates statistical significance when compared to the control cross with Nasrallah; cf Table 4 and Table 5).

	UAS-edlF	UAS-edlX	UAS-Mae34how	UAS-Mae8how	BL edl RNAi	VDRC edl RNAi
pCg-Gal4	↑		↑	↑	↑↑	
pCol-Gal4			↑↑↑	↑↑↑		
E33c						
pHml-Gal4			↑↑↑	↑↑↑	↑↑↑	↑↑↑
pg125-Gal4						

B. Edl as a main character in the resistance mechanism

As *edl* was hypothesized to be the *Rlb* gene and that the YR and YS *Drosophila* lines each carry an allele of *edl*, it became necessary to pursue the characterization of the role for *edl* in the defence reaction against the parasitoid.

To definitively establish the role of *edl* in the resistant/susceptible phenotype, the most direct approach would be to invalidate this gene. However, since it is necessary for many developmental processes, its KO is lethal, animals dying as late embryos or early larvae (Yamada, 2003). To get around this problem, we hypothesized that hemocytes or the tissue involved in their larval production, the lymph gland, could be the primary target of *edl* since : i) the wasp egg encapsulation requires the mobilization of the different hemocytes types (mainly plasmatocytes and lamellocytes) and ii) the physiological basis of the YR/YS differential response was related to the timing of hemocytes production after parasitism, the peak in the number of hemocytes occurring earlier in YR than YS larvae (Russo *et al.*, 2001). We therefore decided to use different promoter-Gal-4 lines inducing either overexpression or the *edl* alleles or their inhibition (RNAi) in the lymph gland or the hemocytes (Table 12).

When overexpressed, the *edl* allele corresponding to a susceptible phenotype in the *rlb* *Drosophila* strain still gives a susceptible phenotype (Table 4), indicating that the susceptible phenotype would not be linked to a quantitative under-expression of this allele (Knight, 2004; Pastinen, 2010).

Conversely, the overexpression of the *edl* resistant allele in the corresponding transgenic lines induced a resistant phenotype mainly when it occurred in hemocytes (Table 4). *Hemolectin* (*pHml-Gal4*) is expressed in larval hemocytes (in a subpopulation of plasmatocytes and crystal cells but not in lamellocytes) (Goto *et al.*, 2003), suggesting overexpression of *edl* occurs in these cells both from the sessile compartments, the lymph gland and in circulation. It could be interesting to look at a specific expression in plasmatocytes to see if *edl* could be implicated in the transdifferentiation of plasmatocytes in lamellocytes. Resistant occurs when expressed in the PSC indicating *edl* could be implicated in the control of the differentiation of the prohemocytes in the lymph gland, as the lymph gland is implicated in the signalling for prohemocytes' differentiation (Crozatier *et al.*, 2004). With *pCg-Gal4* (*Collagen-GAL4*; expressed in larval hemocytes, fat body and lymph gland), a lower but still significant increase in resistance occurred. These data thus suggest that overexpression of *edl*-R in hemocytes and prohemocytes in the lymph gland is enough to induce the increase in resistance.

The lack of effect with *PG125/dome-Gal4* (expression in LG MZ, Hematopoietic progenitors; (Bourbon *et al.*, 2002)) and *e33C-Gal4* (expression in circulating hemocytes and lymph glands, (Asha

et al., 2003)) is unexpected. One explanation would be their expression in subpopulation of hemocytes unaffected or not heavily involved in the immune response against the wasp egg or in an already differentiated state that did not allow *edl* expression to stimulates their role in encapsulation.

Interestingly, the interference with *edl* expression resulted in resistance mainly when done in the mature hemocytes and not in the PSC (Table 5). Inhibition of *edl* in the hemocytes with *pCg-* or *pHml-gal4* indeed increases the resistant phenotype although only with one RNAi line for pCg-Gal4. One possible explanation is based on how *edl* works. In normal conditions, the quantity of this protein is fine-tuned, and an increase or decrease will inhibit or allow other factors such as *aop* and Pointed P2 to play their roles in genes regulation (Vivekanand *et al.*, 2004). Moreover, the level of this switch may vary depending on the state of the targeted cell or its environment. Thus, a low quantity of *edl* could under certain conditions have an effect opposite to that intuitively expected.

Overall, the specific overexpression of *edlR* but also the subexpression in the hemocytes increases the resistant phenotype possibly by acting on their multiplication/differentiation but this may occur only in certain sub-populations of hemocytes and it may depend on their activation state and their environment. A count of the change in the number of hemocytes (in the hemolymph and the lymph gland) and their different categories will be now necessary to complete these experiments and have a better picture of the resulting effect.

C. Edl integration in the signalisation pathways implicated in the immune response

Edl is implicated in the eye development (Vivekanand *et al.*, 2004), the muscle development (Dubois *et al.*, 2016) and cardioblasts diversification (Schwarz *et al.*, 2018). It is known to interact with aop and pnt P2 (Vivekanand *et al.*, 2004; Schwarz *et al.*, 2018). The crystallization of the interaction between aop and edl has been done in 2004 (Qiao *et al.*, 2004). Implication of pnt and aop in the differentiation of hemocytes have been described in Zettervall *et al.*, (2004).

As each allele of *edl* differs on 2 SNPs in their CDS (Figure 31) and both alleles lead to distinct phenotypes in the matter of the resistance against *L. boularidi* G486: the one in YR leading to a resistant phenotype and the one in YS to a susceptible one (Carton *et al.*, 1992). The first SNP at the position 107 induces a change in the codon and thus flies can have a Serine (S) or an Asparagine (N) at the protein position 36. These 2 amino acids are not substitutable as they have different properties. The change is located outside of edl SAM domain and, therefore, do not interfere directly with edl capacity to interact with its partners although. Nonetheless, serine is phosphorylable and not asparagine. As phosphorylation is one of the main changes in protein that affects their functions and many ETS family members are subject to S/T phosphorylation in response to a variety of upstream signals, these modifications exert a broad spectrum of effects on their activity. In aop, one MAPK phosphorylation sites, S127, is required for RAS/ERK pathway responsiveness, phosphorylation at the other sites appears important for amplifying and modulating the response (Rebay & Rubin, 1995). Studies on pnt P2 by mutagenesis have indicated that phosphorylation of Thr151 is critically required for the in vivo function (Tootle & Rebay, 2005; Lau *et al.*, 2012). To our knowledge no study has searched for edl phosphorylation, thus, this potential edl modification type deserves further investigations.

Nevertheless, the interference of 2 partners of edl, pnt p2 and aop, affects the reaction of the larvae against parasitism, mainly in the mature hemocytes for *pnt* and the fatbody for *aop*. The main resistance-induced phenotype is observed with almost the same Gal4 drivers as for *edl*, suggesting an interplay in the same type of hemocytes/cells. A role of the fat body in the resistance phenotype could not be ruled out. The fat body is implicated in many defence reactions against pathogens as it produces AMPs after activation of the Toll and IMD pathways (Ben-Ami *et al.*, 2009; Vogel, 2012). Aop have be described as implicated in the JNK pathway in the fatbody (Zettervall *et al.*, 2004). Wasp parasitism has also been shown to rapidly induce in the larval FB the expression of a gene *edin* (elevated during infection) that encodes for a small secreted protein (related to the Attacin/Diptericin superfamily) required for the larva to mount a normal encapsulation response (Vanha-Aho *et al.*, 2015; Vanha-aho *et al.*, 2016).

Aop and pnt are both ETS transcription factors and the ETS transcription factors are conserved throughout the metazoans (Seth & Watson, 2005). All the members share a conserved DNA-binding domain and are subdivided in subfamilies on the basis of sequence similarities in the ETS binding domain and the presence of additional conserved domains (Seth & Watson, 2005). One such region is the Pointed (PNT) domain. The functions of this domain are multiple: homo-oligomerization, hetero-dimerization and docking site for signalling pathway as the MAPK cascade (Oikawa & Yamada, 2003). They play important roles in cell development, cell differentiation, cell proliferation, apoptosis and tissue remodelling. ETS family (ETS) transcription factors play important roles in cell development, cell differentiation, cell proliferation, apoptosis and tissue remodelling. Most of them are downstream nuclear targets of Ras-MAP kinase signalling, and the deregulation of ETS genes results in the malignant transformation of cells. Several ETS genes are rearranged in human leukemia and Ewing tumours to produce chimeric oncoproteins. Furthermore, the aberrant expression of several ETS genes is often observed in various types of human malignant tumours (Cooper *et al.*, 2014). TEL, the human ortholog of aop, is implicated in the development of leukemia in humans (Dittmer & Nordheim, 1998) and in the dendritic cells differentiation (Lau *et al.*, 2018). This could mean that aop could be implicated in some processes linked to hemocytes proliferation.

Whether pnt and aop are the only interactants of edl involved in the reaction leading to resistance is still to be determined. From my bioinformatic search and the correspondence between the amino acids implicated in the interaction between the SAM-domain of edl and aop (Table 9, Table 10), I choose ETS transcription factors that could potentially interact with edl. I tested two of them, Ets97D and l(3)mbt, and knockdown their expression *via* RNAi expression, showing an increase in resistance when their RNAi were expressed in hemocytes. Although quite preliminary, these results are of interest in order to expand the regulatory network around edl, implicated in the resistant phenotype.

L(3)mbt, could have the ability to interact with edl through its SAM domain. l(3)mbt have been implicated in cellular overproliferation in the brain causing malignant brain tumours (Gateff *et al.*, 1993; Feichtinger *et al.*, 2014), and is thought to be a chromatin reader (Bonasio *et al.*, 2010). It secures tissue identity by preventing the simultaneous expression of identity markers and tissue-specific misexpression signatures (Coux *et al.*, 2018). MacGrogan *et al.* (2004) hypothesizes that the human ortholog to l(3)mbt could be important in some case of myeloid leukemia and is a transcriptional repressor that interacts physically and functionally with TEL (ETV6) via their respective SAM domains (Bocconi *et al.*, 2003). This strongly support that l(3)mbt could have the ability to interact with edl *in vivo* through its SAM domain and play a role in the edl control of

resistance. Interestingly, genetic screens have identified histone H4K20 mono-methylase Pr-set7 (Badenhorst, 2014) as a regulator of hematopoiesis. Pr-set7 is required to maintain PSC hub cells of the larval hematopoietic niche (Tokusumi *et al.*, 2012) and Pr-set7 mutants develop melanotic tumors like gain-of-function JAK/STAT mutants (Minakhina & Steward, 2006). The H4K20(Me)1 mark functions by allowing the recruitment of binding partners such as the tumor suppressor l(3)mbt. L(3)mbt complex with core histones H1 and HP1 is speculated to act as a “chromatin lock” to negatively regulate gene transcription (Trojer *et al.*, 2007). This joins the knowledge of the function of aop and Pnt that can control the expression of a large region of chromatin, increasing the complexity of the role of edl as a chromosomal gene expression regulator.

The second potential partner tested was Ets97D. Ets97D has a pointed and an ETS domain and is the *Drosophila* homolog of the alpha subunit of mammalian NRF-2/GABP, a transcription factor required for proper expression of most genes encoding mitochondrial proteins. NRF-2/GABP role may be by an indirect the regulation of the expression of Tfb1m, a methyltransferase that modifies ribosomal rRNA required for mitochondrial protein translation both in human and *Drosophila* cells (Yang *et al.*, 2014). In *Drosophila*, Ets97D is required for the correct localization of oskar and gurken mRNA in the *Drosophila* oocyte and for the migration of follicle cells, a crucial process for the correct embryo development. In its absence, eggs are smaller and the egg chamber is abnormal (Schulz *et al.*, 1993; Gajewski & A Schulz, 1995). Again, if edl binds *in vivo* to Ets97D, its regulatory function would be expanded to the mitochondrial genes expression and the control of the energetic metabolism.

At the time of the writing of this manuscript, 8 genes can be transcribed into Ets transcription factors in *Drosophila* (cf Uniprot.org). The transcription factors from the ETS family have been described as having many important roles during development as described previously. Among them (Table 9), 5 contain a SAM domain: *Ets98B*, *Ets97D*, *Ets21C*, *pnt* and *aop*. Knowing the implication of the Ets transcription factors in human cancer (Seth & Watson, 2005), edl, interacting with ETS transcription factors, could promote the overproliferation of hemocytes in order to enhance the resistance against parasitoids. This gene could be a major crossroad in the immune reaction against parasitoids.

At this stage we can make the hypothesis that *edl* has a strategic role in the encapsulation response to parasitoid eggs and the resistance phenotype, but further work is needed to understand the molecular interactions in place. The demonstration of direct binding with the different factors requires the development of different tools such as antibodies, cells lines etc.

Whether *edl* could be a universal gene participating in the resistance of *Drosophila* across species is a long shot but its function in development could allow it to conserve its function across species (King Jordan *et al.*, 2002). We can hypothesize that *edl* could have a strategic role in the resistance reaction against parasitoid eggs, but further work is needed to understand the molecular interactions in place. At the protein level, the co-immunoprecipitation experiment started during my work could be carried on in test whether *edl* have other interactants than *aop* and *pnt* P2.

It could be interesting to do several transcriptome comparisons between the resistant and the susceptible lines to answer different questions. One would be on the lamelloblasts before 24h after parasitism, a more precise timing will have to be determined by flow cytometry. Lamelloblasts could be detected if double reporter lines are created and sorted using flow cytometry coupled with FACS (Fluorescence Activated Cell Sorting) by selecting the population of interest (low GFP and no Cherry, Anderl *et al.*, 2016). This would give more information on what is going during a plasmatocytes transdifferentiation into lamellocytes. A second one could be on lymph glands, between 12 and 15h after parasitism for the resistant line. Even better would a single cell transcriptome at the level of the lymph gland, therefore the differences the 3 zones of the lymph gland could be distinguished. This would be useful to understand the signalisation leading to the differentiation of hemocytes in this organ.

To my knowledge this is the most detailed comparison of 2 lines coming from the same original population on the response against parasitoids. The characterization of the phenotypical response of the *Drosophila* larvae is essential even though molecular mechanisms are important to understand. It allows us to know what set the resistant larvae from the susceptible one. Due to its basic function to regulate transcription factors, *edl* is an interesting element to study. But the complex networks in which it is integrated and the diversity of effects downstream of the EGF pathway make it difficult to unravel the *in vivo* contexts in which these complex transcriptional regulatory networks are implicated.

V. Conclusion

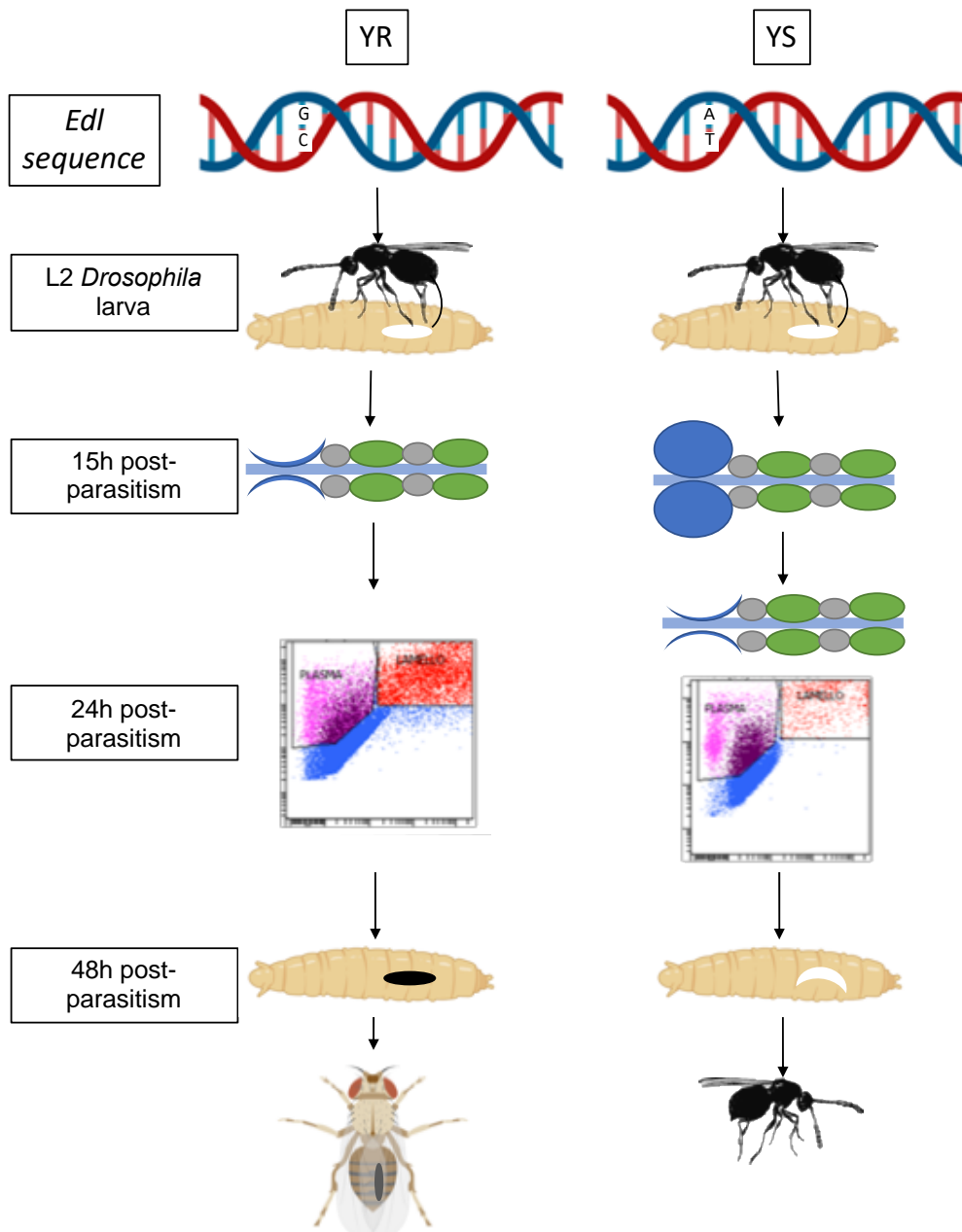


Figure 44: Schematic representation of some of the main results from this project. YR and YS are vertically represented. *Edl* sequence shows the SNP of interest implicated in the amino acid change. L2 *Drosophila* larva represents the stage at which the *Drosophila* is parasitized. 15h post-parasitism: the lymph gland in YR is burst but not in YS. 24h post-parasitism: the number of lamellocytes increases in both strains and the lymph gland of YS bursts. 48h post-parasitism: a capsule is present in the body of YR larvae allowing the survival of the *Drosophila*; the parasitoid egg has hatched in the YS larvae leading to the development of the adult parasitoid.

At the beginning of this work, more than 10 years had passed since the last publication of Hita *et al.*, (2006) indicating *edl* as a possible candidate as the major gene involved in the genetic resistance of the *D. melanogaster* YR strain to the ISy line of the *L. bouleardi* parasitoid. I had to reassemble the pieces and try to complete the puzzle.

The *edl* role has been mainly studied since it regulates transcription factors after activation of the EGF pathway. This led to the description of a subtle quantitative balance between this factor and the two antagonistic transcription factors *aop* and *pointed*, based mainly on a binding equilibrium through the interactions of their SAM domains. Here, I have confirmed that the resistant allele of *edl* (*edlR*) is responsible of the encapsulation phenotype in the YR compared to YS strain, and shown that, in our model, the regulatory situation may be more complex. The main difference between the resistant allele and the susceptible allele of *edl* coding region is a SNP that changes only one aa in the protein: this change may introduce a new phosphorylation site into the *edlR* protein, a regulation important in the protein function, but this is only an *in silico* prediction needing confirmation.

The main feature of *Drosophila* immune resistance to parasitoid egg is the formation of a multicellular, melanised capsule, and lamellocytes are the main cells involved. Thus, the larger and faster the production of lamellocytes, the greater the efficiency of the encapsulation should be. I observed that a transient peak of lamellocytes occurred 22-24h after parasitism in the YR strain larvae, possibly related to the accelerated bursting of the lymph gland a few hours earlier, events not taking place in larvae of the YS strain. This is a clear phenotypic difference between the two strain that may explain the encapsulation. While the YS strain is responding to parasitism, it looks like a slower response, *EdlR* thus may more rapidly activate lymph gland hemocytes maturation and liberation and participate in the transformation of this hemocytes and/or circulating hemocytes in lamellocytes. Due to its lack of DNA binding domain, *edl* functions *via* its action on other transcription factors making them essential elements to study. Indeed, I have shown that interference of the expression of the known and putative interactants of *edl* may also increase the resistant phenotype. Since some of the RNAi sequence may encompassed the SAM-domain region, the different effects may be taken cautiously due to possible off target effect by SAM domain binding to several factors or eliminating several proteins with SAM-domain.

At this time, it is thus difficult to unravel the *in vivo* contexts in which the *edl* complex transcriptional regulatory network is implicated. We have to bear in mind that a very large panel of factors have been shown directly or indirectly implicated in the larval hematopoiesis. Overexpression of certain genes, such as *hopscotch* or *Ras* lead to the formation of lamellocytes. Other genes may control the cell number, such as *EGFR* and *Ras*, as their expression produced a

massive in increase hemocytes. A third group of genes, including, e.g. Alk, Rac1 and Pvr give a mixed response, promoting both hemocyte proliferation and activation. Moreover, other players such as micro RNA, metabolic pathways, and factors from tissues such as the fat body and the muscle have been shown to dialog with the hemocyte production/differentiation mechanism and interfere with the encapsulation mechanism. In conclusion: the control of blood cells in larval hematopoiesis, and during parasitic wasp attacks, is complex and may involve multiple pathways, and we are far from having explored all.

Nevertheless, this work has shown that *edlR* is implicated in the resistant phenotype in the *Drosophila* resistant strain where more lamellocytes and an earlier bursting of the lymph gland giving an advantage when facing the parasitoid egg. We also show that a new factor must be considered, the timing of the response, as it seems essential for the larva successful defense against the parasitoid.

Last but not least, *edl* interacts with ETS transcription factors and considering that some ETS transcription factors are involved in malignant transformation and tumor progression, including invasion, metastasis and neo-angiogenesis through the activation of cancer-related genes, studying the *edl* regulation of those transcription factors could lead to a better understanding of the mechanisms implicated in those diseases and could help in finding potential molecular targets for selective cancer therapy.

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Annexes

Explanation about the creation of the UAS-edlR lines

Following the identification of *edl* as the main candidate for the *R/b* gene, experiments were set up to test the actual involvement of this gene in the resistance of the YR strain.

Production of transgenic strains for edl (from the resistant strain): work mainly performed by Dr. Pascal Vaudin as a post-doctoral researcher in the team.

From the complete *edl* cDNA sequence recovered from Genbank (EST CG15085, GenBank Accession number NM_079062), we designed primers to amplify a region (nucleotide 224 to 1247) containing the first complete exon (863 bp) with part of the UTR (125 bp) and about half of the second exon (335 out of 591bp). The first exon contains the entire open reading frame for the Edl protein, while the second exon is transcribed but not translated into protein. [RT-PCR was performed using the primers 5' GAAGCCAATTGCAGTAGC 3' and 5' ACTGCTCGTCTTGATTCC 3' and mRNA extracted from late second instar larvae of the YR and YS strains. Reverse transcription and amplification were performed respectively with reverse transcriptase SuperscriptTM RnaseH⁻ (InvitrogenTM) and a polydT primer, and the TaKaRa Ex TaqTM polymerase. PCR products were cloned in the pGEM-T Easy Vector (Promega) and clones were sequenced with ABI 3100 AVANT (Applied Biosystem)]. To produce transgenic strains, the cDNA sequence of *mae/edl* (nt 224 to 1247) amplified from the resistant Y1088 strain was cloned into the pUAST plasmid and clones controlled by sequencing. The construct was then injected into embryos of a W118 strain (gift from Y. Carton; susceptible to ISy parasitoids, 0-8% encapsulation) using laboratory facilities (*Drosophila* injection system, IRBI, University of Tours, France). Several transgenic lines were obtained most of which were made homozygous.

Test of the transgenic lines for induction of the resistance phenotype: performed in parallel in the team (IRBI, University of Tours) and by Françoise Frey and Y. Carton (CNRS, Gif sur Yvette).

We first confirmed the expression of the *mae* R transgene by crossing some of the transgenic lines with a *ptc*-Gal4 driver line (induction of expression in the border of imaginal discs, susceptible strain, encapsulation at 2% of ISy parasitoids): [Offspring from W118 x *ptc*-Gal4 had no developmental defect while that of the strain 8 how x *ptc*-Gal4, as an example, showed various development defects notably in the development of the eye].

To test the possible induction of resistance in the transgenic lines, a conditional expression was carried out using an hsp-Gal4 driver line (susceptible, encapsulation at 8% of ISy parasitoids). Following crosses between UAS-*mae* R strains with hsp-Gal4, pools of F1 second instar larvae were heat shocked (37°C for 60 minutes) and returned to 25°C. Parasitism was performed with two ISy parasitoid females for two hours, 30 minutes, 2 hrs, 4hrs or 8hrs after heat shock to identify the best experimental condition. The parasitism assays reported below were carried out 4 hrs after the heat shock with, in control, pools of F1 larvae from the same cross treated in the same way except for the absence of heat shock. Some of the results are provided in the Table below. They highlight a variation in the resistance of transgenic strains prior to induction, certainly because of the different location of the inserts. All the lines tested showed an increase in resistance after induction, of variable level, some being close to 100% encapsulation of ISy parasitoids. Overall, the data supported the hypothesis that *edl/mae* is the *R/b* gene, although additional approaches were necessary to confirm this hypothesis.

Table summarizing some of the results obtained with transgenic strains for *mae* R. Encapsulation rates are provided for strains or F1 larvae from crosses without heat shock or after heat shock for induction of systemic expression of the transgene. In bold, results for transgenic strains 8how and 34 how, subsequently used in the project.

	Without Heat Shock	After Heat Shock
Strain or F1 from a cross		
hsp-Gal4	7.69% (52)	8% (50)
hsp-Gal4 x W118	4.3% (92)	4.25% (94)
hsp-Gal4 x strain 7	35.1 (37)	90.8% (65)
hsp-Gal4 x strain 8	9.3 (43)	73.9 (46)
hsp-Gal4 x strain 20	53.9% (63)	95.8% (24)
hsp-Gal4 x strain 22	12.9 % (31)	86.1 % (65)
hsp-Gal4 x strain 24	7.14 % (56)	95.8 % (24)
hsp-Gal4 x strain 34	25% (52)	86.1% (65)
hsp-Gal4 x strain 39	20.97 % (62)	43.58 % (39)

The strains 3.1 how, 34 how and 8 how, used in this thesis, were also tested for the localisation of the *mae* transgene using *in situ* hybridization on chromosomes (collaboration with Dr. F. Lemeunier, Gif sur Yvette, France). Strain 8 was labelled on X chromosome and 3R in 89 B1-3, strain 3.1 on 2R, 3L in 79 E8-F1 and X, and strain 34 had two marking on X (one in 10 B15-16).

Transgenic strains for edl (sequence of the susceptible allele):

Transgenic strains with part of the cDNA sequence of *mae/edl* from the susceptible Y1089 strain were not produced since we were gifted the UAS-*edl*.X and UAS-*edl*.F by the laboratory of Dr.

Hiromi (Yamada et al., 2003). These strains bear the coding sequence of *mae*, identical to that of *mae* in the Y1089 strain, inserted on the X chromosome (UAS-edl.X) and the second chromosome (UAS-edl.F). Parasitism assays showed that both strains were completely susceptible to ISy parasitoids. After crossing with hsp-Gal4 and heat shock of the F1 larvae, the encapsulation level did not change much, ranging from 0% to 10%. The induction of systemic expression has therefore led to totally different phenotypes depending on the *edl/mae* sequence inserted into the transgenic strains (Y1088 or Y1089 type).

Table 13: Data from the flow cytometry experiment, the 3 replicates are reported in the table.

Time post-parasitism	Strain	Replicate	Number of dissected larvae	Total beads count	Plamastocytes/ul/larva	Lamellocytes/u/larva	Hemocytes/u/larva
10H	YR	1	5	24516	1786	73	1859
10H	YS	1	6	20957	4002	36	4038
10H	YR	2	6	35037	2406	109	2515
10H	YS	2	3	31080	2266	154	2420
10H	YR	3	6	31770	1900	30	1930
10H	YS	3	7	31768	2063	46	2109
12H	YR	1	10	33815	2930	157	3087
12H	YS	1	10	25765	2134	34	2168
12H	YR	2	2	23312	909	9	918
12H	YS	2	5	25875	3732	95	3827
12H	YR	3	9	25130	3420	136	3556
12H	YS	3	10	38304	2113	140	2252
15H	YR	1	8	30848	1364	95	1459
15H	YS	1	10	13066	3729	90	3819
15H	YR	2	5	19851	1760	27	1787
15H	YS	2	7	16685	4407	323	4730
15H	YR	3	10	18462	3987	93	4080
15H	YS	3	10	16518	3428	174	3601
24H	YR	1	10	25919	744	16	760
24H	YS	1	7	25502	1540	469	2009
24H	YR	2	7	33249	2549	1085	3634
24H	YS	2	10	28736	2616	694	3310
24H	YR	3	10	25283	4356	2597	6953
24H	YS	3	10	42509	874	436	1310
28H	YR	1	7	24657	989	2437	3426
28H	YS	1	4	37577	837	61	898
28H	YR	2	6	26255	2295	1280	3575
28H	YS	2	9	16074	4941	884	5825
28H	YR	3	9	23794	1327	2094	3422
28H	YS	3	8	31792	1135	1119	2254
30H	YR	1	10	34496	1658	400	2058
30H	YS	1	10	35560	1271	887	2157
30H	YR	2	7	17205	2354	1002	3356
30H	YS	2	4	16581	4201	1300	5501
30H	YR	3	5	18741	2801	834	3635
30H	YS	3	9	13240	1731	1117	2848
48H	YR	1	7	13439	3511	2891	6402
48H	YS	1	10	22042	3277	1192	4468
48H	YR	2	7	11213	10704	1210	11914
48H	YS	2	10	25920	5793	1370	7163
48H	YR	3	4	13965	4633	2290	6923
48H	YS	3	4	15488	9962	1135	11096

Table 14: Number of total dissected larvae for the lymph gland bursting experiment (3 replicates pooled together)

Time post-parasitism (h)	Strain	Number of parasitized larvae dissected	Number of non-parasitized larvae dissected
10	YR	17	18
12		20	31
15		15	31
24		18	16
28		22	23
30		19	16
10	YS	15	21
12		13	29
15		11	19
24		21	21
28		36	30
30		16	16

Table 15: Number of larvae dissected per cross for the UAS x GAL4

	Nasrallah	UAS edlF	UAS edlX	UAS Mae 34 how	UAS Mae 8 how	Bl edl RNAi	VDRG edl RNAi	VDRG Pit P2 RNAi	Bl pit RNAi	Bl 26759 yan RNAi	Bl 35404 yan RNAi
Nasrallah	16	33	14	66	71	16	47	91	80	89	64
PCg	55	92	76	63	63	50	37	0	42	53	41
pCol	53	36	29	47	48	30	39	71	31	32	29
e33c	71	58	19	53	68	40	53	31	58	40	50
pHml	64	69	35	45	52	70	34	28	32	43	52
pg125	78	44	31	47	24	24	30	40	56	59	38