Beyond *Drosophila:* RNAi In Vivo and Functional Genomics in Insects

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Abstract

The increasing availability of insect genomes has revealed a large number of genes with unknown functions and the resulting problem of how to discover these functions. The RNA interference (RNAi) technique, which generates loss-of-function phenotypes by depletion of a chosen transcript, can help to overcome this challenge. RNAi can unveil the functions of new genes, lead to the discovery of new functions for old genes, and find the genes for old functions. Moreover, the possibility of studying the functions of homologous genes in different species can allow comparisons of the genetic networks regulating a given function in different insect groups, thereby facilitating an evolutionary insight into developmental processes. RNAi also has drawbacks and obscure points, however, such as those related to differences in species sensitivity. Disentangling these differences is one of the main challenges in the RNAi field.

INTRODUCTION

The ability to sequence DNA at high speed and low cost is one of the most significant technical advances in modern biology. Consequently, the race to sequence entire genomes in eukaryote species, which started with the fruit fly, *Drosophila melanogaster*, and humans, *Homo sapiens*, is advancing at formidable speed. At the time of writing this manuscript (July 2009), 50 insect genome projects either finished or are in progress (see **http://www.ncbi.nlm.nih.gov/sites/entrez?db=genomeprj**), and it is likely that in the near future all significant phylogenetic groups, perhaps every order, will have at least one representative with its genome sequenced. In parallel, the growing availability of genomes is revealing a large array of genes with unknown functions, and this is especially dramatic in the amazingly complex insect world. However, these discoveries lead to the problem of how to unveil the functions of this avalanche of new genes. The RNA interference (RNAi) technique has arrived just in time to help us face this singular challenge. This review deals with the great potential offered by RNAi for studying gene function in the seemingly endless world of insects.

RNA interference (RNAi): cellular process by which an mRNA is targeted for degradation by a dsRNA with a strand complementary to a fragment of such mRNA

Double-stranded RNA (dsRNA): in RNAi experiments, the molecule delivered to the experimental system, which is then cleaved by Dicer enzyme into siRNAs

Small interfering RNA (siRNA): in RNAi experiments, fragments derived from the delivered dsRNA, which bind the complementary region of the target mRNA as a key step for its degradation

RISC: RNA-induced silencing complex

A SHORT HISTORY OF RNAi

Toward the end of the 1980s, Jorgensen and colleagues (51) were studying the role of chalcone synthase in the biosynthetic pathway of anthocyanin in plants. Anthocyanin gives the violet color to petunias, and Jorgensen's team overexpressed chalcone synthase with the aim of obtaining petunias with a deeper violet color, although they unexpectedly obtained whitish flowers. Expression of chalcone synthase in these transgenic whitish petunias was some 50 times lower than in the wild type, suggesting that transgenic chalcone synthase had in some way suppressed the endogenous gene (51).

In animals, the first clues about RNAi were obtained by Guo and Kemphues (25) in a series of experiments conducted with RNA antisense on the nematode *Caenorhabditis elegans*. Thus, while investigating the gene *par-1*, these authors observed that it was not expressed when nematodes were treated with RNA antisense for *par-1*, as expected. However, *par-1* expression was also impaired in control experiments with the RNA sense. This paradoxical result was subsequently explained by the experiments of Fire, Mello, and coworkers (21), who carefully purified the RNA antisense, RNA sense, and double-stranded RNA (dsRNA) for the gene *unc-22* of *C. elegans*. The results of *unc-22* interference showed that single-stranded RNAs (either sense or antisense) were between 10 and 100 times less effective than dsRNA. Indeed, single-stranded sense RNAs were effective only if delivered just after the antisense or vice versa, which suggested that both strands hybridized in vivo to form a dsRNA, which appeared to be responsible for the activity (21).

Although it was presumed that both strands of the dsRNA had to unwind before coupling to the target RNA, the complete antisense strand had never been detected. This led plant virologists Hamilton and Baulcombe to search for fragments of antisense RNA derived from the dsRNA. They detected RNAs of approximately 25 nucleotides and proposed that this was the length necessary to produce the RNAi (26). Soon afterward, two independent teams (27, 70) working on *D. melanogaster* cells purified RNA fragments of approximately 21–23 nucleotides thought to be responsible for the RNAi and proposed that the dsRNA is converted into these shorter sequences (called small interfering RNAs, or siRNAs) that are able to bind the complementary region of the target mRNA, thus leading to its degradation.

The next steps involved studying the cleavage of dsRNA into siRNAs and the degradation mechanisms of the target mRNA. Again using *D. melanogaster* cells, Bernstein, Hannon, and coworkers (6) showed that there was an enzymatic activity for each of the two mechanisms, and that both could be isolated by ultracentrifugation. The activity fragmenting the target mRNA, called RNA-induced silencing complex, or RISC, was found in the pellet, whereas that fragmenting the dsRNA into siRNAs remained in the supernatant. The study of the

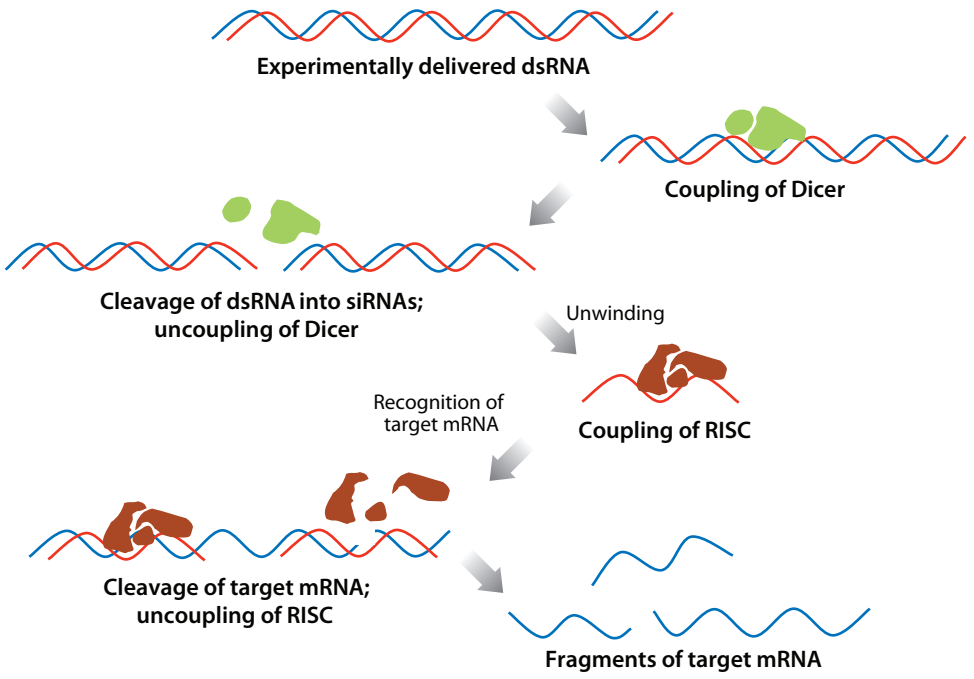

Figure 1

Basic mechanisms of RNA interference (RNAi). The double-stranded RNA (dsRNA) is cleaved into fragments of ~21 nucleotides (the small interfering RNAs, or siRNAs) by the enzyme Dicer. The siRNAs unwind, and the antisense strand couples to the RNA-induced silencing complex (RISC) and conveys it to the target mRNA. Then RISC couples to the target mRNA, blocking and degrading it.

enzyme that cleaves the dsRNA was undertaken on *D. melanogaster* S2 cells by the same team, who found that it was a type III RNase codified by the gene CG4792, which they called Dicer (5). The study of RISC initiated by Tuschl's group using extracts of human HeLa cells allowed them to discover the first Argonaute proteins, which are responsible for the cleavage of the target mRNA (45). **Figure 1** summarizes the basic mechanisms of RNAi. Subsequent research has elucidated other mechanistic details, such as the specific functions of different forms of Dicer and Argonaute, how the siRNAs are unwound and incorporated into RISC, and the composition of RISC (57).

RNAi AND REVERSE FUNCTIONAL GENOMICS

In classical functional genomics, the function of interest is chosen first and then the genes determining it are identified. The publication of the first genomes (*D. melanogaster*, *H. sapiens*) led to the discovery of a large number of genes with unknown functions, which led to reverse functional genomics, whereby the gene is chosen first and then its function studied. *D. melanogaster* again became a paradigm, as it can be genetically transformed through conventional techniques, which allows mutant phenotypes for the chosen genes to be studied.

The advent of RNAi represents a new paradigm, as it opens the door to the study of nontransformable species. The first step in this process involves conveying a dsRNA with a strand complementary to a fragment of the target mRNA into the cells. After checking that target mRNA levels have lowered, the study of the phenotype illuminates the corresponding functions. Experiments can be carried out either in vitro or in vivo.

UAS: upstream activator sequence

Interference In Vitro

The easiest system involves incubating the cells with the dsRNA added to the medium, as described for the first time in *D. melanogaster* (16). Due to the ease of this approach and the availability of libraries, most *D. melanogaster* RNAi screens have been performed using cell-based tests. Screenings can be carried out for a reduced number of genes or through genome-wide applications (10). An example is the screen for tyrosine kinase receptor regulators in *D. melanogaster* cells, reported by Friedman and Perrimon (22).

Interference In Vivo

In genetically transformable species, RNAi can be triggered by the expression of a long double-stranded hairpin RNA from a transgene containing a gene fragment cloned as an inverted repeat. The expression of such transgenes under the control of a generic promoter containing the GAL4-responsive upstream activator sequence (UAS) element can target RNAi to any cell type at any stage of the insect for which a suitable GAL4 driver line is available. Approaches may be on a one-by-one gene basis, as in the work of Kennerdell and Carthew on *D. melanogaster* embryos (32), or at a genome-wide level, using libraries of transgenic RNAi strains. Libraries of transgenic *D. melanogaster* harboring UAS-driven RNAi hairpins have been generated on a genome-wide scale (19), and a number of papers have been published using them, such as that of Dickson and coworkers, which led to the identification of the sex peptide receptor of *D. melanogaster* (69).

A more straightforward approach, which is applicable to nonmodel species for which systematic recovery of mutants is not feasible, consists of delivering the dsRNA to the chosen stage (from egg to adult) of the experimental specimen and then examining the resulting phenotype. This approach can change the landscape of reverse functional genomics in nonmodel insect species more profoundly, and the comments that follow are based exclusively on it.

TWELVE YEARS OF RNAi IN VIVO IN INSECTS

In 1998 Kennerdell and Carthew (31) were the first to use RNAi in vivo to study the genes *frizzled* and *frizzled 2* in *D. melanogaster*. A year later, Brown and coworkers (12) carried out functional studies of Hox genes in a holometabolous species, the beetle *Tribolium castaneum*. A year later, RNAi was used for the first time in a hemimetabolous species, the bug *Oncopeltus fasciatus*, in which Hughes and Kaufman (29) also studied the function of Hox genes. There has since been such a deluge of papers in this field that an exhaustive treatment would be inappropriate here. What follows is a selection of emblematic cases with the aim of covering representative functions and species.

Studies of Embryonic Development

Insects present a remarkable flexibility in embryonic development. In the ancestral short-germ-band mode, head and thorax arise from the egg's posterior, and abdominal segments form progressively from a posterior growth zone. By contrast, in the more derived long-germ-band mode, all segments emerge simultaneously in a syncytial environment, with head and thorax at the egg's anterior (11).

D. melanogaster has remained the more widely studied species among long-germ-band insects. As early as 1998, the pioneering work of Kennerdell and Carthew (31) demonstrated with RNAi that the genes *frizzled* and *frizzled 2* belong to the *wingless* pathway. Embryo injection has been widely used to study individual genes and small-scale screens, such as those carried out for genes involved in nervous system development (33) and embryo cellularization (55). However, there is considerable morphological and functional diversity among long-germ-band species, therefore it would be of interest to compare dipterans with other orders. To this end, Desplan and coworkers (11) have investigated the hymenopteran *Nasonia vitripennis*, a long-germ-band species that lacks the gene *bicoid*, which in dipterans organizes the

structural pattern of the anterior body region. RNAi showed that a repressor system of maternal origin localized in the anterior part of the embryo inhibits the genes determining the formation of the posterior part; the head and the thorax of *N. vitripennis* can therefore be formed correctly with the products of the *orthodenticle*, *hunchback*, and *giant* genes, in the absence of *bicoid* (11).

Among insects with intermediate germ band, the first species studied were the bug *O. fasciatus* and the cricket *Gryllus bimaculatus*. In *O. fasciatus*, Kaufman and coworkers used RNAi to silence Hox genes (**Figure 2*a–c***) and genes involved in segmentation and segment specification (1, 29, 38), and Noji and coworkers also studied genes involved in segmentation and segment specification in *G. bimaculatus*

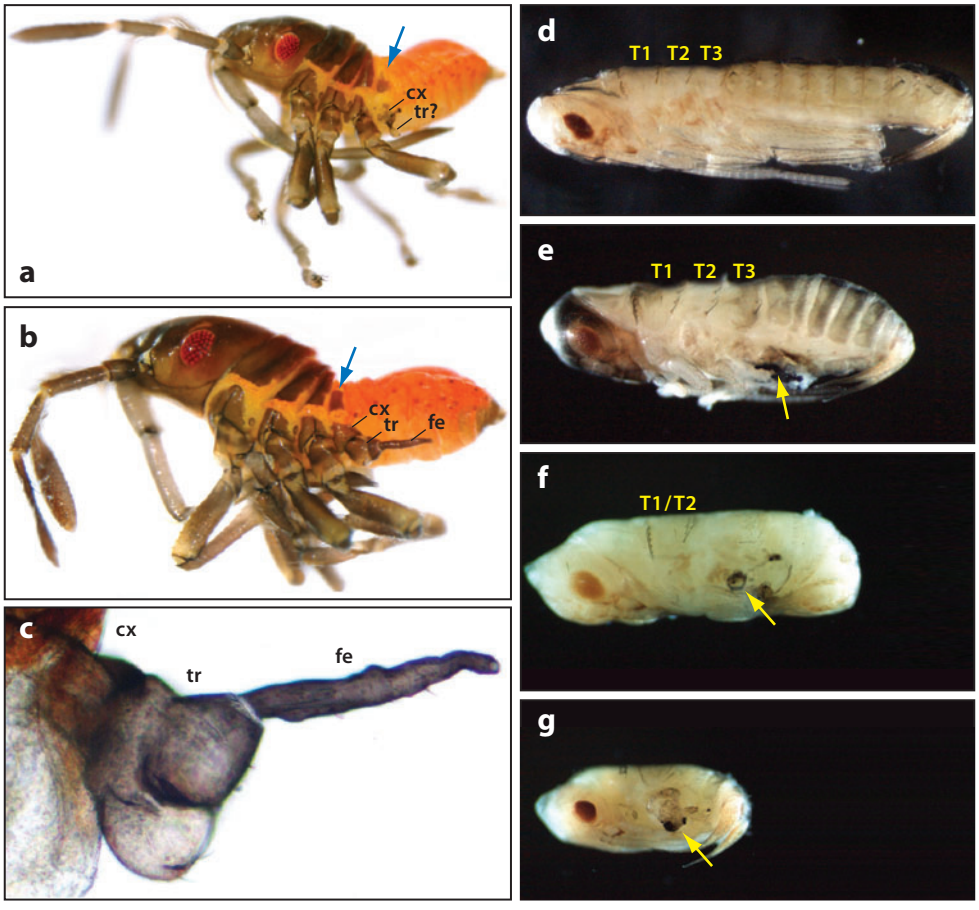

Figure 2

(*a–c*) RNAi of the Hox gene *ultrabithorax* in the embryo of the hemipteran *Oncopeltus fasciatus* leads to the formation of larvae with thorax-like color and ectopic legs in the first abdominal segment. Panels *a* and *b* show the general aspect, and panel *c* shows an ectopic abdominal leg, with the corresponding coxa (cx), trochanter (tr), and femur (fe) (1). (*d–g*) In the cricket *Gryllus bimaculatus*, RNAi of the gap gene *Krüppel* results in embryos with abdominal and thoracic segments fused or absent. Panel *d* shows a normal embryo with the three thoracic segments (T1, T2, and T3) indicated, whereas panels *e–g* show phenotypes with segments fused (49). Photos courtesy of Thom Kaufman (*a–c*) and Sumihare Noji (*d–g*).

Parental RNAi: delivery of dsRNA to the mother, which results in RNAi in offspring embryos

JH: juvenile hormone

(49) (**Figure 2*d*–*g***). The results in both species showed a considerable functional conservation with respect to *D. melanogaster*, although some differences were observed. Even the comparison between *O. fasciatus* and *G. bimaculatus* afforded subtle differences, for example, in the regulation of *Krüppel* by Hox genes.

Among short-germ-band species, *T. castaneum* has been thoroughly studied by a number of research groups investigating segmentation and Hox genes (9, 12, 59). Moreover, Bucher and colleagues have demonstrated the occurrence of parental RNAi in *T. castaneum*, by silencing a number of genes (*Distalless*, *maxillopedia*, and *giant*) in the embryo after treating the mother with the corresponding dsRNAs (13). The role of *hunchback* in segmentation has been investigated in the locust *Locusta migratoria* (28), whereas RNAi in the cockroach *Periplaneta americana*, a short-germ-band and basal species, revealed that segmentation involves mechanisms similar to those in vertebrates. Thus, segment formation is induced by cyclic segmental stripes of *hairy* and *Delta* expression, which suggests that Notch-mediated segmentation is the ancestral mechanism for segment formation in insects (56).

Postembryonic Development

The most frequently studied processes during postembryonic development are related to molting, especially to metamorphic molts. The RNAi of the Hox gene *ultrabithorax* in *T. castaneum*, for example, promotes the hindwings, which are normally membranous, to become elytra, like the forewings (**Figure 3**). This work showed that elytra are formed in the absence of *ultrabithorax* (65). This finding is interesting because it shows a contrasting situation to *D. melanogaster*, for which *ultrabithorax* promotes haltere identity in the hindwings.

Further RNAi studies in *T. castaneum* afforded a number of clues for understanding the antimetamorphic action of juvenile hormone (JH). Konopova and Jindra (34), for example, have shown that interference of the gene *Met* (the product of which is a transcription factor involved in resistance to JH) in larval stages provokes a precocious transition to the pupal stage. This indicates that *Met* mediates the inhibitory action of JH on metamorphosis. More recent papers based on RNAi experiments have shown that the transcription factor Broad (35, 54, 62) and Krüppel homolog 1 (48) are also involved in the antimetamorphic action of JH.

Blattella germanica has become a convenient model for RNAi approaches among hemimetabolous species. Thus, studies carried out on this cockroach have revealed the conserved role of a number of nuclear receptors involved in the molecular action of ecdysteroids in molting. Knockdown of the ecdysone receptor itself (BgEcR and BgRXR/USP), along with BgHR3 and BgFTZ-F1, results in ecdysis impairment, whereas knockdown of BgE75 impairs apolysis (see, for example, References 18 and 43).

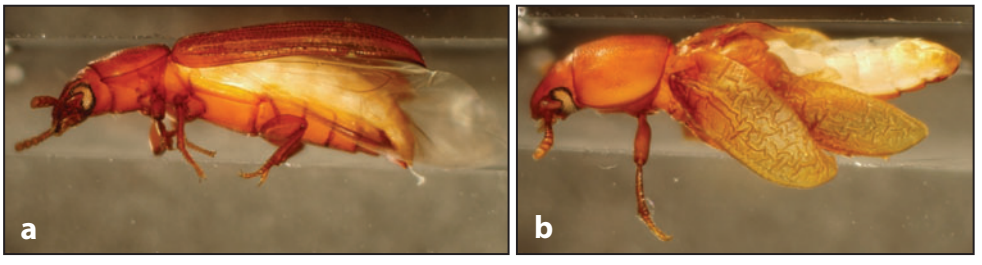

Figure 3

RNAi of the Hox gene *ultrabithorax* in larvae of the beetle *Tribolium castaneum* leads to the formation of adults with two pairs of elytra. (*a*) A normal adult. (*b*) The double-elytra phenotype (65). Photos courtesy of Yoshi Tomoyasu and Rob Denell.

Both cockroaches and crickets are suitable models for studying appendage regeneration. In *G. bimaculatus*, for example, RNAi has allowed a number of steps in the signaling cascades required for regenerating a leg to be elucidated, and also provided general clues that are also relevant in vertebrate limb regeneration (50).

Reproduction and Vitellogenesis

The most important protein in vitellogenesis is vitellogenin (Vg), which is generally produced in the fat body, then released to the hemolymph, and incorporated into growing oocytes through a receptor. RNAi has allowed the disruption of Vg expression in the honey bee, *Apis mellifera*, and in *B. germanica*. In *A. mellifera*, production of JH was higher in workers whose Vg levels had been lowered by RNAi (24), which points to the occurrence of a sort of feedback mechanism specific to this eusocial species. In *B. germanica*, RNAi of Vg inhibited Vg production in the fat body and impaired oocyte growth (44), whereas RNAi of Vg receptor (15) (**Figure 4**) produced a similar phenotype, with the oocytes permanently immature. In this nonsocial cockroach, however, lowered levels of Vg did not stimulate JH production.

Vg: vitellogenin

Another interesting aspect of vitellogenesis is the study of mechanisms mediating the transduction of a nutritional signal into a reproductive output. In *B. germanica*, for example, RNAi has shown that the protein TOR (target of rapamycin) is crucial for the transformation of nutritional signals into production of JH, which is essential for vitellogenesis and reproduction in this species (42).

Behavior

Hematophagous insects require an arsenal of bioactive proteins in the salivary glands to be efficient bloodsuckers. In the hemipteran *Rhodnius prolixus*, for example, which is a vector of Chagas disease, RNAi of Nitrophorin 2 provokes a decrease of anticoagulant activity and less efficient feeding behavior (3). Similarly, Boisson and associates (8) used RNAi to silence the apyrase AgApy in the salivary glands of the malaria mosquito *Anopheles gambiae* and thereby showed its important role in host probing

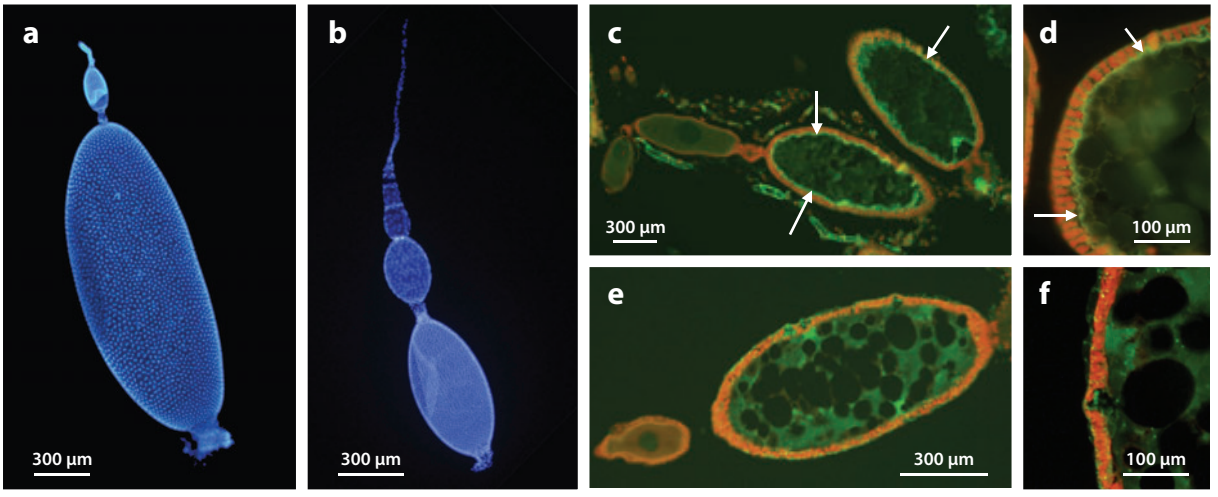

Figure 4

RNAi of vitellogenin receptor (VgR) of the cockroach *Blattella germanica* prevents the accumulation of vitellogenin by the basal oocytes. (*a*) Ovariole from a control six-day-old female with basal oocytes. (*b*) Ovariole from a knockdown six-day-old female. Immunocytochemistry of VgR in three-day-old adults indicates that, in controls, the receptor localizes in the cortex of basal oocytes (*c*, *d*, *arrows*), whereas it is absent in knockdowns (*e*, *f*) (15). Photos courtesy of Maria-Dolors Piulachs.

behavior. RNAi experiments have demonstrated the role of a number of molecules in behavior outside the salivary glands. The enzyme 3-hydroxy-3-methylglutaryl CoA reductase, for example, which is involved in the mevalonate pathway and the synthesis of JH, has been implicated in the control of sexual dimorphism of locomotor activity in *D. melanogaster* (4). Likewise, RNAi studies in *B. germanica* have demonstrated the role of the neuropeptide pigment-dispersing factor in the regulation of locomotor circadian rhythms. dsRNA treatments caused rhythmic males to become arrhythmic in light-dark cycles or in constant darkness (37).

Complex Biosynthetic Pathways

One of the biosynthetic pathways studied with RNAi is that of chitin, a key component of the exoskeleton and the midgut peritrophic matrix. RNAi experiments in *T. castaneum* have shown that chitin synthases CHS1 and CHS2 play anatomically specialized roles. Thus, whereas CHS1 is needed for the formation of the epidermal cuticle, CHS2 is essential for depositing chitin into the peritrophic matrix (2). The same team (2) has shown that the protein Laccase 2 is crucial for cuticle tanning in *T. castaneum* (**Figure 5**).

Sexual pheromone pathways have also been studied with RNAi. Matsumoto and coworkers (52) used RNAi to disrupt the expression of enzymes involved in the synthesis of bombykol, the main component of *Bombyx mori* sexual pheromone, as well as pheromone-binding proteins and the receptor of the pheromone biosynthesis activator neuropeptide. The results indicated that these proteins are essential for bombykol synthesis and action. In the lepidopteran *Epiphyas postvittana*, RNAi experiments allowed the pheromone-binding protein of the antennae (66) to be silenced and, in addition to provide functional information, involved the methodological novelty of applying the dsRNA by feeding.

Resistance Against Biological Agents

Insects possess a defense system against pathogens based on cellular and humoral reactions, and RNAi approaches have helped to unveil several details of them. One of the more thoroughly studied models has been *Anopheles-Plasmodium* relationships. The first study with RNAi was carried out on defensin, an antimicrobial peptide, on the mosquito *A. gambiae*. The results showed that defensin is important for protecting the mosquito against infections of Gram-positive bacteria (7). Other RNAi studies conducted by Kafatos and coworkers showed that *A. gambiae* expresses proteins with antagonistic effects on *Plasmodium berghei*, a murine model for malaria studies. One of

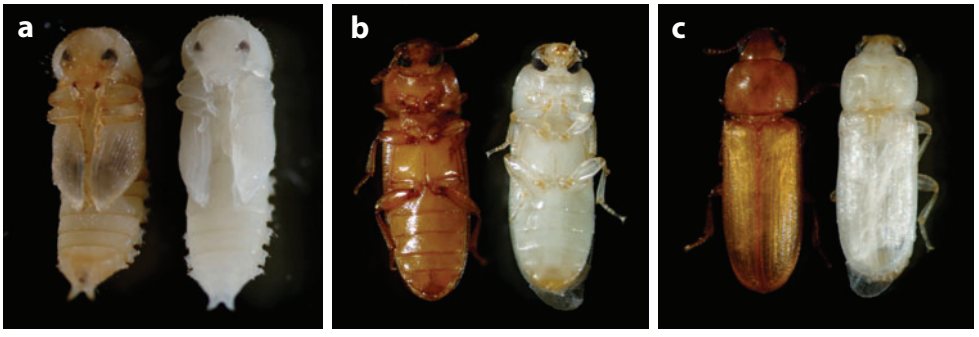

Figure 5

RNAi of the gene *Laccase 2*, which expresses a phenoloxidase in larvae of *Tribolium castaneum*, prevents tanning after the pupal (*a*) and imaginal (*b*, *c*) molts (2). In each photograph the control is shown at the left and the RNAi knockdown at the right. Photos courtesy of Yas Arakane.

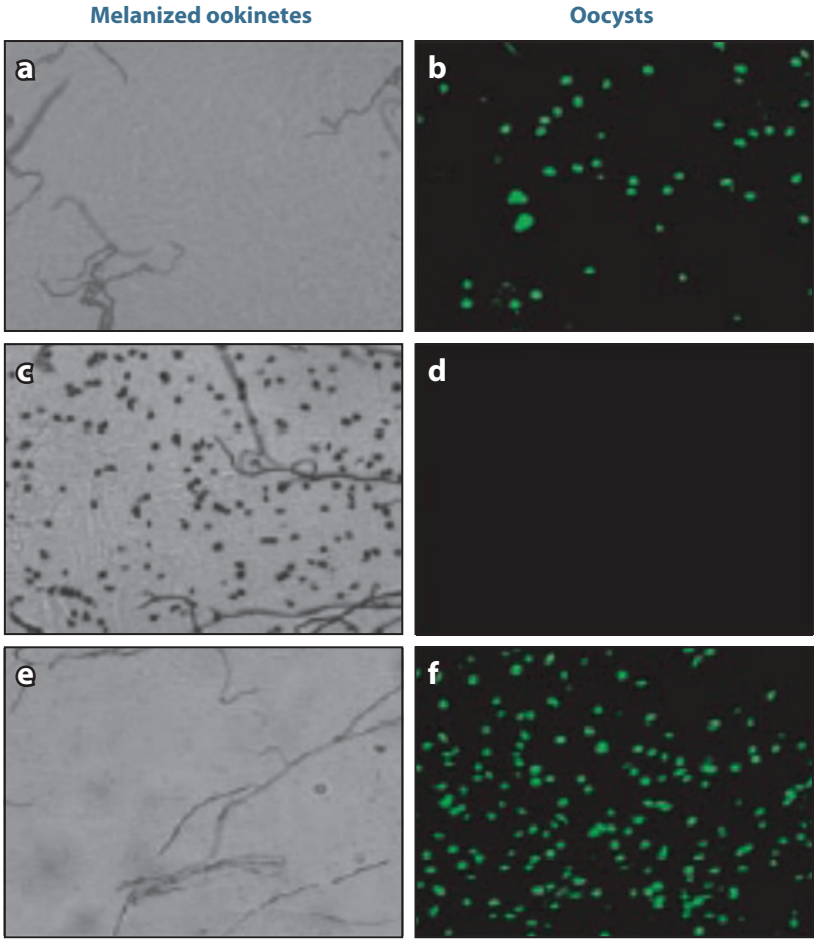

Figure 6

Systematic experiments of RNAi in the malaria mosquito *Anopheles gambiae* demonstrated that it expresses a type-C lectin (CTL4), which plays a protective role on *Plasmodium*, and a leucine-rich protein (LRM1), which impairs the transition from ookinetes to oocysts in *Plasmodium*. Images show melanized ookinetes and oocysts of *Plasmodium* in controls (*a*, *b*) and in specimens interfered with for CTL4 (*c*, *d*) and for LRM1 (*e*, *f*) (53). Photos courtesy of Fotis Kafatos.

these proteins is LRM1, which is leucine rich and which induces mortality of *Plasmodium* ookinetes. Conversely, *A. gambiae* expresses two type-C lectins that protect *Plasmodium* against the mosquito immune defenses (53) (**Figure 6**).

Another important pathway in insect immunology is Toll, which signalizes the immune response against fungi and gram-positive bacteria. Selective interference of the components of the Toll pathway in *D. melanogaster* led to determining the relative importance of each component in the immune response (23). Likewise, Reynolds and colleagues (20) have studied bacterial recognition with RNAi in the lepidopteran *Manduca sexta*. They showed that a nonpathogenic strain of *Escherichia coli* induced the expression of hemolin and peptidoglycan recognition protein in *M. sexta* hemocytes, whereas this upregulation was prevented by RNAi. Knockdown of hemolin decreased the ability of insects to clear *E. coli* from the hemolymph, caused a reduction in the number

BT: *Bacillus thuringiensis*

Systemic properties: in RNAi of *C. elegans*, the spread of RNAi effects from cell to cell and from tissue to tissue in the whole animal

Transitive properties: in RNAi of *C. elegans*, production of secondary siRNAs from mRNAs targeted by the RNAi mechanism under the priming action of the primary siRNAs derived from the delivered dsRNA

RdRP: RNA-dependent RNA polymerase

of free hemocytes, and reduced their ability to engulf bacteria. These results elegantly demonstrate the key role of hemolin in the *M. sexta* immune response.

Resistance Against Chemicals: Pest Control

Detoxification mechanisms have been studied with special interest in the case of insecticides, with particular emphasis on the cytochrome P450 protein family, which is involved in insecticide resistance. These proteins use NADPH cytochrome P450 reductase for correct performance, and RNAi of this reductase increases the sensitivity of mosquitoes to pyrethroids (39). An insecticide that is currently gaining in importance is *Bacillus thuringiensis* (BT) toxin. BT toxin interacts with receptors located in the digestive system, although the identity of these receptors has remained elusive. RNAi of the aminopeptidase M localized in the midgut of the lepidopterans *Spodoptera litura* and *Helicoverpa armigera* resulted in a decrease of sensitivity to BT toxin (61). Given that aminopeptidase M interacts directly with BT toxin (61), these experiments suggest that it plays the role of BT toxin receptor.

The above results suggest that RNAi can help when studying mechanisms of action of known insecticides, and they point to new targets for new insecticides. RNAi itself could even be envisaged as an insect control tool through targeting vital genes, although efficient systems of dsRNA formulation and delivery must be developed. The approach of administering dsRNAs by feeding, as seen in *E. postvittana* (66) and *R. prolixus* (3), paves the way in this field, and the possibility of delivering the dsRNA by soaking, as in *C. elegans* (63), should also be considered, for example, in particularly permeable stages of aquatic insects.

THE PROBLEM OF SPECIES SENSITIVITY

The diversity of species and functions studied by systemic RNAi is considerable (**Table 1**). There is a good representation of pterygote orders, either polyneopterans (Orthoptera, Blattaria and Isoptera), paraneopterans (Hemiptera) or oligoneopterans (Coleoptera, Neuroptera, Hymenoptera, Lepidoptera and Diptera). However, no studies have been reported on groups phylogenetically as important as Entognatha, Archaeognatha, Zygentoma, Ephemeroptera and Odonata. Although many positive results have been published, not all reported species show the same degree of sensitivity toward RNAi (64, 67), and as negative results are often not reported, it is highly likely that a number of poorly sensitive species have been tested but not published. Although it is too early to establish trends, it seems that less-derived species are more sensitive to systemic RNAi. The polyneopterans and paraneopterans tested are fully sensitive (*B. germanica*, *G. bimaculatus* and *O. fasciatus*), whereas within the oligoneopterans, less-derived species (*T. castaneum*) are much more sensitive than those that are more derived (Lepidoptera and Diptera). The best known of the poorly sensitive species is *D. melanogaster*, for which dsRNA penetrates a number of tissues in adults and embryos (64), whereas in larvae it is incorporated only in hemocytes (47). In lepidopterans, such as *Bombyx mori* and *Manduca sexta*, hemocytes are also an easy target for RNAi, whereas other tissues seem rather resistant to respond (20, 47).

The most RNAi-sensitive animal reported to date is not an insect but the nematode *C. elegans*, in which interference has transitive and systemic properties (46). Transitive properties derive from the production of secondary siRNAs from the action of an RNA-dependent RNA polymerase (RdRP) on the mRNAs targeted by the RNAi mechanism, and on the priming action of the primary siRNAs derived from the introduced dsRNA (60). Systemic properties, on the other hand, refer to the spread of RNAi effects from cell to cell and from tissue to tissue in the whole organism. These systemic properties largely rely on the gene *Sid-1*, which encodes a multitransmembrane domain protein that seems to act as a channel for dsRNA (68).

Table 1 Insect species tested with RNAi in vivo

Order	Species	Studied functions
Orthoptera	*Locusta migratoria*	Embryonic development (segmentation)
Orthoptera	*Gryllus bimaculatus*	Embryonic development (segmentation, formation of the digestive tube), postembryonic development (leg regeneration), allatostatins and juvenile hormone, behavior (locomotor rhythms)
Dictyoptera	*Blattella germanica*	Embryonic development (segmentation), postembryonic development (molt), reproduction (vitellogenin, vitellogenin receptor, oogenesis, allatostatins, and juvenile hormone), transduction of nutritional signals (TOR and juvenile hormone), behavior (locomotor rhythms)
Dictyoptera	*Periplaneta americana*	Embryonic development (segmentation), synaptic specificity
Dictyoptera	*Diploptera punctata*	Allatostatin receptor
Isoptera	*Reticulitermes flavipes*	Social behavior (hexamerins and polyphenism)
Hemiptera	*Oncopeltus fasciatus*	Embryonic development (pattern formation, segmentation, appendages)
Hemiptera	*Rhodnius prolixus*	Salivary glands (anticoagulant agents, sucking behavior)
Hemiptera	*Bemisia tabaci*	Actin-based dynamics in oocytes (*chickadee* gene)
Coleoptera	*Tribolium castaneum*	Embryonic development (pattern formation, appendages, segmentation), postembryonic development (microsculpture and specialized pubescence, elytra formation, molecular action of juvenile hormone), chitin synthesis, odor perception
Coleoptera	*Apriona germari*	Resistance to physical agents (protection against heat stress)
Coleoptera	*Harmonia axyridis*	Methodology
Coleoptera	*Gastrophysa atrocyanea*	Diapause
Neuroptera	*Chrysopa perla*	Postembryonic development (nuclear receptors and metamorphosis)
Hymenoptera	*Nasonia vitripennis*	Embryonic development (pattern formation, segmentation)
Hymenoptera	*Apis mellifera*	Embryonic development (Hox genes, integument), postembryonic development (nuclear receptors), vitellogenesis (vitellogenin), social behavior (vitellogenin, regulation of juvenile hormone), biogenic amine receptors
Lepidoptera	*Hyalophora cecropia*	Immune system (antimicrobial peptides, melanization)
Lepidoptera	*Bombyx mori*	Embryonic development (pair-rule genes), postembryonic development (wing unfolding, metamorphosis), pheromone synthesis, coloration
Lepidoptera	*Manduca sexta*	Immune system (protection against *Photorhabdus* infections), resistance to physical agents (encapsulation)
Lepidoptera	*Helicoverpa armigera*	Resistance to chemical agents (receptor of BT toxin), acetylcholinesterase
Lepidoptera	*Spodoptera litura*	Resistance to chemical agents (receptor of BT toxin)
Lepidoptera	*Spodoptera littoralis*	Sperm release
Lepidoptera	*Spodoptera exigua*	Chitin synthesis
Lepidoptera	*Spodoptera frugiperda*	Allatostatins, juvenile hormone
Lepidoptera	*Epiphyas postvittana*	Gut carboxylesterase, pheromone binding
Lepidoptera	*Plodia interpunctella*	Eye coloration in embryo
Diptera	*Aedes aegypti*	Vitellogenesis (transduction of nutritional signals), immune system (Toll pathway)
Diptera	*Anopheles gambiae*	Salivary glands (probing and sucking behavior), immune system (susceptibility to *Plasmodium* infection), resistance to chemical agents (pyrethroid insecticides)
Diptera	*Drosophila melanogaster*	Embryonic development (e.g., pattern formation, segmentation, muscle formation, synaptic stabilization, Notch pathway), oogenesis and chorion formation, immune system (Toll pathway), resistance to chemical agents (effects of ethanol), behavior
Diptera	*Lucilia sericata*	Embryonic development (segmentation)
Diptera	*Sarcophaga peregrina*	Receptor of low-density lipoproteins, hemocyte proteins
Diptera	*Episyrphus balteatus*	Embryonic development (pattern formation)

A comparison of RNAi genes in *C. elegans* and insects (64) was rather disappointing because insects (at least those species with a genome reported) do not possess homologues of *C. elegans RdRP*, and it is not clear whether insect sequences similar to *Sid-1* are true homologues of it, which points to scarce conservation of amplification and spread of RNAi mechanisms between *C. elegans* and insects. Conversely, the core machinery of RNAi, such as the cleaving enzymes Dicer and Argonaute, is basically conserved in *C. elegans* and insects (64). Indeed, overexpression of dsRNAs within cells using hairpin RNAs readily triggers RNAi in *D. melanogaster* tissues resistant to systemic RNAi (47), which suggests that poor sensitivity is not related to the RNAi core machinery, but rather to the penetration and transmission of the interfering signal through cells and tissues.

D. melanogaster S2 cells spontaneously take up dsRNA and use it for RNAi. The endocytic pathway mediates dsRNA uptake, and scavenger receptors also play a significant role (58). Orthologous genes of this endocytosis machinery are also important for systemic RNAi in *T. castaneum* (64). However, the core machinery for endocytosis is too generic for making useful comparisons of species sensitivity toward RNAi. Moreover, it is possible that uptake of dsRNA by *D. melanogaster* S2 cells in vitro follows a mechanism similar to that of hemocytes in vivo, which does not help to explain the inability of cells from other *D. melanogaster* tissues to take up dsRNA.

Other reasons that might account for differences in species sensitivity could be the occurrence of degradation mechanisms that are able to remove alien RNA. Species possessing highly efficient systems of this kind should, in general, be less sensitive to RNAi. Moreover, differences of response of core RNAi genes (*Dicer* and *Argonaute*, for example) after dsRNA treatment might also explain differences in species sensitivity.

The type of tissue can also influence RNAi efficiency. For example, when using RNAi for silencing the lipophorin receptor of *B. germanica*, we observed that the response was much faster and more transient in the fat body than in the ovary (14), which may point to differences in tissue penetrability. Salivary glands are comparatively less sensitive to RNAi than other tissues. For example, RNAi of salivary gland genes in the mosquito *A. gambiae* required high doses of dsRNA, which is in agreement with the expression of *Dicer* and *Argonaute* genes being lower in these glands than in other tissues (8). The type of gene can also influence RNAi results. Thus, genes with efficient feedback mechanisms of regulation might readily counteract depletion of mRNA levels with higher rates of transcription. The fast recovery of mRNA levels in the fat body after RNAi of the lipophorin receptor mentioned above (14) reflects this possibility. There are also genes whose transient expression would make the completion of the sequential steps for RNAi impossible (**Figure 1**). An example of this might be the *yellow-g* gene in the ovary of *B. germanica*. *yellow-g* seems to be involved in choriogenesis, and it is expressed as an acute mRNA peak lasting only a few hours, but even though many other genes have been silenced in the ovary of *B. germanica* by systemic RNAi, *yellow-g* was not knocked down by conventional procedures (30). **Table 2** summarizes the possible reasons for insensitivity toward RNAi at species, tissue, and gene levels.

ADVANTAGES AND DISADVANTAGES OF SYSTEMIC RNAi

Advances in the functional characterization of insect genes have been based on forward genetic screens in *D. melanogaster*. Mutations are generated at random, phenotypes of interest are scored, and the mutated gene is identified thereafter. This traditional approach has been formidably successful, but it remains restricted to genetically transformable models and limited by intrinsic biases in mutagenesis methodology, by the large set of mutants that must be examined, and by the time-consuming effort required for the identification of the mutated gene. With RNAi, the expression of a given gene can be disrupted, and the phenotypic

Table 2 Possible causes of RNAi insensitivity in insects

Intrinsic of the species	Alien dsRNA are efficiently degraded
	Deficient amplification and spreading of the RNA signal
	Low response of core RNAi genes after dsRNA treatment
Intrinsic of the tissue	The tissue is hardly permeable to dsRNAs
	The elements of the RNAi machinery are mildly expressed in the tissue
Intrinsic of the gene	The particular dsRNA is efficiently degraded
	The gene efficiently counteracts RNAi by increasing transcription rates
	The target mRNA is too transient
	The target mRNA is protected against RNases

effects shed light on its function; thus, a phenotype is automatically linked to a precise DNA sequence. Systemic RNAi is the easiest and least tedious approach, can be done by most entomology laboratories, and enables the study of nonmodel species representing diverse insect orders.

Another advantage of systemic RNAi is that it allows a higher versatility of silencing, as the inactivation is sequence specific and not locus specific. If the aim is to simultaneously silence related mRNAs in the same specimen, it is relatively straightforward to design a dsRNA targeting a region common to all of them. Conversely, it is possible to silence a particular isoform using a dsRNA specific for it. One example of this approach is the RNAi of the main isoforms of the nuclear receptor HR3, either collectively or individually, reported in *B. germanica* (17). Systemic RNAi also enables the study of gene functions at chosen stages by delivering the corresponding dsRNA just before the stage under study. This allows the separate study of functions in particular stages of the embryo, the larvae, the pupae, or the adult. Parental RNAi makes possible gene silencing in the embryo through the technically easy dsRNA delivery to the mother.

RNAi can be advantageous for discovering hidden functions of genes whose knockout by mutation is deleterious. Conversely, partial suppression of the transcript becomes a drawback when the absence of phenotype does not lead to conclusive data. In *B. germanica*, for instance, RNAi of the precursor of allatostatins (peptides that inhibit JH synthesis) reduced target mRNA by 90% but did not result in an increase in JH production (41). This ambiguous result left unanswered the question of whether allatostatins are true physiological regulators of JH synthesis in *B. germanica*.

RNAi experiments might also suffer from off-target effects, leading to knockdown of genes with structural similarity to the delivered dsRNA (36, 40). The design and stringent quality control of long dsRNAs therefore remain important issues. Using two dsRNAs encompassing two different regions of the target mRNA and assessing whether both induce the same phenotype is a safe and technically easy precaution, as taken, for example, in RNAi of the HR3 nuclear receptor in *B. germanica* mentioned above (17).

FUTURE DIRECTIONS: OLD AND NEW CHALLENGES

More research is needed on RNAi mechanisms, not so much on the core machinery, which seems to be universal and highly conserved across the animal kingdom, but on the amplification and spread of the interfering signal. Unveiling the factors involved in the amplification and spread in fully and poorly sensitive insect species will shed light on the reasons for insensitivity in the latter. This information might then help researchers find a way to improve systemic RNAi in particularly important insensitive species. Moreover, comparative data regarding RNAi mechanisms in

species representing a large range of sensitivity could provide an insight into the evolution of these mechanisms in insects.

Functional genomics, either gene-by-gene or through genome-wide screenings (10), will continue to be the most obvious RNAi field of application. RNAi will be used not only for unveiling the functions of given genes, but also for determining the interactions among genes, thus establishing the topology of gene networks. Genome-wide screenings can now be carried out in the highly derived dipteran *D. melanogaster* and in less-derived species, such as *T. castaneum*, *A. mellifera*, and *B. mori*, and they will soon be available in hemimetabolous species, such as the human body louse, *Pediculus humanus*, or the pea aphid, *Acyrthosiphon pisum*. Indeed, an RNAi screen will soon be only the first step in the comprehensive analysis of biological processes. To paraphrase Boutros and Ahringer (10), the end of the screen will be only the beginning of the experiment.

The potential application of the RNAi technique to any gene and any species could lead to comparative studies of the function of a gene, or gene network, in species covering a large spectrum of insect orders. This could provide significant insight into the evolutionary processes that have modeled these functions and, in this respect, developmental processes will receive preferential attention. In regard to embryonic development, comparative data obtained in a handful of species with long, intermediate, and short germ bands have already afforded a number of surprises. The extension of data to more gene networks involved in developmental processes and to species representing key, as yet unstudied insect orders will lead to more robust hypotheses about the evolution of body building. Moreover, RNAi can facilitate comparative studies and evolutionary insight into other processes, such as social behaviors, reproductive strategies, and host-parasite interactions, to mention only a few exciting examples. The insect world, with its more than 30 orders, about 1 million described species, and perhaps 30 million still awaiting taxonomic discovery, still hides many genetic black boxes, and RNAi appears to be a formidable key for opening a large number of them.

SUMMARY POINTS

1. The discovery of RNAi in the 1990s opened a new avenue for extending studies of functional genomics to many nonmodel insects.
2. RNAi can be used under different approaches in vivo and in vitro. However, systemic RNAi in vivo is the approach that can foster more dramatically the field of functional genomics in nonmodel insects.
3. Systemic RNAi in vivo has been used successfully for studying a variety of functions related to development, reproduction, behavior, immunology, and other complex biochemical patterns.
4. So far, RNAi has been used for investigating a number of genes in some 30 species of insects representing a variety of orders, including Orthoptera, Dictyoptera, Isoptera, Hemiptera, Coleoptera, Neuroptera, Hymenoptera, Lepidoptera, and Diptera.
5. Results obtained indicate that not all species are equally sensitive to systemic RNAi in vivo; less-derived species are generally more sensitive than more-derived species.
6. Differences of sensitivity seem related to differences of amplification and spreading of the RNAi signal. The study of the mechanisms underlying these processes is one of the challenges in the field.

7. Given that RNAi can be used in a large variety of species representing different groups, comparative and evolutionary studies will be the great beneficiaries of this approach.

DISCLOSURE STATEMENT

The author is not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

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Contents

Indexes

Errata

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