RNA interference in Lepidoptera: an overview of successful and unsuccessful studies and implications for experimental design

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Abstract

Gene silencing through RNA interference (RNAi) has revolutionized the study of gene function, particularly in non-model insects. However, in Lepidoptera (moths and butterflies) RNAi has many times proven to be difficult to achieve. Most of the negative results have been anecdotal and the positive experiments have not been collected in such a way that they are possible to analyze. In this review, we have collected detailed data from more than 150 experiments including all to date published and many unpublished experiments. Despite a large variation in the data, trends that are found are that RNAi is particularly successful in the family Saturniidae and in genes involved in immunity. On the contrary, gene expression in epidermal tissues seems to be most difficult to silence. In addition, gene silencing by feeding dsRNA requires high concentrations for success. Possible causes for the variability of success in RNAi experiments in Lepidoptera are discussed. The review also points to a need to further investigate the mechanism of RNAi in lepidopteran insects and its possible connection to the innate immune response. Our general understanding of RNAi in Lepidoptera will be further aided in the future as our public database at http://insectacentral.org/RNAi will continue to gather information on RNAi experiments.

1. Preface

RNA interference in Lepidoptera was first reported in 2001 at the 5th International Workshop on Molecular Biology and Genetics of the Lepidoptera (Bettencourt et al., 2002), generating considerable interest in the possibility of utilizing reverse genetics to investigate gene function in Lepidoptera. During subsequent years, performing RNAi in Lepidoptera has proven not as straight-forward as shown for other insects. During the 8th International Workshop on Molecular Biology and Genetics of the Lepidoptera in 2009 (http://bio.demokritos.gr/Leps/leps.htm), it was recognized that it would be worthwhile to integrate published and unpublished results and investigate this peculiar phenomenon in depth. First, a survey was set up to acquire detailed experimental information for all studies, regardless of publication status and outcome. Subsequently, exploration of this community dataset allowed us to address the question of whether RNAi for the lepidopteran clade is any different from other insects and what solutions can be recommended for the future.

2. Introduction

2.1. Short overview of RNAi silencing pathway

Most eukaryotic organisms, including insects, possess common machinery for sequence-specific post-transcriptional gene silencing that is triggered by the presence of double-stranded RNA (dsRNA), resulting in the degradation of the targeted mRNA (Fire et al., 1998). This process, RNA interference (RNAi) in animals (Hannon, 2002) and post-transcriptional gene silencing in plants (Baulcombe, 2004), is a type of highly specific defense

reaction. It depends on the specific Watson-Crick pairing formed by the small RNAs that trigger gene silencing and their target mRNAs. Different types of small RNAs have been described in insects and other multicellular organisms including short interfering RNAs (siRNAs), piwi-interacting RNAs (piRNAs), endogenous siRNAs (endo-siRNAs or esiRNAs), and microRNAs (miRNAs) (Siomi and Siomi, 2009; Brodersen and Voinnet, 2009).

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

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 (Lipard and Paterson, 2010). Indeed small RNA deep sequencing studies in *D. melanogaster* identified

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

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

2.3. Description of database used in the paper

The database was custom built after community consultation and using the MIARE (Minimum Information Criteria for RNAi Experiments) specifications. Database support was

provided by gmod_dbsf (http://www.gmod.org/gmod_dbsf; Papanicolaou and Heckel, 2010) and hosted at http://insectacentral.org/RNAi (accessed 2 November 2010). Content was built from published data found in table 1 and unpublished experiments provided by the authors. The database includes information on target and construct sequences, procedures for making the constructs, procedures used for delivery of dsRNA, how the RNAi efficacy was assayed and information on the animals used. References to the supplementary table are written as ST:XXX corresponding to the InsectaCentral ID.

3. A brief summary of successful RNAi experiments in Lepidoptera

Successful RNAi experiments have been carried out in a number of lepidopteran species to date (Table 1). The first lepidopteran RNAi publications appeared in June 2002; one reported the knockdown of a pigment gene following dsRNA injection into *B. mori* embryos (Quan et al., 2002), another targeted a pattern recognition protein, hemolin, in *Hyalophora cecropia* embryos by heritable RNAi (Bettencourt et al., 2002) and a third targeted a putative *Bacillus thuringiensis* toxin receptor in *Spodoptera litura* larvae (Rajagopal et al., 2002). In the succeeding years, RNAi experiments using lepidopterans were consistently published with publication numbers increasing from 2006 with nine different studies being published in 2009.

A large proportion of RNAi studies have taken place using *B. mori* and *Manduca sexta* (31% and 25%, respectively), although a number of other insect species are represented, including many members of the Noctuidae (6 out of the 14 lepidopteran species represented were members of this family, although these insects only represent 10 out of 37 publications). RNAi experiments have already advanced our understanding in a number of systems; in

particular developmental processes and immunity (see Table 1).

4. Variation in RNAi efficiency related to species

4.1. Different methods of delivery

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

Micro-injection of dsRNA into embryos has most often been used for *B. mori*, and in all cases successfully, although the silencing levels varied (Quan et al., 2002; Liu et al., 2008; Matsumoto et al., 2009; Pan et al., 2009; Tomita and Kikuchi, 2009; Fig. 1). High levels of silencing of the target gene were also described in two studies of transgenic *B. mori* expressing hairpin RNAs (Kanginakudru et al., 2007; Dai et al., 2008). Injections of dsRNA into embryos of other lepidopteran species were also reported, notably in *Mamestra configurata*, *Plodia interpunctella*, *Helicoverpa armigera* and *S. exigua* (Fabrick et al., 2004;

Tsuzuki et al., 2005; D. Collinge and A. Williams, unpublished results cited in Gordon et al., 2009; Herrero, ST:14). While silencing was observed in the three former species, this was not the case for *S. exigua* (Fig. 1). The cause of the latter failure is unknown but could be related to injection at a stage after cellularization since systemic RNAi was also not effective in this species (see section 4.1.1. and section 7).

4.1.1. Systemic RNAi

Systemic RNAi involves specific gene silencing following injection of dsRNA into the insect (Huvenne and Smagghe, 2010). A great variation exists among different lepidopteran species with respect to their sensitivity to systemic RNAi and high or no silencing can occur at very different concentrations of dsRNA (Table 2). In a few species, including *H. cecropia*, *Antheraea pernyi* and *M. sexta*, high levels of silencing can be achieved by application of very low amounts of dsRNA (less than 10 ng per mg tissue; Bettencourt et al., 2002; Hirai et al., 2004; Terenius et al., 2007; Kanost, ST:7,34,36,50,51,53,54,180,198). In *H. cecropia*, heritable RNAi effects on the embryos of the next generation were also reported following injection of dsRNA into pupae (Bettencourt et al., 2002), which could suggest that the injected dsRNA had entered into the gonads of the developing pupae. In these cases, success may be explained by the fact that immunity-related genes were silenced (see Section 5). Likewise, in *M. sexta* studies, concentrations of applied dsRNA were rather low (less than 10-100 ng per mg tissue) and targeted genes and tissues examined were related to the investigation of the immune response but effects varied greatly (Levin et al., 2005; Eleftherianos et al., 2006, 2009; Zhuang et al., 2007, 2008; Eleftherianos, ST:137,185-196; Kanost, ST:7,34,36,50,51,53,54,180,198). However, it should be stressed that no systematic investigation was undertaken to evaluate differences in sensitivity between species in more

detail. In most studies, a "standard" range of amounts of dsRNA is injected, which varies between 1 and 100 μg. Since the same range is routinely injected in small and large species, the calculated sensitivity to RNAi (amount of dsRNA administered per mg tissue required to achieve silencing) appears higher in large species. For instance, a high level of silencing has been achieved when a dose of 100 μg of dsRNA was injected into larvae of *Antheraea mylitta* (Gandhe et al., 2007). Such dose corresponds to a high concentration for most lepidopteran larvae but in the case of *A. mylitta* last instar larvae, that could weigh up to 5 g, it would calculate as only 20 ng/mg. Further studies are required to establish a clear dose-response relationship between the concentration of dsRNA and the amount of silencing to more accurately determine the sensitivity of RNAi. For instance, in *Laphygma exigua*, uniform levels of high silencing have been achieved with concentrations of dsRNA at 0.3-0.5 μg/mg tissue (Zhang, ST:166,171-173,177,220,222), but dose-response relationships using lower concentrations of dsRNA to establish the sensitivity to RNAi were not reported. Clearly, in this species even lower concentrations of dsRNA could be effective, which is beneficial since it is predicted to cause fewer off-target effects. It is therefore recommended that in future studies researchers use different doses and specify the concentrations of dsRNA (as opposed to amounts of dsRNA) in the descriptions of their experiments. Furthermore, by determining the dose-response relationship a concentration could be chosen at which efficiency is maintained but at minimal risk of non-specific effects.

With a range of studies integrated in this review, we can relate silencing effect with phylogeny and investigate if the biology of a particular species may have an effect (Figure 2). There are indeed species with a high resistance to application of dsRNA. Very high amounts of dsRNA (more than 1 μg per mg of tissue) did not result in any silencing effects in *Bicyclus anynana*, *Chrysodeixis includens* and *Spodoptera littoralis* (Popadic, ST:9; Saenko, ST:98;

Strand, ST:141-144; see also Iga and Smagghe, 2010; Marcus, 2005). Target tissues in these cases were epidermis (*Bicyclus* larval and pupal wings), endocrine glands (*Spodoptera* prothoracic glands) and tissues primarily involved in the immune response (*Chrysodeixis* hemocytes, fat body, gut), suggesting that resistance to RNAi is not restricted to particular tissues.

Saturniidae stands out as the only family in which multiple species (within the genera *Antheraea* and *Hyalophora*) consistently showed evidence of effective RNAi. This phylogenetic signal may be due to limited amount of studies. In the two best studied species, *B. mori* and *M.* sexta, we see a large variation in RNAi efficiency and other factors may be at play. In *B. mori*, injection of dsRNA targeting the ecdysone receptor or other target genes in the epidermis did not result in silencing effects (Swevers, ST:233; Futahashi, ST:88,89). Another study of the regulation of *B. mori* metamorphosis by ecdysone and juvenile hormone required the use of a viral vector for delivery of RNAi (Uhlirova et al., 2003). On the other hand, silencing effects were reported for genes involved in the regulation of the immune response, for genes expressed in the silk gland and the pheromone gland and, unlike in *C. elegans*, also for genes expressed in the nervous system (Tabunoki et al., 2004; Ohnishi et al., 2006, 2009; Gandhe et al., 2007; Huang et al., 2007; Hossain et al., 2008; Mrinal and Nagaraju, 2008; Ohnishi, ST:210-213,215-218). Overall, it appears that high to very high doses of dsRNA (0.1µg/mg - > 1µg/mg) have a larger chance of success than lower doses in *B. mori*. In four examples, dsRNA was injected in *B. mori* pupae for uptake in developing eggs and to investigate effects during embryonic development but all these attempts were unsuccessful (Niimi, ST:41-44). However, in another study, silencing of antibacterial genes in embryos was achieved after injection of larvae of the previous generation (Mrinal and Nagaraju, 2008).

A variant of injection into whole organisms is to culture insect organs *in vitro* in the presence of dsRNA and monitor the levels of silencing. Compared to *in vivo,* degradation and clearance of dsRNA for *in vitro* cultures is minimal and the process of RNAi is expected to be more robust. This approach was applied in *M. configurata* where successful silencing was observed for midgut tissue cultured in the presence of very high amounts (more than 1 μg per mg tissue) of dsRNA (Hegedus, ST:33,47-49). Similarly, efficient silencing was obtained when high amounts of dsRNA were directly injected in vas deferens tissue of *S. littoralis* cultured *in vitro* (Gvakharia et al., 2003). Notably, this approach was successful only by direct injection and could not be obtained by soaking of the tissue or hemocoel injection. Also pheromone glands of *Heliothis virescens* were cultured in the presence of very high amounts of dsRNA but in this case the absence of silencing could be caused by the short period of application (3 hours; Barthel, ST:158). Another alternative method that was used to achieve gene silencing in the pheromone gland comprised of direct injection of dsRNA into the pheromone glands of the adult in *B. mori* (Hull et al., 2010). Although in this method high concentrations of dsRNA can be applied locally (calculated as 0.1 μg/mg tissue; ST:231,232,234-239), gene silencing did not appear more effective than systemic injection in this species (4:4, no silencing:high silencing for direct injection; 8:12, no silencing:high silencing for hemocoel injection; see also Fig. 2).

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

group of insects. To more fully address this possibility, further studies on the mechanisms of dsRNA uptake and metabolism are needed. Overall, the genes examined are expressed in many different tissues, and although no particular tissue was observed to be refractory to injected dsRNA, some tissues may still limit dsRNA uptake (see Section 5). Another factor that could affect the potency of RNAi is the amount of mRNA present. High levels might be due to high transcription rates or to stable mRNA. Some of the variation across the studies in our database may therefore be accounted for by differences in each gene's susceptibility to RNAi (see Section 6), rather than to intrinsic mechanistic differences in the RNAi machinery in different species.

4.1.2. Environmental RNAi

Feeding of dsRNA is an even more attractive approach than hemocoel injection because it is non-invasive and furthermore opens the possibility of developing new methods of pest control through the production of species-specific hairpin RNAs against pests in transgenic plants (Price and Gatehouse, 2008). Interest for this approach received a great boost after the high profile publications of its feasibility in several pest insect species, including the lepidopteran *H. armigera* (Baum et al., 2007; Gordon and Waterhouse, 2007; Mao et al., 2007).

Feeding of dsRNA has been applied with greatest effect in *Plutella xylostella*, *S. exigua* and *M. sexta* (Bautista et al., 2009; Tian et al., 2009; Whyard et al., 2009; Yang et al., 2009; Gómez, ST:179), while low levels of silencing could also be obtained in *Epiphyas postvittana* and *Trichoplusia ni* (Turner et al., 2006; Wang, ST:241; Figure 3). A recent study in *S. exigua* even established gene silencing after mixing bacteria expressing target dsRNAs in their food (Tian et al., 2009). Effects of gene silencing in *T. ni* larvae were variable as only a small

percentage of the treated larvae showed detectable reduction in expression (Wang, ST:241). By contrast, a series of unsuccessful attempts were reported in the noctuid pest insects *H. armigera* and *Spodoptera frugiperda* (Collinge, ST:56,68-73; Nowara, ST:75-80,91-93), but in two publications efficient targeting of gene transcripts in midgut and brain tissue by feeding was reported in *S. frugiperda* (Griebler et al., 2008; Rodríguez-Cabrera et al., 2010). In the latter publication, it was discussed that efficient silencing by dsRNA by feeding was greatly enhanced when larvae were used immediately after the molt and in addition were subjected to starvation for 24 hrs. In such starved larvae, dsRNA-degrading activity in the midgut was greatly decreased and could be an important factor for the increased sensitivity to dsRNA (Rodríguez-Cabrera et al., 2010). As mentioned above, in one study of *H. armigera,* effective silencing of an inducible gene involved in plant toxin detoxification was achieved (Mao et al., 2007).

Feeding of dsRNA seems to be successful only at high amounts: in general, there seems to be a correlation between amount of dsRNA and degree of silencing (Table 2). Interestingly, while feeding of long dsRNA to *H. armigera* larvae was generally not very successful (Collinge ST:56,68-73), high levels of silencing could be achieved using a custom-designed siRNA nucleotide (Kumar et al., 2009), suggesting different efficiencies between short siRNAs and long dsRNAs in this species.

5. Variation in RNAi efficiency related to tissue

Most studies in this review involved investigations of the immune response and defense against exogenous substances and its main effector organs hemocytes, fat body and midgut (Bettencourt et al., 2002; Hirai et al., 2004; Eleftherianos et al., 2006, 2009; Gandhe et al.,

2007; Mao et al., 2007; Soberón et al., 2007; Terenius et al., 2007; Zhuang et al., 2007; Mrinal and Nagaraju, 2008; Whyard et al., 2009; Yang et al., 2009; Duvic, ST:25,37; Eleftherianos, ST:137,185-192; Garbutt, ST:135; Herrero, ST:4; Kanost, ST:7,34-36,49-51, 53, 54,180, 181, 185-194, 198; Strand, ST:141-144). In these cases, silencing effects among genes could differ considerably (Fig. 4). In *M. sexta*, it was striking that efficient silencing of immune genes could be achieved after injection of low to intermediate doses of dsRNA in the hemocoel (Eleftherianos et al., 2006, 2009; Eleftherianos, ST:137,185-186). It has previously been reported that expression of *Hemolin* (a pattern recognition molecule exclusive to Lepidoptera) in the silkmoth *A. pernyi* is up-regulated in response to dsRNA as such, and that silencing of *Hemolin* affects the progress of virus infection (Hirai et al., 2004). These results allow us to speculate that sensitivity to RNAi can be coupled by other immunogenic factors. Interestingly, a recent study has highlighted the critical role of immune cells in the tumor microenvironment and showed how RNAi can be used to restore an efficient antitumor immune response in mice (Kortylewski et al., 2009). This finding emphasizes the possibility that combined activation of RNAi and innate immunity can have synergistic effects.

In contrast, epidermal tissue (larval epidermis and pupal wing) seems to be rather refractory to RNAi as only low silencing was obtained and that in only one study (Futahashi, ST:83) whereas in nine other studies no silencing was obtained (Futahashi, ST:87-89; Oostra, ST:46; Popadic, ST:9; Saenko, ST:97-99; Swevers, ST:233; Fig. 4). However, a recent series of experiments showed successful disruption of the regulatory pathway controlling chitin synthesis during molting in *S. exigua* (with epidermis as likely target tissue although catalogued under "whole organism" in Fig. 4; Chen et al., 2008; Zhang, ST:222). Rather sensitive to RNAi is brain tissue, which is unexpected because of the documented refractoriness of brain tissue to RNAi in nematodes that are prone to RNAi (Kennedy et al.,

2004). In *C. elegans* this refractoriness is caused by the expression of the *eri-1* nuclease in brain tissue and differential expression of nucleases should also be considered as a contributing factor to the resistance of RNAi in different lepidopteran species.

Further, in *H. cecropia* and *B. mori*, injection of dsRNA in the pupa can result in their uptake by the developing oocytes in the ovary and the observation of phenotypic effects in developing embryos (Bettencourt et al., 2002; Mrinal and Nagaraju, 2008). However, in four other studies in *B. mori*, heritable RNAi was not successful (Niimi, ST:41-44; Fig. 4).

When studying RNAi in tissues, *in vitro* cultures of dissected tissues can be of great use as this technique allows the effectiveness of dsRNA constructs to be rapidly examined outside of the complexity of a total organism. As already mentioned above, this has been performed in *M. configurata* where several structural genes that contribute proteins to the midgut peritrophic membrane (insect intestinal mucins, non-mucin structural proteins and chitin deacetylase) were tested *in vitro* (Hegedus, ST:33,47-49). Comparison of *in vitro* working situations to non-working *in vivo* situations may reveal compounds or enzymes in the intact organism that destroy the dsRNA *in vivo*, for example, a dsRNA-degrading enzyme in the hemolymph. The *M. configurata* midgut expresses a dsRNAase gene; however, recent studies demonstrated that feeding of chitin deacetylase (*McCDA1)* dsRNA to neonate or fourth instar larvae totally eliminated the cognate transcript within 24-48 hours, respectively (D. Hegedus, unpublished results).

To address the issue of uptake of dsRNA by insect cells, two cell lines from lepidopteran pests, Hi5 (derived from *T. ni*) and Sl2 (derived from *S. littoralis*) were incubated with high concentrations (ca. 100 μg/ml) of dsRNA labeled with fluorescein (Ambion Silencer SiRNA Labeling Kit). Although the specific fluorescence of the dsRNAs was not very high (1 molecule of fluorescein per 100-150 nucleotides), significant, and

sometimes intense, internalization of FAM-dsRNA in the cells was observed (Fig. 5), indicating that uptake of dsRNA by lepidopteran cells is not a limiting factor. However, efficient uptake of dsRNA is not synonymous with efficient silencing as similar levels of dsRNA uptake were observed in *Bombyx*-derived Bm5 cells which are known to be defective in homologous gene silencing when dsRNA is just added in the culture medium of the cells (Hannan et al., 2009; L. Swevers, H. Huvenne, G. Smagghe, unpublished results). Thus, physiological processes downstream of dsRNA uptake are necessary to conduct efficient RNAi in particular lepidopteran cell lines, and, presumably, in particular tissues of lepidopteran species as well.

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

In the case of feeding, the midgut obviously is the primary target organ, representing environmental RNAi. It would also be very interesting if the silencing signal could spread from the midgut to other tissues in the insect, causing systemic RNAi. While generally cases of efficient silencing of genes in midgut tissue were predominant (inset in Fig. 4: Turner et al., 2006; Sivakumar et al., 2007; Whyard et al., 2009; Gómez, ST:179), silencing signals spread to the remaining tissues of the insect only in a limited number of cases (Meyering-Vos 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/cm^2), with 67 of these showing significant mortality and/or reduced growth rate at the lower dose (5.2 ng/cm^2). 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

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

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

7. Similar experiments with different outcomes

There are several cases in which similar RNAi experiments produced conflicting results. In *H. armigera* two groups carried out near identical RNAi experiments where they injected dsRNA into the hemocoel of the larva and assayed for the knockdown of their target genes in the gut using qPCR. One group (targeting the aminopeptidase-N gene product; Sivakumar, ST:127) found a high degree of silencing in midgut tissue, whilst the other group (targeting the cadherin gene product; Wee, ST:162) reported no silencing of their gene. Both of these proteins are found on the outside of midgut epithelial cells, as glycoprotein components of the cell membrane. Aminopeptidase-N proteins are involved in dietary protein digestion (Terra and Ferreira, 1994; Angelucci et al., 2008), whereas proteins in the classical cadherin family are involved in calcium-dependent cell-cell adhesion. However, the lepidopteran cadherin-like

proteins are present primarily in midgut columnar cell apical membranes (Wang et al., 2005; Aimanova et al., 2006); while their function is unclear, they are of importance as Bt toxin binding proteins, as are the aminopeptidases. It is unclear why the aminopeptidase-N gene is more susceptible to silencing than the cadherin gene, particularly in the absence of comparative data on gene expression and mRNA turnover. However, attempts to knockdown aminopeptidase-N genes in other lepidopterans, including *Ostrinia nubilalis*, *S. exigua* and *E. postvittana*, have not been successful (Crava, ST:81; Herrero, ST:4,14; Gatehouse, ST:67), suggesting that there is no general susceptibility of aminopeptidase genes to RNAi.

There are also a number of cases in which successful experiments have been reported in one life stage of insect and not others. For example, one group achieved a high degree of knockdown of the *S. littoralis* period gene by injecting dsRNA into the adult hemocoel (Bebas, ST:18). Another group carried out a similar experiment in *S. littoralis* larvae and was unsuccessful in achieving a knockdown of the Halloween gene (Iga, ST:57-61). This result may reflect differences in the types of genes targeted (as discussed above) but it may also be caused by underlying and unexplained differences in the susceptibility of different life stages of the same insect species to RNAi. The same pattern was observed in the closely related species *S. frugiperda*, in which Meyering-Vos (ST:38,101,102) achieved a high degree of knockdown of allostatin and allotropin genes in adult insects whereas Lundmark (ST:13) was unsuccessful in an attempt to knock down five different genes in larvae. The fact that many different genes were targeted in these studies makes it more likely that the insect is refractory to RNAi in its larval stage, rather than the result being due to any differences in the target genes and their transcripts.

8. Features of the dsRNA

From the wealth of data submitted to the database, several technical aspects on the dsRNA production and time from injection to detection have been possible to analyze. The conclusion is that there seem to be no correlation between success rate and time from dsRNA injection to time of detection of silencing, methods for annealing and purification, or kits used. However, whether dsRNA is added before or at the same time as a gene is turned on can have large impact on the outcome. As illustrated in Fig. 7, simultaneous transfection of dsRNA and virus into Sf9 cells does not inhibit virus proliferation, while prior incubation with dsRNA does. Also, preparations of dsRNA with cationic lipid reagents to stimulate uptake generally does not result in increase in functional uptake when injected *in vivo* (Niimi, ST:41-43; Ohnishi, ST:210-218). Most of the studies have used dsRNA for silencing and only a few hairpin or siRNA. Of the latter, both methods have resulted in high silencing. Another feature of dsRNA that was discussed early in the use of RNAi was the impact of the length of the dsRNA and it has been determined that in *Drosophila* S2-cells the minimum length of uptake of dsRNA was 211 bp (Saleh et al., 2006). Likewise, when silencing P450 in *H. armigera* with transgenic plants producing dsRNA, experiments with *Arabidopsis* Dicer mutants showed long, unprocessed dsRNA fragments to be more effective than the siRNA products of Dicer activity (Mao et al., 2007). However, in our dataset the success rate is independent of length of dsRNA (Fig. S1).

9. Conclusion

The results deposited in the database illustrate the high variability of success of RNAi experiments in lepidopteran insects. Only a few solid predictions have emerged and the

questions remain what the cause is for the high variability at the molecular and cellular level and if techniques can be adapted to increase the efficiency of RNAi.

On a theoretical basis, differences in efficacy of systemic or environmental RNAi can be caused by several different mechanisms. One obvious cause is that the Lepidoptera, like other insects, lack clear functional homologs of the two types of genes required for systemic RNAi in *C. elegans*, i.e. the canonical RdRP and the RNA-transporter sid-1. That other genes in insects (and other animals) appear to be able to compensate to a degree for these absent genes is one of the surprising findings of recent work on RNAi; however the extent of their ability to do this is unclear and, based on many experimental observations such as those in this review, appears quite limited. However the presence of an intracellular RdRP-based anti-viral RNA-immunity would help explain the presence of genes that encode the core RNAi machinery, such as Dicer-2, dsRNA-binding proteins and Argonautes, in these species. Another possible factor is that competition can also occur between the siRNA- and miRNA-programmed effector complexes (Tomari et al., 2007), providing an alternative if less compelling explanation for the retention of these genes in the absence of the RdRP. Thus, studies that look for expression of elements of the core RNAi machinery could provide an explanation for some of the differences observed. Second, a barrier can exist at the level of uptake of dsRNA. While dsRNA may be taken up efficiently, as shown in figure 5, it may not be sorted correctly during endosome trafficking and fail to reach the appropriate dsRNA-processing machinery. Recently, it was found that RISC function is linked to the endocytic pathway (Siomi and Siomi, 2009; Lee et al., 2009) and it is possible that there is a direct functional connection between uptake of dsRNA by endosomes and processing by RISC at organelles dedicated to mRNA degradation such as GW-bodies (Schneider et al., 2006). Such a barrier could be revealed by co-localization studies of fluorescent dsRNA and antibody

staining of RISC components by fluorescence microscopy. Third, dsRNA-degrading nucleases can be present in particular tissues, hemolymph or gut lumen to inactivate administered dsRNA (Arimatsu et al., 2007). However, the fate of injected or ingested dsRNA remains uncharted territory up to present.

The data in this review suggest that the genes most likely to be susceptible to dsRNA-induced RNAi are those active in immunity, supporting similar findings published previously (e.g. Hirai et al., 2004; Eleftherianos et al., 2006, 2009). Moreover, a study in *D. melanogaster* showed that only lymphocytes were able to take up injected dsRNA, resulting in silencing of a transgene expressed only in those cells (Miller et al 2009). While no comparable specific study has been undertaken in Lepidoptera, the silencing of immune-response genes known to be expressed in hemocytes is consistent with the *D. melanogaster* work. Furthermore, in *D. melanogaster*, dsRNA uptake by hemocytes has been shown to be required for RNAi-based immunity to RNA viruses (Saleh et al., 2009). While there have been studies in Lepidoptera of hemocyte-expressed genes that are induced by virus infection (Shelby and Popham, 2009) these have not yet extended to the RNA viruses now being increasingly analyzed in *D. melanogaster* (Ding and Voinnet, 2007). Nonetheless, the observations now accumulating of immune genes being vulnerable to dsRNA-induced RNAi suggests that an endocytic pathway enabling dsRNA-induction of RNAi immunity may exist in Lepidoptera as already shown in *D. melanogaster*.

It is noted that high amounts of dsRNA are used in many studies, raising the question of the specificity of effects. For comparison, successful studies in mammals without significant side-effects are accomplished by doses as low as 50 ng per mg tissue (Li et al., 2010). Although the incorporation of modifications in the applied RNA oligonucleotides may be an important factor to explain differences between studies employing mammals and insects, it is

clear that the issue of specificity remains to be addressed satisfactorily in insects, especially in cases where high amounts of dsRNA are used to achieve silencing. As already mentioned above, non-specific activation of the innate immune response could account (partially) for some of the effects observed. An early study in *A. pernyi* showed that injection of dsGFP induced the expression of *Hemolin* dose-dependently, but that the anti-bacterial response was left unaffected (Hirai et al., 2004). To corroborate these data, further investigations of how administration of dsRNA affects the immune response are necessary. In the same vein, conclusive proof of the involvement of the RNAi pathway in the observed effects, for example through detection of specific siRNAs derived from the injected or ingested dsRNA, has not been reported in any of the studies. For instance, sequencing of *B. mori* small RNA libraries have revealed the existence of piRNAs and miRNAs but not siRNAs (Jagadeeswaran et al., 2008; Kawaoka et al., 2008; Zhang et al., 2009). It would be considered a major breakthrough if such results can be presented.

10. Future directions

RNAi is thought to have developed as a protection against virus infections. In the co-evolutionary race between host and microbe, viruses have developed ways to avoid the silencing by suppressing RNAi (Li and Ding, 2006; Ding and Voinnet, 2007). The presence of viral infections in lepidopterans could cause difficulties in silencing. Only a limited number of lepidopteran RNA viruses have been identified to date (Gordon and Waterhouse 2006; Zeddam et al., 2010), including some that appear to cause unapparent infections and may be subject to anti-viral immunity as found for *D. melanogaster*. Similar studies to those of Wu et al. (2010) performed in Diptera would in Lepidoptera likely be very rewarding and may make

this a more productive approach than specifically searching for the few if any viruses known *a priori* from any particular species. There are also no reports of virus-infected colonies of moths or butterflies in the database; however, it is possible that this is due to the lack of investigation rather than of infection. Therefore, it would be advisable to screen for viruses known for RNAi inhibition such as the Flock House virus in *Galleria mellonella*.

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

The issue of validity of RNAi has been discussed extensively in *D. melanogaster* research which has resulted in recommendations to ensure that results obtained in RNAi experiments are valid. In systematic screens that involve transgenic *D. melanogaster*, the false positive rate is estimated to be 5-7% and analysis indicated that sequence identities stretching

over 12 bp could already generate off-target effects (Mummery-Widmer et al., 2009; Schnorrer et al., 2010). Ideally, association of an RNAi phenotype with a previously observed phenotype should be confirmed by an independent method. While the generation of classical mutants or the generation of transgenic insects to rescue the RNAi phenotype with RNAi-resistant transgenes is far beyond the boundary of possibilities in most insects, the recommendation to use a second dsRNA that targets a different region of the same gene can be easily applied (Langer et al., 2010). To control for specificity of dsRNA effects, quantitative PCR experiments can also be carried out to check expression of household genes such as actin or tubulin and verify that administration of dsRNA does not affect general cell physiology.

A major theme in the history of RNAi in Lepidoptera is the inconsistency of the silencing. This paper has highlighted areas that could explain some of the difficulties encountered, however much is still unexplained and therefore, a way to certify that the method is working is warranted. We propose that a gold standard is set up with a gene that seems to be possible to silence in several species and with a protocol that has been developed and used in several laboratories. With such a tool in hand, it would be possible to distinguish between failures due to technical issues and those due to biology. In the longer term, it may be possible to envisage lepidopteran models (e.g., *B. mori*) expressing the key *C .elegans* genes for systemic RNAi and therefore able to allow genome-wide RNAi screens such as those carried out in other model systems such as *C. elegans* (e.g., Kamath and Ahringer, 2003) and 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	*Bombyx mori*	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 *E. coli* from hemolymph[11]), sex pheromone synthesis[12,13,14,15]
Crambidae	*Diatraea saccharalis*	Bt toxin receptor (aminopeptidase-N)[16]
Crambidae	*Ostrinia nubilalis*	Role of chitinase genes in regulating chitin content of midgut peritrophic matrix[17]
Noctuidae	*Spodoptera exigua*	Ecdysis[18], chitin synthesis pathway[18,19,20], trehalose synthesis pathway[20,21], role of storage hexamerins in development[22]
Noctuidae	*Spodoptera frugiperda*	Juvenile hormone titer (effect of allatotropins and allatostatins)[23,24], defense against Bt Cry toxin[25]
Noctuidae	*Spodoptera littoralis*	Circadian rhythm of sperm release[26,27]
Noctuidae	*Spodoptera litura*	Bt toxin receptor (aminopeptidase-N)[28]
Noctuidae	*Mamestra brassicae*	Embryonic development (formation of bilateral procephalic lobes)[29]

Noctuidae	*Helicoverpa armigera*	Acetylcholinesterase (role in regulation of differentiation and development)[30], Bt toxin receptor (aminopeptidase-N)[31]
Plutellidae	*Plutella xylostella*	Insecticide resistance (role of cytochrome P450 in resistance to permethrin)[32], role of cadherin in larval growth and development)[33]
Pyralidae	*Plodia interpunctella*	Embryonic development (eye-color pigmentation)[34]
Saturniidae	*Antheraea assama*	Sex determination[35]
Saturniidae	*Antheraea mylitta*	Immune system (nodule formation)[36], sex determination[35]
Saturniidae	*Antheraea pernyi*	Immune system[37]
Saturniidae	*Hyalophora cecropia*	Embryonic development[38], immune system[39] (roles of pattern recognition protein hemolin)
Sphingidae	*Manduca sexta*	Immune system (hemocyte adhesion and encapsulation[40,41,42,43], phagocytosis and melanotic nodule formation & clearance of *E. coli* from the hemolymph[44], protective effect of prior *E. coli* infection on *Photorhabdus luminescens* infection[45], role of plasmatocyte-spreading peptide (PSP) during *E. coli* and *P. luminescens* infection[46], effect of a *P. luminescens* antibiotic on phenoloxidase[47], role of pattern recognition proteins in resistance to

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

Numbers refer to the publication list found in supplementary data

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

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

Figure Legends

Fig. 1. RNAi efficiency following dsRNA injections in embryos of different lepidopteran species. Experiments that have achieved high silencing are indicated in black, those that achieved low silencing in grey and unsuccessful experiments in white. Degree of silencing in individual experiments is derived from supplementary table 1 and is a subjective measure of silencing as provided by the database submitter.

Fig. 2. RNAi silencing efficiency following hemocoel injection of dsRNA (intact or digested) in different lepidopteran species. Color codes and data source as in Fig. 1.

Fig. 3. RNAi silencing efficiency after feeding dsRNA in different lepidopteran species. Color codes and data source as in Fig. 1. Experiments in which successful silencing was achieved by feeding synthetic siRNA in *Helicoverpa armigera* (Kumar et al., 2009, section 4.1.2) are not included in this figure.

Fig. 4. RNAi silencing efficiency in different tissues of lepidopteran species after hemocoel injection (main figure) or feeding (insert). Color codes and data source as in Fig. 1. Experiments in which successful silencing was achieved by feeding synthetic siRNA in *Helicoverpa armigera* (Kumar et al., 2009, section 4.1.2) are not included in this figure.

Fig. 5. Fluorescence microscope images of *Trichoplusia ni*-derived Hi5 or *Spodoptera littoralis*-derived Sl2 cells after soaking in high concentrations (~100 μg/ml) of fluorescein (FAM)-labeled dsRNA for 24 h. Control cells were left untreated. Uptake of FAM-labeled

dsRNA by individual cells showed considerable variation that resulted in the observation of intense signals in some cells and the absence of fluorescence in other cells. In general, higher fluorescence signals were observed in Hi5 cells than in Sl2 cells. Shown are selected treated cells in which strong internalization of FAM-labeled dsRNA is detected. The experiment suggests that lepidopteran cells are able to take up dsRNA molecules.

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

Fig. 7. RNAi efficiency depending on time for addition of dsRNA. The level of the viral coat protein GP64 indicates that simultaneous transfection of dsRNA for the baculovirus gene ie-1 (dsie-1) with viral infection (SI) did not trigger viral suppression while there was 3-fold decrease when the cells were pre-incubated with dsie-1 (PI), suggesting the necessity for prior activation of the host RNAi machinery. One µg of dsie-1 was transfected to half a million of Sf9 cells either 24 h prior to the *Ac*NPV viral challenge (PI) or along with the virus (SI). A viral dose of 5 Multiplicity of infection (MOI) was used.

C indicates mock infection and was set to 100%.

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

Supplementary Fig. 1. Impact of dsRNA length on RNAi silencing efficiency in Lepidoptera. There is no obvious correlation between the length of dsRNA used in the experiments and successful gene knockdown.

Figure(s)

Figure 1

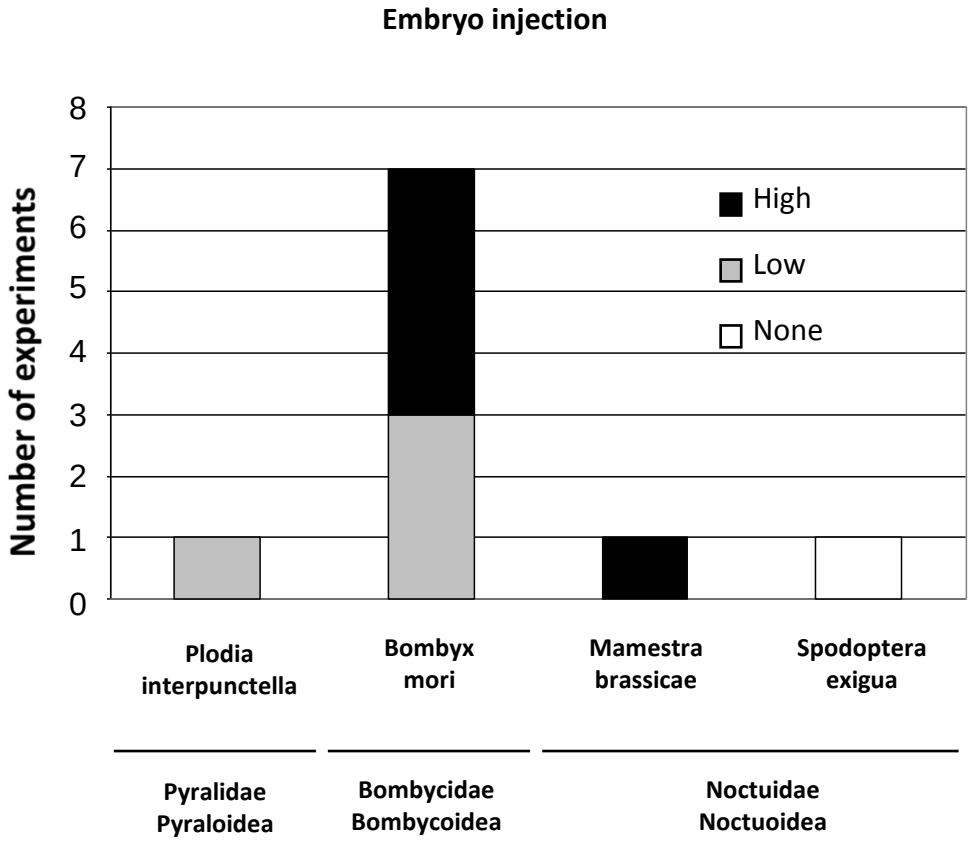


Figure 2

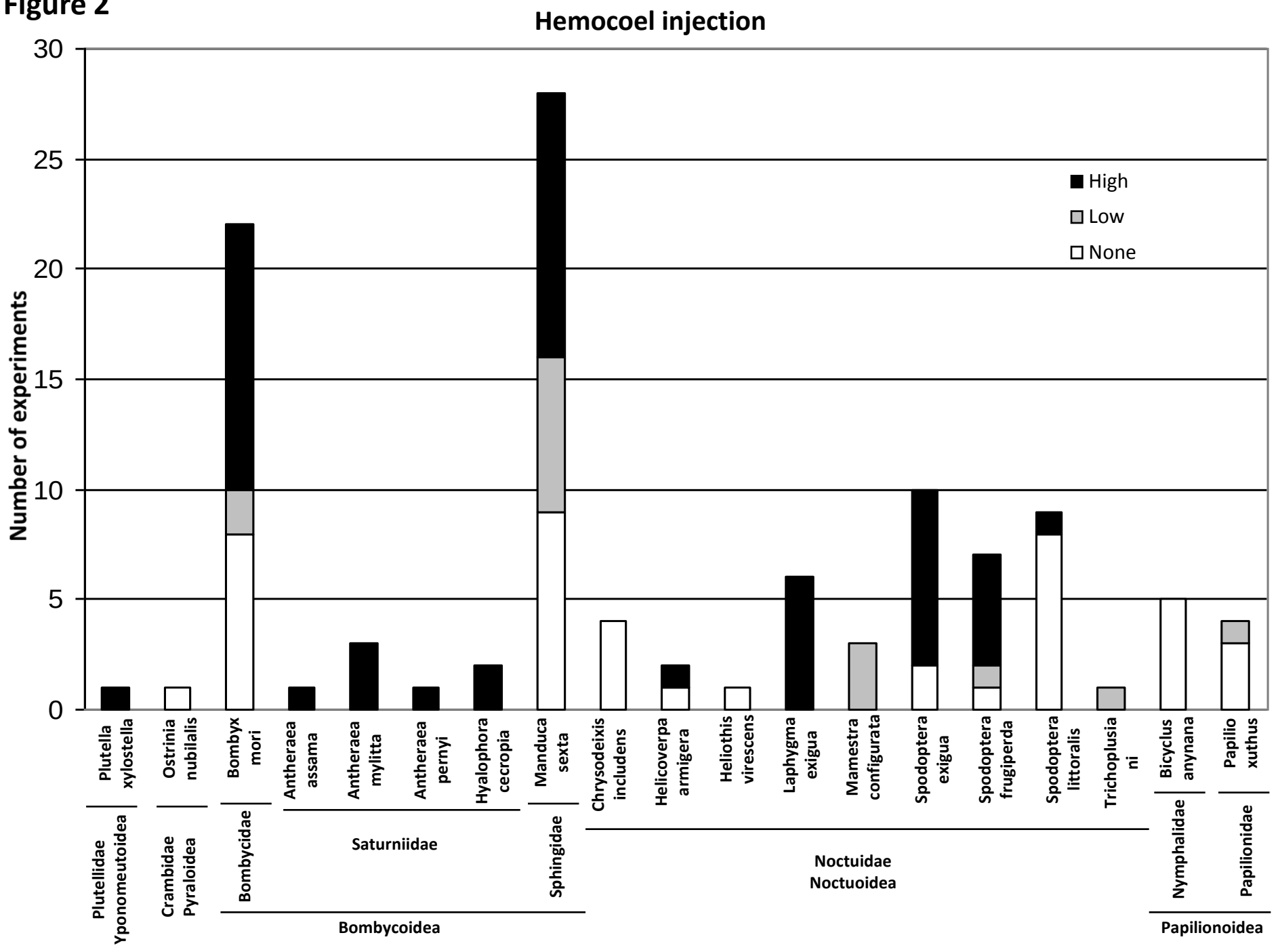

Hemocoel injection
Number of experiments
30
25
20
15
10
5
0
High
Low
None
Plutella xylostella
Ostrinia nubilalis
Bombyx mori
Antheraea assama
Antheraea mylitta
Antheraea pernyi
Hyalophora cecropia
Manduca sexta
Chrysodeixis includens
Helicoverpa armigera
Heliothis virescens
Laphygma exigua
Mamestra configurata
Spodoptera exigua
Spodoptera frugiperda
Spodoptera littoralis
Trichoplusia ni
Bicyclus anynana
Papilio xuthus
Plutellidae
Yponomeutoidea
Crambidae
Pyraloidea
Bombycidae
Saturniidae
Sphingidae
Noctuidae
Noctuoidea
Nymphalidae
Papilionidae
Bombycoidea
Papilionoidea

Figure 3

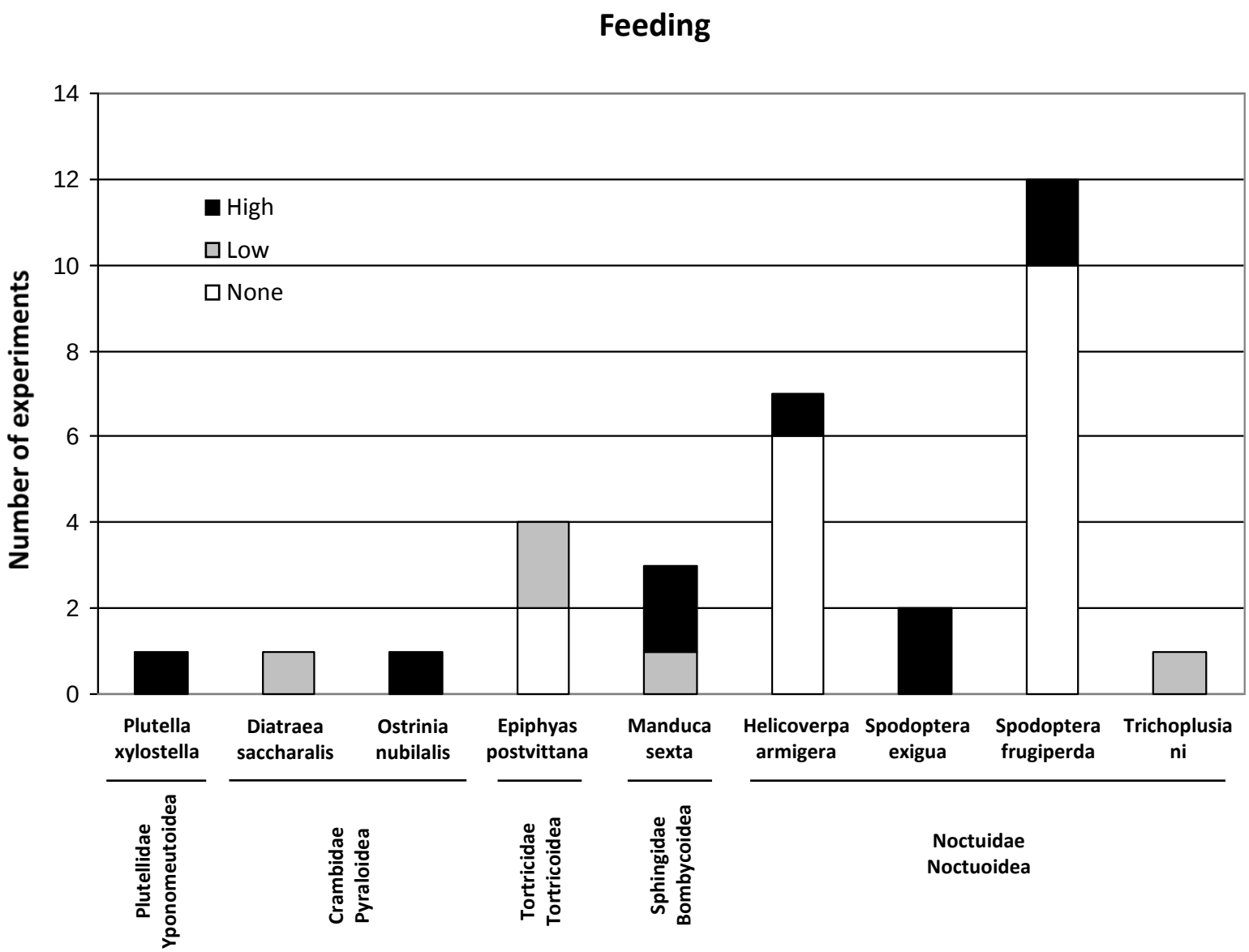
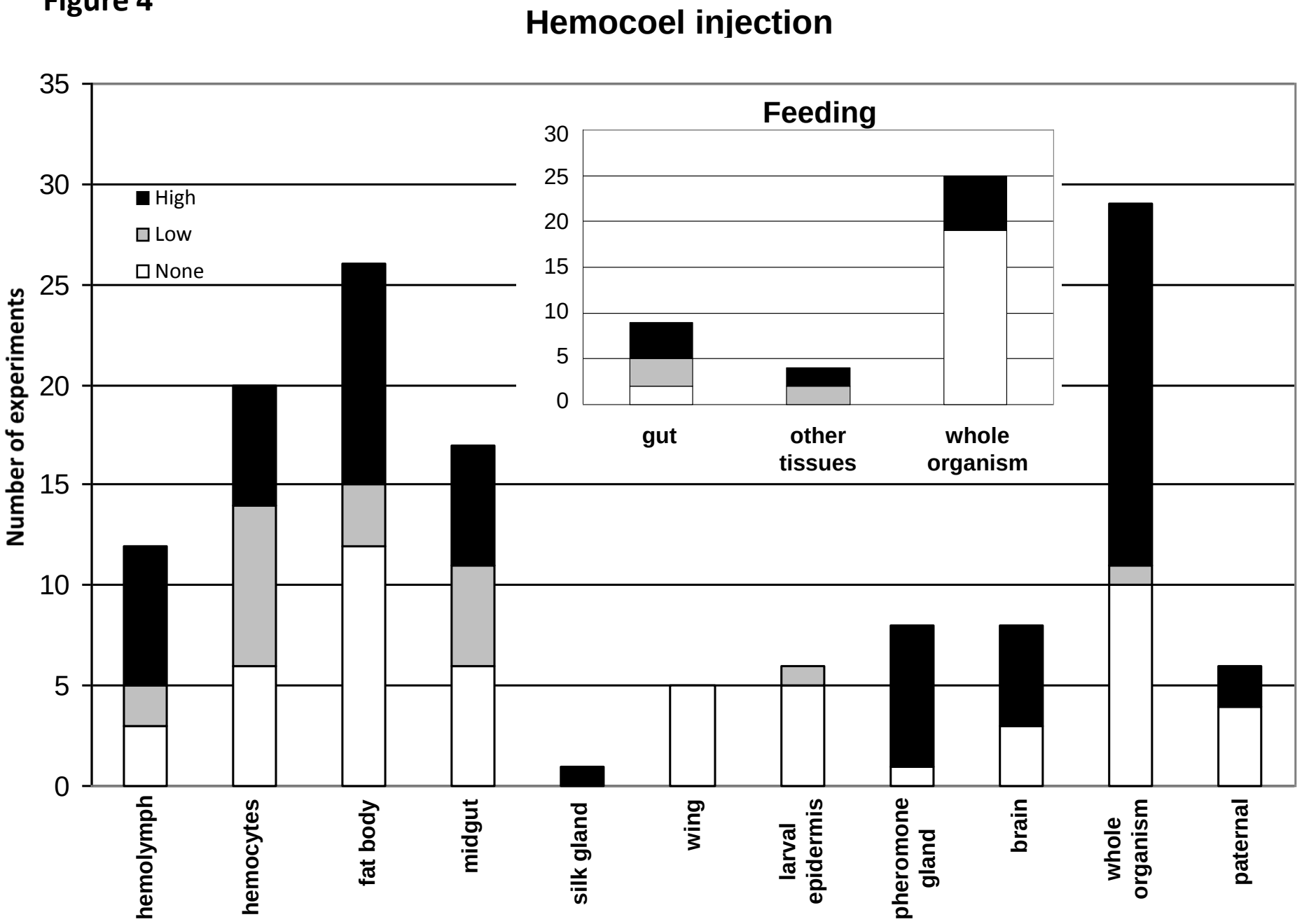
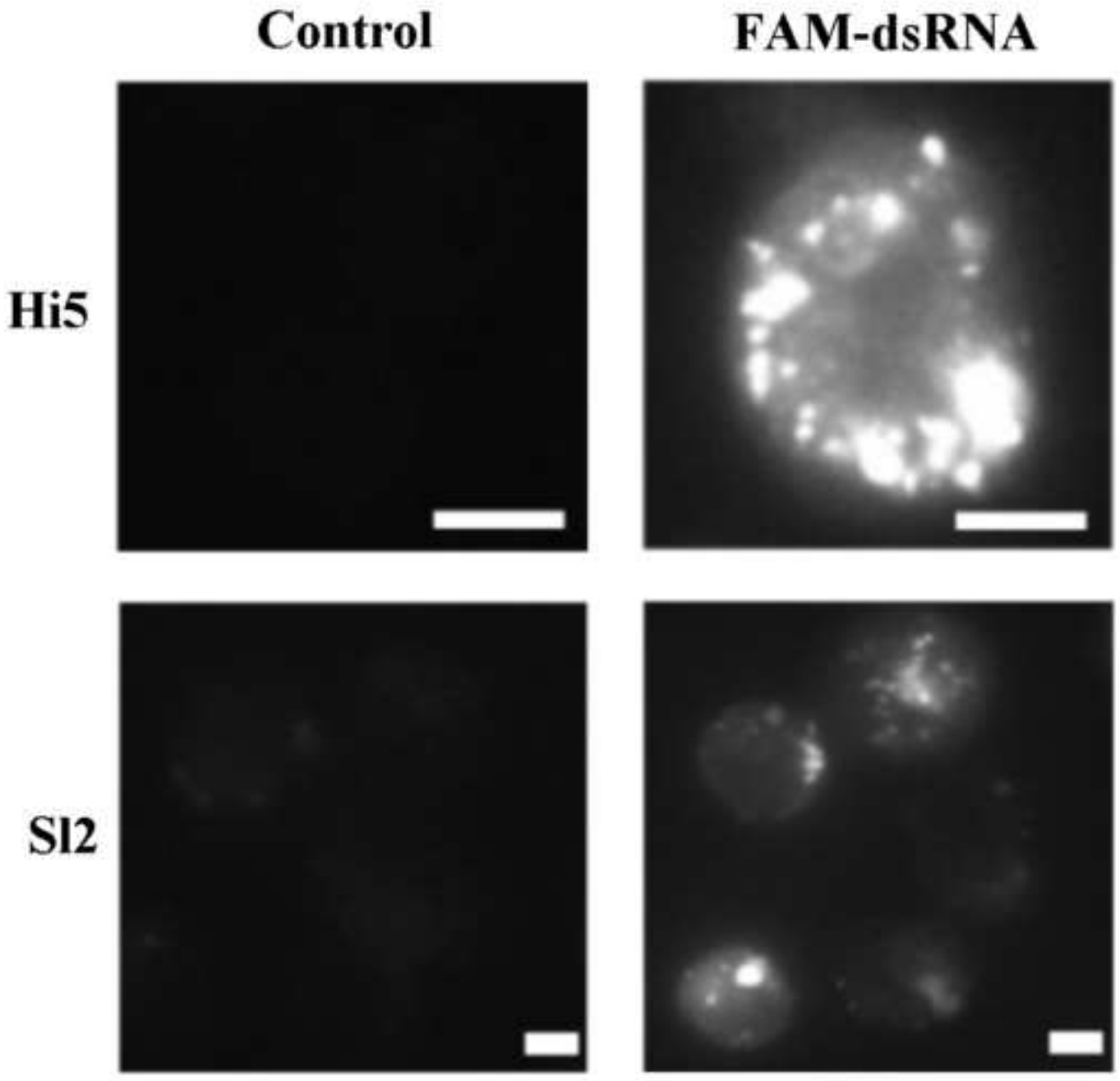

Feeding
Number of experiments
14
12
10
8
6
4
2
0
High
Low
None
Plutella xylostella
Diatraea saccharalis
Ostrinia nubilalis
Epiphyas postvittana
Manduca sexta
Helicoverpa armigera
Spodoptera exigua
Spodoptera frugiperda
Trichoplusia ni
Plutellidae Yponomeutoidea
Crambidae Pyraloidea
Tortricidae Tortricoidea
Sphingidae Bombycoidea
Noctuidae Noctuoidea

Figure 4


Hemocoel injection
Number of experiments
35
30
25
20
15
10
5
0
High
Low
None
hemolymph
hemocytes
fat body
midgut
silk gland
wing
larval epidermis
pheromone gland
brain
whole organism
paternal
Feeding
30
25
20
15
10
5
0
gut
other tissues
whole organism

Figure(s)
Click here to download high resolution image



Figure(s)

Figure 6

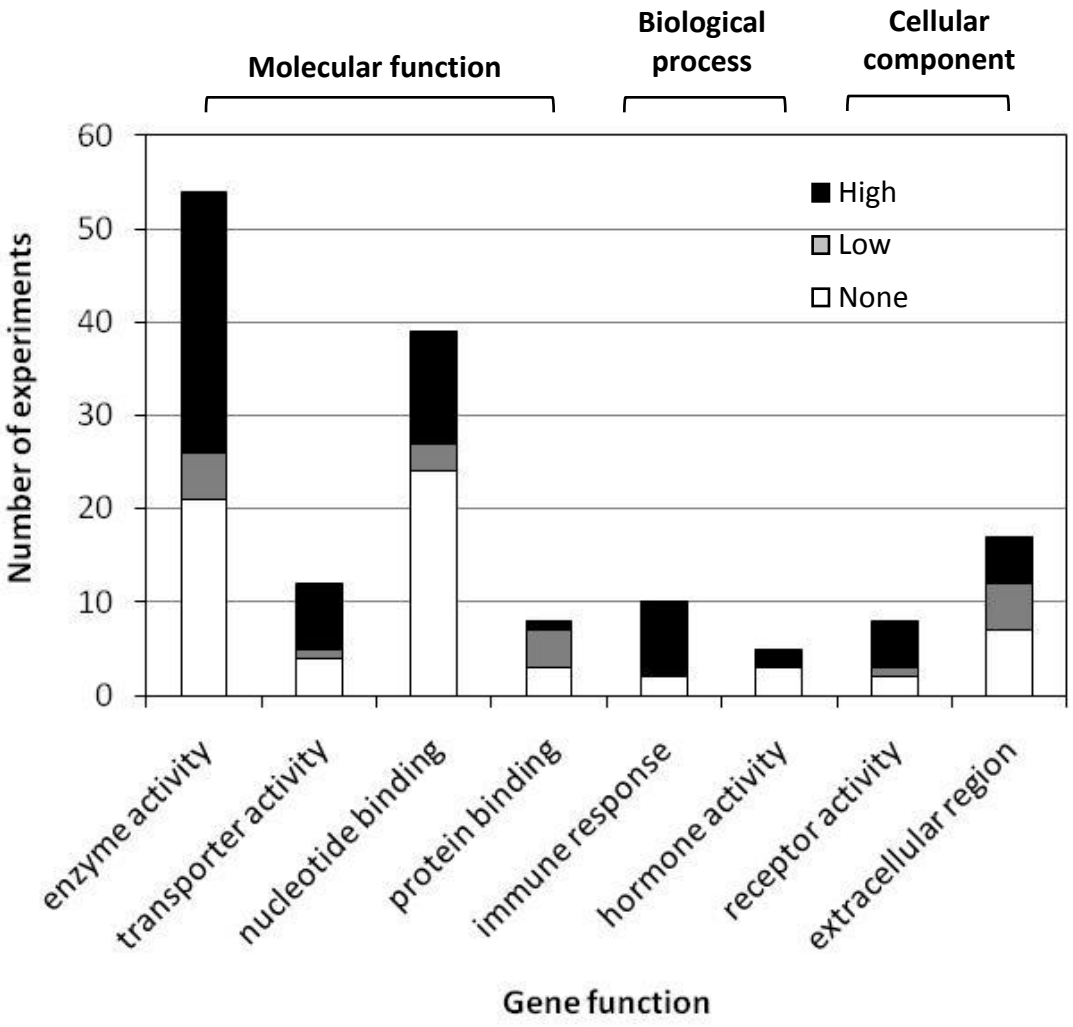


Figure 7

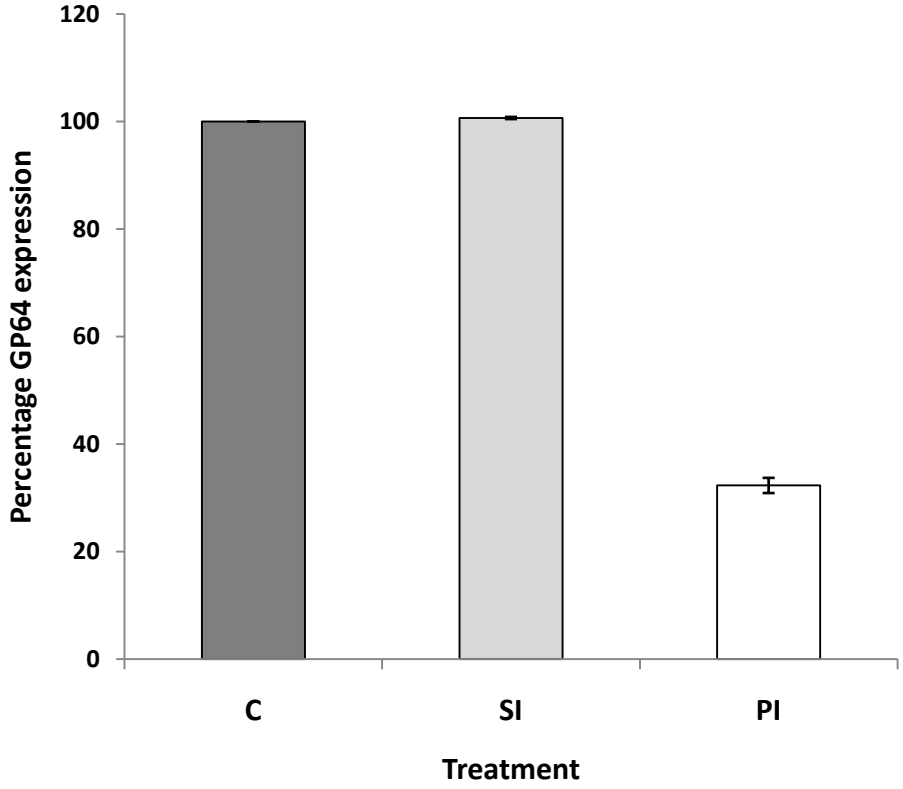
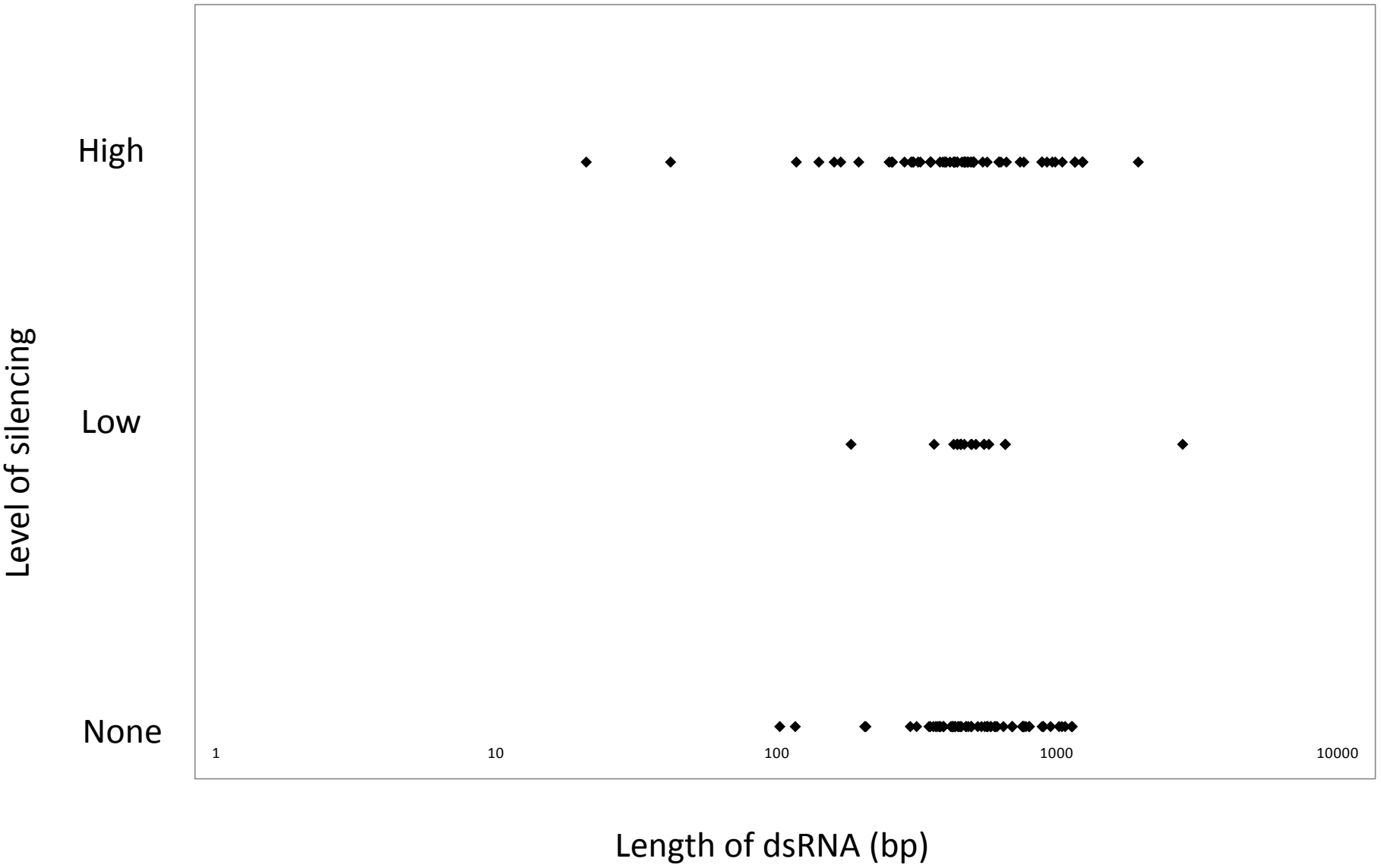

120
100
80
60
40
20
0
Percentage GP64 expression
C
SI
PI
Treatment

Figure S1


High
Low
None
Level of silencing
1
10
100
1000
10000
Length of dsRNA (bp)

e-component

Click here to download e-component: References to Table 1 2010.10.29.docx

e-component

Click here to download e-component: Supp table 1 101102.xls