

EFSA workshop «Risk assessment considerations for  
RNAi-based GM plants»

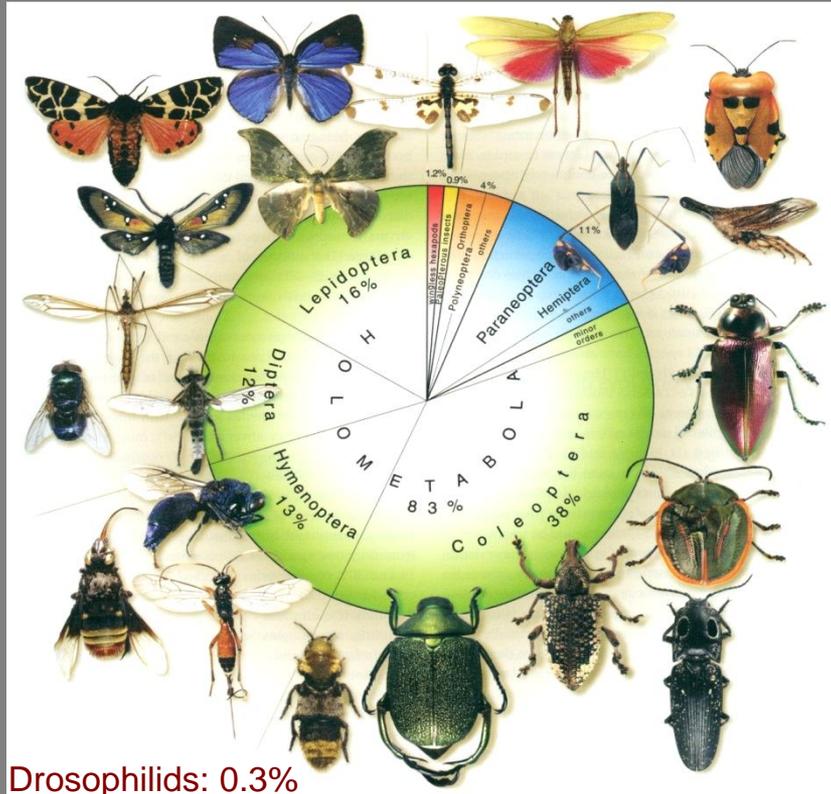
4-5 June 2014 Brussels, Belgium

# RNAi in insects

Xavier Bellés

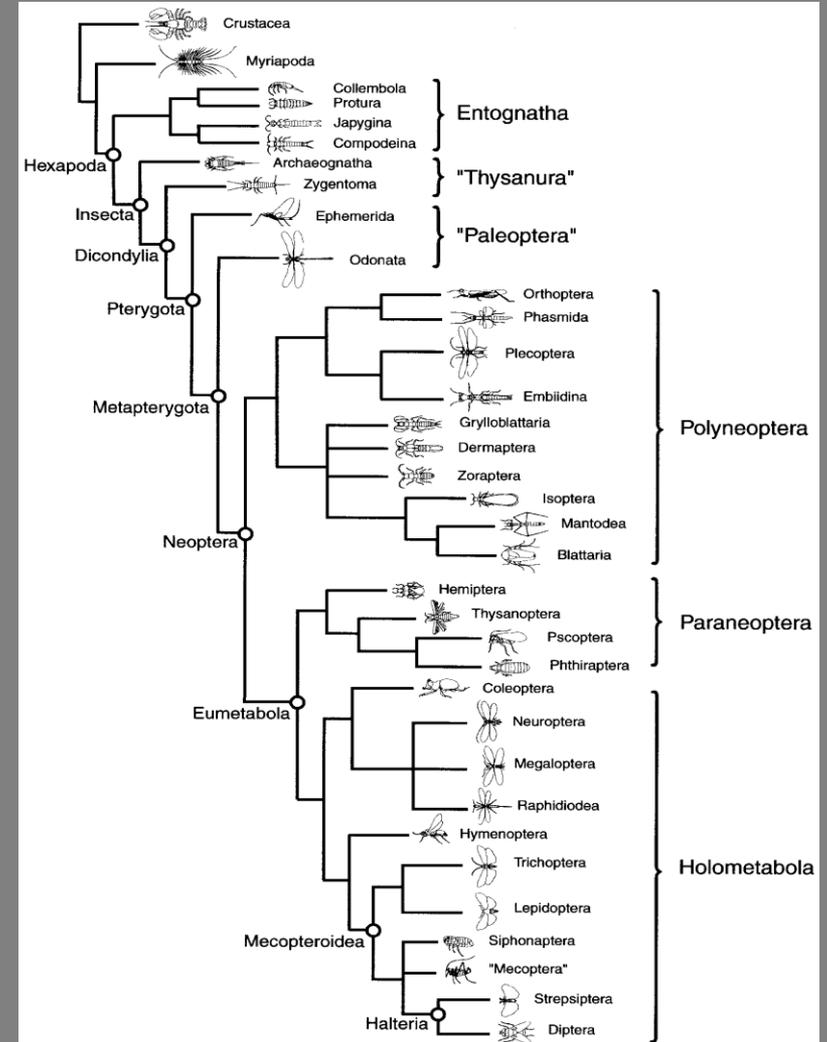
Institute of Evolutionary Biology  
(CSIC-UPF, Barcelona, Spain)

# The extraordinary diversity of insects, and the poor representativity of *Drosophila*



*Evolution of the Insects. Grimaldi & Engel*

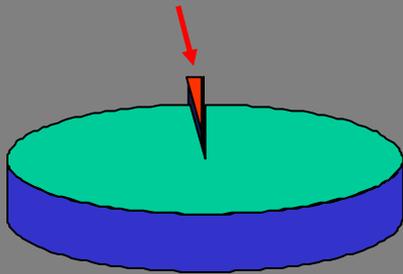
There are ca. 200 insects species for which the genome sequencing is finished or progressing, and the genome of ca. 5000 other species will be over the next 5 years. This has lead to the identification of a high number of genes for which no functions are known. How to deal with the functions of them? RNAi is the solution.



*Wheeler et al. 2001. The Phylogeny of the Extant Hexapod Orders*

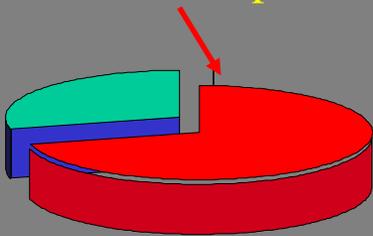
# Towards the end of *Drosophila* dictatorship

Drosophilids

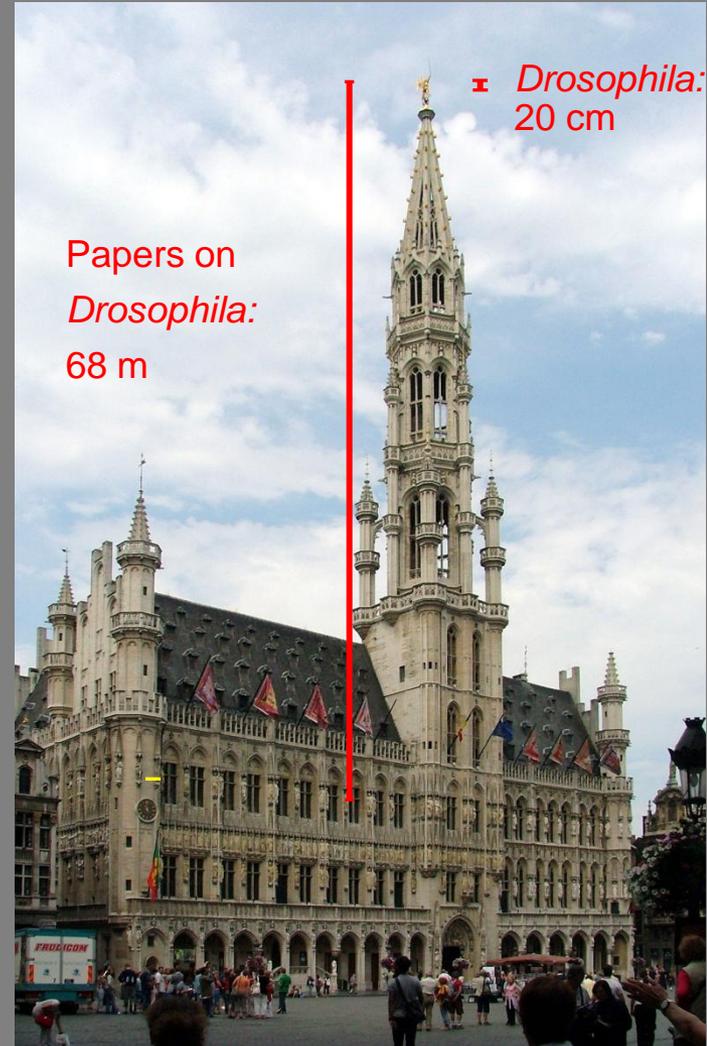


Drosophilids represent 0.3% of total described insect species

Papers on Drosophilids



Papers on Drosophilids represent 71% of total papers published on insect functional genomics (PubMed, 1980-2007)



Brussels City Hall Tower, 96 m

# The advent of RNAi

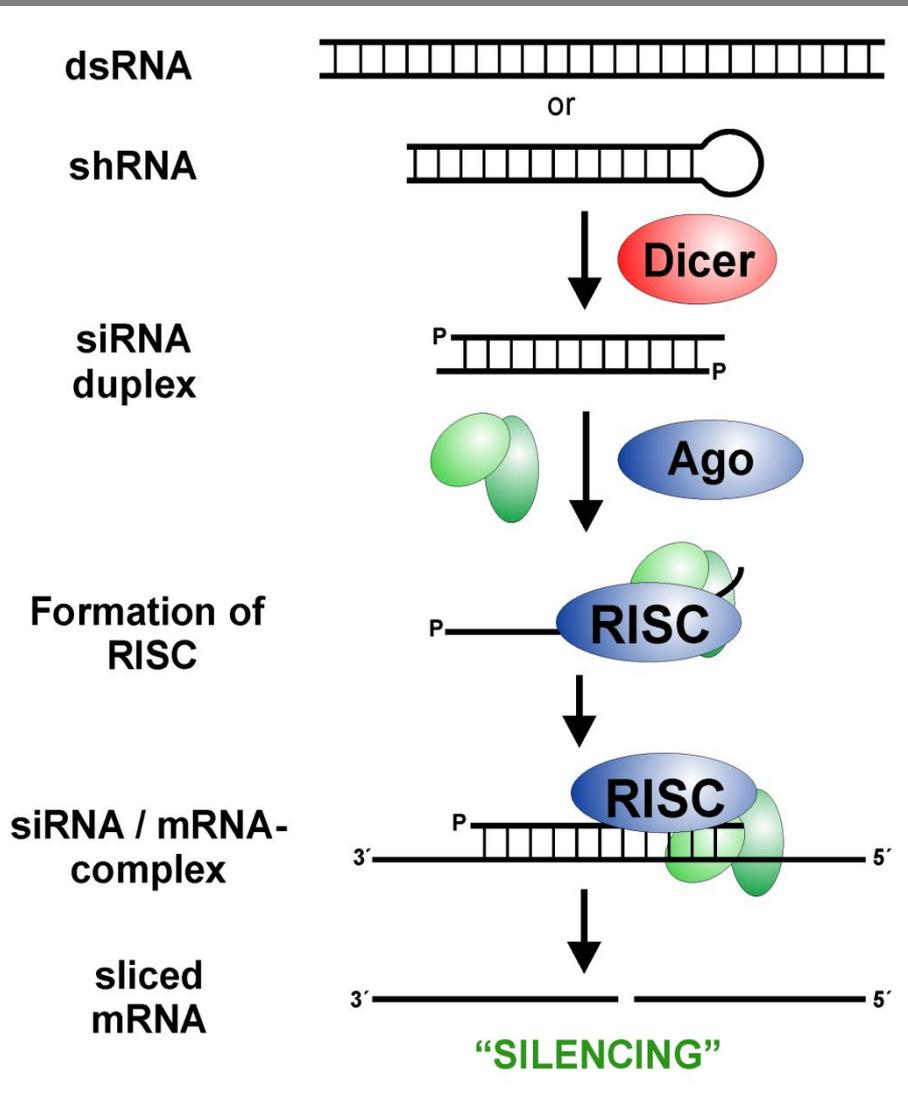
Working on the nematode *Caenorhabditis elegans*, Fire, Mello and associates showed that exogenous double-stranded RNA triggered gene silencing, thus, they discovered RNAi.

And, RNAi was a wonderful present to entomologists, who were then able to do functional genomics in non model species, beyond *Drosophila*.



Andrew Z. Fire and Craig C. Mello were awarded by the Nobel Prize in Physiology or Medicine in 2006

# Summary of RNAi mechanisms



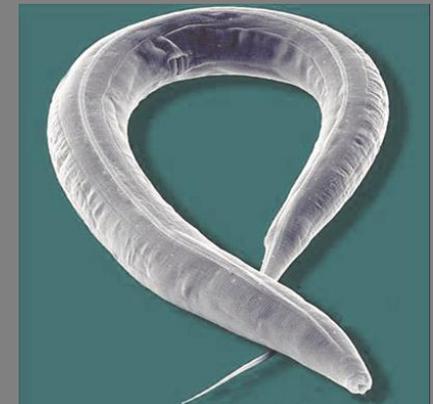
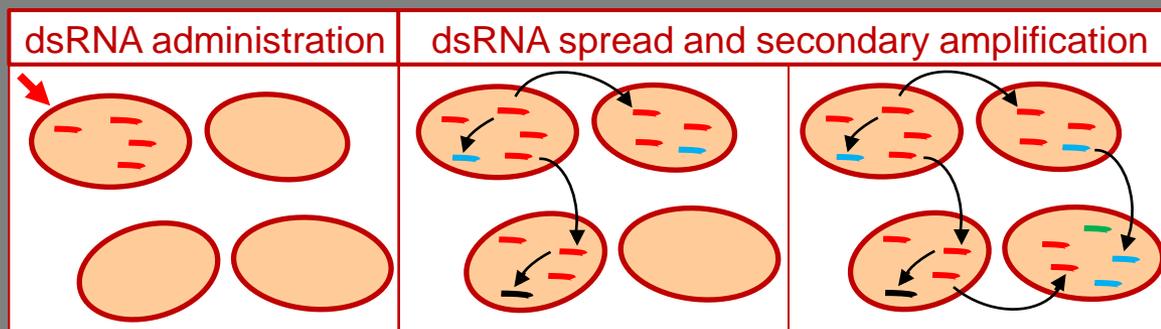
The double stranded RNA is processed by the enzyme Dicer into ca. 21-nucleotide siRNA duplexes. The produced siRNAs are loaded onto Argonaute (Ago) proteins within the RNA induced silencing complex (RISC), where one strand of the siRNA duplex is eliminated, and where the other one guides the AGO protein to the target RNA and induces its degradation.

## ***C. elegans*, one of the most sensitive and favourable models for RNAi**

After the discovery of RNAi in *Caenorhabditis elegans*, this nematode became a most favorable model for RNAi research, in part due to two remarkable features of RNAi uncovered in this species.

1. Uptake and spread of the signal. dsRNA easily penetrates the cells and the resulting siRNAs spread to other cells and tissues. **Key factor: SID-1** a transmembrane protein that functions as a dsRNA pore or channel.

2. Amplification of the signal. siRNA complementary to sequences upstream of or downstream from the initial trigger region in the target mRNA are secondarily produced. **Key factor: RdRp** (RNA-dependent RNA polymerase ) that allow the synthesis of these secondary siRNAs.



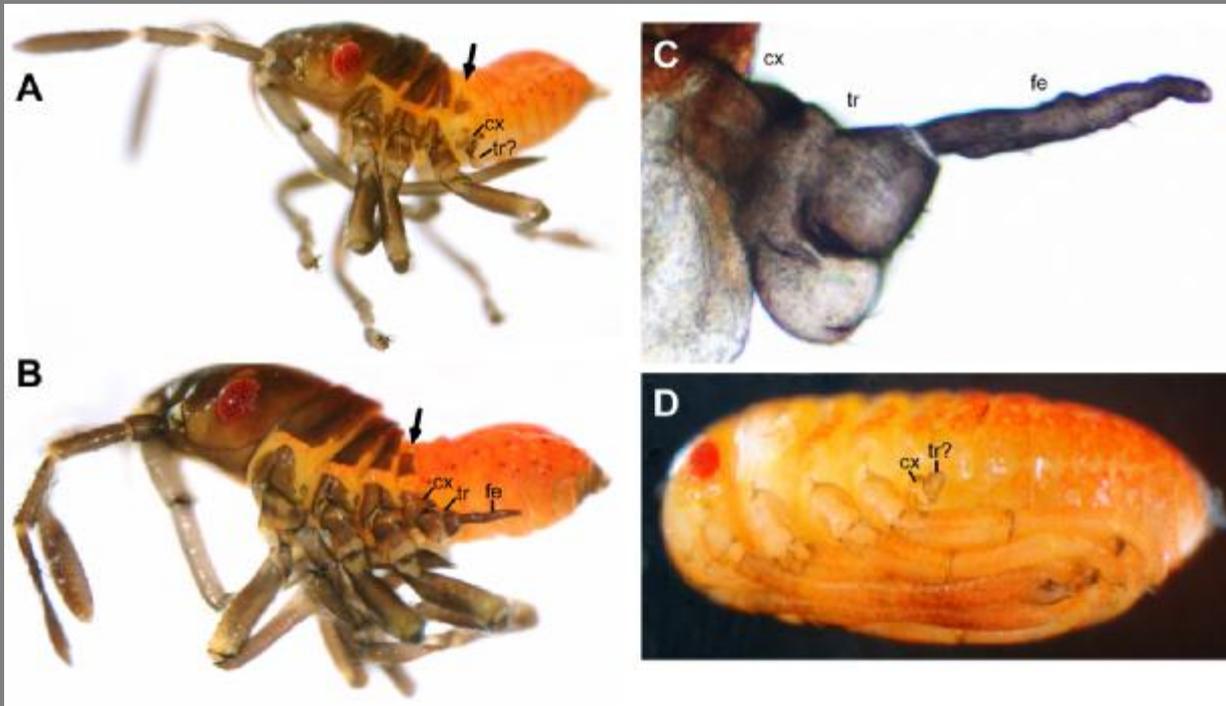
# **Beyond Drosophila**

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**10 years of RNAi in  
non-model insects**

# Embryo development

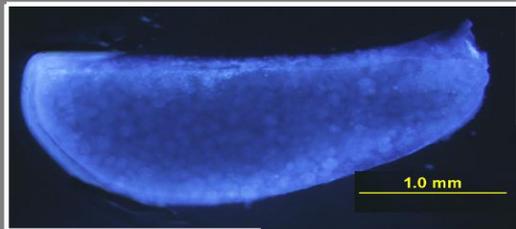
RNAi of a Hox gene (*ultrabithorax*) gives embryos with ectopic dorsal pigmentation and ectopic appendages on the first abdominal segment of *Oncopeltus fasciatus* (Angelini et al., 2005)



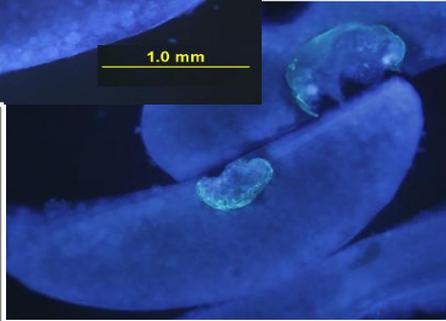
dsUbx-treated embryos of *Oncopeltus fasciatus*

# Embryo development

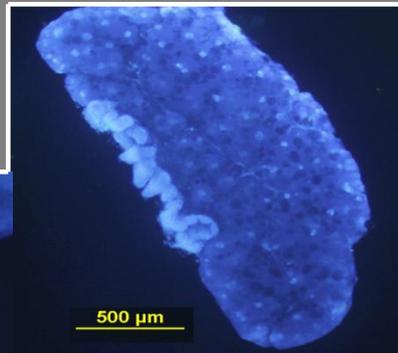
Parental RNAi of Broad-complex results in severe to mild morphological deficiencies in the embryo of *Blattella germanica* (Pagone, Bellés & Piulachs, 2010)



Germinal band not formed (22%)



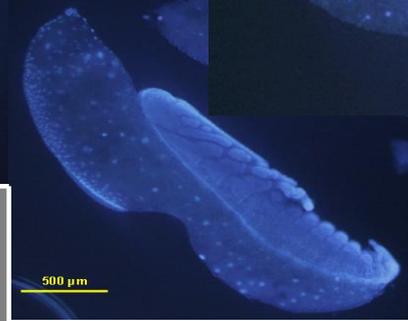
Amorph mass on the ventral side (20%)



Embryo with segmentation incorrect (12%)



Control at 35% development



Embryo with unusually elongated shape (do not hatch) (46%).

Embryos of *Blattella germanica* from females treated with dsBgBR-C. A case of parental RNAi

# Postembryonic development

RNAi of a Hox gene (*ultrabithorax*) gives adults with two pairs of elytra after pupal and imaginal molt in *Tribolium castaneum* (Tomoyasu et al, 2005)



*Ultrabithorax* is required for membranous wing identity in *Tribolium castaneum*

dsUbx-treated adults of *Tribolium castaneum*



# Vitellogenin endocytosis



Sequence analysis of BgVgR indicated that it is a lipoprotein receptor belonging to the subgroup of insect vitellogenin receptors.

RNAi of BgVgR led to a phenotype characterized by low yolk content in the basal oocyte and high vitellogenin concentration in the haemolymph.

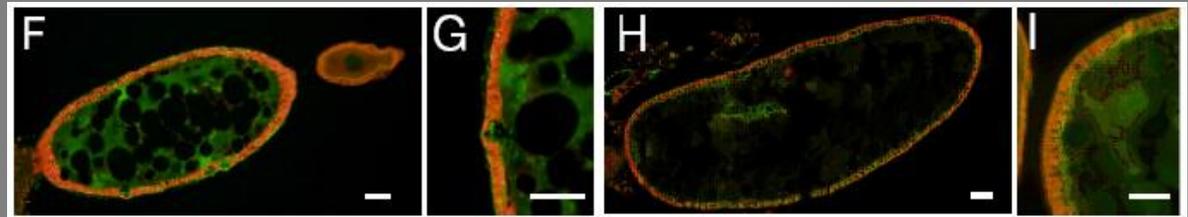
dsBgVgR      Control



dsBgVgR

Control

Immunocytochemistry reveals that VgR is localized in the cortex of the oocyte. dsBgVgR-treated specimens do not show VgR immunoreactivity.

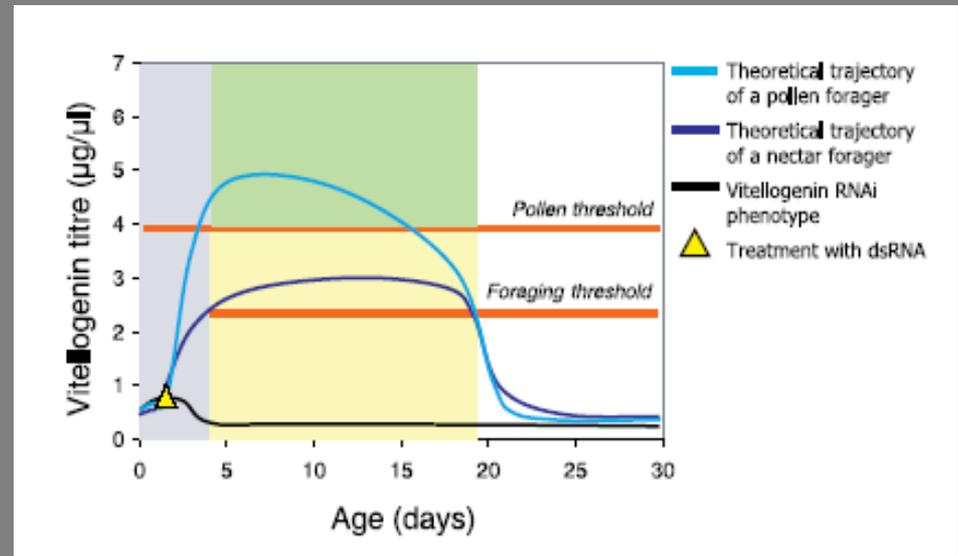


# JH, vitellogenin and social organization in honeybees



Vitellogenin is a major reproductive protein in insects, in general, and seems to be a sort of endocrine factor in honeybees, driving behavior.

RNAi of AmVg results in workers that start foraging flights earlier than controls, and collect larger loads of nectar, as in control low-vitellogenin content workers.



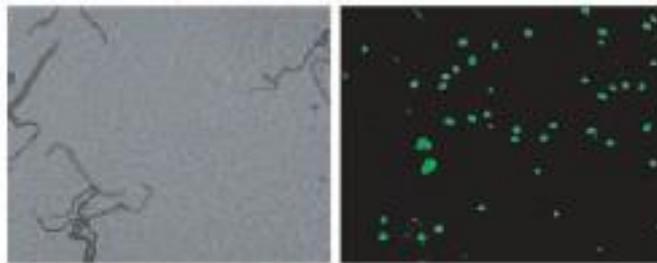
Vitellogenin gene activity paces onset of foraging behaviour and primes bees for specialized foraging tasks.

# Mosquito genes and *Plasmodium* development

MELANIZED  
OOKINETES

OOCYSTS

Control



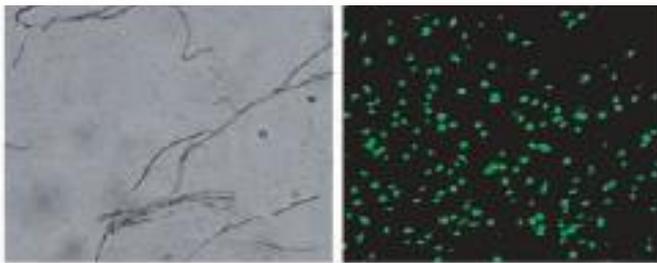
RNAi CTL4.

Good for *Plasmodium*



RNAi LRIM1.

Bad for *Plasmodium*



Systematic RNAi experiments have shown that an *Anopheles gambiae* C-type lectine (CTL4) act as protective agonist and one leucine rich-repeat protein (LRIM1) acts as an antagonist on the development of *Plasmodium* ookinetes to oocysts.

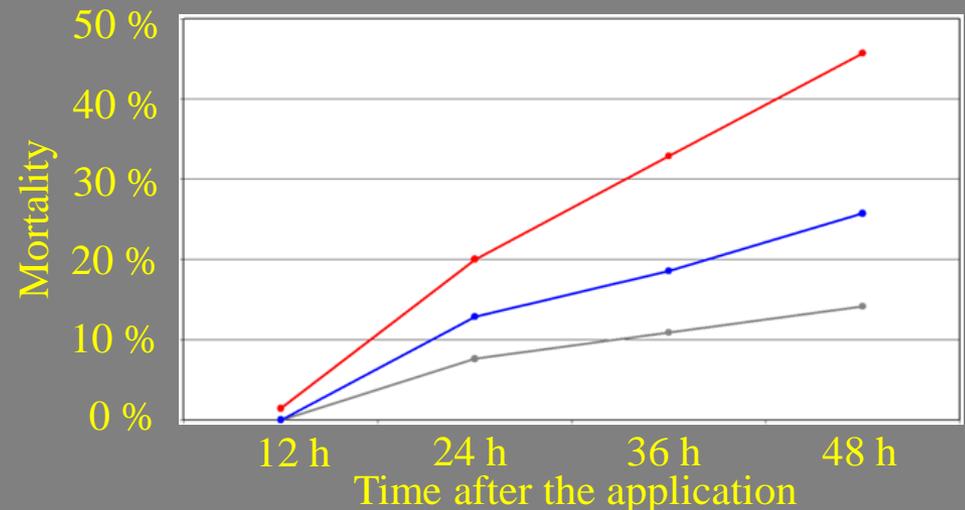
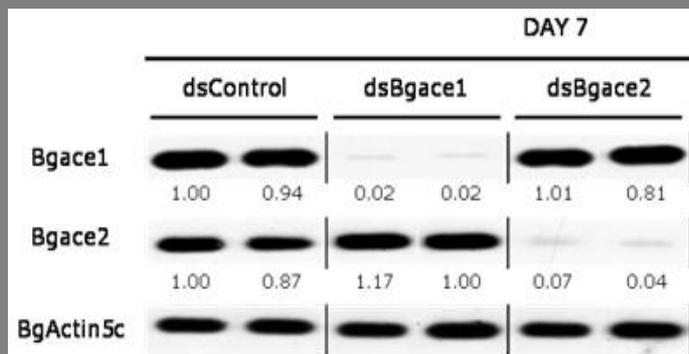
*Plasmodium* melanized ookinetes and oocysts in control and dsCTL4- and dsLRM1-treated females of *Anopheles gambiae* (Osta et al., 2004)

# Susceptibility to insecticides. The case of ACE

Acetylcholinesterase (ACE) is a key enzyme in neuronal synapsis, and it is the target of a number of insecticides, like Chlorpyrifos. In the cockroach *Blattella germanica* there are two ACE isoforms (ACE1 and ACE2) that work in an independent way.



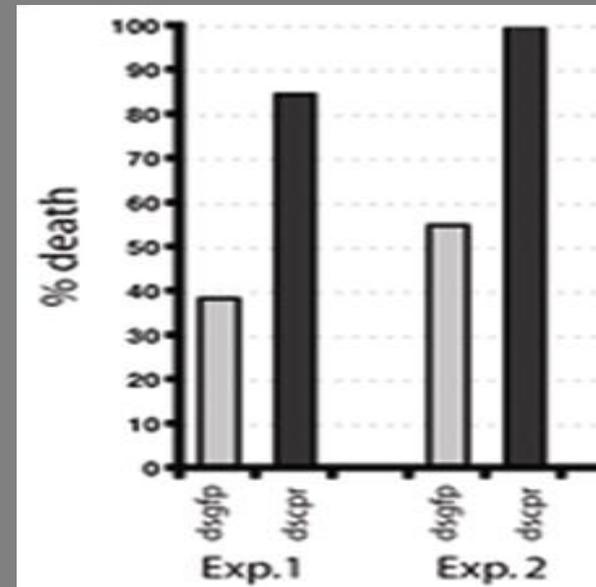
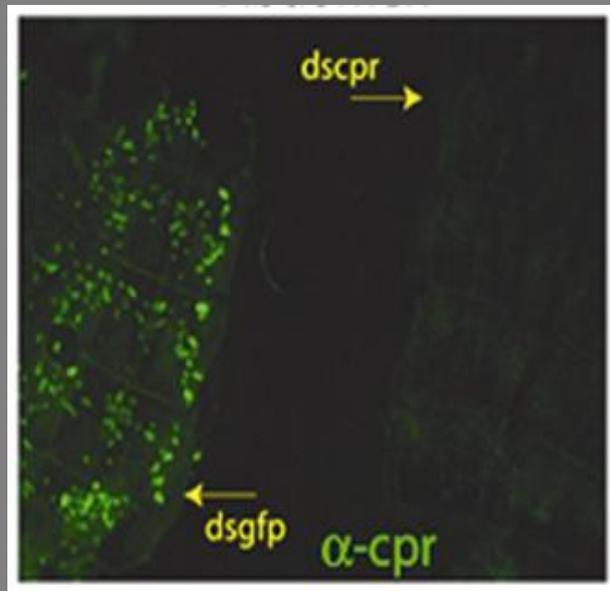
Depletion of ACE expression by RNAi increases the sensitivity towards toxic effects of Chlorpyrifos In Vivo. There is a differential response of ACE1 and ACE2.



● dsControl ● dsBgACE1 ● dsBgACE2

# Susceptibility to insecticides. The case of CPR

RNAi of Cytochrome P450 reductase (CPR) increases susceptibility to permethrin in malaria mosquito, *Anopheles gambiae*



Whole mount abdomen stains taken from control (dsgfp) or dsCPR injected mosquitoes. Oenocyte staining is drastically reduced, and susceptibility to permethrin increased (Lycett et al., 2006)

# What have we learned?

Araneae  
*Cupiennius salei*

1. Given a new gene, find the function (unveil the functions of new genes)
2. Given an old gene, assess presumed functions, or unveil new ones
3. Given a function, find the gene (candidate gene approach)

## Acari

*Dermacentor variabilis*  
*Ixodes scapularis*  
*Rhipicephalus sanguineus*  
*Amblyomma americanum*  
*Tetranychus urticae*

## Dictyoptera

*Blattella germanica*  
*Periplaneta americana*  
*Diploptera punctata*

## Orthoptera

*Locusta migratoria*  
*Gryllus bimaculatus*

## Isoptera

*Reticulitermes flavipes*

## Hemiptera

*Oncopeltus fasciatus*  
*Rhodnius prolixus*  
*Bemisia tabaci*

## Coleoptera

*Tribolium castaneum*  
*Apriona germari*  
*Harmonia axyridis*  
*Gastrophysa atrocyanea*

## Neuroptera

*Chrysopa perla*

## Hymenoptera

*Nasonia vitripennis*  
*Apis mellifera*

## Lepidoptera

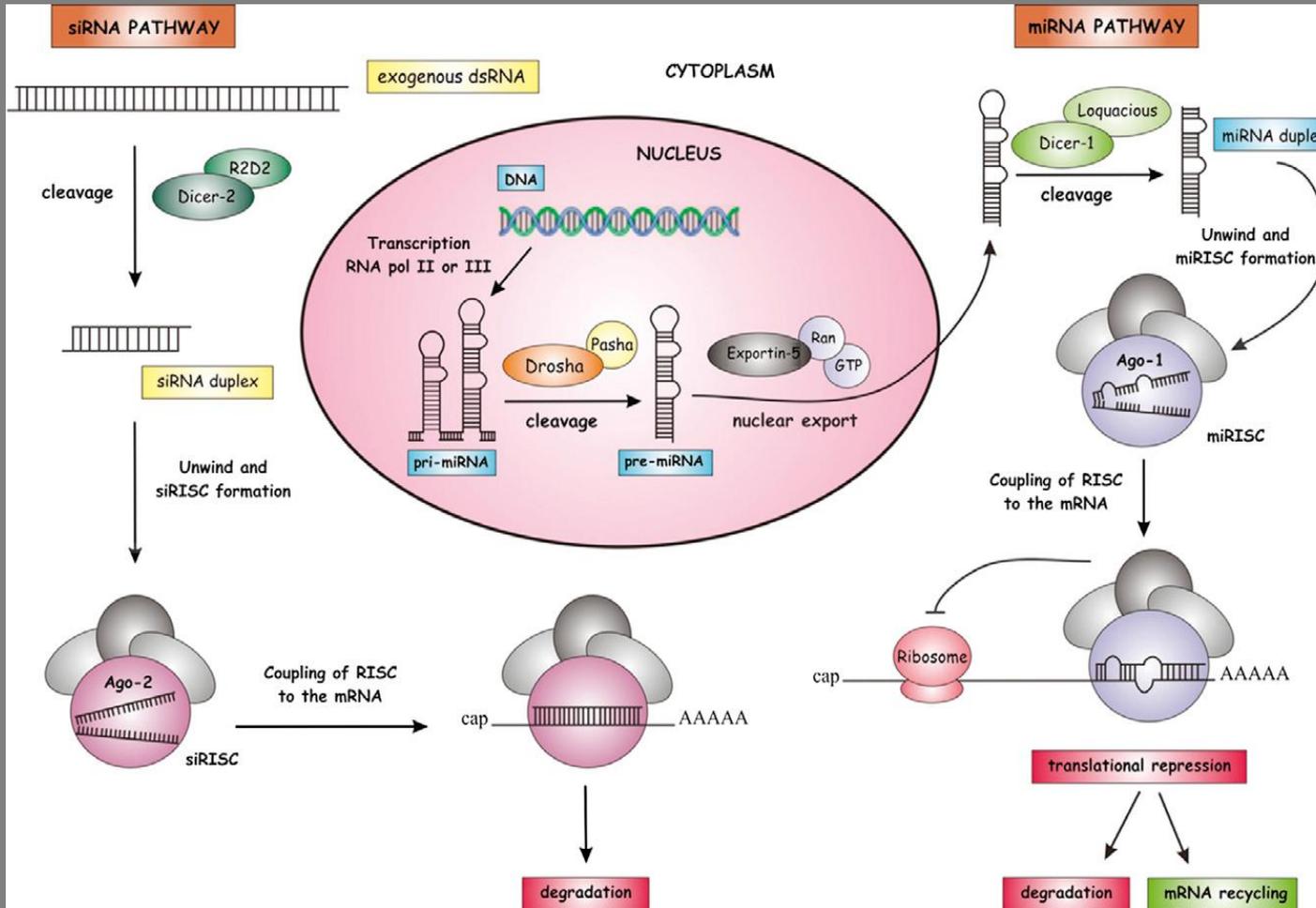
*Hyalophora cecropia*  
*Bombyx mori*  
*Manduca sexta*  
*Helicoverpa armigera*  
*Spodoptera litura*  
*Spodoptera exigua*  
*Spodoptera frugiperda*  
*Epiphyas postvittana*  
*Plodia interpunctella*

## Diptera

*Aedes aegypti*  
*Anopheles gambiae*  
*Drosophila melanogaster*  
*Lucilia sericata*  
*Sarcophaga peregrina*  
*Episyrphus balteatus*

# Lessons concerning mechanisms

# Insects have two Dicers



Dicer 1 (and AGO 1) specialized in miRNA pathways.

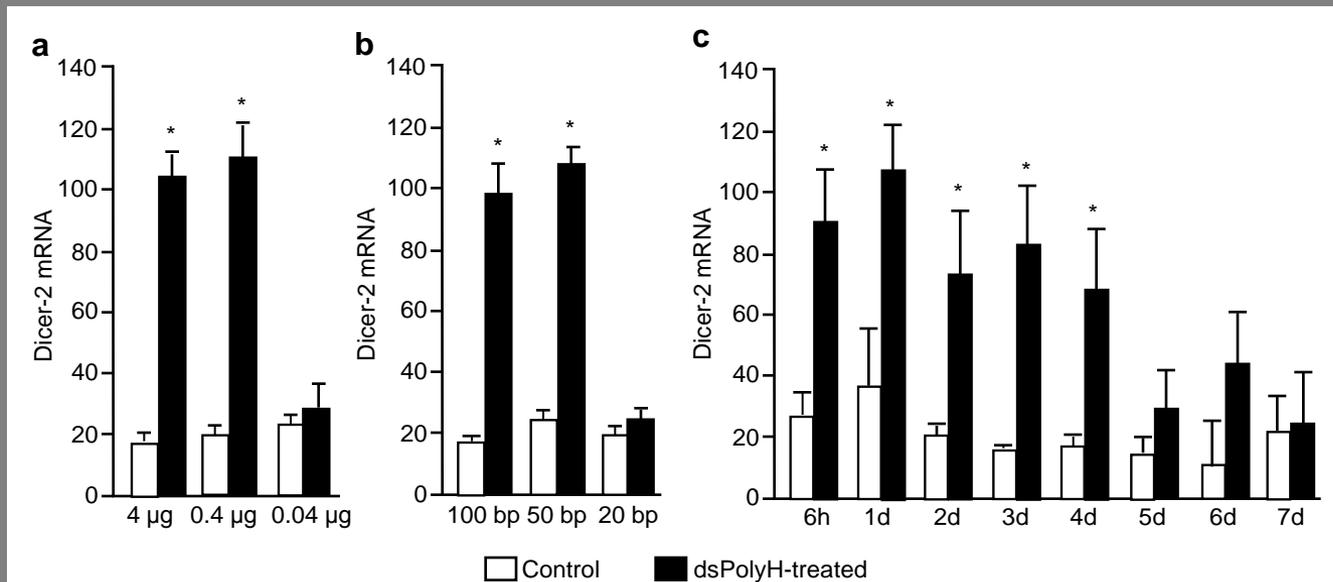
Dicer 2 (and AGO 2) specialized in RNAi pathways

Interesting because while RNAi is operating (using Dicer1), the miRNA pathway is not affected, and post-translational regulation of transcripts by miRNAs continues normally.

# Dicer-2, a sensor of alien RNA

On attempting to knockdown Dicer-2 expression in *Blattella germanica* by RNAi, we found that treatment with Dicer-2 dsRNA upregulated the targeted mRNA.

Dicer-2 upregulation was also observed after treating with a nucleopolyhedrovirus dsRNA. Experiments with this alien dsRNA showed an all-or-none response with a threshold for inducing Dicer-2 upregulation between 0.4 and 0.04  $\mu\text{g}$  in terms of dsRNA concentration and between 50 and 20 bp in terms of dsRNA length.



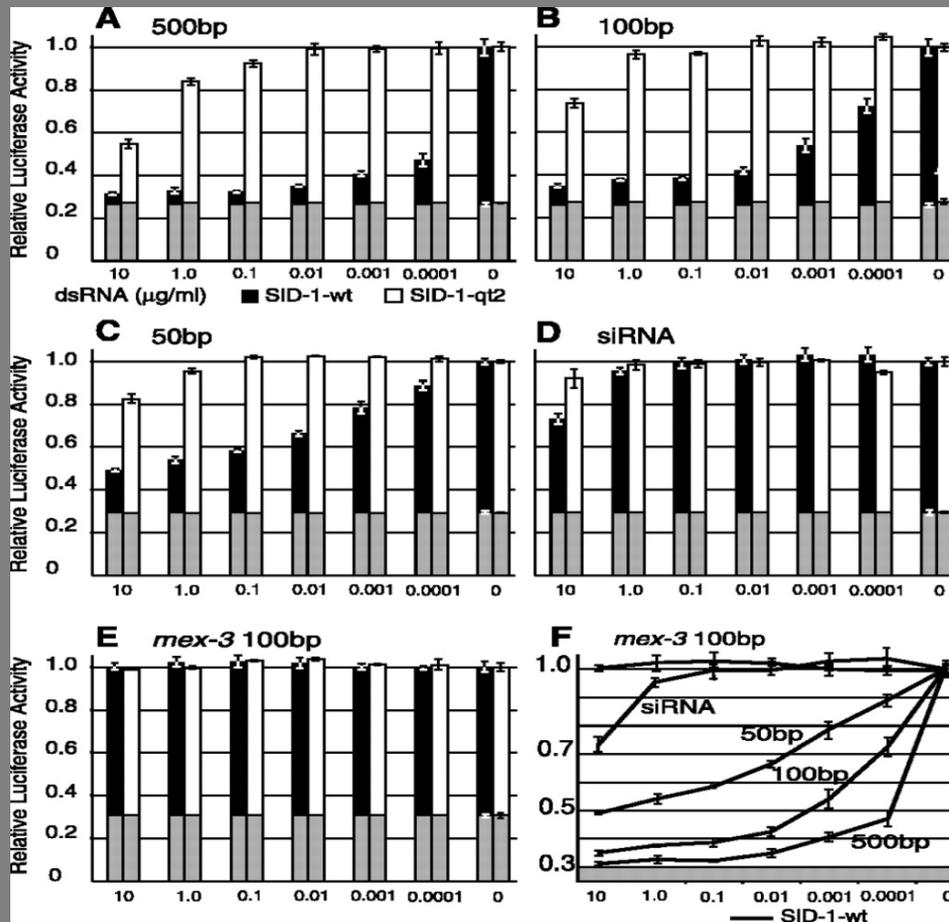
## Uptake and signal spread. Sid-1 in insects

In *C. elegans*, once dsRNA is introduced, RNAi silencing spreads throughout the organism and even into its progeny. This systemic spread of silencing does not occur in *sid-1* mutants, although silencing is observed within the cell where dsRNA is injected or expressed. Not all insects possess Sid-1 orthologs

Presence of *Sid-1* orthologs in different insect species

Species	Expression	Reference
<i>Tribolium castaneum</i>	+	Tomoyasu et al. (2008)
<i>Aedes aegypti</i>	/	Tomoyasu et al. (2008)
<i>Anopheles gambiae</i>	/	Tomoyasu et al. (2008)
<i>Culex quinquefasciatus</i>	/	NCBI Blastn
<i>Acrythosiphon pisum</i>	/	XM 001951872.1
<i>Aphis gossypii</i>	/	Xu and Han (2008)
<i>Rhodnius prolixus</i>	/	NCBI Blastn
<i>Rhopalosiphum padi</i>	/	EF533712
<i>Sitobion avenae</i>	/	Xu and Han (2008)
<i>Apis mellifera</i>	+	Aronstein et al. (2006)
<i>Nasonia vitripennis</i>	/	Tomoyasu et al. (2008)
<i>Bombyx mori</i>	/	Tomoyasu et al. (2008)
<i>Spodoptera exigua</i>	/	FJ619650.1
<i>Schistocerca americana</i>	+	Dong and Friedrich (2005)
<i>Pediculus humanus</i>	/	XM_002427838

# Transport of dsRNA into *Drosophila* S2 cells by the *C. elegans* protein Sid-1



*Drosophila* lacks a SID-1 homolog and do not show systemic RNAi. Yet *Drosophila* S2 cells can initiate RNAi triggered by dsRNA present in the growth medium.

*Drosophila* S2 cells expressing *C. elegans* SID-1 initiate RNAi in response to at least a 100,000-fold lower dsRNA concentration and in contrast to endogenous uptake, internalize dsRNA in a rapid, largely energy-independent manner.

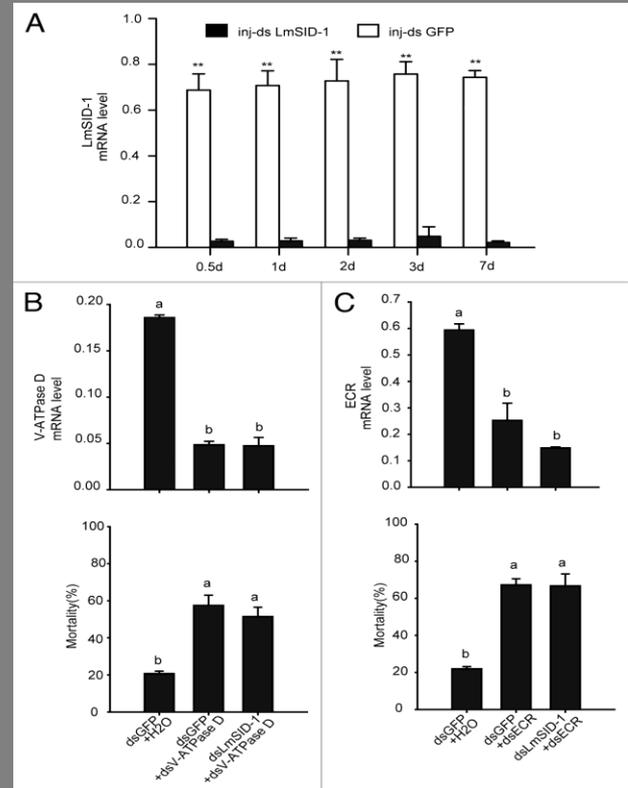
These results indicate that rather than a pump or a receptor, SID-1 functions as a dsRNA pore or channel, which enables transfer of dsRNA across the cell membrane into the cytoplasm.

# Sid-1 is not necessary in *Locusta*

Silencing of LmSID-1 gene did not influence RNAi effects of other genes.



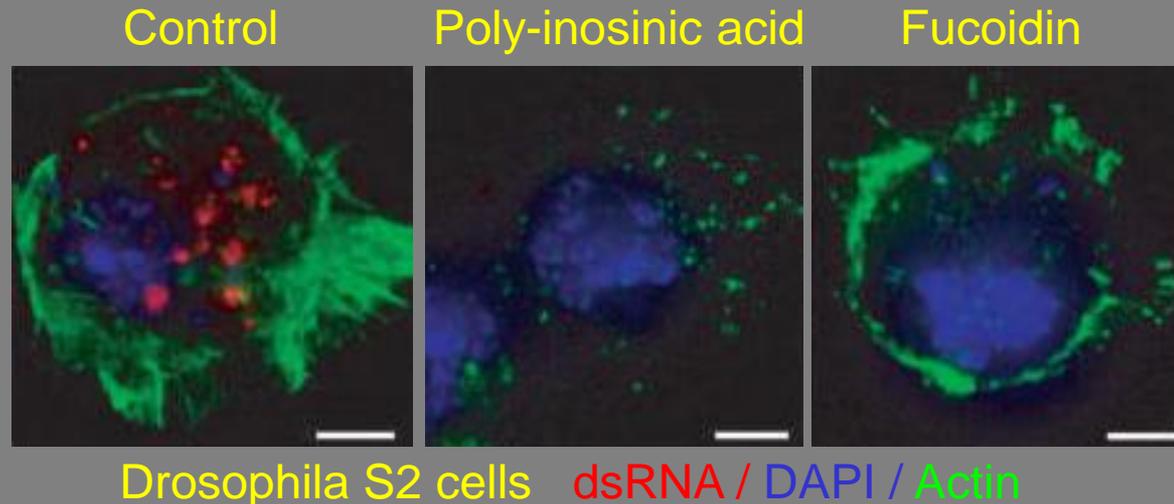
Experiment in vitro showed that expression of the LmSID-1 protein in *Drosophila* S2 cells do not enhance dsRNA uptake.



These findings imply the existence of alternative mechanisms underlying insect systemic RNAi, which may be different from *Caenorhabditis elegans*

## Endocytic pathway. Scavenger receptors

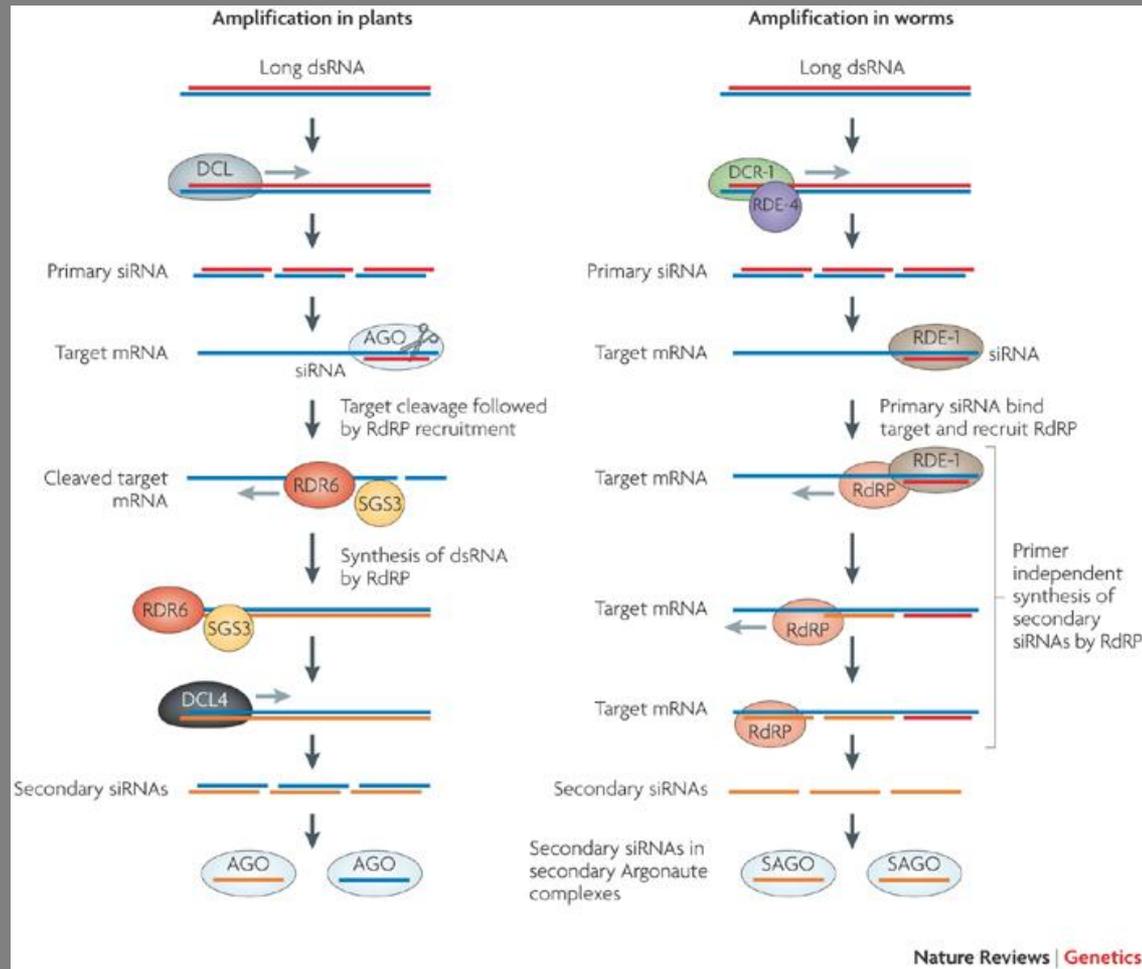
The role of endocytosis in dsRNA uptake was shown using drugs that impair this process. The experiments of Saleh et al. (2006) showed that dsRNA was associated with vesicles, suggesting that the dsRNA fragments were taken up by receptor-mediated endocytosis, and that a combination of scavenger receptors participated in dsRNA uptake.



In total, 23 out of the 7216 genes screened by Saleh et al. (2006) were found to be involved in dsRNA uptake and processing. Some of these sequences are known to be involved in endocytosis, encoding for proteins of the vesicle mediated transport, conserved oligomeric Golgi complex family, cytoskeleton organization and protein transport.

# Signal amplification: RdRp

RNA dependent RNA polymerase (RdRp) allows independent synthesis of secondary siRNAs. Insects do not possess RdRp orthologs



But in most cases the RNAi response is very robust and lasts a long time (to allow parental RNAi, for example), which suggests that there are efficient mechanisms of signal amplification in insects

# Lessons concerning idiosyncrasies

A number of species have shown to be remarkably reluctant to RNAi, notably, among lepidoptera, diptera and hymenoptera.  
Why?

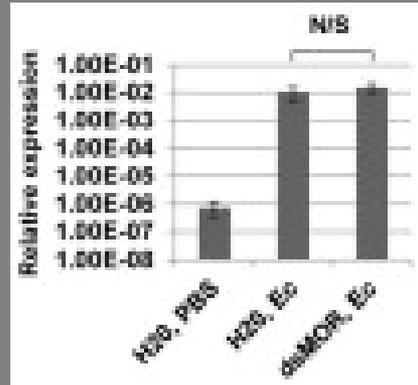
# Possible causes accounting for RNAi insensitivity in insects

<b>Intrinsic of the species</b>	<b>Deficient amplification and spreading of the RNA signal.</b>
	<b>Alien dsRNA is efficiently degraded.</b>
<b>Intrinsic of the tissue</b>	<b>The tissue is hardly permeable to dsRNAs</b>
	<b>The elements of the RNAi machinery are mildly expressed in the tissue.</b>
<b>Intrinsic of the gene</b>	<b>The particular dsRNA is efficiently degraded.</b>
	<b>The gene efficiently counteracts RNAi by increasing transcription rates.</b>

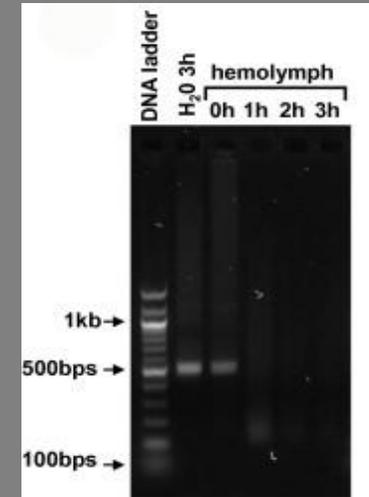
# Alien dsRNA is differently degraded in different species



*Manduca sexta*



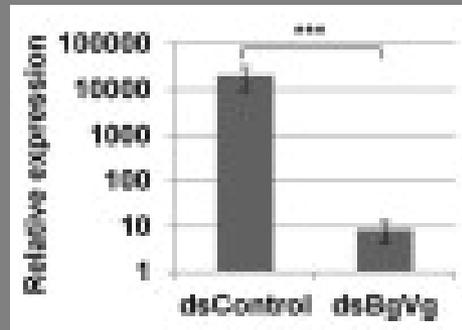
Low silencing efficiency



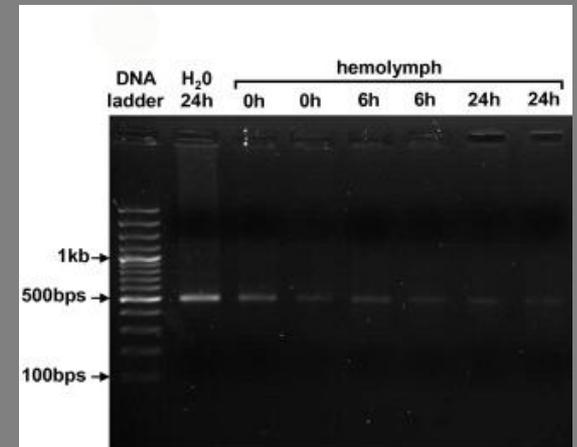
Low stability of dsRNA



*Blattella germanica*

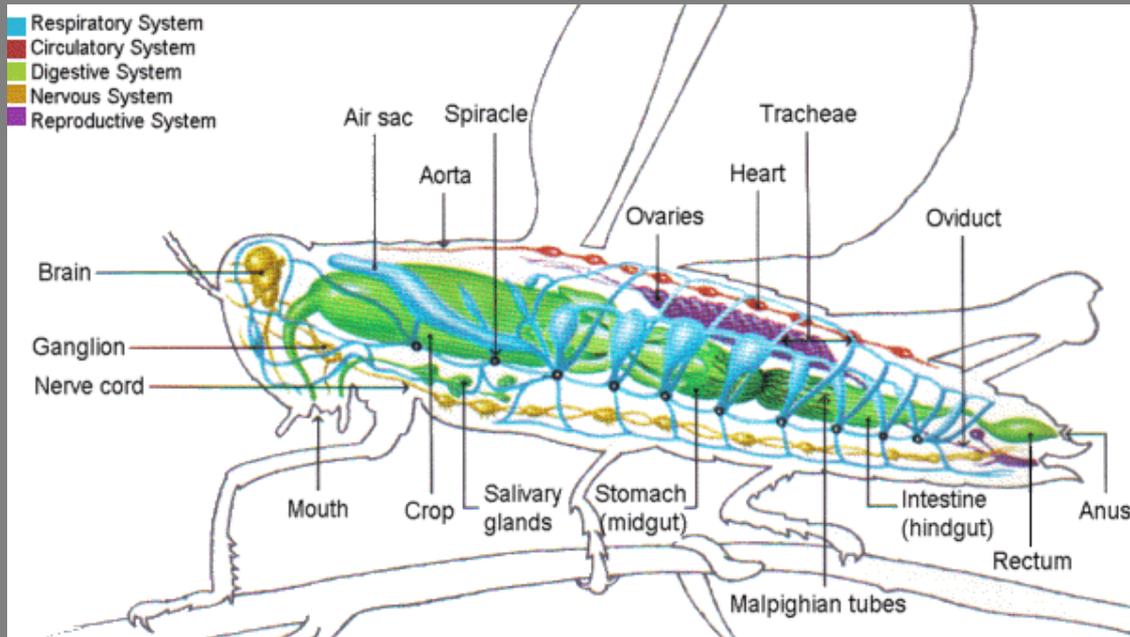


High silencing efficiency



High stability of dsRNA

# Different permeability of different tissues



Often the more sensitive tissues are those directly in contact with hemolymph. The fat body is an example.

In the case of the ovary, the more internal ovarioles show a transcript decrease lower than those located in the periphery.

## Future directions

### Subjects promising interesting results in insect RNAi research

1. Mechanisms of dsRNA uptake and siRNA spread (are there functional equivalents of SID-1 in insects?).
2. Mechanisms of siRNA amplification (are there functional equivalents of RdRp in insects?) .
3. Mechanisms of dsRNA degradation.
4. Technology for dsRNA or siRNA delivery.