

Terenius et al. RNAi in Lepidoptera

- 1 RNA interference in Lepidoptera: an overview of successful and unsuccessful studies and
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3 implications for experimental design
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51
52 94 **Keywords:** RNA interference; Lepidoptera; delivery methods; tissue uptake; gene function;
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54 95 dsRNA properties
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1 97 **Abstract**

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3 98 Gene silencing through RNA interference (RNAi) has revolutionized the study of gene
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6 99 function, particularly in non-model insects. However, in Lepidoptera (moths and butterflies)
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8 100 RNAi has many times proven to be difficult to achieve. Most of the negative results have been
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11 101 anecdotal and the positive experiments have not been collected in such a way that they are
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13 102 possible to analyze. In this review, we have collected detailed data from more than 150
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15 103 experiments including all to date published and many unpublished experiments. Despite a
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18 104 large variation in the data, trends that are found are that RNAi is particularly successful in the
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20 105 family Saturniidae and in genes involved in immunity. On the contrary, gene expression in
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23 106 epidermal tissues seems to be most difficult to silence. In addition, gene silencing by feeding
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25 107 dsRNA requires high concentrations for success. Possible causes for the variability of success
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28 108 in RNAi experiments in Lepidoptera are discussed. The review also points to a need to further
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30 109 investigate the mechanism of RNAi in lepidopteran insects and its possible connection to the
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33 110 innate immune response. Our general understanding of RNAi in Lepidoptera will be further
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35 111 aided in the future as our public database at <http://insectacentral.org/RNAi> will continue to
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37 112 gather information on RNAi experiments.
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1 114 **1. Preface**

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6 116 RNA interference in Lepidoptera was first reported in 2001 at the 5th International
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8 117 Workshop on Molecular Biology and Genetics of the Lepidoptera (Bettencourt et al., 2002),
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10 118 generating considerable interest in the possibility of utilizing reverse genetics to investigate
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12 119 gene function in Lepidoptera. During subsequent years, performing RNAi in Lepidoptera has
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15 120 proven not as straight-forward as shown for other insects. During the 8th International
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18 121 Workshop on Molecular Biology and Genetics of the Lepidoptera in 2009
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20 122 (<http://bio.demokritos.gr/Leps/leps.htm>), it was recognized that it would be worthwhile to
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22 123 integrate published and unpublished results and investigate this peculiar phenomenon in
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25 124 depth. First, a survey was set up to acquire detailed experimental information for all studies,
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27 125 regardless of publication status and outcome. Subsequently, exploration of this community
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30 126 dataset allowed us to address the question of whether RNAi for the lepidopteran clade is any
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32 127 different from other insects and what solutions can be recommended for the future.
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37 129 **2. Introduction**

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42 131 *2.1. Short overview of RNAi silencing pathway*

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47 133 Most eukaryotic organisms, including insects, possess common machinery for
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49 134 sequence-specific post-transcriptional gene silencing that is triggered by the presence of
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51 135 double-stranded RNA (dsRNA), resulting in the degradation of the targeted mRNA (Fire et
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53 136 al., 1998). This process, RNA interference (RNAi) in animals (Hannon, 2002) and post-
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56 137 transcriptional gene silencing in plants (Baulcombe, 2004), is a type of highly specific defense
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1 138 reaction. It depends on the specific Watson-Crick pairing formed by the small RNAs that
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3 139 trigger gene silencing and their target mRNAs. Different types of small RNAs have been
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5 140 described in insects and other multicellular organisms including short interfering RNAs
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8 141 (siRNAs), piwi-interacting RNAs (piRNAs), endogenous siRNAs (endo-siRNAs or
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10 142 esiRNAs), and microRNAs (miRNAs) (Siomi and Siomi, 2009; Brodersen and Voinnet,
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13 143 2009).

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15 144 The basic RNAi process can be divided into three main steps (Tomari and Zamore,
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18 145 2005). First, a long endogenous or exogenous dsRNA molecule that is expressed in, or
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20 146 introduced into, the cell is processed into small RNA duplexes by Dicer, a ribonuclease III
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23 147 (RNase III) enzyme. Depending on the organism, there may be one or more than one Dicer,
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25 148 each responsible for a different type of short dsRNA product (Meister and Tuschl, 2004). For
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28 149 example, in *Drosophila melanogaster*, Dicer-1 is mainly used to produce miRNAs, while
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30 150 Dicer-2 is responsible for the processing of long dsRNAs into siRNAs (Lee et al., 2004).
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33 151 During the second step of the RNAi mechanism, these duplexes are unwound and one strand,
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35 152 the loaded single-stranded RNA (ssRNA) that is called the guide strand, is preferentially
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38 153 loaded into a protein complex known as the RNA-induced silencing complex (RISC). Third,
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40 154 the RISC complex finds potential target messenger RNAs (mRNA) sharing complete or
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42 155 partial sequence homology to the dsRNA. The guide strand directs a RISC-bound
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45 156 endonuclease (called ‘slicer’, an Argonaute protein) to lead to the cleavage of the target, a
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48 157 messenger RNA (mRNA). Overall, the discovery that cells respond to dsRNA by silencing
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50 158 the target has changed our view of gene regulation and provided a transformative new
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52 159 technology for reverse functional genomics. Implementing it in a clade with few mutant
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55 160 stocks and long generation times is, therefore, of the utmost importance.
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2.2. RNAi in arthropods

Detailed definitions of RNAi in insects have previously been described (Huvenne and Smagghe, 2010). Despite the fact that the RNAi pathways operate by highly conserved strategies in different organisms, they comprise different proteins and mechanisms. The foremost example is the amplification of exogenous dsRNA which in nematodes, plants and fungi, functions through a cellular RNA-dependent RNA polymerase (RdRP) that amplifies the effect of gene silencing by transitive generation of target gene-derived secondary siRNAs induced by the injected or orally administered dsRNA (Sijen et al., 2001, Pak and Fire, 2007; Sijen et al., 2007). RdRP is probably responsible for the robust response of dsRNA-mediated RNAi in these organisms but homologs of these canonical RdRPs are not present in insect genomes, although they have been identified in genomes of basal arthropods such as the tick genome (Gordon and Waterhouse, 2007; Kurscheid et al., 2009; Obbard et al., 2009). Genes encoding the canonical RdRP are also found in basal genomes of the Deuterostomia, including cephalochordates and some tunicates, but not in vertebrates (Obbard et al., 2009). In insects, early studies identified an RdRP-like activity in cell-free extracts from *Drosophila* embryos (Lipardi et al., 2001), although there was no evidence for replication of exogenous dsRNA in Diptera or Lepidoptera (Roignant et al., 2003). These findings led to suggestions that the multiple-turnover RISC may mediate RNA silencing in the absence of RdRP in insects (Schwarz et al., 2002). Recent studies in *D. melanogaster* have now shown that the *D-elp1* gene, a subunit of the cellular RNA polymerase II, has RdRP activity and is involved in immunity via RNAi (Lipardi et al., 2009). This gene is universal in animals and so may be involved in enabling RNAi under specific conditions in animals generally (Lipardi and Paterson, 2010). Indeed small RNA deep sequencing studies in *D. melanogaster* identified

1 186 siRNAs derived from several viruses (Wu et al., 2010), a finding consistent with an active
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3 187 RNAi pathway conferring immunity. The *Caenorhabditis elegans* RdRPs have now been
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6 188 introduced into *D. melanogaster* (Duan et al., 2010) in order to study their ability to enhance
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8 189 RNAi. In contrast to the above differences, the core machinery of RNAi including the
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11 190 cleaving enzymes Dicer and Argonaute is conserved in *C. elegans* and all insects with a
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13 191 published genome sequence (Tomoyasu et al., 2008).

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15 192 Further, the RNAi silencing signal is capable of intercellular movement in eukaryotic
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18 193 organisms. Mobility of RNAi effects is consistently observed in *C. elegans* (Voinnet and
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20 194 Baulcombe, 1997). Homologues of the *C. elegans* systemic RNA interference deficient-1 (*sid-*
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22 195 *1*) gene (Winston et al., 2002), which are responsible for the systemic spread of dsRNAs in
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24 196 the worm, have been identified in the moths *Bombyx mori* and *Spodoptera exigua*, as well as
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26 197 in other insects such as *Apis mellifera*, *Tribolium castaneum* and aphids, but not in the *D.*
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28 198 *melanogaster* genome (Winston et al., 2002; Honeybee Genome Sequencing Consortium,
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30 199 2006; Xu and Han, 2008; Tian et al., 2009). Closer analysis of these insect *sid-1*-like (*sil*)
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32 200 proteins (Tomoyasu et al., 2008) showed them to be more similar to another *C. elegans*
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34 201 protein (tag-130) which is not involved in RNAi. Moreover, the *sil* proteins were found not to
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36 202 be required for RNAi in the beetle (Tomoyasu et al., 2008). It is of interest that no
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38 203 homologues of the closely related *sid-2* gene from *C. elegans* (Winston et al., 2007) have
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40 204 been identified in any insect species thus far.

41 42 43 44 45 205 46 47 206 *2.3. Description of database used in the paper*

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52 208 The database was custom built after community consultation and using the MIARE
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54 209 (Minimum Information Criteria for RNAi Experiments) specifications. Database support was
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1 210 provided by gmod_dbsf (http://www.gmod.org/gmod_dbsf; Papanicolaou and Heckel, 2010)
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3 211 and hosted at <http://insectacentral.org/RNAi> (accessed 2 November 2010). Content was built
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5 212 from published data found in table 1 and unpublished experiments provided by the authors.
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8 213 The database includes information on target and construct sequences, procedures for making
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10 214 the constructs, procedures used for delivery of dsRNA, how the RNAi efficacy was assayed
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12 215 and information on the animals used. References to the supplementary table are written as
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14 216 ST:XXX corresponding to the InsectaCentral ID.
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21 218 **3. A brief summary of successful RNAi experiments in Lepidoptera**

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25 220 Successful RNAi experiments have been carried out in a number of lepidopteran species
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27 221 to date (Table 1). The first lepidopteran RNAi publications appeared in June 2002; one
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29 222 reported the knockdown of a pigment gene following dsRNA injection into *B. mori* embryos
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31 223 (Quan et al., 2002), another targeted a pattern recognition protein, hemolin, in *Hyalophora*
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33 224 *cecropia* embryos by heritable RNAi (Bettencourt et al., 2002) and a third targeted a putative
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35 225 *Bacillus thuringiensis* toxin receptor in *Spodoptera litura* larvae (Rajagopal et al., 2002). In
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37 226 the succeeding years, RNAi experiments using lepidopterans were consistently published with
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39 227 publication numbers increasing from 2006 with nine different studies being published in
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41 228 2009.
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47 229 A large proportion of RNAi studies have taken place using *B. mori* and *Manduca sexta*
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49 230 (31% and 25%, respectively), although a number of other insect species are represented,
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51 231 including many members of the Noctuidae (6 out of the 14 lepidopteran species represented
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53 232 were members of this family, although these insects only represent 10 out of 37 publications).
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56 233 RNAi experiments have already advanced our understanding in a number of systems; in
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1 234 particular developmental processes and immunity (see Table 1).

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4 236 **4. Variation in RNAi efficiency related to species**

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7 8 238 *4.1. Different methods of delivery*

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13 240 To evaluate the silencing efficiency of RNAi, one has to take into account the method of
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15 241 delivery. It is generally assumed that RNAi will always occur once dsRNA is delivered inside
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18 242 the cell and that the limiting factor exists at the level of its functional uptake. Indeed, when
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20 243 dsRNA is delivered by injection into early pre-differentiated embryos the efficiency of RNAi
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22 244 solely depends on the availability of the core RNAi machinery. Similarly, introduction of
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24 245 nucleic acid (including dsRNA) in tissue culture cells is greatly stimulated by transfection
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26 246 using cationic lipids as delivery agents and therefore corresponds to “intracellular RNAi” as
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28 247 opposed to “systemic RNAi”. Expression of hairpin RNAs by transgenes is also considered
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30 248 efficient because it takes place inside the cells. It is, therefore, of no surprise that the majority
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32 249 of experiments that employed embryo injection, transfection of cell lines or expression of
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34 250 hairpin RNAs by transgenes were successful.

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36 251 Micro-injection of dsRNA into embryos has most often been used for *B. mori*, and in all
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38 252 cases successfully, although the silencing levels varied (Quan et al., 2002; Liu et al., 2008;
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40 253 Matsumoto et al., 2009; Pan et al., 2009; Tomita and Kikuchi, 2009; Fig. 1). High levels of
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42 254 silencing of the target gene were also described in two studies of transgenic *B. mori*
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44 255 expressing hairpin RNAs (Kanginakudru et al., 2007; Dai et al., 2008). Injections of dsRNA
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46 256 into embryos of other lepidopteran species were also reported, notably in *Mamestra*
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57 257 *configurata*, *Plodia interpunctella*, *Helicoverpa armigera* and *S. exigua* (Fabrick et al., 2004;
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1 258 Tsuzuki et al., 2005; D. Collinge and A. Williams, unpublished results cited in Gordon et al.,
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3 259 2009; Herrero, ST:14). While silencing was observed in the three former species, this was not
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6 260 the case for *S. exigua* (Fig. 1). The cause of the latter failure is unknown but could be related
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8 261 to injection at a stage after cellularization since systemic RNAi was also not effective in this
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11 262 species (see section 4.1.1. and section 7).

12 13 263 14 15 264 *4.1.1. Systemic RNAi*

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18 265 Systemic RNAi involves specific gene silencing following injection of dsRNA into the
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20 266 insect (Huvenne and Smagghe, 2010). A great variation exists among different lepidopteran
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23 267 species with respect to their sensitivity to systemic RNAi and high or no silencing can occur
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25 268 at very different concentrations of dsRNA (Table 2). In a few species, including *H. cecropia*,
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27 269 *Antheraea pernyi* and *M. sexta*, high levels of silencing can be achieved by application of very
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30 270 low amounts of dsRNA (less than 10 ng per mg tissue; Bettencourt et al., 2002; Hirai et al.,
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33 271 2004; Terenius et al., 2007; Kanost, ST:7,34,36,50,51,53,54,180,198). In *H. cecropia*,
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35 272 heritable RNAi effects on the embryos of the next generation were also reported following
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38 273 injection of dsRNA into pupae (Bettencourt et al., 2002), which could suggest that the
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40 274 injected dsRNA had entered into the gonads of the developing pupae. In these cases, success
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42 275 may be explained by the fact that immunity-related genes were silenced (see Section 5).
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45 276 Likewise, in *M. sexta* studies, concentrations of applied dsRNA were rather low (less than 10-
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47 277 100 ng per mg tissue) and targeted genes and tissues examined were related to the
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50 278 investigation of the immune response but effects varied greatly (Levin et al., 2005;
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52 279 Eleftherianos et al., 2006, 2009; Zhuang et al., 2007, 2008; Eleftherianos, ST:137,185-196;
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54 280 Kanost, ST:7,34,36,50,51,53,54,180,198). However, it should be stressed that no systematic
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57 281 investigation was undertaken to evaluate differences in sensitivity between species in more
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1 282 detail. In most studies, a “standard” range of amounts of dsRNA is injected, which varies
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3 283 between 1 and 100 µg. Since the same range is routinely injected in small and large species,
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5 284 the calculated sensitivity to RNAi (amount of dsRNA administered per mg tissue required to
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7 285 achieve silencing) appears higher in large species. For instance, a high level of silencing has
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9 286 been achieved when a dose of 100 µg of dsRNA was injected into larvae of *Antheraea mylitta*
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11 287 (Gandhe et al., 2007). Such dose corresponds to a high concentration for most lepidopteran
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13 288 larvae but in the case of *A. mylitta* last instar larvae, that could weigh up to 5 g, it would
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15 289 calculate as only 20 ng/mg. Further studies are required to establish a clear dose-response
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17 290 relationship between the concentration of dsRNA and the amount of silencing to more
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19 291 accurately determine the sensitivity of RNAi. For instance, in *Laphygma exigua*, uniform
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21 292 levels of high silencing have been achieved with concentrations of dsRNA at 0.3-0.5 µg/mg
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23 293 tissue (Zhang, ST:166,171-173,177,220,222), but dose-response relationships using lower
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25 294 concentrations of dsRNA to establish the sensitivity to RNAi were not reported. Clearly, in
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27 295 this species even lower concentrations of dsRNA could be effective, which is beneficial since
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29 296 it is predicted to cause fewer off-target effects. It is therefore recommended that in future
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31 297 studies researchers use different doses and specify the concentrations of dsRNA (as opposed
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33 298 to amounts of dsRNA) in the descriptions of their experiments. Furthermore, by determining
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35 299 the dose-response relationship a concentration could be chosen at which efficiency is
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37 300 maintained but at minimal risk of non-specific effects.

38 301 With a range of studies integrated in this review, we can relate silencing effect with
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40 302 phylogeny and investigate if the biology of a particular species may have an effect (Figure 2).
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42 303 There are indeed species with a high resistance to application of dsRNA. Very high amounts
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44 304 of dsRNA (more than 1 µg per mg of tissue) did not result in any silencing effects in *Bicyclus*
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46 305 *anyana*, *Chrysodeixis includens* and *Spodoptera littoralis* (Popadic, ST:9; Saenko, ST:98;
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1 306 Strand, ST:141-144; see also Iga and Smagghe, 2010; Marcus, 2005). Target tissues in these
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3 307 cases were epidermis (*Bicyclus* larval and pupal wings), endocrine glands (*Spodoptera*
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5 308 prothoracic glands) and tissues primarily involved in the immune response (*Chrysodeixis*
6
7
8 309 hemocytes, fat body, gut), suggesting that resistance to RNAi is not restricted to particular
9
10 310 tissues.

11
12
13 311 Saturniidae stands out as the only family in which multiple species (within the genera
14
15 312 *Antheraea* and *Hyalophora*) consistently showed evidence of effective RNAi. This
16
17
18 313 phylogenetic signal may be due to limited amount of studies. In the two best studied species,
19
20 314 *B. mori* and *M. sexta*, we see a large variation in RNAi efficiency and other factors may be at
21
22
23 315 play. In *B. mori*, injection of dsRNA targeting the ecdysone receptor or other target genes in
24
25 316 the epidermis did not result in silencing effects (Swevers, ST:233; Futahashi, ST:88,89).

26
27 317 Another study of the regulation of *B. mori* metamorphosis by ecdysone and juvenile hormone
28
29
30 318 required the use of a viral vector for delivery of RNAi (Uhlirva et al., 2003). On the other
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32
33 319 hand, silencing effects were reported for genes involved in the regulation of the immune
34
35 320 response, for genes expressed in the silk gland and the pheromone gland and, unlike in *C.*
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37 321 *elegans*, also for genes expressed in the nervous system (Tabunoki et al., 2004; Ohnishi et al.,
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40 322 2006, 2009; Gandhe et al., 2007; Huang et al., 2007; Hossain et al., 2008; Mrinal and
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42 323 Nagaraju, 2008; Ohnishi, ST:210-213,215-218). Overall, it appears that high to very high
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45 324 doses of dsRNA (0.1 µg/mg - > 1 µg/mg) have a larger chance of success than lower doses in
46
47 325 *B. mori*. In four examples, dsRNA was injected in *B. mori* pupae for uptake in developing
48
49
50 326 eggs and to investigate effects during embryonic development but all these attempts were
51
52 327 unsuccessful (Niimi, ST:41-44). However, in another study, silencing of antibacterial genes in
53
54 328 embryos was achieved after injection of larvae of the previous generation (Mrinal and
55
56
57 329 Nagaraju, 2008).

1 330 A variant of injection into whole organisms is to culture insect organs *in vitro* in the
2
3 331 presence of dsRNA and monitor the levels of silencing. Compared to *in vivo*, degradation and
4
5 332 clearance of dsRNA for *in vitro* cultures is minimal and the process of RNAi is expected to be
6
7
8 333 more robust. This approach was applied in *M. configurata* where successful silencing was
9
10 334 observed for midgut tissue cultured in the presence of very high amounts (more than 1 µg per
11
12 335 mg tissue) of dsRNA (Hegedus, ST:33,47-49). Similarly, efficient silencing was obtained
13
14 336 when high amounts of dsRNA were directly injected in vas deferens tissue of *S. littoralis*
15
16 337 cultured *in vitro* (Gvakharia et al., 2003). Notably, this approach was successful only by direct
17
18 338 injection and could not be obtained by soaking of the tissue or hemocoel injection. Also
19
20 339 pheromone glands of *Heliothis virescens* were cultured in the presence of very high amounts
21
22 340 of dsRNA but in this case the absence of silencing could be caused by the short period of
23
24 341 application (3 hours; Barthel, ST:158). Another alternative method that was used to achieve
25
26 342 gene silencing in the pheromone gland comprised of direct injection of dsRNA into the
27
28 343 pheromone glands of the adult in *B. mori* (Hull et al., 2010). Although in this method high
29
30 344 concentrations of dsRNA can be applied locally (calculated as 0.1 µg/mg tissue;
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32 345 ST:231,232,234-239), gene silencing did not appear more effective than systemic injection in
33
34 346 this species (4:4, no silencing:high silencing for direct injection; 8:12, no silencing:high
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36 347 silencing for hemocoel injection; see also Fig. 2).

348 Analysis of the studies from our database that reported the concentration of injected
349 dsRNA showed no obvious correlation with the degree of silencing in Lepidoptera (table 2).
350 In studies involving dsRNA injection into other species of insects, direct correlations between
351 dose and the potency of RNAi have been noted (e.g. Arakane et al., 2005; Boisson et al.,
352 2006; Whyard et al., 2009). The lack of correlation between dsRNA dose and RNAi in
353 Lepidoptera may reflect differences in the sensitivity/resistance to systemic RNAi in this

1 354 group of insects. To more fully address this possibility, further studies on the mechanisms of
2
3 355 dsRNA uptake and metabolism are needed. Overall, the genes examined are expressed in
4
5 356 many different tissues, and although no particular tissue was observed to be refractory to
6
7
8 357 injected dsRNA, some tissues may still limit dsRNA uptake (see Section 5). Another factor
9
10 358 that could affect the potency of RNAi is the amount of mRNA present. High levels might be
11
12
13 359 due to high transcription rates or to stable mRNA. Some of the variation across the studies in
14
15 360 our database may therefore be accounted for by differences in each gene's susceptibility to
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18 361 RNAi (see Section 6), rather than to intrinsic mechanistic differences in the RNAi machinery
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20 362 in different species.
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23 363 24 25 364 *4.1.2. Environmental RNAi*

26
27 365 Feeding of dsRNA is an even more attractive approach than hemocoel injection because
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30 366 it is non-invasive and furthermore opens the possibility of developing new methods of pest
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32 367 control through the production of species-specific hairpin RNAs against pests in transgenic
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35 368 plants (Price and Gatehouse, 2008). Interest for this approach received a great boost after the
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37 369 high profile publications of its feasibility in several pest insect species, including the
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40 370 lepidopteran *H. armigera* (Baum et al., 2007; Gordon and Waterhouse, 2007; Mao et al.,
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42 371 2007).
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45 372 Feeding of dsRNA has been applied with greatest effect in *Plutella xylostella*, *S. exigua*
46
47 373 and *M. sexta* (Bautista et al., 2009; Tian et al., 2009; Whyard et al., 2009; Yang et al., 2009;
48
49 374 Gómez, ST:179), while low levels of silencing could also be obtained in *Epiphyas postvittana*
50
51
52 375 and *Trichoplusia ni* (Turner et al., 2006; Wang, ST:241; Figure 3). A recent study in *S. exigua*
53
54 376 even established gene silencing after mixing bacteria expressing target dsRNAs in their food
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57 377 (Tian et al., 2009). Effects of gene silencing in *T. ni* larvae were variable as only a small
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1 378 percentage of the treated larvae showed detectable reduction in expression (Wang, ST:241).
2
3 379 By contrast, a series of unsuccessful attempts were reported in the noctuid pest insects *H.*
4
5 380 *armigera* and *Spodoptera frugiperda* (Collinge, ST:56,68-73; Nowara, ST:75-80,91-93), but
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7
8 381 in two publications efficient targeting of gene transcripts in midgut and brain tissue by
9
10 382 feeding was reported in *S. frugiperda* (Griebler et al., 2008; Rodríguez-Cabrera et al., 2010).
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12 383 In the latter publication, it was discussed that efficient silencing by dsRNA by feeding was
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14
15 384 greatly enhanced when larvae were used immediately after the molt and in addition were
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17
18 385 subjected to starvation for 24 hrs. In such starved larvae, dsRNA-degrading activity in the
19
20 386 midgut was greatly decreased and could be an important factor for the increased sensitivity to
21
22
23 387 dsRNA (Rodríguez-Cabrera et al., 2010). As mentioned above, in one study of *H. armigera*,
24
25 388 effective silencing of an inducible gene involved in plant toxin detoxification was achieved
26
27
28 389 (Mao et al., 2007).

30 390 Feeding of dsRNA seems to be successful only at high amounts: in general, there seems
31
32 391 to be a correlation between amount of dsRNA and degree of silencing (Table 2). Interestingly,
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34
35 392 while feeding of long dsRNA to *H. armigera* larvae was generally not very successful
36
37
38 393 (Collinge ST:56,68-73), high levels of silencing could be achieved using a custom-designed
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40 394 siRNA nucleotide (Kumar et al., 2009), suggesting different efficiencies between short
41
42 395 siRNAs and long dsRNAs in this species.

47 397 **5. Variation in RNAi efficiency related to tissue**

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52 399 Most studies in this review involved investigations of the immune response and defense
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54 400 against exogenous substances and its main effector organs hemocytes, fat body and midgut
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57 401 (Bettencourt et al., 2002; Hirai et al., 2004; Eleftherianos et al., 2006, 2009; Gandhe et al.,
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1 402 2007; Mao et al., 2007; Soberón et al., 2007; Terenius et al., 2007; Zhuang et al., 2007;
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3 403 Mrinal and Nagaraju, 2008; Whyard et al., 2009; Yang et al., 2009; Duvic, ST:25,37;
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5 404 Eleftherianos, ST:137,185-192; Garbutt, ST:135; Herrero, ST:4; Kanost, ST:7,34-36,49-51,
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8 405 53, 54,180, 181, 185-194, 198; Strand, ST:141-144). In these cases, silencing effects among
9
10 406 genes could differ considerably (Fig. 4). In *M. sexta*, it was striking that efficient silencing of
11
12 407 immune genes could be achieved after injection of low to intermediate doses of dsRNA in the
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14
15 408 hemocoel (Eleftherianos et al., 2006, 2009; Eleftherianos, ST:137,185-186). It has previously
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17
18 409 been reported that expression of *Hemolin* (a pattern recognition molecule exclusive to
19
20 410 Lepidoptera) in the silkworm *A. pernyi* is up-regulated in response to dsRNA as such, and that
21
22 411 silencing of *Hemolin* affects the progress of virus infection (Hirai et al., 2004). These results
23
24
25 412 allow us to speculate that sensitivity to RNAi can be coupled by other immunogenic factors.
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27 413 Interestingly, a recent study has highlighted the critical role of immune cells in the tumor
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29 414 microenvironment and showed how RNAi can be used to restore an efficient antitumor
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31
32 415 immune response in mice (Kortylewski et al., 2009). This finding emphasizes the possibility
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35 416 that combined activation of RNAi and innate immunity can have synergistic effects.
36

37 417 In contrast, epidermal tissue (larval epidermis and pupal wing) seems to be rather
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39 418 refractory to RNAi as only low silencing was obtained and that in only one study (Futahashi,
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41
42 419 ST:83) whereas in nine other studies no silencing was obtained (Futahashi, ST:87-89; Oostra,
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44 420 ST:46; Popadic, ST:9; Saenko, ST:97-99; Swevers, ST:233; Fig. 4). However, a recent series
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46
47 421 of experiments showed successful disruption of the regulatory pathway controlling chitin
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49 422 synthesis during molting in *S. exigua* (with epidermis as likely target tissue although
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52 423 catalogued under “whole organism” in Fig. 4; Chen et al., 2008; Zhang, ST:222). Rather
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54 424 sensitive to RNAi is brain tissue, which is unexpected because of the documented
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57 425 refractoriness of brain tissue to RNAi in nematodes that are prone to RNAi (Kennedy et al.,
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1 426 2004). In *C. elegans* this refractoriness is caused by the expression of the *eri-1* nuclease in
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3 427 brain tissue and differential expression of nucleases should also be considered as a
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6 428 contributing factor to the resistance of RNAi in different lepidopteran species.
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8 429 Further, in *H. cecropia* and *B. mori*, injection of dsRNA in the pupa can result in their
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10 430 uptake by the developing oocytes in the ovary and the observation of phenotypic effects in
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12
13 431 developing embryos (Bettencourt et al., 2002; Mrinal and Nagaraju, 2008). However, in four
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15
16 432 other studies in *B. mori*, heritable RNAi was not successful (Niimi, ST:41-44; Fig. 4).
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18 433 When studying RNAi in tissues, *in vitro* cultures of dissected tissues can be of great use
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20 434 as this technique allows the effectiveness of dsRNA constructs to be rapidly examined outside
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22
23 435 of the complexity of a total organism. As already mentioned above, this has been performed
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25 436 in *M. configurata* where several structural genes that contribute proteins to the midgut
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27
28 437 peritrophic membrane (insect intestinal mucins, non-mucin structural proteins and chitin
29
30 438 deacetylase) were tested *in vitro* (Hegedus, ST:33,47-49). Comparison of *in vitro* working
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33 439 situations to non-working *in vivo* situations may reveal compounds or enzymes in the intact
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35 440 organism that destroy the dsRNA *in vivo*, for example, a dsRNA-degrading enzyme in the
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37
38 441 hemolymph. The *M. configurata* midgut expresses a dsRNAase gene; however, recent studies
39
40 442 demonstrated that feeding of chitin deacetylase (*McCDAl*) dsRNA to neonate or fourth instar
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42 443 larvae totally eliminated the cognate transcript within 24-48 hours, respectively (D. Hegedus,
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44
45 444 unpublished results).
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47 445 To address the issue of uptake of dsRNA by insect cells, two cell lines from
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49
50 446 lepidopteran pests, Hi5 (derived from *T. ni*) and S12 (derived from *S. littoralis*) were
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52 447 incubated with high concentrations (ca. 100 µg/ml) of dsRNA labeled with fluorescein
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55 448 (Ambion Silencer SiRNA Labeling Kit). Although the specific fluorescence of the dsRNAs
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57 449 was not very high (1 molecule of fluorescein per 100-150 nucleotides), significant, and
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1 450 sometimes intense, internalization of FAM-dsRNA in the cells was observed (Fig. 5),
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3 451 indicating that uptake of dsRNA by lepidopteran cells is not a limiting factor. However,
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5 452 efficient uptake of dsRNA is not synonymous with efficient silencing as similar levels of
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7
8 453 dsRNA uptake were observed in *Bombyx*-derived Bm5 cells which are known to be defective
9
10 454 in homologous gene silencing when dsRNA is just added in the culture medium of the cells
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12
13 455 (Hannan et al., 2009; L. Swevers, H. Huvenne, G. Smaghe, unpublished results). Thus,
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15 456 physiological processes downstream of dsRNA uptake are necessary to conduct efficient
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18 457 RNAi in particular lepidopteran cell lines, and, presumably, in particular tissues of
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20 458 lepidopteran species as well.

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23 459 Earlier observations using Hi5 cells have also shown that dsRNA is not efficiently taken
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25 460 up from the cell culture medium, while intracellular RNAi, obtained after transfection of
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27 461 dsRNA, was very efficient, even at low dose (Beck and Strand, 2003, 2005). Similar results
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29
30 462 were obtained for a cell line derived from *C. includens* (Johnson et al., 2010), for which
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32 463 systemic RNAi *in vivo* was not observed (see above), indicating that in this species the
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35 464 process of functional uptake from the culture medium or the hemolymph rather than the
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37 465 efficiency of the intracellular RNAi machinery is the limiting factor, at least for some tissues.

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39
40 466 In the case of feeding, the midgut obviously is the primary target organ, representing
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42 467 environmental RNAi. It would also be very interesting if the silencing signal could spread
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44 468 from the midgut to other tissues in the insect, causing systemic RNAi. While generally cases
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46
47 469 of efficient silencing of genes in midgut tissue were predominant (inset in Fig. 4: Turner et
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49 470 al., 2006; Sivakumar et al., 2007; Whyard et al., 2009; Gómez, ST:179), silencing signals
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51
52 471 spread to the remaining tissues of the insect only in a limited number of cases (Meyering-Vos
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54 472 et al., 2006; Bautista et al., 2009; Tian et al., 2009).

6. Variation in RNAi efficiency related to gene function

The type of gene to be silenced can significantly affect the outcome of an RNAi experiment. Here, we used Gene Ontology for functional categorization of lepidopteran genes that have been attempted to be knocked down by systemic RNAi (Fig. 6). In total, out of 130 genes used for the analysis, only 38% were silenced at high levels while 48% and 14% of the genes failed to be silenced or they were silenced at low levels, respectively. Although it is difficult to establish trends from the current data, it seems that immune-related genes are more sensitive to systemic RNAi (80% success rate). In contrast, we found that genes from the protein binding group and the transporter activity group were refractory to silencing.

In their study of RNAi in the Western corn rootworm (Coleoptera: Chrysomelidae), Baum et al. (2007) examined 290 genes using a systematic oral delivery protocol involving the application of two dsRNA doses on artificial diet. They reported considerable variation in the target genes' susceptibility to RNAi: 125 were found to show significant larval mortality and/or reduced growth rate at the higher dose (52 ng/cm²), with 67 of these showing significant mortality and/or reduced growth rate at the lower dose (5.2 ng/cm²). The most susceptible genes are listed in their paper, but unfortunately the remaining genes were not identified. Some of the genes identified by Baum et al. (2007) as being most susceptible are among those described in the current review. These include the v-ATPases subunits A and D, and the ribosomal protein S4; none of which had any lethal phenotype during feeding trials. It might be helpful if genes identified as being particularly susceptible to RNAi were tested with alternative delivery protocols.

In some cases the efficiency of RNAi-mediated knockdown appears to depend on the identity and nature of the target gene. Possible explanations are that the dsRNA reagents or

1 498 the resulting siRNA molecules may be subject to sequence-specific degradation, or that the
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3 499 silencing specificity depends on the stability of the mRNA in question. Thus, genes with
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6 500 efficient feedback mechanisms of regulation might prevent depletion of mRNA levels with
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8 501 higher rates of transcription. Possible reasons for insensitivity toward systemic RNAi at
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11 502 species, tissue and gene levels have recently been reviewed (Bellés, 2010).

13 503 The present report describes the post-transcriptional silencing of genes through dsRNA-
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15 504 induced mRNA degradation. There is also a possibility that silencing of particular genes may
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18 505 occur in Lepidoptera by inhibition of transcription, which is a distinct effect from both
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20 506 classical and miRNA-mediated RNAi and is associated with heterochromatin maintenance. It
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22
23 507 has previously been reported that this mode of gene silencing exists in yeast, plants and
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25 508 nematodes, but has not been confirmed to occur in insects (Lippman and Martienssen, 2004).

30 510 **7. Similar experiments with different outcomes**

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34
35 512 There are several cases in which similar RNAi experiments produced conflicting results.
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37 513 In *H. armigera* two groups carried out near identical RNAi experiments where they injected
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40 514 dsRNA into the hemocoel of the larva and assayed for the knockdown of their target genes in
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42 515 the gut using qPCR. One group (targeting the aminopeptidase-N gene product; Sivakumar,
43
44 516 ST:127) found a high degree of silencing in midgut tissue, whilst the other group (targeting
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46
47 517 the cadherin gene product; Wee, ST:162) reported no silencing of their gene. Both of these
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49
50 518 proteins are found on the outside of midgut epithelial cells, as glycoprotein components of the
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52 519 cell membrane. Aminopeptidase-N proteins are involved in dietary protein digestion (Terra
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54 520 and Ferreira, 1994; Angelucci et al., 2008), whereas proteins in the classical cadherin family
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57 521 are involved in calcium-dependent cell-cell adhesion. However, the lepidopteran cadherin-like
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1 522 proteins are present primarily in midgut columnar cell apical membranes (Wang et al., 2005;
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3 523 Aimanova et al., 2006); while their function is unclear, they are of importance as Bt toxin
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5 524 binding proteins, as are the aminopeptidases. It is unclear why the aminopeptidase-N gene is
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7
8 525 more susceptible to silencing than the cadherin gene, particularly in the absence of
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11 526 comparative data on gene expression and mRNA turnover. However, attempts to knockdown
12
13 527 aminopeptidase-N genes in other lepidopterans, including *Ostrinia nubilalis*, *S. exigua* and *E.*
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15 528 *postvittana*, have not been successful (Crava, ST:81; Herrero, ST:4,14; Gatehouse, ST:67),
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17
18 529 suggesting that there is no general susceptibility of aminopeptidase genes to RNAi.

19
20 530 There are also a number of cases in which successful experiments have been reported in
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22
23 531 one life stage of insect and not others. For example, one group achieved a high degree of
24
25 532 knockdown of the *S. littoralis* period gene by injecting dsRNA into the adult hemocoel
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27
28 533 (Bebas, ST:18). Another group carried out a similar experiment in *S. littoralis* larvae and was
29
30 534 unsuccessful in achieving a knockdown of the Halloween gene (Iga, ST:57-61). This result
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32
33 535 may reflect differences in the types of genes targeted (as discussed above) but it may also be
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35 536 caused by underlying and unexplained differences in the susceptibility of different life stages
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38 537 of the same insect species to RNAi. The same pattern was observed in the closely related
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40 538 species *S. frugiperda*, in which Meyering-Vos (ST:38,101,102) achieved a high degree of
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42 539 knockdown of allostatin and allotropin genes in adult insects whereas Lundmark (ST:13) was
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44
45 540 unsuccessful in an attempt to knock down five different genes in larvae. The fact that many
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47 541 different genes were targeted in these studies makes it more likely that the insect is refractory
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49
50 542 to RNAi in its larval stage, rather than the result being due to any differences in the target
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52 543 genes and their transcripts.

53 544

57 545 **8. Features of the dsRNA**

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3 547 From the wealth of data submitted to the database, several technical aspects on the
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6 548 dsRNA production and time from injection to detection have been possible to analyze. The
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8 549 conclusion is that there seem to be no correlation between success rate and time from dsRNA
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11 550 injection to time of detection of silencing, methods for annealing and purification, or kits
12
13 551 used. However, whether dsRNA is added before or at the same time as a gene is turned on can
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15
16 552 have large impact on the outcome. As illustrated in Fig. 7, simultaneous transfection of
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18 553 dsRNA and virus into Sf9 cells does not inhibit virus proliferation, while prior incubation
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20
21 554 with dsRNA does. Also, preparations of dsRNA with cationic lipid reagents to stimulate
22
23 555 uptake generally does not result in increase in functional uptake when injected *in vivo* (Niimi,
24
25 556 ST:41-43; Ohnishi, ST:210-218). Most of the studies have used dsRNA for silencing and only
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27
28 557 a few hairpin or siRNA. Of the latter, both methods have resulted in high silencing. Another
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30 558 feature of dsRNA that was discussed early in the use of RNAi was the impact of the length of
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32
33 559 the dsRNA and it has been determined that in *Drosophila* S2-cells the minimum length of
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35 560 uptake of dsRNA was 211 bp (Saleh et al., 2006). Likewise, when silencing P450 in *H.*
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37 561 *armigera* with transgenic plants producing dsRNA, experiments with *Arabidopsis* Dicer
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40 562 mutants showed long, unprocessed dsRNA fragments to be more effective than the siRNA
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42 563 products of Dicer activity (Mao et al., 2007). However, in our dataset the success rate is
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45 564 independent of length of dsRNA (Fig. S1).

46 47 565 48 49 566 **9. Conclusion**

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54 568 The results deposited in the database illustrate the high variability of success of RNAi
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57 569 experiments in lepidopteran insects. Only a few solid predictions have emerged and the
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1 570 questions remain what the cause is for the high variability at the molecular and cellular level
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3 571 and if techniques can be adapted to increase the efficiency of RNAi.
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5
6 572 On a theoretical basis, differences in efficacy of systemic or environmental RNAi can
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8 573 be caused by several different mechanisms. One obvious cause is that the Lepidoptera, like
9
10 574 other insects, lack clear functional homologs of the two types of genes required for systemic
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12 575 RNAi in *C. elegans*, i.e. the canonical RdRP and the RNA-transporter sid-1. That other genes
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14 576 in insects (and other animals) appear to be able to compensate to a degree for these absent
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16 577 genes is one of the surprising findings of recent work on RNAi; however the extent of their
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18 578 ability to do this is unclear and, based on many experimental observations such as those in
19
20 579 this review, appears quite limited. However the presence of an intracellular RdRP-based anti-
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22 580 viral RNA-immunity would help explain the presence of genes that encode the core RNAi
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24 581 machinery, such as Dicer-2, dsRNA-binding proteins and Argonautes, in these species.
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30 582 Another possible factor is that competition can also occur between the siRNA- and miRNA-
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32 583 programmed effector complexes (Tomari et al., 2007), providing an alternative if less
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34 584 compelling explanation for the retention of these genes in the absence of the RdRP. Thus,
35
36 585 studies that look for expression of elements of the core RNAi machinery could provide an
37
38 586 explanation for some of the differences observed. Second, a barrier can exist at the level of
39
40 587 uptake of dsRNA. While dsRNA may be taken up efficiently, as shown in figure 5, it may not
41
42 588 be sorted correctly during endosome trafficking and fail to reach the appropriate dsRNA-
43
44 589 processing machinery. Recently, it was found that RISC function is linked to the endocytic
45
46 590 pathway (Siomi and Siomi, 2009; Lee et al., 2009) and it is possible that there is a direct
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48 591 functional connection between uptake of dsRNA by endosomes and processing by RISC at
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50 592 organelles dedicated to mRNA degradation such as GW-bodies (Schneider et al., 2006). Such
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52 593 a barrier could be revealed by co-localization studies of fluorescent dsRNA and antibody
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1 594 staining of RISC components by fluorescence microscopy. Third, dsRNA-degrading
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3 595 nucleases can be present in particular tissues, hemolymph or gut lumen to inactivate
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5
6 596 administered dsRNA (Arimatsu et al., 2007). However, the fate of injected or ingested
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8 597 dsRNA remains uncharted territory up to present.
9

10
11 598 The data in this review suggest that the genes most likely to be susceptible to dsRNA-
12
13 599 induced RNAi are those active in immunity, supporting similar findings published previously
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15 600 (e.g. Hirai et al., 2004; Eleftherianos et al., 2006, 2009). Moreover, a study in *D.*
16
17 601 *melanogaster* showed that only lymphocytes were able to take up injected dsRNA, resulting
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19
20 602 in silencing of a transgene expressed only in those cells (Miller et al 2009). While no
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22 603 comparable specific study has been undertaken in Lepidoptera, the silencing of immune-
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24
25 604 response genes known to be expressed in hemocytes is consistent with the *D. melanogaster*
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27 605 work. Furthermore, in *D. melanogaster*, dsRNA uptake by hemocytes has been shown to be
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29
30 606 required for RNAi-based immunity to RNA viruses (Saleh et al., 2009). While there have
31
32 607 been studies in Lepidoptera of hemocyte-expressed genes that are induced by virus infection
33
34
35 608 (Shelby and Popham, 2009) these have not yet extended to the RNA viruses now being
36
37 609 increasingly analyzed in *D. melanogaster* (Ding and Voinnet, 2007). Nonetheless, the
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40 610 observations now accumulating of immune genes being vulnerable to dsRNA-induced RNAi
41
42 611 suggests that an endocytic pathway enabling dsRNA-induction of RNAi immunity may exist
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44
45 612 in Lepidoptera as already shown in *D. melanogaster*.
46

47 613 It is noted that high amounts of dsRNA are used in many studies, raising the question of
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49
50 614 the specificity of effects. For comparison, successful studies in mammals without significant
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52 615 side-effects are accomplished by doses as low as 50 ng per mg tissue (Li et al., 2010).
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54 616 Although the incorporation of modifications in the applied RNA oligonucleotides may be an
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57 617 important factor to explain differences between studies employing mammals and insects, it is
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1 618 clear that the issue of specificity remains to be addressed satisfactorily in insects, especially in
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3 619 cases where high amounts of dsRNA are used to achieve silencing. As already mentioned
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5 620 above, non-specific activation of the innate immune response could account (partially) for
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8 621 some of the effects observed. An early study in *A. pernyi* showed that injection of dsGFP
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10 622 induced the expression of *Hemolin* dose-dependently, but that the anti-bacterial response was
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12 623 left unaffected (Hirai et al., 2004). To corroborate these data, further investigations of how
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14 624 administration of dsRNA affects the immune response are necessary. In the same vein,
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16 625 conclusive proof of the involvement of the RNAi pathway in the observed effects, for
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18 626 example through detection of specific siRNAs derived from the injected or ingested dsRNA,
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20 627 has not been reported in any of the studies. For instance, sequencing of *B. mori* small RNA
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22 628 libraries have revealed the existence of piRNAs and miRNAs but not siRNAs (Jagadeeswaran
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24 629 et al., 2008; Kawaoka et al., 2008; Zhang et al., 2009). It would be considered a major
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26 630 breakthrough if such results can be presented.
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35 632 **10. Future directions**

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40 634 RNAi is thought to have developed as a protection against virus infections. In the co-
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42 635 evolutionary race between host and microbe, viruses have developed ways to avoid the
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44 636 silencing by suppressing RNAi (Li and Ding, 2006; Ding and Voinnet, 2007). The presence
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46 637 of viral infections in lepidopterans could cause difficulties in silencing. Only a limited number
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48 638 of lepidopteran RNA viruses have been identified to date (Gordon and Waterhouse 2006;
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50 639 Zeddiam et al., 2010), including some that appear to cause unapparent infections and may be
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52 640 subject to anti-viral immunity as found for *D. melanogaster*. Similar studies to those of Wu et
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54 641 al. (2010) performed in Diptera would in Lepidoptera likely be very rewarding and may make
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1 642 this a more productive approach than specifically searching for the few if any viruses known *a*
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3 643 *priori* from any particular species. There are also no reports of virus-infected colonies of
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5 644 moths or butterflies in the database; however, it is possible that this is due to the lack of
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8 645 investigation rather than of infection. Therefore, it would be advisable to screen for viruses
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11 646 known for RNAi inhibition such as the Flock House virus in *Galleria mellonella*.

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13 647 Several aspects deserve careful attention when performing RNAi experiments in
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15 648 Lepidoptera. First, the mode of regulation of the gene in question should also be taken into
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18 649 account. For example, it has sometimes been observed that particular genes are resistant to
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21 650 RNAi when other genes expressed in the same tissue are not, which could imply that some
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23 651 genes are regulated by post-translational mechanisms. Second, it is important to consider that
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25 652 the outcome of an RNAi experiment depends on the dynamics of mRNA synthesis and
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28 653 breakdown of the target gene. Third, it is always a concern that based on the mechanism of
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30 654 gene silencing, RNAi treatments may in some cases induce off-target effects. For instance, it
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33 655 is known that siRNAs produced can interfere with other small RNA pathways such as the
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35 656 miRNA pathway (Brodersen and Voinnet, 2009) and that dsRNA can induce the innate
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38 657 immune response through interaction with Toll-like receptors, at least in vertebrates.
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40 658 Therefore, it is important to use a control dsRNA that is not linked to the physiological
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43 659 process to check the specificity of gene targeting; in many cases dsRNAs corresponding to
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45 660 GFP or luciferase are used. This procedure controls for non-specific effects that are caused by
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47 661 both the structure and the sequence of dsRNA.

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49 662 The issue of validity of RNAi has been discussed extensively in *D. melanogaster*
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52 663 research which has resulted in recommendations to ensure that results obtained in RNAi
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55 664 experiments are valid. In systematic screens that involve transgenic *D. melanogaster*, the false
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57 665 positive rate is estimated to be 5-7% and analysis indicated that sequence identities stretching
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1 666 over 12 bp could already generate off-target effects (Mummery-Widmer et al., 2009;
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3 667 Schnorrer et al., 2010). Ideally, association of an RNAi phenotype with a previously observed
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5 668 phenotype should be confirmed by an independent method. While the generation of classical
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8 669 mutants or the generation of transgenic insects to rescue the RNAi phenotype with RNAi-
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10 670 resistant transgenes is far beyond the boundary of possibilities in most insects, the
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13 671 recommendation to use a second dsRNA that targets a different region of the same gene can
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15 672 be easily applied (Langer et al., 2010). To control for specificity of dsRNA effects,
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18 673 quantitative PCR experiments can also be carried out to check expression of household genes
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20 674 such as actin or tubulin and verify that administration of dsRNA does not affect general cell
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23 675 physiology.

25 676 A major theme in the history of RNAi in Lepidoptera is the inconsistency of the
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27 677 silencing. This paper has highlighted areas that could explain some of the difficulties
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30 678 encountered, however much is still unexplained and therefore, a way to certify that the
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33 679 method is working is warranted. We propose that a gold standard is set up with a gene that
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35 680 seems to be possible to silence in several species and with a protocol that has been developed
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37 681 and used in several laboratories. With such a tool in hand, it would be possible to distinguish
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40 682 between failures due to technical issues and those due to biology. In the longer term, it may be
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42 683 possible to envisage lepidopteran models (e.g., *B. mori*) expressing the key *C. elegans* genes
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45 684 for systemic RNAi and therefore able to allow genome-wide RNAi screens such as those
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47 685 carried out in other model systems such as *C. elegans* (e.g., Kamath and Ahringer, 2003) and
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49
50 686 even in *D. melanogaster* cells (Perrimon and Mathey-Perot, 2007).

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Table 1. Successful RNAi experiments in Lepidoptera.

Family	Species	Studied functions
Bombycidae	<i>Bombyx mori</i>	Embryonic development (appendage formation ^{1,2} , segment pre-patterning, elongation ³ , antimicrobial peptides ⁴), postembryonic development (ecdysis ⁵ , larval-pupal molt ⁶ , metamorphosis ⁷ , wing expansion ⁸ , adult wing and leg formation ⁶), embryonic and larval coloration ⁹ , cocoon pigmentation ¹⁰ , immune system (clearance of <i>E. coli</i> from hemolymph ¹¹), sex pheromone synthesis ^{12,13,14,15}
Crambidae	<i>Diatraea saccharalis</i>	Bt toxin receptor (aminopeptidase-N) ¹⁶
Crambidae	<i>Ostrinia nubilalis</i>	Role of chitinase genes in regulating chitin content of midgut peritrophic matrix ¹⁷
Noctuidae	<i>Spodoptera exigua</i>	Ecdysis ¹⁸ , chitin synthesis pathway ^{18,19,20} , trehalose synthesis pathway ^{20,21} , role of storage hexamerins in development ²²
Noctuidae	<i>Spodoptera frugiperda</i>	Juvenile hormone titer (effect of allatotropins and allatostatins) ^{23,24} , defense against Bt Cry toxin ²⁵
Noctuidae	<i>Spodoptera littoralis</i>	Circadian rhythm of sperm release ^{26,27}
Noctuidae	<i>Spodoptera litura</i>	Bt toxin receptor (aminopeptidase-N) ²⁸
Noctuidae	<i>Mamestra brassicae</i>	Embryonic development (formation of bilateral procephalic lobes) ²⁹

1 2 3 4 5 6 7	Noctuidae	<i>Helicoverpa armigera</i>	Acetylcholinesterase (role in regulation of differentiation and development) ³⁰ , Bt toxin receptor (aminopeptidase-N) ³¹
8 9 10 11 12 13 14	Plutellidae	<i>Plutella xylostella</i>	Insecticide resistance (role of cytochrome P450 in resistance to permethrin) ³² , role of cadherin in larval growth and development) ³³
15 16 17 18 19	Pyralidae	<i>Plodia interpunctella</i>	Embryonic development (eye-color pigmentation) ³⁴
20 21 22	Saturniidae	<i>Antheraea assama</i>	Sex determination ³⁵
23 24 25 26 27	Saturniidae	<i>Antheraea mylitta</i>	Immune system (nodule formation) ³⁶ , sex determination ³⁵
28 29	Saturniidae	<i>Antheraea pernyi</i>	Immune system ³⁷
30 31 32 33 34	Saturniidae	<i>Hyalophora cecropia</i>	Embryonic development ³⁸ , immune system ³⁹ (roles of pattern recognition protein hemolin)
35 36 37 38 39 40 41 42 43 44 45 46 47 48 49 50 51 52 53 54 55 56 57 58 59	Sphingidae	<i>Manduca sexta</i>	Immune system (hemocyte adhesion and encapsulation ^{40,41,42,43} , phagocytosis and melanotic nodule formation & clearance of <i>E. coli</i> from the hemolymph ⁴⁴ , protective effect of prior <i>E. coli</i> infection on <i>Photographus luminescens</i> infection ⁴⁵ , role of plasmatocyte-spreading peptide (PSP) during <i>E. coli</i> and <i>P. luminescens</i> infection ⁴⁶ , effect of a <i>P. luminescens</i> antibiotic on phenoloxidase ⁴⁷ , role of pattern recognition proteins in resistance to

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		<i>Photorhabdus</i> infection ⁴⁸ , protective effect of nitric oxide synthesis on oral <i>Photorhabdus</i> infection) ⁴⁹ , Bt toxin receptor (cadherin) ⁵⁰ , defense against Bt Cry toxins ⁵¹ , pesticide development (vATPase target) ⁵²
Tortricidae	<i>Epiphyas postvittana</i>	Larval gut carboxylesterase, pheromone binding ⁵³

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1011 Numbers refer to the publication list found in supplementary data

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Table 2. Number of experiments at which a particular degree of silencing was achieved depending on dsRNA concentration ^{a,b}.

dsRNA concentration (μg per mg tissue)	Hemocoel injection			Feeding		
	None	low	high	none	low	high
Low ($< 0.01\mu\text{g}/\text{mg}$)	9	5	4	N.A.	N.A.	N.A.
Intermediate ($0.01\mu\text{g}/\text{mg} - < 0.1\mu\text{g}/\text{mg}$)	16	1	10	8	0	0
High ($0.1\mu\text{g}/\text{mg} - < 1\mu\text{g}/\text{mg}$)	2	2	2	0	2	1
Very high ($> 1\mu\text{g}/\text{mg}$)	7	3	5	0	1	1

^a Only experiments are included in which the amount of dsRNA administered could be calculated exactly.

^b The experiments in which successful silencing was achieved by feeding synthetic siRNA in *H. armigera* (Kumar et al., 2009, section 4.1.2) are not included in this table.

N.A. = not applicable.

1 1024 **Figure Legends**

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6 1026 **Fig. 1.** RNAi efficiency following dsRNA injections in embryos of different lepidopteran

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8 1027 species. Experiments that have achieved high silencing are indicated in black, those that

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10 1028 achieved low silencing in grey and unsuccessful experiments in white. Degree of silencing in

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12 1029 individual experiments is derived from supplementary table 1 and is a subjective measure of

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14 1030 silencing as provided by the database submitter.

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18 1032 **Fig. 2.** RNAi silencing efficiency following hemocoel injection of dsRNA (intact or digested)

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20 1033 in different lepidopteran species. Color codes and data source as in Fig. 1.

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24 1035 **Fig. 3.** RNAi silencing efficiency after feeding dsRNA in different lepidopteran species.

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26 1036 Color codes and data source as in Fig. 1. Experiments in which successful silencing was

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28 1037 achieved by feeding synthetic siRNA in *Helicoverpa armigera* (Kumar et al., 2009, section

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30 1038 4.1.2) are not included in this figure.

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34 1040 **Fig. 4.** RNAi silencing efficiency in different tissues of lepidopteran species after hemocoel

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36 1041 injection (main figure) or feeding (insert). Color codes and data source as in Fig. 1.

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38 1042 Experiments in which successful silencing was achieved by feeding synthetic siRNA in

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40 1043 *Helicoverpa armigera* (Kumar et al., 2009, section 4.1.2) are not included in this figure.

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44 1045 **Fig. 5.** Fluorescence microscope images of *Trichoplusia ni*-derived Hi5 or *Spodoptera*

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46 1046 *littoralis*-derived S12 cells after soaking in high concentrations (~100 µg/ml) of fluorescein

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48 1047 (FAM)-labeled dsRNA for 24 h. Control cells were left untreated. Uptake of FAM-labeled

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1 1048 dsRNA by individual cells showed considerable variation that resulted in the observation of
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3 1049 intense signals in some cells and the absence of fluorescence in other cells. In general, higher
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6 1050 fluorescence signals were observed in Hi5 cells than in S12 cells. Shown are selected treated
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8 1051 cells in which strong internalization of FAM-labeled dsRNA is detected. The experiment
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11 1052 suggests that lepidopteran cells are able to take up dsRNA molecules.

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16 1054 **Fig. 6.** RNAi efficiency related to gene function. Lepidopteran genes that were targeted by
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18 1055 systemic RNAi silencing were functionally categorized according to Gene Ontology. Color
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20 1056 codes and data source as in Fig. 1.

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25 1058 **Fig. 7.** RNAi efficiency depending on time for addition of dsRNA. The level of the viral coat
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28 1059 protein GP64 indicates that simultaneous transfection of dsRNA for the baculovirus gene ie-1
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30 1060 (dsie-1) with viral infection (SI) did not trigger viral suppression while there was 3-fold
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33 1061 decrease when the cells were pre-incubated with dsie-1 (PI), suggesting the necessity for prior
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35 1062 activation of the host RNAi machinery. One μ g of dsie-1 was transfected to half a million of
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38 1063 Sf9 cells either 24 h prior to the *AcNPV* viral challenge (PI) or along with the virus (SI). A
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40 1064 viral dose of 5 Multiplicity of infection (MOI) was used.
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42 1065 C indicates mock infection and was set to 100%.

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45 1066 The bars indicate standard deviation from the reading of two different Western blots.

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47 1067
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50 1068 **Supplementary Fig. 1.** Impact of dsRNA length on RNAi silencing efficiency in
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52 1069 Lepidoptera. There is no obvious correlation between the length of dsRNA used in the
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55 1070 experiments and successful gene knockdown.

Figure 1

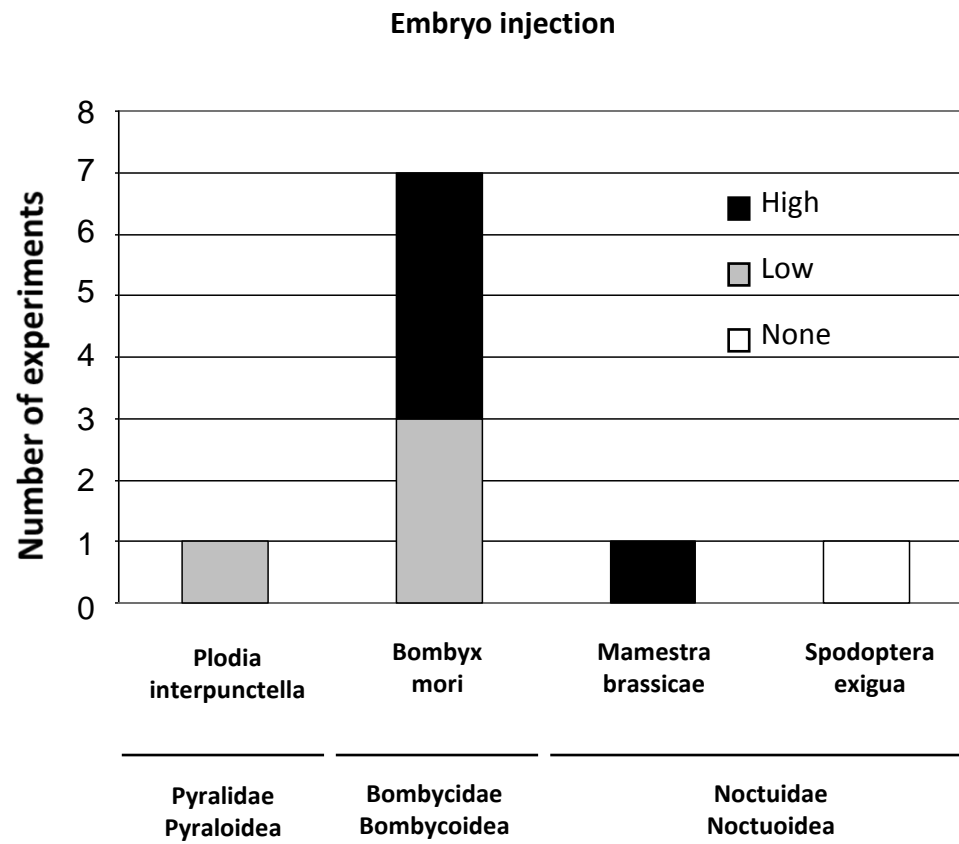


Figure 2

Hemocoel injection

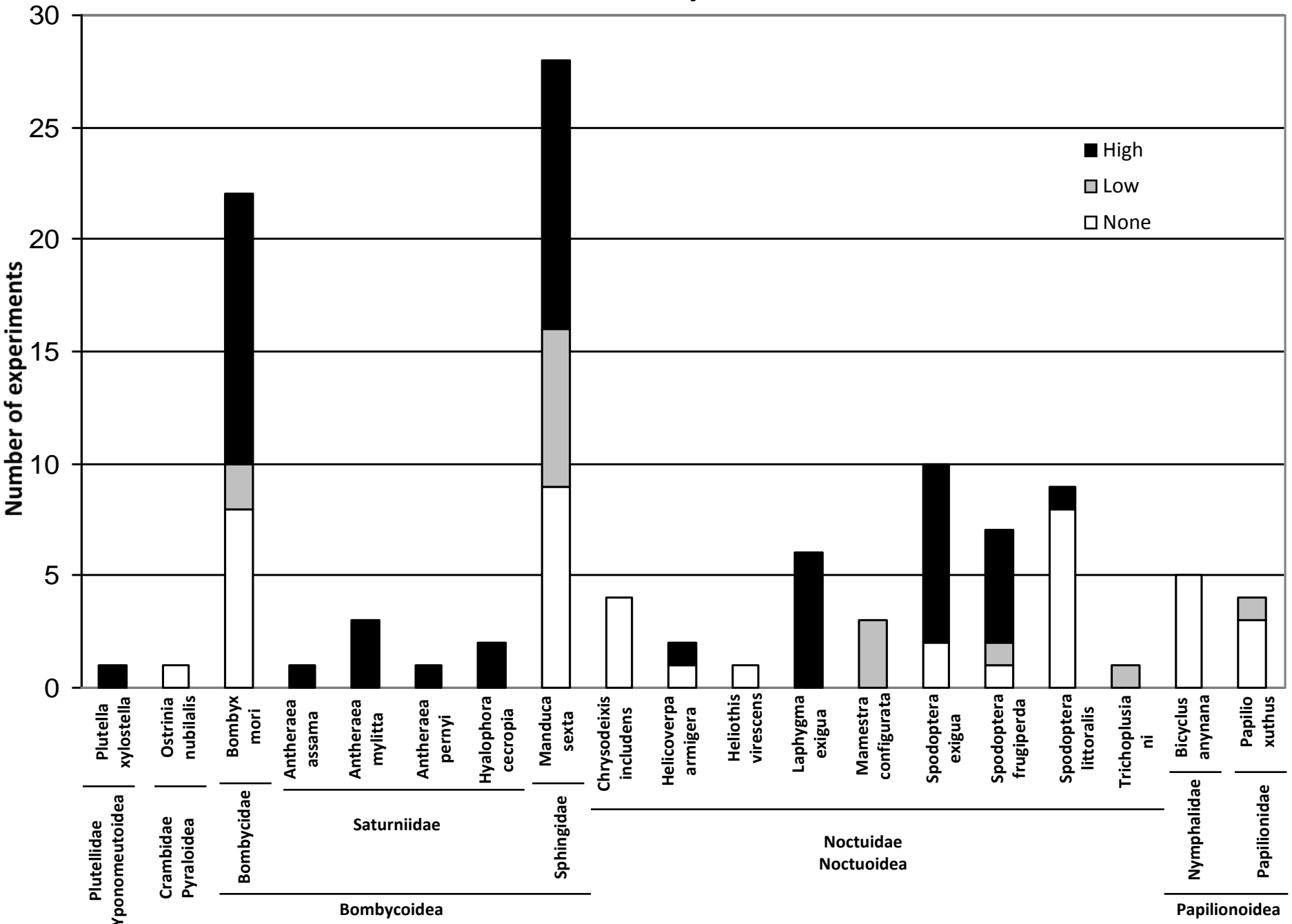


Figure 3

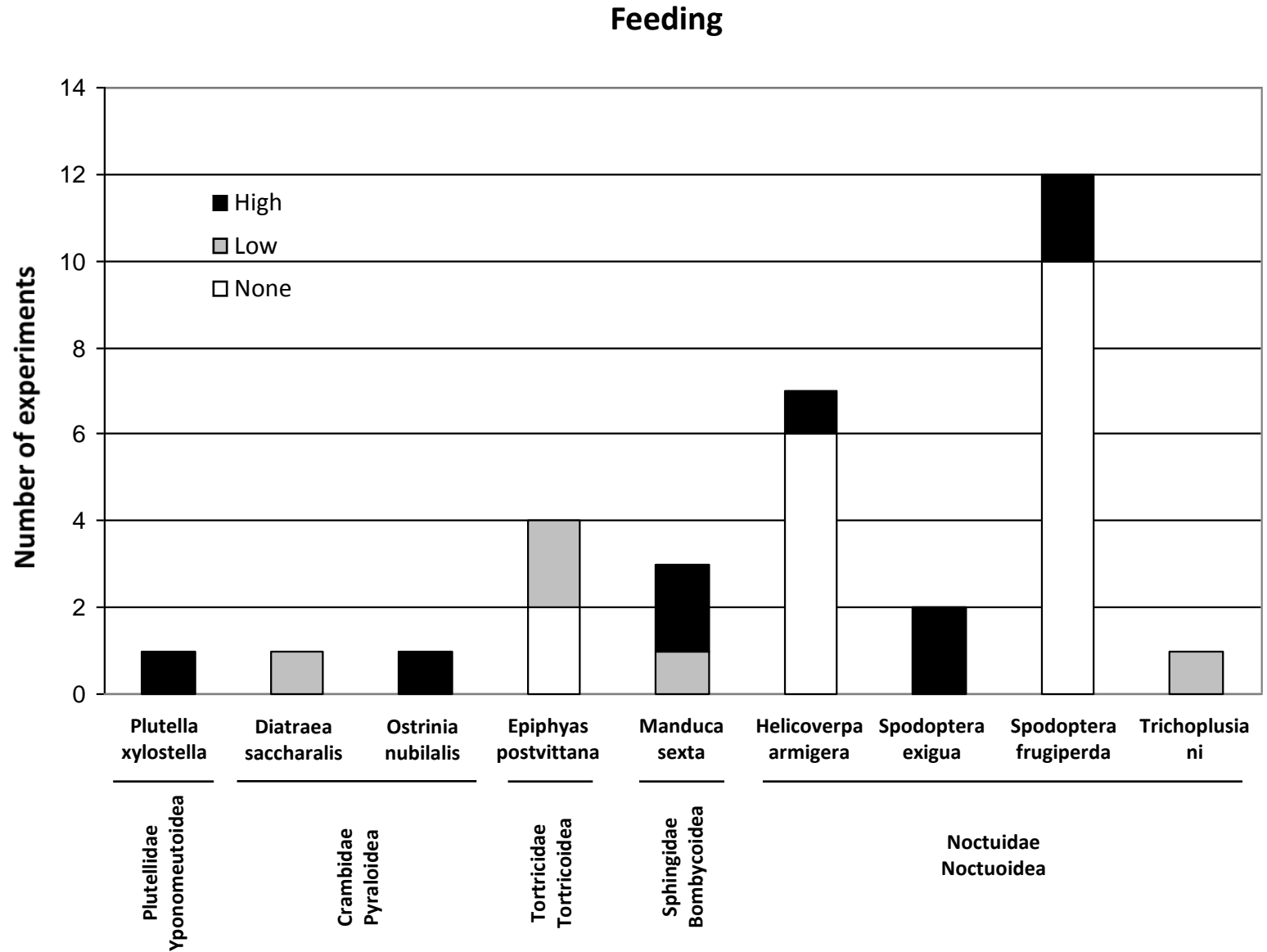
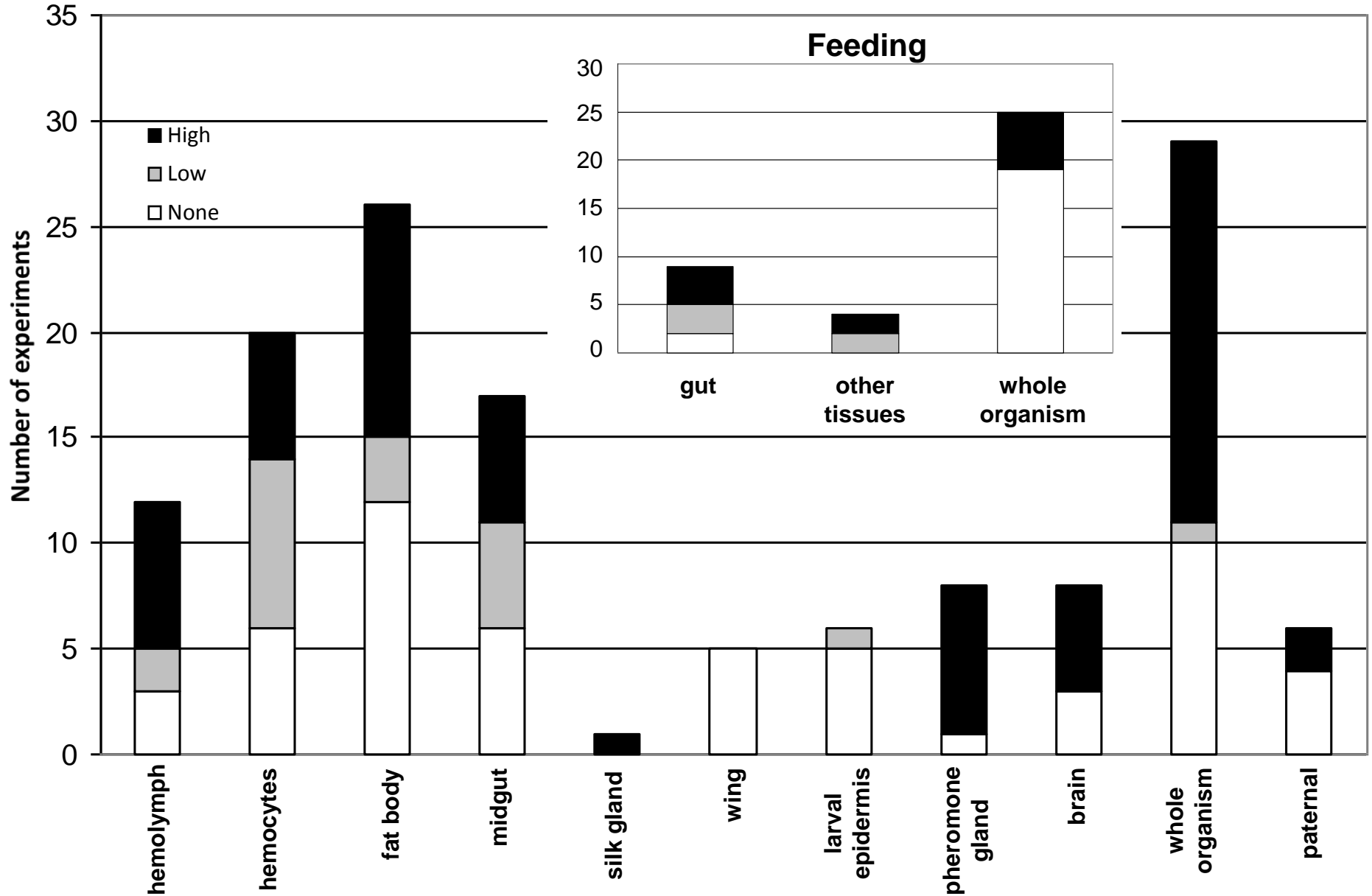
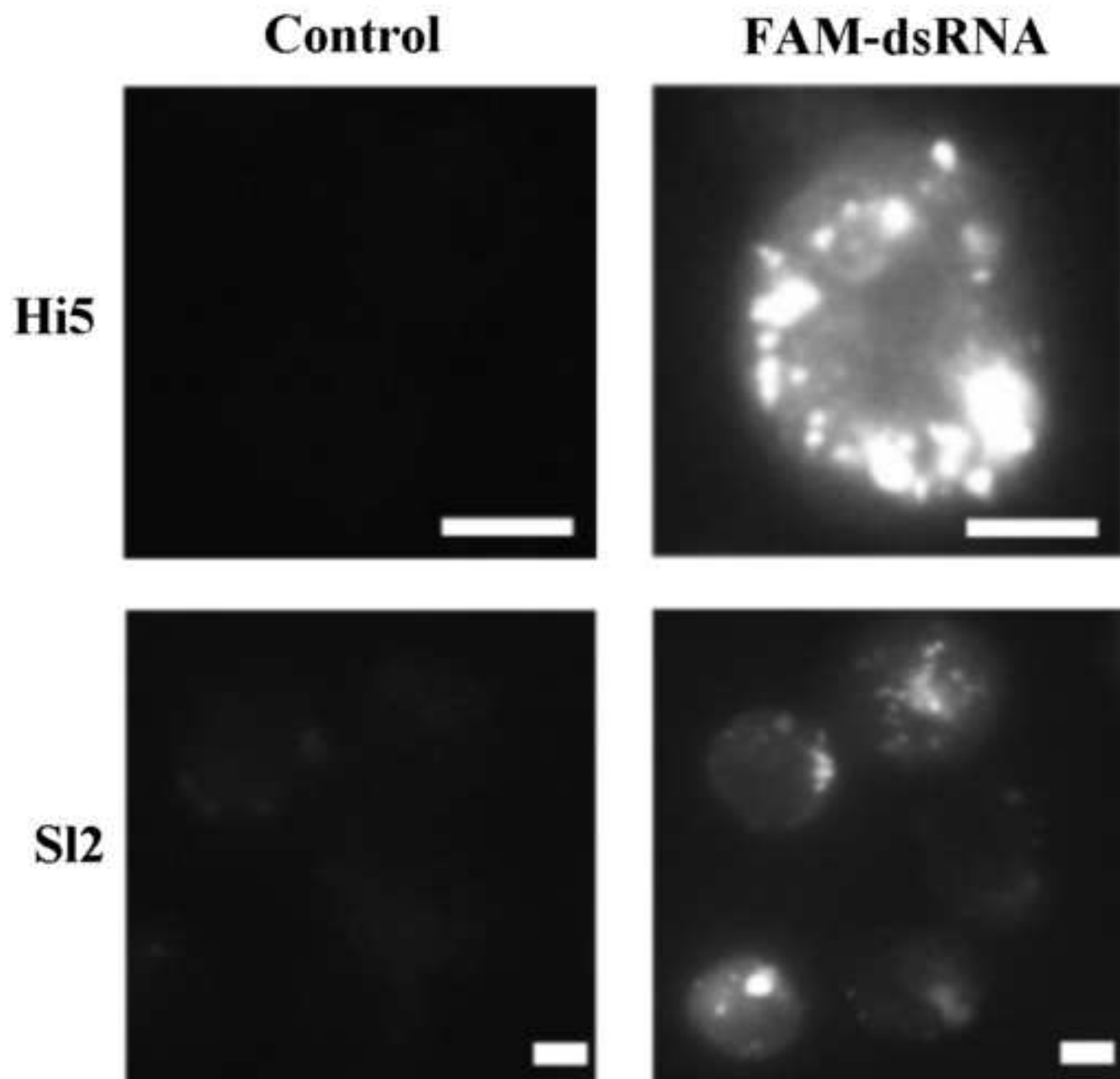


Figure 4

Hemocoel injection





Figure(s)
Figure 6

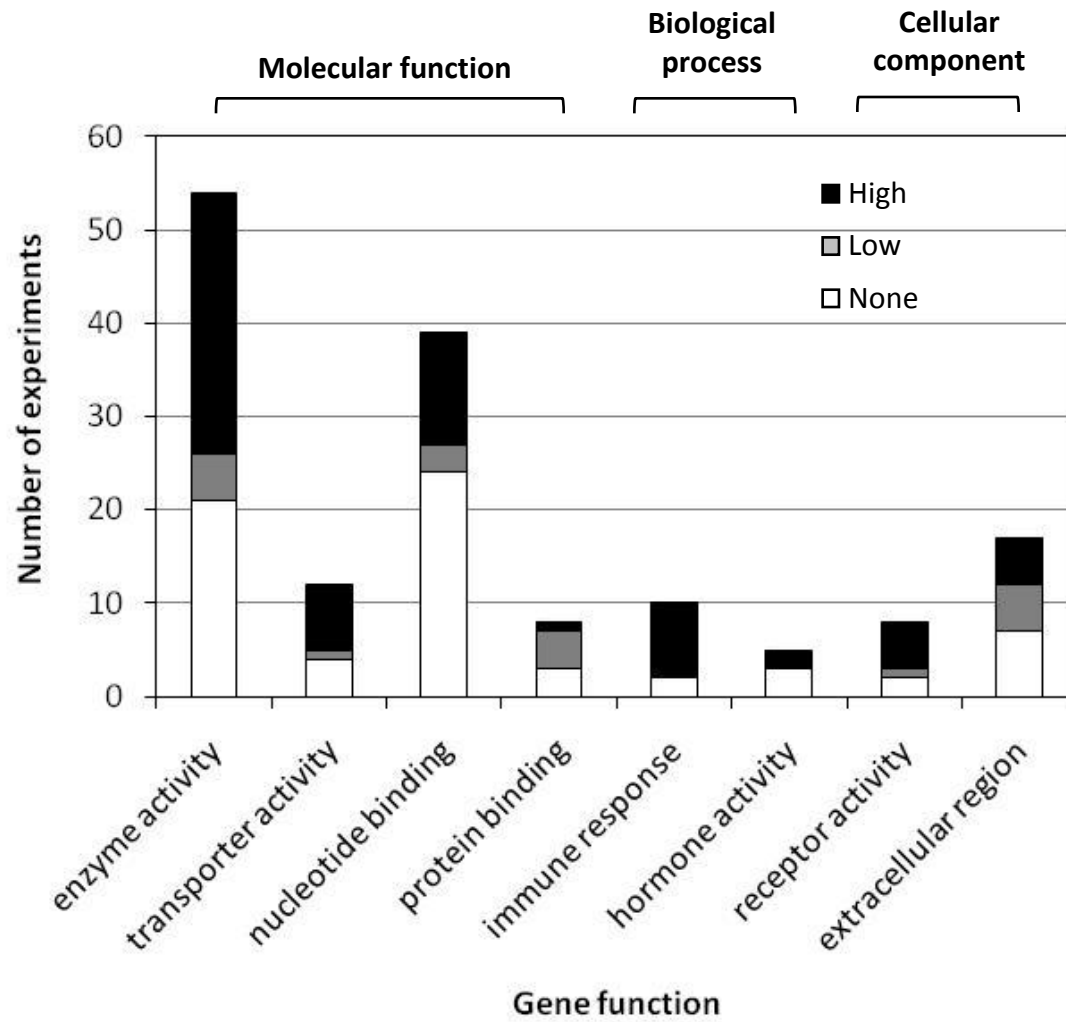


Figure 7

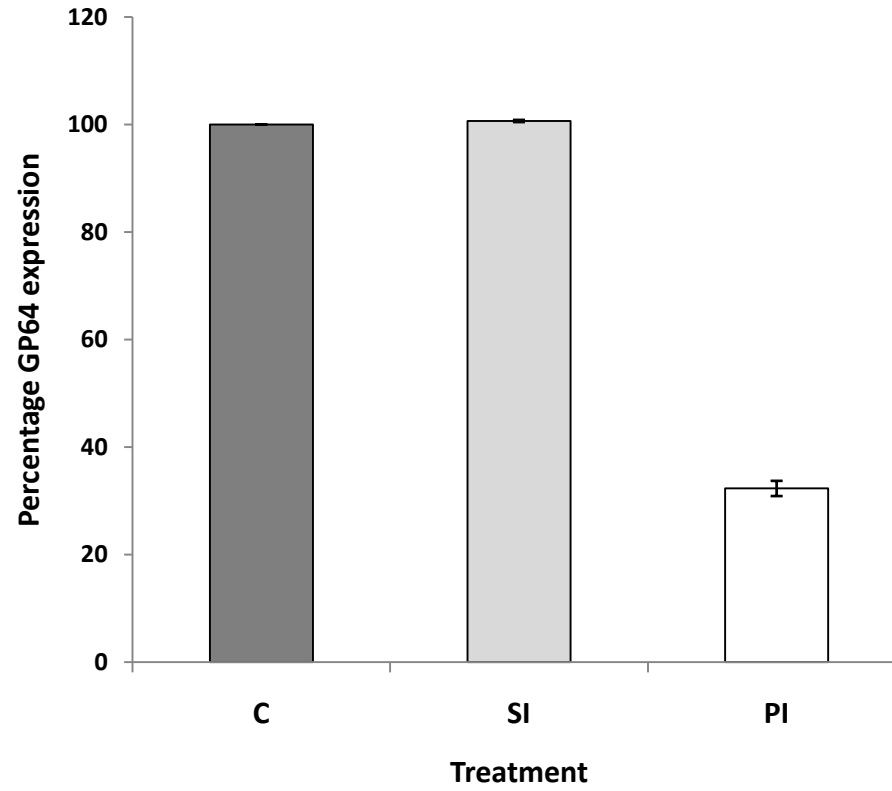
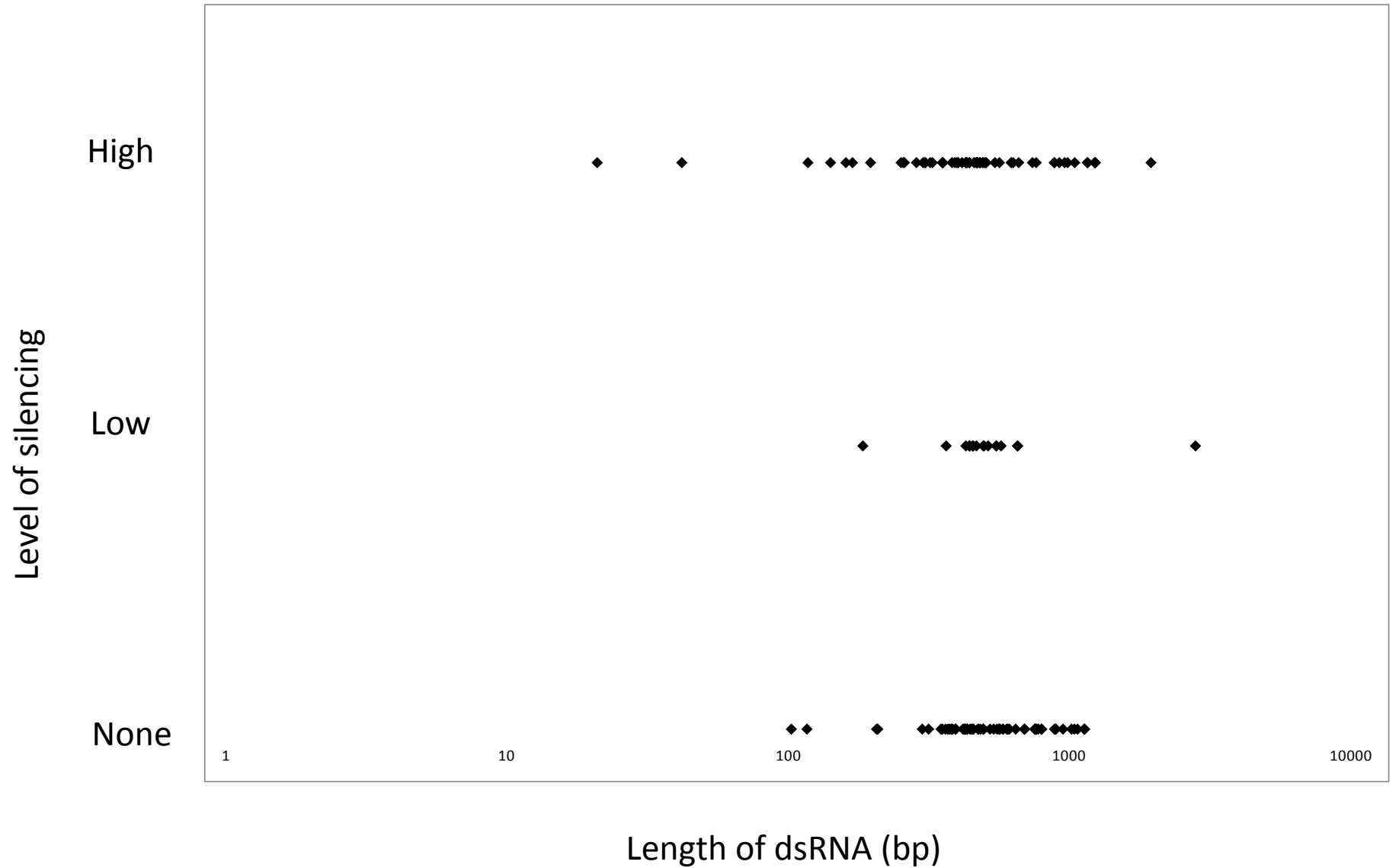


Figure S1



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