RNA interference in honeybees: off-target effects caused by dsRNA

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Abstract

RNA interference involves the targeted knockdown of mRNA triggered by complementary dsRNA molecules applied to an experimental organism. Although this technique has been successfully used in honeybees (*Apis mellifera*), it remains unclear whether the application of dsRNA leads to unintended expression knockdown in unspecific, non-targeted genes. Therefore, we studied the gene expression of four non-target genes coding for proteins that are involved in different physiological processes after treatment with three dsRNAs in two abdominal tissues. We found unspecific gene downregulation depending on both the dsRNA used and the different tissues. Hence, RNAi experiments in the honeybee require rigid controls and carefully selected dsRNA sequences to avoid misinterpretation of RNAi-derived phenotypes.

Keywords

RNA interference ; honeybees ; off-target effects ; real-time PCR

1. Introduction

After the honeybee (*Apis mellifera*) became a model organism for the study of the genetic basis of eusociality, it was important to have a well-established, specific system to knock down genes. Presently, knockout mutants cannot be produced in *Apis*; thus, RNA interference (RNAi) appeared as a powerful tool for such functional gene studies by inducing loss-of-function phenotypes through target complementary short double-stranded RNA (dsRNA) molecules. Since its discovery in *Caenorhabditis elegans* (Fire et al. 1998), RNAi has become the predominant reverse genetic method in a variety of non-model organisms. Moreover, as honeybees are among the few recognized beneficial insects with a large economical and ecological impact, the use of RNAi is increasingly used as a tool for fighting pests and pathogens in apiculture (Maori et al. 2009; Paldi et al. 2010).

In light of the high potential power of RNAi for understanding honeybee genetics, it is surprising how few studies have been conducted using RNAi in *A. mellifera*. Some of them manipulated eggs or applied dsRNA to larvae either by feeding or injections (Aronstein and Saldivar 2005; Beye et al. 2003; Aronstein et al. 2006; Patel et al. 2007; Kucharski et al. 2008; Nunes and Simões 2009; Maori et al. 2009), whereas there are only very few reports on the successful manipulation of adult individuals (Amdam et al. 2003; Farooqui et al. 2004; Seehuus et al. 2006; Schlüns and Crozier 2007; Gatehouse et al. 2004; Müßig et al. 2010; Mustard et al. 2010).

Apart from the study of Müßig and colleagues, who use a combination of siRNAs and dsRNAs, all studies used target-specific dsRNA rather than siRNAs, the 21–23-nucleotide (nt) molecules processed out of longer dsRNAs, to manipulate gene function. However, dsRNAs have repeatedly been shown to cause off-target effects in higher animals. Studies in mammalian cells have shown that RNAi can cause the degradation of untargeted mRNAs by cross-hybridization regions towards the processed siRNAs (Jackson et al. 2003; Scacheri et al. 2004) or by siRNAs acting as miRNAs (Jackson et al. 2006). Additionally, dsRNAs may also alter gene expressions in a sequence-independent manner, such as activating antiviral mechanisms (Kumar and Carmichael 1998). Hence, the introduction of exogenous dsRNA molecules into mammalian cells often results in a global, nonspecific suppression of gene expression. This is achieved by the activation of two independent RNAi-activated pathways: the dsRNA recognition protein PKR (dsRNA-dependent protein kinase; Nanduri et al. 1998) and the 2′,5′-oligoadenylate synthetase. Both pathways lead to a general inhibition of protein synthesis (Sledz and Williams 2004). Double-stranded RNA also initiates a signalling cascade leading to the production of interferons (Williams 1999). Cytokines, which represent the first line of defence against viral infections, trigger the upregulation of interferon-stimulated genes and consequently lead to altered protein synthesis. Such sequence-dependent off-target effects as well as sequence-independent reactions towards dsRNA were also found in higher non-mammalian vertebrates (Oates et al. 2000; Zhao et al. 2001) and insects (Kulkarni et al. 2006), suggesting that the phenomenon of off-target RNAi reactions is not restricted to mammals.

To assess whether such off-target effects also occur in adult honeybees treated with dsRNAs, we analysed the gene expression of four non-target genes in two different abdominal tissues, the fat body and the ovaries, to compare whether different tissues treated with the same dsRNA show tissue-specific responses. The selected tissues are of prime interest for understanding honeybee biology because they are closely linked to the control of reproduction (ovaries) and are central to the honeybee's immune system (fat body). Furthermore, we chose one dsRNA (dsGFP) that has no known honeybee homologue and two dsRNAs (dsGPDH and dsVG) from the honeybee transcriptome. In particular, the dsVG sequence used in this study has been shown to successfully knock down its targeted gene in the honeybee fat body (Amdam et al. 2003).

To quantify the impact on expression levels, four non-target genes that lacked similarities with any of the injected dsRNAs were chosen: (1) *AmSID-I*: This is the honeybee homologue of the *SID-I* transmembrane channel protein. It is involved in dsRNA internalization in *C. elegans* and humans and facilitates systemic RNAi responses (Winston et al. 2002; Feinberg and Hunter 2003). This gene is particularly suited because Aronstein et al. (2006) report on a

correlation between *amSID-1* expression and the application of dsRNA in adult honeybees. (2) *amATF-2*: This gene shares homologies with the mammalian *ATF-2* transcription factor. Among others, genes targeted by *amATF-2* regulate transcription factors and proteins engaged in stress and DNA damage response (Bhoumik et al. 2007). (3) *amDHAP-AT*: Dihydroxy acetone phosphate acyl transferase is involved in lipid metabolism, facilitating the production of triacylglycerides (TAG). TAGs are used in eukaryotes as energy storages and repository of essential and non-essential fatty acids (Coleman and Lee 2004). (4) *amCPR*: NADPH-dependent cytochrome P450 reductase belongs to cytochrome P450 enzymes. These enzymes are involved in the detoxification of xenobiotics and are therefore commonly used as stress biomarkers. In insects, endogenous functions of these enzymes include the metabolism of ecdysteroids, juvenile hormones and pheromones (Feyereisen 1999). As these four selected genes code for proteins that cover very different physiological functions, they are particularly suited to screen a variety of different gene cascades for unspecific RNAi effects in the organism.

2. Materials and methods

2.1 BLAST analyses of dsRNA sequences

All three selected dsRNA sequences were compared with the honeybee genome during the design process using the Basic Local Alignment Tool. None of the dsRNAs shared sequence similarities with any of the evaluated non-target genes or contain any 20-bp segment identical to any known bee sequence. As dsRNAs are processed by the dicer complex into a cocktail of siRNAs 19–21 nt in length, the absence of 20-nt stretches of homology minimizes the possibility of off-target effects.

2.2 Production of dsRNA

To generate templates for dsRNA production, we cloned the *amVG* and the *amGPDH* part into pGem-T easy vectors (Promega). The respective fragments were obtained by standard PCRs using approximately 100-ng genomic DNA obtained by chloroform–phenol extraction (e.g. Maniatis et al. 1982; for primers, see Table I). As there are several *Apis GPDH* isoforms, there is the danger of getting a mixture of different PCR products for *amGPDH*. Therefore, we chose two primers in a region lacking the conserved domains (dsGPDH position within the *amGPDH* gene, 636–816). Furthermore, we checked the product identity by direct sequencing. In the case of *amVG*, we used primers from a well-established protocol (Amdam et al. 2003). The obtained vectors containing *amGPDH* and *amVG*, as well as the pGFP vector (GenBank ID: U17997, Clontech) were cloned into JM109 competent cells according to the manufacturer's instructions (Promega). Plasmids were purified after Del Sal et al. (1988). One of the obtained *amGPDH* and *amVG* clones, as well as one clone carrying the *GFP* encoding sequence, was used for PCRs producing the dsRNA templates. PCRs were adapted to the Biotherm™ DNA Polymerase (Genecraft) using 0.2 mM dNTPs, 0.3 µM of T7 promoter-added primer (see Table I), 1.5 mM $MgCl_2$ and 5 U Taq polymerase in a total reaction volume of 100 µL. PCR protocols consisted of 5-min DNA denaturation and Taq activation at 95°C, followed by 40 cycles of 30 s at 95°C, 30 s at 56°C for *GFP* and 54°C for *amGpdh* and *amVG*, and 1 min at 72°C. A final extension of 20 min at 72°C completed the protocol. The resulting PCR products were purified with the QIAquick PCR Purification Kit

(Qiagen). Subsequently, dsRNA from GFP, *amVG* and *amGpdh* was derived using the T7 Ribomax™ Express RNAi System (Promega) with an extended transcription time of 5 h at 32°C. The resulting dsRNA was purified by a Qiazol chloroform treatment and the pellet resolved in nuclease-free water. The dsRNA quality was verified in 1.8% agarose gels and its concentration photometrically quantified. dsRNA concentrations were adjusted to 5 µg/µL by diluting with insect ringer (see Section 2.3.) right before the injection.

Table I. Primer sequences and corresponding product sizes (all primers except the *amVG* primers were derived using Primer3; Rozen and Skaletsky 2000).

Method	Gene (accession no.)	Primer	Sequence (5'→3')	Product size (bp)
RNAi	*GFP* (M62653)	GFPI	TAATACGACTCACTATAGGGCGATTTCCATGGCCAACACTTGTCA	501
		GFPII	TAATACGACTCACTATAGGGCGATCAAGAAGGACCATGTGGTC	
	amGPDH (NM_001014994)	GPDH-T7I	TACGACTCACTATAGGGCGATGCTGGTTTCATCGATGGTTT	180
		GPDH-T7II	TAATACGACTCACTATAGGGCGATACGATTTCGACCACCGTAAC	
	amVg (NM_001011578)	VGI	TAATACGACTCACTATAGGGCGAACGACTCGACCAACGACTT	494
		VGII	TAATACGACTCACTATAGGGCGAAACGAAAGGAACGGTCAATTCC	
	amRp49 (NM_001011587)	Rp49I	TCGTCACCAGAGTGATCGTT	243
		Rp49II	CCATGAGCAATTTCAGCACA	
qPCR	*amSID-1* (XP_395167)	amSID-1I	GCTCGGGCATCAGTTACATT	296
		amSID-1II	ACTGCAAGAGCAATGTTCCA	
	amATF-2 (XP_393896)	amATF-2I	GATTGGACGAAATCGAAGGA	169
		amATF-2II	TGGTATCCCCTTTCGTCTTG	
	amDHAP-AT (XP_396018)	amDHAPI	ATTGCAAGTGGAATGGATTT	463
		amDHAPII	ATTGGCATGCAGAAATAGGT	
	amCPR (XP_001119949)	amCPRI	AATTGAAGGTGCAGGAGAAG	464
		amCPRII	GAACATGAGTGCGTGGATTA	
	amGPDH	GPDHIII	ACGGGCAAGAAAATCTCTGA	172
		GPDHIV	CCATAGGCATTGTCTCACCA	

2.3 Injection and incubation

Brood combs from one *Apis mellifera carnica* colony from the apiary of the Martin-Luther-University (Halle/Saale) were incubated at 34°C and 60% humidity. Newly emerged workers were anaesthetized by cooling on ice and subsequently injected with 5 µg of each dsRNA with a microsyringe (Hamilton, 10 µL) between the fifth and sixth abdominal segments following established protocols (Amdam et al. 2003). Negative controls were injected with insect ringer (54 mM NaCl, 24 mM KCl, 7 mM $CaCl_2 \cdot 2H_2O$). Both groups were marked with coloured tags. Injected bees were kept on wax plates until they recovered. Bees not showing haemolymph leakage were kept for 24 h at 34°C with food and water ad libitum together with 25 untreated worker bees. After 24 h, the bees were shock-frozen in liquid nitrogen and stored at −80°C until tissue preparation.

2.4 RNA preparation and real-time measurements

Ovaries and fat bodies were dissected on cooled wax plates using RNAlater (Ambion) in order to avoid RNA degradation. Tissues were manually homogenised using plastic pestles. RNA extraction followed the standard Trizol (Invitrogen) protocol (Chomczynski and Sacchi 1987) with subsequent DNase (Promega) digestion. RNA quality and quantity were assessed by photometry. Aliquots containing 1 µg RNA were immediately reverse-transcribed with M-MLV H-Point Mutant Reverse Transcriptase (Promega) using oligo-dT Primer (0.5 µg/µL, Promega) according to the manufacturer's instructions. Sybr Green assays consisting of 5 µL iQ SYBR Green Supermix (Biorad), 1 µL template and 1 µL of each Primer (1 µM) in a 10-µL reaction volume were run for gene expression studies. Each sample was run in duplicate. The real-time PCR cycling profile consisted of 3-min incubation at 95°C, followed by 39 cycles of 15 s at 95°C and 30 s at 54°C for annealing and 30 s at 72°C for extension and data collection. The following melting curve analysis was performed between 50°C and 90°C, reading the fluorescence at 1°C increments. The purity of the PCR products was additionally checked on 1.8% agarose gels. *C*(t) values were calculated by the Opticon Monitor 3 software (Biorad) using a single standard deviation over cycle range after baseline subtraction using the Global Minimum Trend option.

2.5 Data analyses and statistics

Whenever replicate samples differed in *C*(t) values larger than 0.5, the samples were rerun to obtain more reliable estimates for the average *C*(t) values. For calculating the respective relative gene expressions (RGE), the honeybee ortholog of the ribosomal protein 49 (rp49) was used as a housekeeping gene (Lourenço et al. 2008). The PCR efficiency for every sample was calculated from the linear phase of fluorescence increase due to target duplication (Peccoud and Jacob 1996; Pfaffl 2001a) to control for different PCR efficiencies between different samples and different genes. Relative gene expressions were calculated according to Pfaffl (2001b) using the following equation:

$$RGE = Efficiency_{target}^{-C(t)} / Efficiency_{rp49}^{-C(t)}$$

3. Results

The injection with insect ringer, which was used for dsRNA dilution, had no detectable impact on the gene expression of the four analysed non-target genes (Figure 1) in both of the evaluated tissues. Hence, ringer-injected and untreated bees were pooled to provide the controls for further analyses.

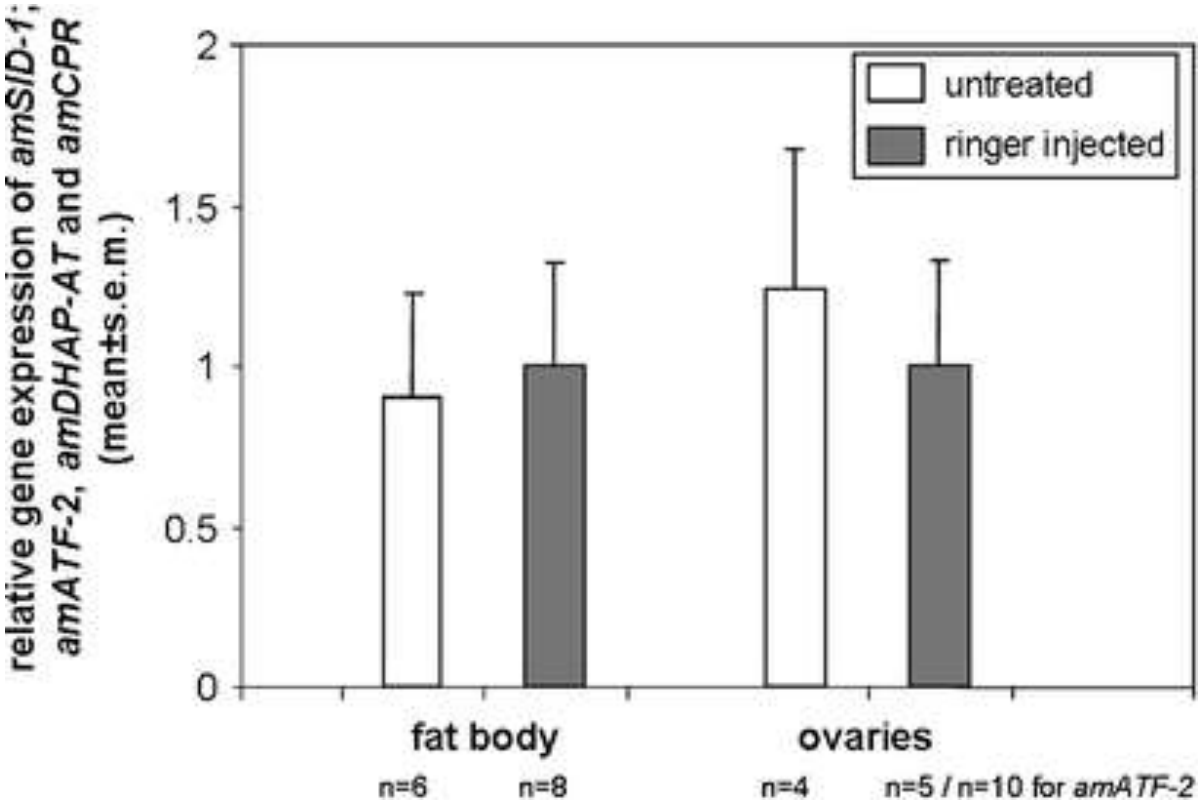

Figure 1. Pooled relative gene expression of four non-target genes in untreated bees compared with bees injected with honeybee ringer in two different tissues. We pooled the data for simplification as none of the individual genes showed an altered gene expression after the injection of ringer solution (*t* tests of log-transformed data, $P_{min} = 0.24$). After pooling, the injected bees did not differ from the untreated bees in their transcript level of the evaluated genes in either tissue (repeated-measures ANOVA of log-transformed data—fat body: $P = 0.642$, $F = 0.649$; ovaries: $P = 0.926$, $F = 0.200$). *N* refers to the number of individual bees. Note that the expression of all four non-target genes of every bee was taken into account when calculating the pooled gene expression.

3.1 Effect of *amGPDH*-specific dsRNA on its target gene in different tissues

Injection of dsGPDH and dsGFP into the body cavity of adult honeybees led to a marked *amGPDH* gene knockdown of 81% and 79%, respectively, in the fat body tissue (Figure 2). The similarity in the extent of the *amGPDH* gene knockdown is surprising as, unlike dsGPDH, dsGFP does not show any sequence similarity towards *amGPDH*. In contrast, neither dsGPDH nor dsGFP affected the *amGPDH* gene expression in ovarian tissue.

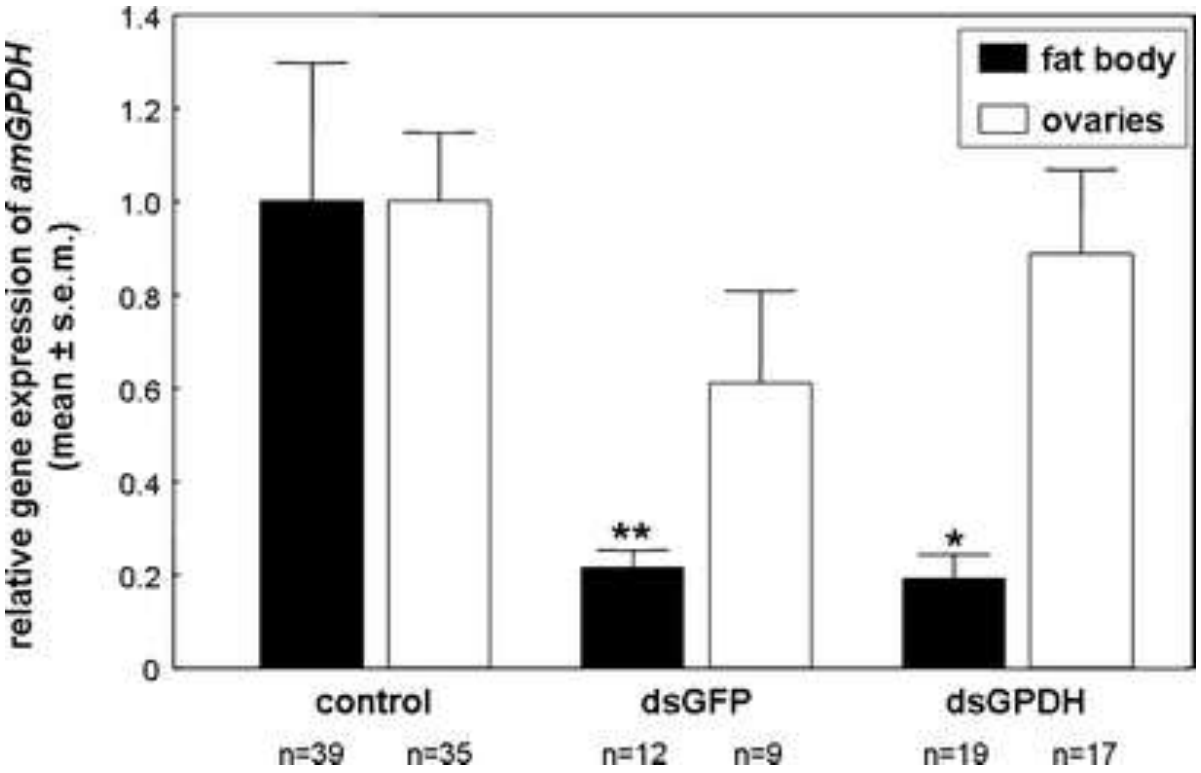

Figure 2. *amGPDH* knockdown in fat body and ovarian tissue after injection of dsRNA directed against *amGPDH* and *GFP*, respectively. Both the *amGPDH* gene expression in the fat bodies and ovaries of untreated and ringer-injected bees did not significantly deviate from each other (*t* tests of log-transformed data: fat body, $P = 0.293$; ovaries, $P = 0.177$). They serve as controls and were set to 1. *Asterisks* indicate significant differences (*$P \leq 0.05$, **$P \leq 0.01$; *t* test of log-transformed data).

3.2 Effects of dsRNA sequences on the overall gene expression in abdominal tissues

The dsRNA sequences had highly variable impacts on the overall gene expression of the non-target genes in ovarian and fat body tissues. The specific dsRNAs for *amVg* and for *GFP* did not alter the overall gene expression of the four non-target genes in either tissue. In contrast, the dsRNA for the honeybee *amGPDH* homologue had a strong impact on the gene expression of the evaluated genes in the fat body. Injection of this dsRNA led to a transcript level decrease of 70% in the fat body compared with the gene expression in the ovaries (Figure 3). To exclude potential differences in endogenous expression of the non-target genes between ovarian and fat body tissues that could confound the observed tissue-specific differences in gene expression, we compared the endogenous tissue-specific expression of every gene in untreated and ringer-injected bees (Figure 4). None of the genes showed a significantly different expression between the fat body and the ovaries in both experimental groups.

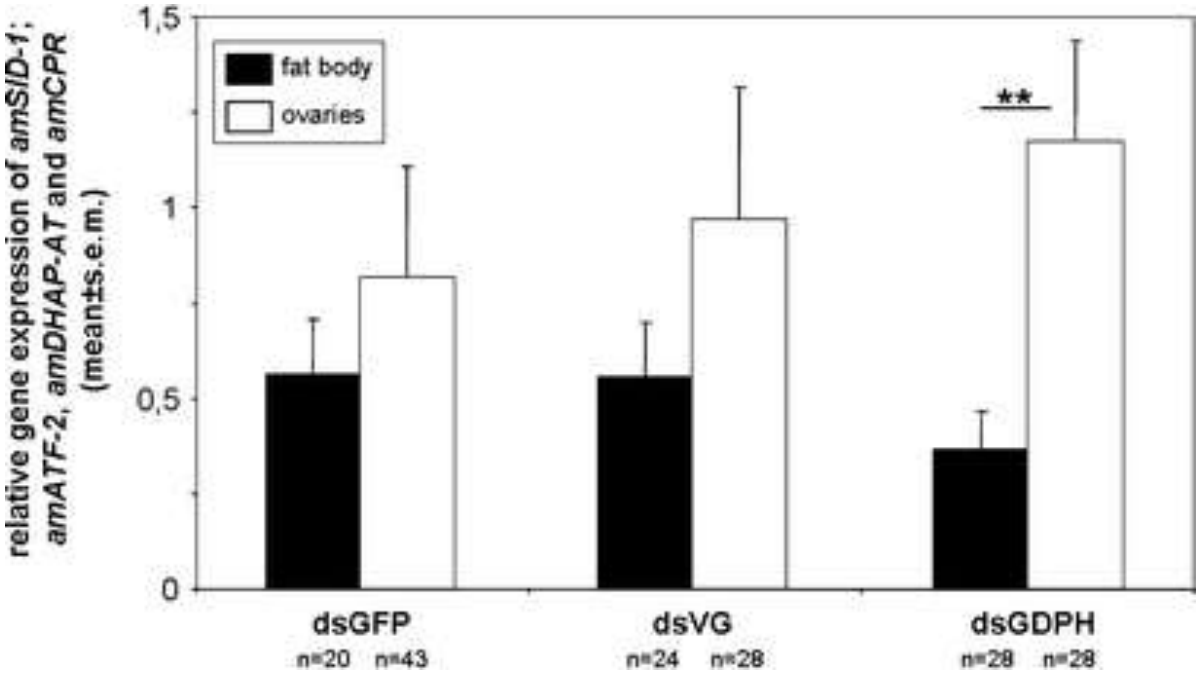

Figure 3. Impact of three different dsRNA sequences on the gene expression of four non-target genes in two abdominal tissues. *N* number of measurements. *Asterisks* indicate significant differences between the gene expression in honeybee ovaries and fat body (repeated-measures ANOVA of log-transformed data: $P = 0.013$, $F = 7.111$). The overall gene expression in the fat body and the ovaries did not differ from the controls (repeated-measures ANOVA of log-transformed data—fat body: $P = 0.380$, $F = 1.109$; ovaries: $P = 0.330$, $F = 1.321$).

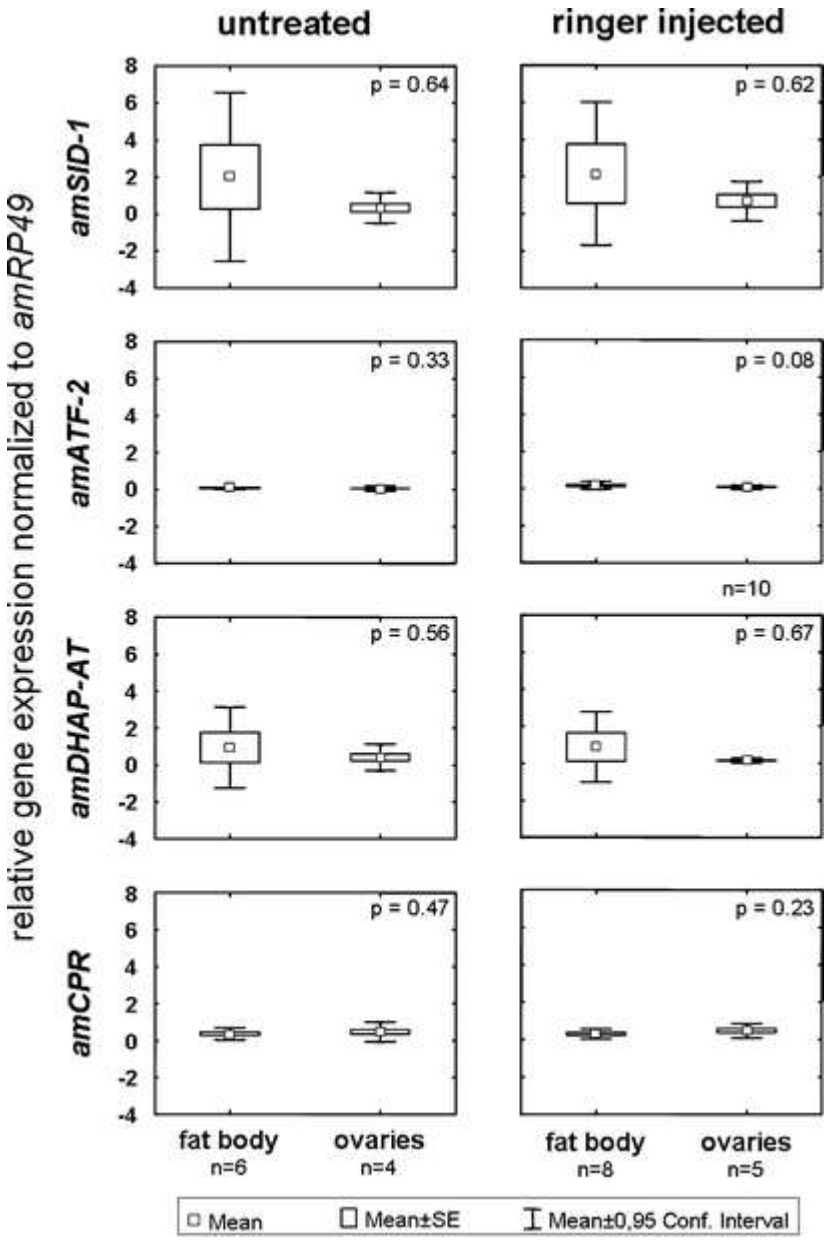

Figure 4. Endogenous gene expression of *amSID-1*, *amATF-2*, *amCPR* and *amDHAP-AT* in ovaries and fat bodies of untreated and ringer-injected honeybees. Individuals of both groups did not show differential expression between the evaluated tissues for any tested gene (*t* test of log-transformed data).

3.3 Tissue-specific effects of dsRNA sequences on selected genes

In parallel to the differences in the overall gene expression across both tissues, we observed gene- and tissue-specific differences in transcript abundances after dsRNA treatment (Figure 5). Compared with the controls, injection of dsGPDH led to an increased *amATF-2* expression in the ovaries and a significantly reduced *amDHAP-AT* expression in the fat body. As the tissue-specific endogenous expression of both genes did not differ (Figure 4), it is clear that the injection of dsGPDHs led to the altered gene expression profile between both abdominal tissues.

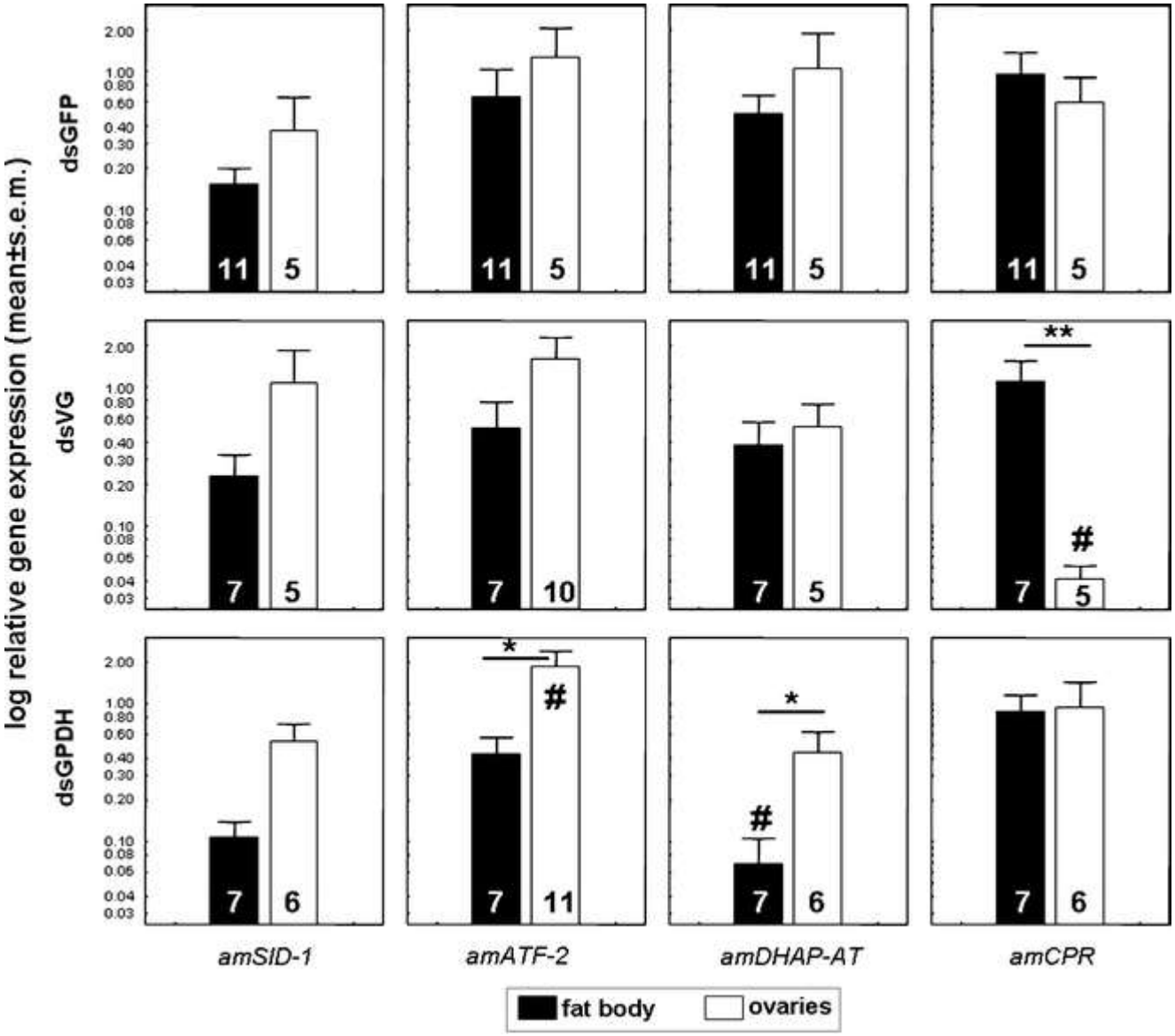

Figure 5. Tissue-dependent transcript level of four non-target genes after the treatment with one out of three different dsRNAs. The relative gene expression values for pairwise comparisons of the injection effects on the four non-target genes in the fat body and the ovaries were normalized by setting the transcript level of the control groups to 1. *Asterisks* indicate significant differences between the relative expression of the non-target gene between the two tissues (*$P \leq 0.05$, **$P \leq 0.01$; *t* test of log-transformed data). *Number sign* indicates significant differences ($^{\#}P < 0.01$; *t* test of log-transformed data) between the respective treatment and the tissue-specific control (untreated and ringer-injected individuals). Note the logarithmic scale of the *y*-axis.

In comparison to dsGPDH and dsGFP, dsVG injection resulted in a marked (90%) and specific knockdown of *amCPR* mRNA in ovarian tissue relative to control bees. The downregulation of *amCPR* in the ovaries was specific for dsVG as the gene expression of *amCPR* within this experimental group significantly differed from all other dsRNA treatments. Hence, *amCPR* expression in the ovaries was significantly different from all other evaluated genes (Newman–Keuls post hoc test, $P \leq 0.05$). Finally, in contrast to dsGPDH and dsVG, dsGFP did not alter transcript abundances in any of these four evaluated genes in either abdominal tissue.

4. Discussion

The injection of the dsRNA solvent did not have any detectable impact on the studied non-target genes. Therefore, our observations were not the result of a wounding or septic reaction in response to the ringer injection, but specific responses to our dsRNA treatments, either caused by sequence homologies or toxicity of the dsRNA molecules.

4.1 Tissue-specific response on dsRNA injections

Initially, we determined the relative expression of the non-target genes in the fat body and the ovaries in untreated and ringer-injected control individuals to ensure that the shifts in transcript abundance after dsRNA treatment were not just caused by the differences in the endogenous expression levels in both tissues. In the fat body, the lack of differential expression amongst the tested genes in control individuals confirms that the overall downregulation was due to the dsRNA treatment (most strongly for dsGPDH). The fat body trophocytes are known for dsRNA uptake (Amdam et al. 2003; Seehuus et al. 2006) since, among other functions, they are central to the detoxification and secretion of substances destined for exportation (de Oliveira and Cruz-Landim 2003). Therefore, the fat body contains a suite of transport mechanisms designed for the rapid uptake and release of an array of substances from the haemolymph. In comparison, ovaries and more particular follicle cells are less accessible for dsRNAs (Jarosch and Moritz 2011) as two dense cellular layers, the ovariole sheath (King et al. 1968) and the follicular epithelial cells (Engels 1968), may act as efficient barriers towards the dsRNA molecules.

4.2 Off-target gene regulation by dsRNA

We recorded four different dsRNA–off-target gene combinations showing altered transcript abundances after the treatment. dsGPDH altered the expression of *amATF-2* and *amDHAP-AT*, dsVG treatment decreased the expression of *amCPR* and injection of dsGFP decreased the expression of *amGPDH*. Clearly, every dsRNA evaluated in this study had an effect on a single gene, and one (dsGPDH) affected two different non-target genes. One of those genes, *amDHAP-AT*, is metabolically related to *amGPDH*. Both the target and the non-target genes code for proteins involved in lipid metabolism. The *Apis* homologue of *GPDH* bridges glycolysis and both the production and degradation of triacylglycerides. Furthermore, it serves as a cytosolic partner in the glycerol-3-phosphate shuttle (Brisson et al. 2001). *amDHAP-AT* facilitates the production of triacylglycerides, which are used as energy stores and a repository of essential and non-essential fatty acids (Coleman and Lee 2004). The downregulation of *amGPDH* and, therefore, the inhibition of glycolysis may have led to a parallel downregulation of *amDHAP-AT*. Especially the downregulation of *amDHAP-AT* within the fat body, the tissue where lipids are stored (de Oliveira and Cruz-Landim 2003), suggests a co-regulation of both enzymes in order to cope with the altered energy budget of the cells. Nevertheless, none of the other dsRNA–gene combinations in this study is physiologically related in a similar manner to *amGPDH* and *amDHAP-AT*, suggesting that they represent true off-target gene regulation.

4.3 Mechanistic reasons for off-target effects

Since the downregulation of *amGPDH* in the fat body cells by dsGPDH was accompanied by several non-target effects, the specificity of both the knockdown and dsGPDH remains questionable. The non-target downregulations may have been caused by sequence-specific cross-hybridizations between the processed secondary siRNAs and the genes. Nevertheless, all three dsRNAs, all specifically designed to have no sequence homology longer than 20 bp with any gene in the honeybee genome, showed at least one unspecific off-target knockdown. Although we cannot completely exclude the possibility of interactions between the secondary siRNAs and the evaluated genes, we still feel it prudent to consider the observed effects, particularly those of dsGFP and dsVG, as sequence-unspecific off-target effects.

In conclusion, we strongly recommend concentrating effort on the design of RNAi effective molecules, combining several dsRNAs for one target gene and using more stringent controls when setting up RNAi protocols in honeybees. To rigorously identify gene functions based on RNAi-derived phenotypes, measuring the mRNA level of RNAi targeted genes relative to a single non-target gene is clearly insufficient. As this study shows, treatments with gene-specific dsRNA can lead to nonspecific effects, which in turn may lead to false interpretations of the observed RNAi-derived phenotypes.

Acknowledgements

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