International Journal of
Molecular Sciences

MDPI

Article Supplementary file

Knockdown of the *Halloween* genes *spook*, *shadow* and *shade* influences oocyte development, egg shape, oviposition and hatching in the desert locust.

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Abstract: Ecdysteroids are widely investigated for their role during the molting cascade in insects; however, they are also involved in the development of the female reproductive system. Ecdysteroids are synthesized from cholesterol, which is further converted via a series of enzymatic steps into the main molting hormone, 20-hydoxyecdysone. Most of these biosynthetic conversion steps involve the activity of cytochrome P450 (CYP) hydroxylases, which are encoded by the *Halloween* genes. Three of these genes, *spook* (*spo*), *phantom* (*phm*) and *shade* (*shd*), were previously characterized in the desert locust, *Schistocerca gregaria*. Based on recent sequencing data, we have now identified the sequences of *disembodied* (*dib*) and *shadow* (*sad*), for which we also analyzed spatiotemporal expression profiles using qRT-PCR. Furthermore, we investigated the possible role(s) of five different *Halloween* genes in the oogenesis process by means of RNA interference mediated knockdown experiments. Our results showed that depleting the expression of *SchgrSpo*, *SchgrSad* and *SchgrShd* had a significant impact on oocyte development, oviposition and hatching of the eggs. Moreover, the shape of the growing oocytes, as well as the deposited eggs, was very drastically altered by the experimental treatments. Consequently, it can be proposed that these three enzymes play an important role in oogenesis.

Keywords: Biosynthesis; Ecdysone; Ecdysteriod; Hemimetabola; Hormone; Insect; Oogenesis; Reproduction

Citation: Lastname, F.; Lastname, F.; Lastname, F. Title. *Int. J. Mol. Sci.* **2022**, *23*, 9232. https://doi.org/10.3390/ijms23169232

Academic Editor: Firstname Lastname

Received: date
Accepted: date
Published: date

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1. Supplementary data

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ATA	GAA	CAA	ACT	GAT	GCT	GTA	ATT	CAA	GAA	TTT	ATA	GAA	CTG	ATC	AGA
I	E	Q	T	D	A	V	I	Q	E	F	I	E	L	I	R
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R	H	S	K	S	Q	K	P	H	D	Y	L	H	D	L	S
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N	G	L	Q	L	W	K	F	M	E	T	P	L	Y	K	K
TTC	AAG	ATA	GCA	TAT	CAT	TTT	ATG	GAG	AGT	GTT	GCA	TTG	GAG	CTG	GTA
F	K	I	A	Y	H	F	M	E	S	V	A	L	E	L	V
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S	Q	K	A	T	A	M	E	K	G	W	K	R	N	D	G
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H	Q	A	S	L	L	E	Q	Y	L	L	S	P	D	L	S
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Supplementary Figure S1. ***SchgrDib*** **nucleotide sequence and translation.** qPCR primers and primers for generating two dsRNA constructs are indicated in red, blue and green, respectively.

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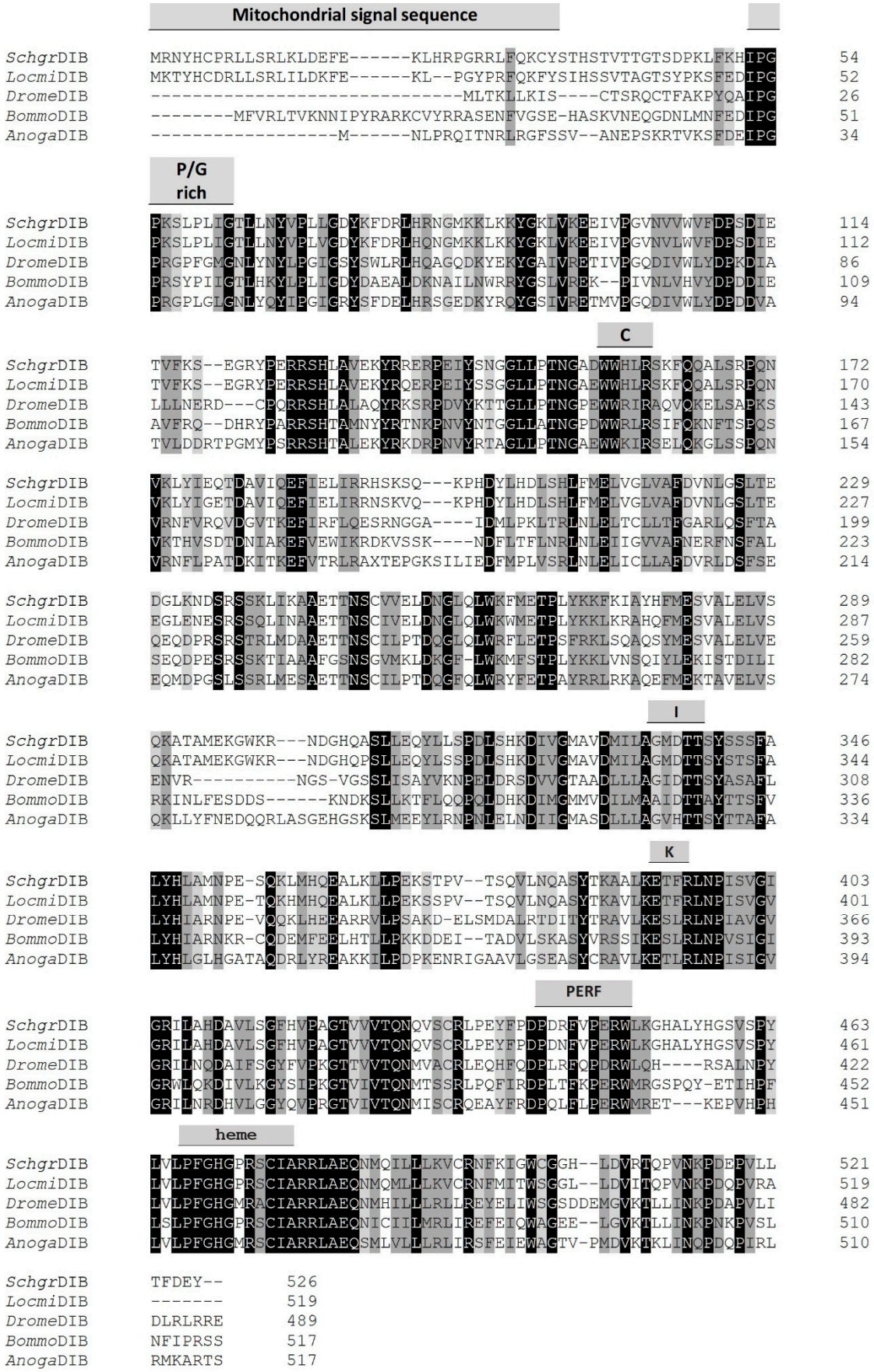
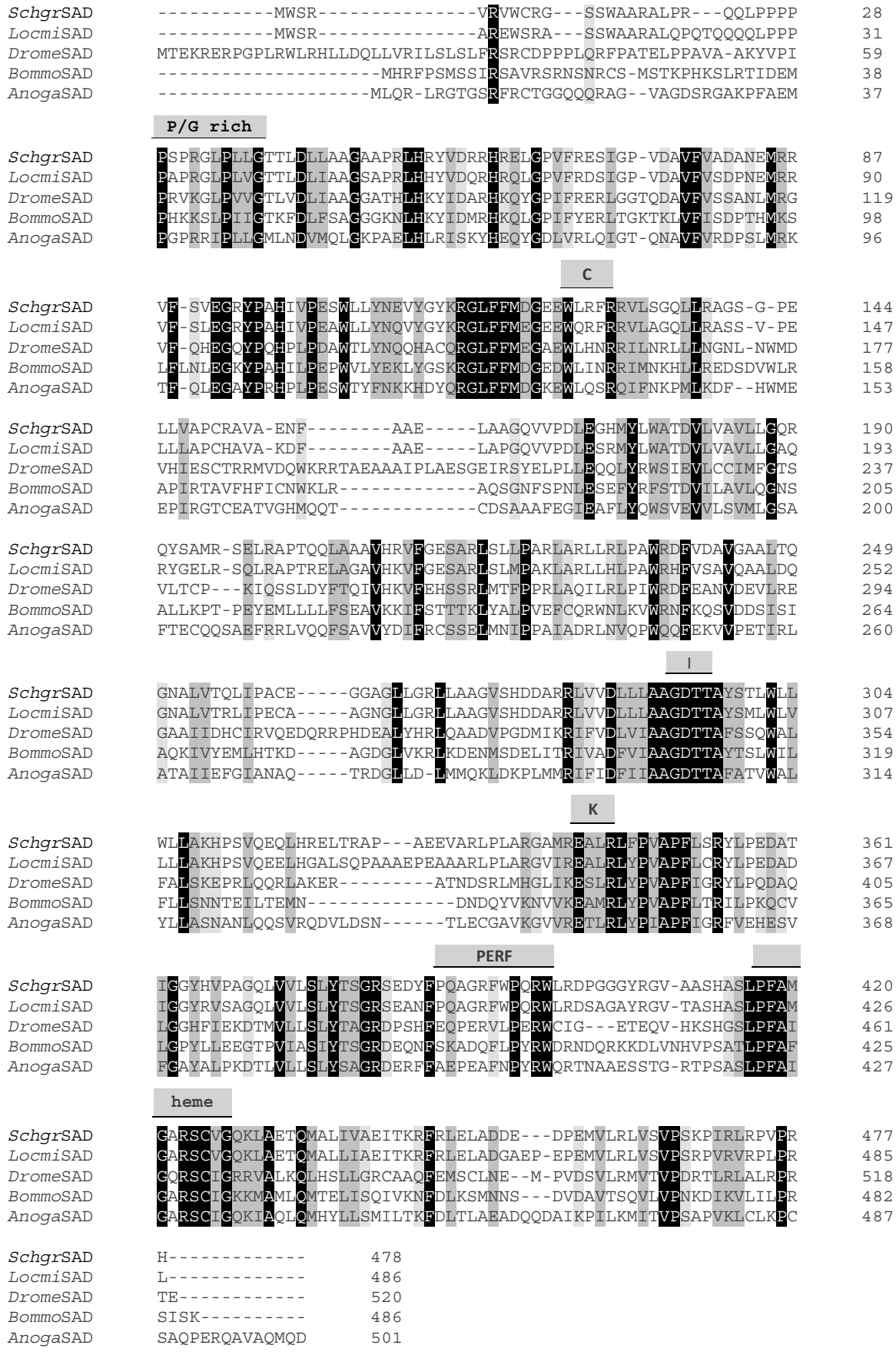
CAC	TGA														
H	*														

Supplementary Figure S2. ***SchgrSad*** **nucleotide sequence and translation.** qPCR primers and primers for generating two dsRNA constructs are indicated in red, blue and green, respectively.



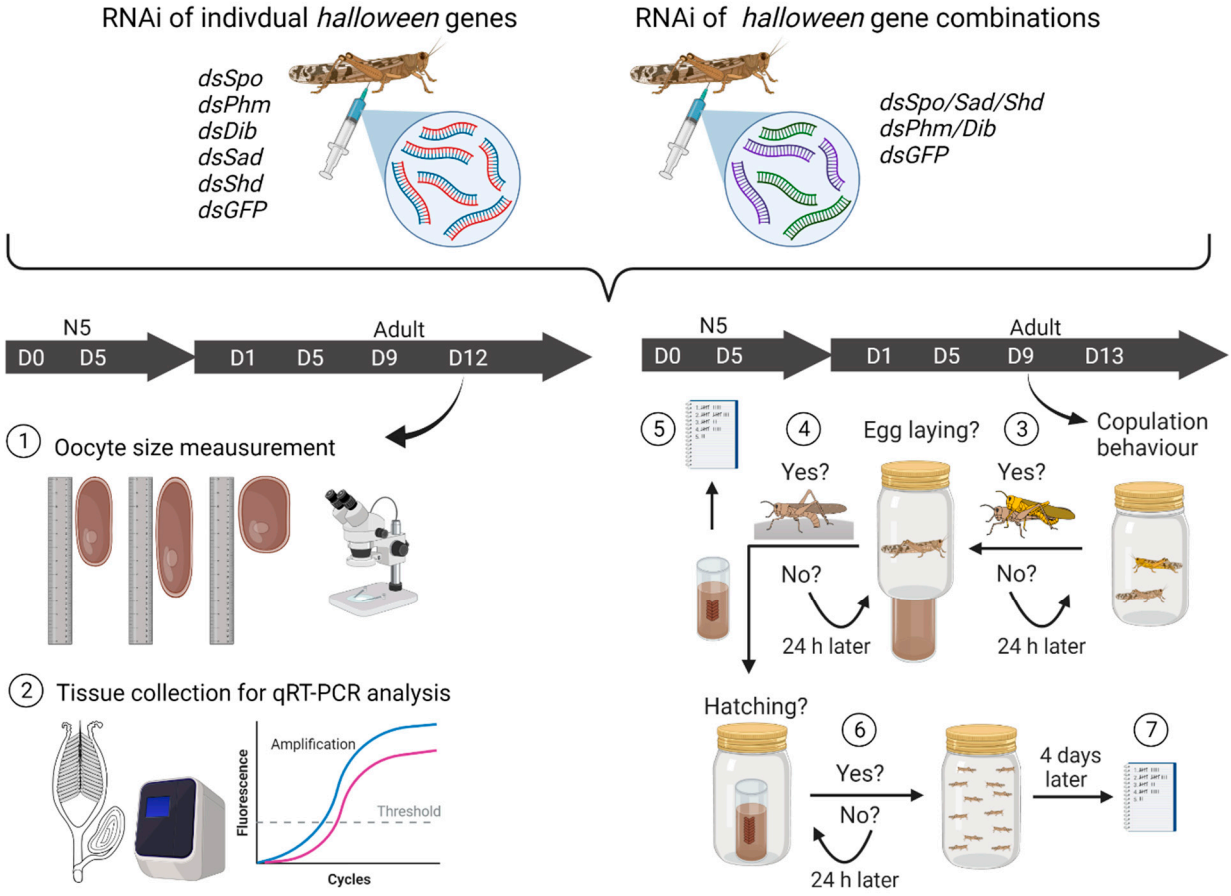
Supplementary Figure S3. Multiple sequence alignment of the cloned *Schgr*DIB (KY404120) with other identified DIB proteins from *Locusta migratoria* (*Locmi*DIB, AVL92819.1), *Drosophila melanogaster* (*Drome*DIB, AAF47831.2),

***Bombyx mori* (*Bommo*DIB, BAD99022.1) and *Anopheles gambiae* (*Anoga*DIB, ABU42523).** The multiple sequence alignment was made using the M-Coffee web server in the default settings (http://tcoffee.crg.cat/apps/tcoffee/do:mcoffee). The degree of conservation was highlighted using Boxshade and is indicated as follows: residues highlighted in black: complete; residues highlighted in dark grey: strong; residues highlighted in light grey: weak. Several key motifs for CYP450 enzymes are shown: mitochondrial signal sequence, P/G rich domain, helix-C, helix-I, helix-K, PERF-motif and the heme-binding domain.

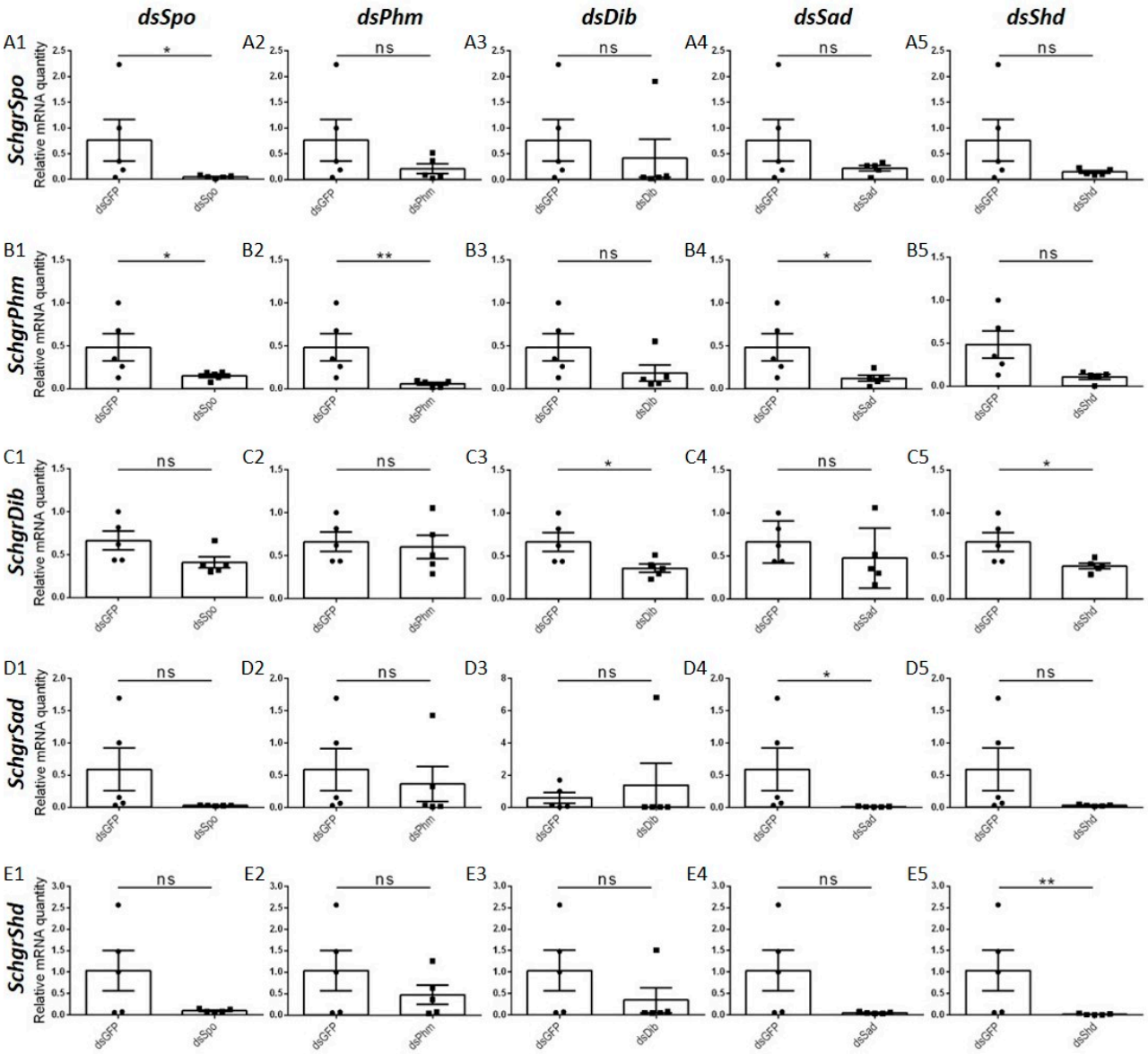


Supplementary Figure S4. Multiple sequence alignment of the *Schgr*SAD (MZ780957) with other identified and predicted SAD proteins from *Locusta migratoria* (*Locmi*SAD, AVL92827), *Drosophila melanogaster* (*Drome*SAD,

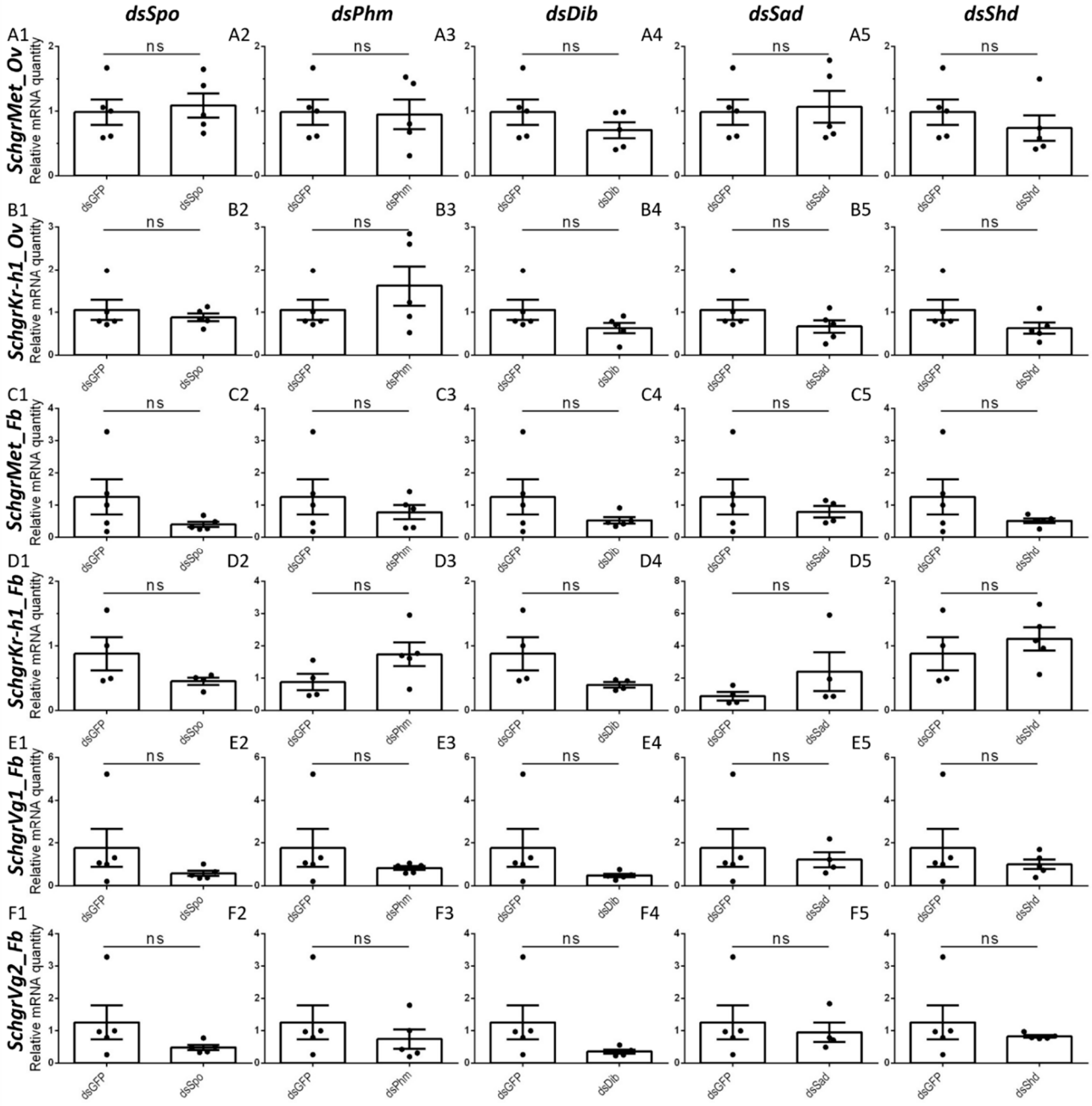
AAF54711), *Bombyx mori* (*Bommo*SAD, NP_001106224) and *Anopheles gambiae* (*Anoga*SAD, ABU42524). The multiple sequence alignment was made using the M-Coffee web server in the default settings (http://tcoffee.crg.cat/apps/tcoffee/do:mcoffee). The degree of conservation was highlighted using Boxshade and is indicated as follows: residues highlighted in black: complete; residues highlighted in dark grey: strong; residues highlighted in light grey: weak. Several key motifs for CYP450 enzymes are shown: P/G rich domain, helix-C, helix-I, helix-K, PERF-motif and the heme-binding domain.



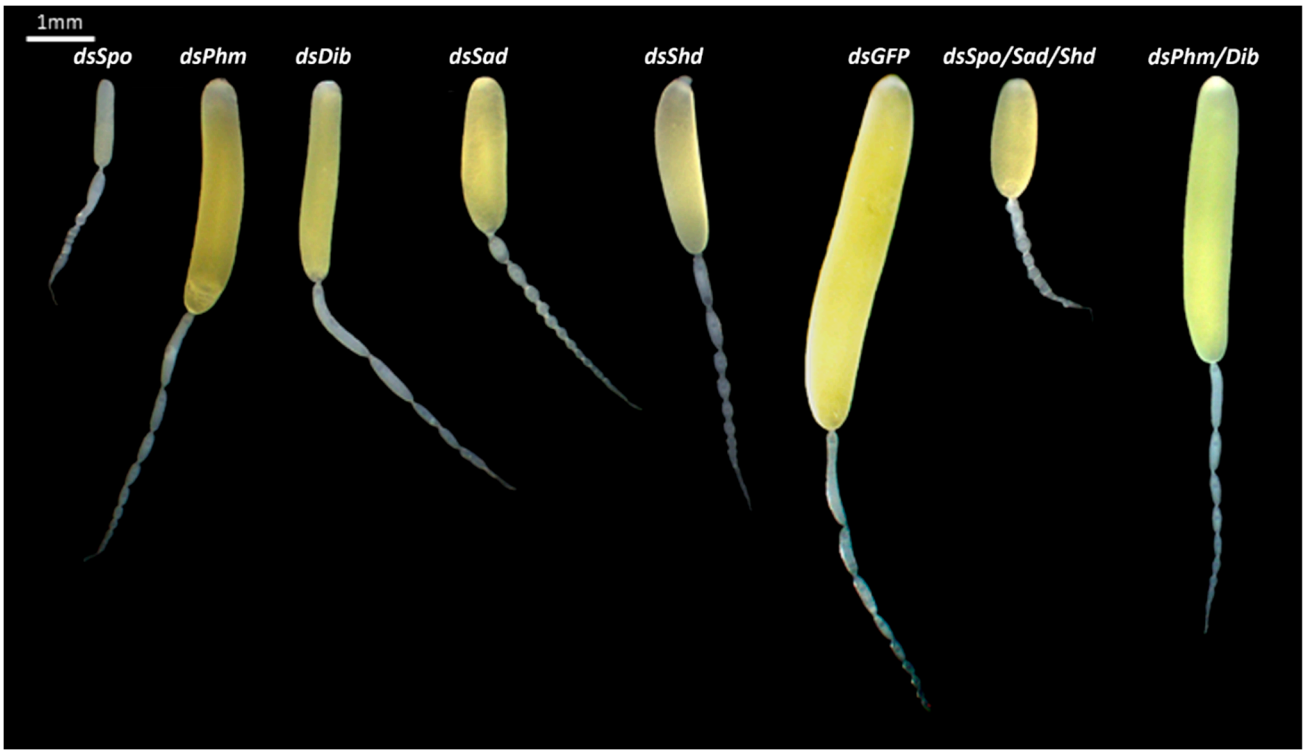
Supplementary Figure S5. Graphical representation of the performed experiments. Two different types of injections were performed. First, each *Halloween* gene was downregulated separately by injecting dsRNA targeting one of the genes of interest (*dsSpo*, *dsPhm*, *dsDib*, *dsSad*, *dsShd* and *dsGFP*). Second, *Halloween* genes *SchgrSpo*, *SchgrSad* and *SchgrShd*, as well as *SchgrPhm* and *SchgrDib*, were downregulated together by injecting a combination of different dsRNA molecules (*dsSpo/Sad/Shd* and *dsPhm/Dib*). Female locusts were injected on day 0 and day 5 of the 5th nymphal stage and day 1, day 5 and day 9 of the adult stage. A first group of female locusts was dissected on day 12 of the adult stage for measuring the size of basal oocytes and for RNA sample preparations. From each individual female, the length and width of five basal oocytes were measured (Observation ①). An ovary was collected to determine transcript levels of genes of interest by means of qRT-PCR (Observation ②). A second group of female locusts was used for an observational analysis of their copulation behavior, fecundity and fertility. From day 9 of the adult stage, female locusts were joined with a virgin male for two hours during which occurrence of copulation was monitored. If copulation did not occur in these two hours, the male was removed from the container and the experiment was repeated the following day. If copulation did occur, the male and female were allowed to stay together for the following 22 hours (Observation ③). Afterwards, the male was removed, and an egg pot was placed under the individual container. Egg pots were controlled daily for the presence of deposited eggs (Observation ④), and if eggs were laid their numbers were counted (Observation ⑤). Lastly, all pots were controlled daily for the appearance of hatchlings (Observation ⑥) and the number of hatchlings was counted four days after hatching (Observation ⑦). The second group of female locusts had received an extra dsRNA injection on day 13 of the adult stage. (This scheme was made using Biorender®)



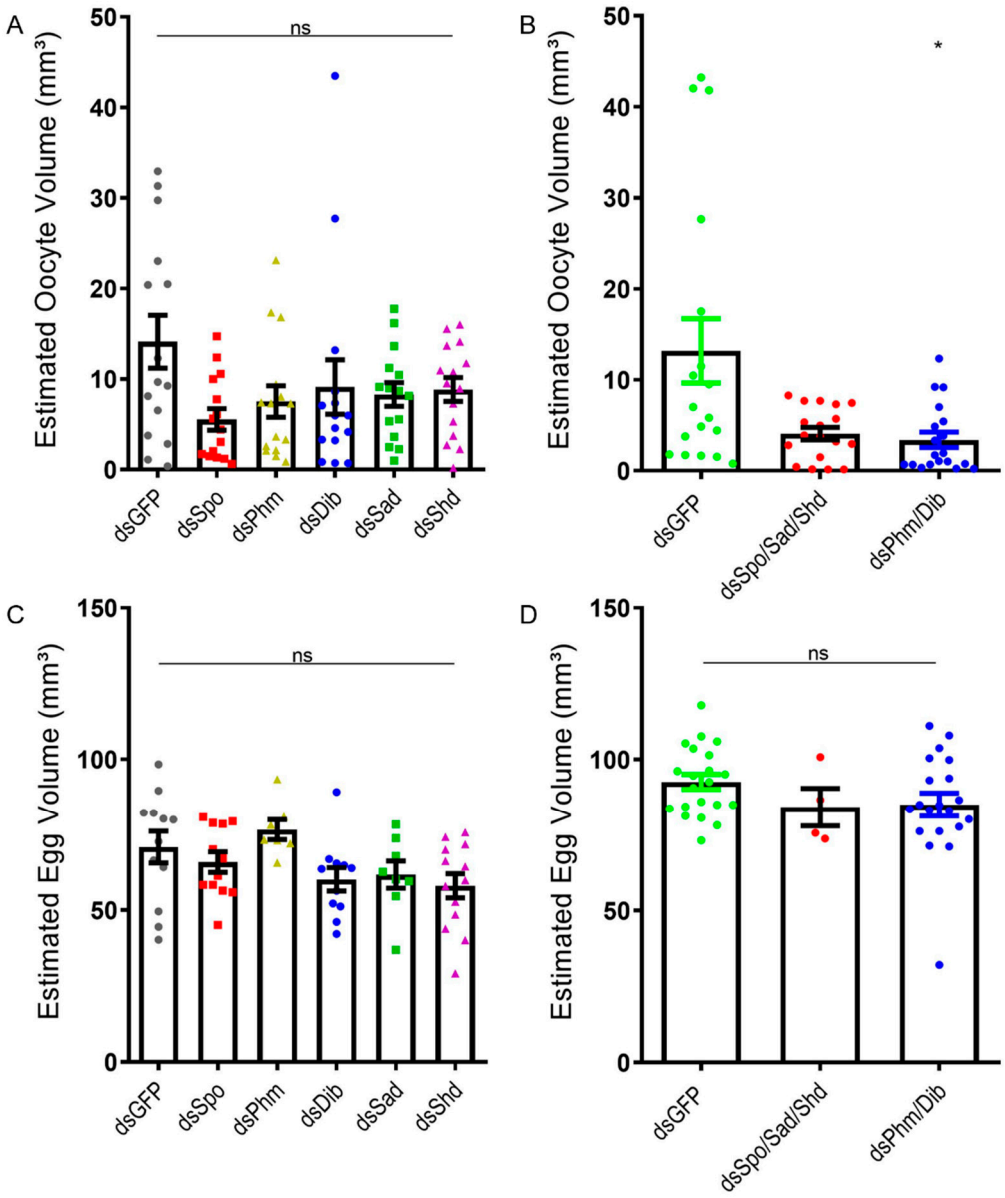
Supplementary Figure S6. Transcript levels of five *Halloween* genes (*SchgrSpo*, *SchgrPhm*, *SchgrDib*, *SchgrSad* and *SchgrShd*) in the ovaries of *dsSpo-*, *dsPhm-*, *dsDib-*, *dsSad-* or *dsShd*-injected adult female locusts. (A1-5) Relative *SchgrSpo* transcript levels measured in the ovaries of *dsSpo-*, *dsPhm-*, *dsDib-*, *dsSad-* and *dsShd-treated* females, respectively. **(B1-5)** Relative *SchgrPhm* transcript levels measured in the ovaries of *dsSpo-*, *dsPhm-*, *dsDib-*, *dsSad-* and *dsShd-treated* females, respectively. **(C1-5)** Relative *SchgrDib* transcript levels measured in the ovaries of *dsSpo-*, *dsPhm-*, *dsDib-*, *dsSad-* and *dsShd-treated* females, respectively. **(D1-5)** Relative *SchgrSad* transcript levels measured in the ovaries of *dsSpo-*, *dsPhm-*, *dsDib-*, *dsSad-* and *dsShd-treated* females, respectively. **(E1-5)** Relative *SchgrShd* transcript levels measured in the ovaries of *dsSpo-*, *dsPhm-*, *dsDib-*, *dsSad-* and *dsShd* -treated females, respectively. All relative transcript levels were measured in the ovaries of female locusts twelve days after the final molt using qRT-PCR. The data are presented as mean ± S.E.M of five independent pools of three locusts, run in duplicate and normalized to *glyceraldehyde 3-phosphate dehydrogenase* (*GAPDH*) and *CG13220* transcript levels; each dot represents one data point. Significant differences in comparison with *dsGFP*-injected females are indicated by (an) asterisk(s) (** $p < 0.01$ and * $p < 0.5$; two-sided Unpaired t-test on log-transformed data including the Welch's correction for comparisons of the *SchgrSpo* transcript levels in *dsShd-* and *dsGFP-treated* females (A5), *SchgrPhm* transcript levels in *dsShd-* and *dsGFP-treated* females (B5), *SchgrSad* transcript levels in *dsSpo-* (D1), *dsSad-* (D4), *dsShd-* (D5) and *dsGFP-treated* females and *SchgrShd* transcript levels in *dsSpo-* (E1), *dsShd-* (E4) and *dsGFP-treated* females).



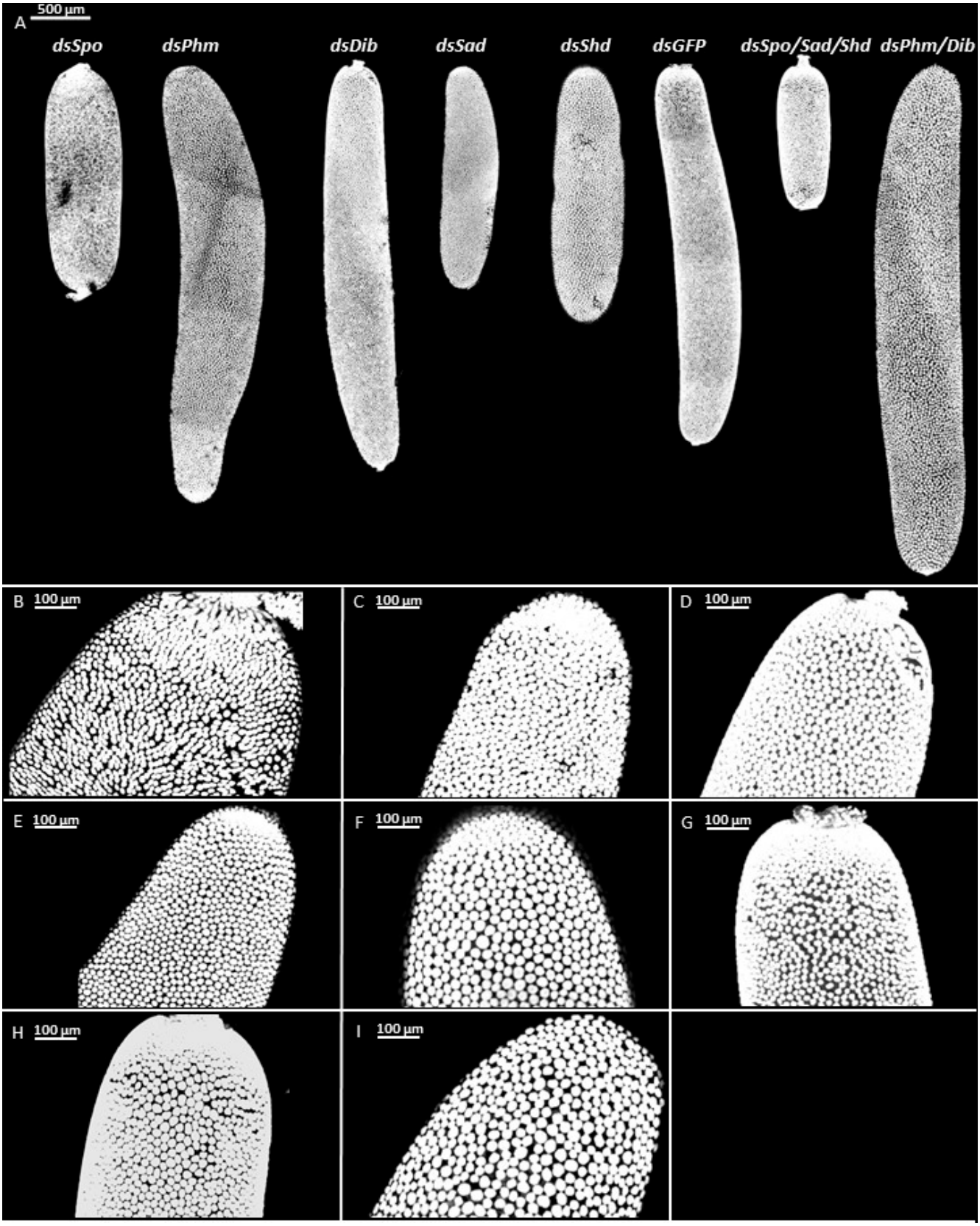
Supplementary Figure S7. Transcript levels of genes involved in vitellogenin (Vg) synthesis in the fat body and JH signalling in the fat body and ovaries of *dsSpo-*, *dsPhm-*, *dsDib-*, *dsSad-* or *dsShd*-injected adult female locusts. (A1-5) Relative *SchgrMet* transcript levels measured in the ovaries of *dsSpo-*, *dsPhm-*, *dsDib-*, *dsSad-* or *dsShd*-treated females, respectively. **(B1-5)** Relative *SchgrKr-h1* transcript levels measured in the ovaries of *dsSpo-*, *dsPhm-*, *dsDib-*, *dsSad-* or *dsShd*-treated females, respectively. **(C1-5)** Relative *SchgrMet* transcript levels measured in the fat body of *dsSpo-*, *dsPhm-*, *dsDib-*, *dsSad-* or *dsShd*-treated females, respectively. **(D1-5)** Relative *SchgrKr-h1* transcript levels measured in the fat body of *dsSpo-*, *dsPhm-*, *dsDib-*, *dsSad-* or *dsShd*-treated females, respectively. **(E1-5)** Relative *SchgrVg1* transcript levels measured in the fat body of *dsSpo-*, *dsPhm-*, *dsDib-*, *dsSad-* or *dsShd*-treated females, respectively. **(F1-5)** Relative *SchgrVg2* transcript levels measured in the fat body of *dsSpo-*, *dsPhm-*, *dsDib-*, *dsSad-* or *dsShd*-treated females, respectively. All relative transcript levels were measured in the fat body or ovaries of female locusts twelve days after the final molt using qRT-PCR. The data represent the mean ± S.E.M of five independent pools of three locusts, run in duplicate and normalized to *CG13220* and *β-actin* transcript levels; each dot represents one data point. No significant differences were observed in comparison with *dsGFP*-injected females (two-sided Unpaired t-test on log-transformed data including the Welsch's correction for the comparison of *SchgrVg1* transcript levels of *dsPhm-* and *dsGFP*-treated females (E2) and of *SchgrVg2* transcript levels in *dsShd-* and *dsGFP*-treated females (F5)).



Supplementary Figure S8. Representative ovarioles of *dsSpo*-, *dsPhm*-, *dsDib*-, *dsSad*-, *dsShd*-, *dsGFP*-, *dsSpo*/*Sad*/*Shd*- and *dsPhm*/*Dib*-injected females. *dsSpo* basal oocyte: length = 1.3 mm – width = 0.3 mm; *dsPhm* basal oocyte: length = 3.5 mm – width = 0.6 mm; *dsDib* basal oocyte: length = 3.0 mm – width = 0.5 mm; *dsSad* basal oocyte: length = 2.3 mm – width = 0.7 mm; *dsShd* basal oocyte: length = 2.6 mm – width = 0.6 mm; *dsGFP* basal oocyte: length = 5.2 mm – width = 0.9 mm; *dsSpo*/*Sad*/*Shd* basal oocyte: length = 1.8 mm – width = 0.7 mm; *dsPhm*/*Dib* basal oocyte: length = 4.2 mm – width = 0.7 mm. Scale bar = 1 mm.

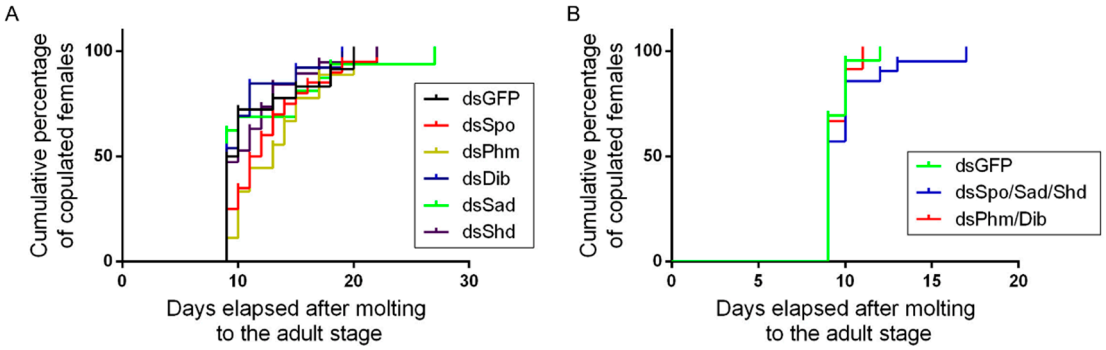


Supplementary Figure S9. Estimated volume (calculated based on length and width measurements) of oocytes and eggs derived from *dsSpo-*, *dsPhm-*, *dsDib-*, *dsSad-*, *dsShd-*, *dsGFP-*, *dsSpo/Sad/Shd-* and *dsPhm/Dib*-injected females. **(A)** The volume of five basal oocytes per *dsSpo-*, *dsPhm-*, *dsDib-*, *dsSad-*, *dsShd-* or *dsGFP*-injected female locust (n = 15 for all conditions) is represented as mean ± S.E.M.; each dot represents one data point (Kruskal-Wallis test with Dunn's multiple comparison test) **(B)** The volume of five basal oocytes per *dsSpo/Sad/Shd-*, *dsPhm/Dib-* or *dsGFP*-injected female locust (n = 15 for all conditions) is represented as mean ± S.E.M.; each dot represents one data point. Significant differences are indicated by an asterisk (* $p < 0.05$; Kruskal-Wallis test with Dunn's Multiple Comparison Test). **(C)** The volume of 20 eggs per *dsSpo-* (n = 12), *dsPhm-* (n = 7), *dsDib-* (n = 11), *dsSad-* (n = 8), *dsShd-* (n = 13) or *dsGFP*-injected (n = 12) female locust is represented as mean ± S.E.M;. each dot represents one data point (One-way ANOVA test with Dunnett's multiple comparison test) **(D)** The volume of seven eggs per *dsSpo/Sad/Shd-* (n = 4), *dsPhm/Dib-* (n = 24) or *dsGFP*-injected (n = 23) female locust is represented as mean ± S.E.M;. each dot represents one data point (Kruskal-Wallis test with Dunn's multiple comparison test).

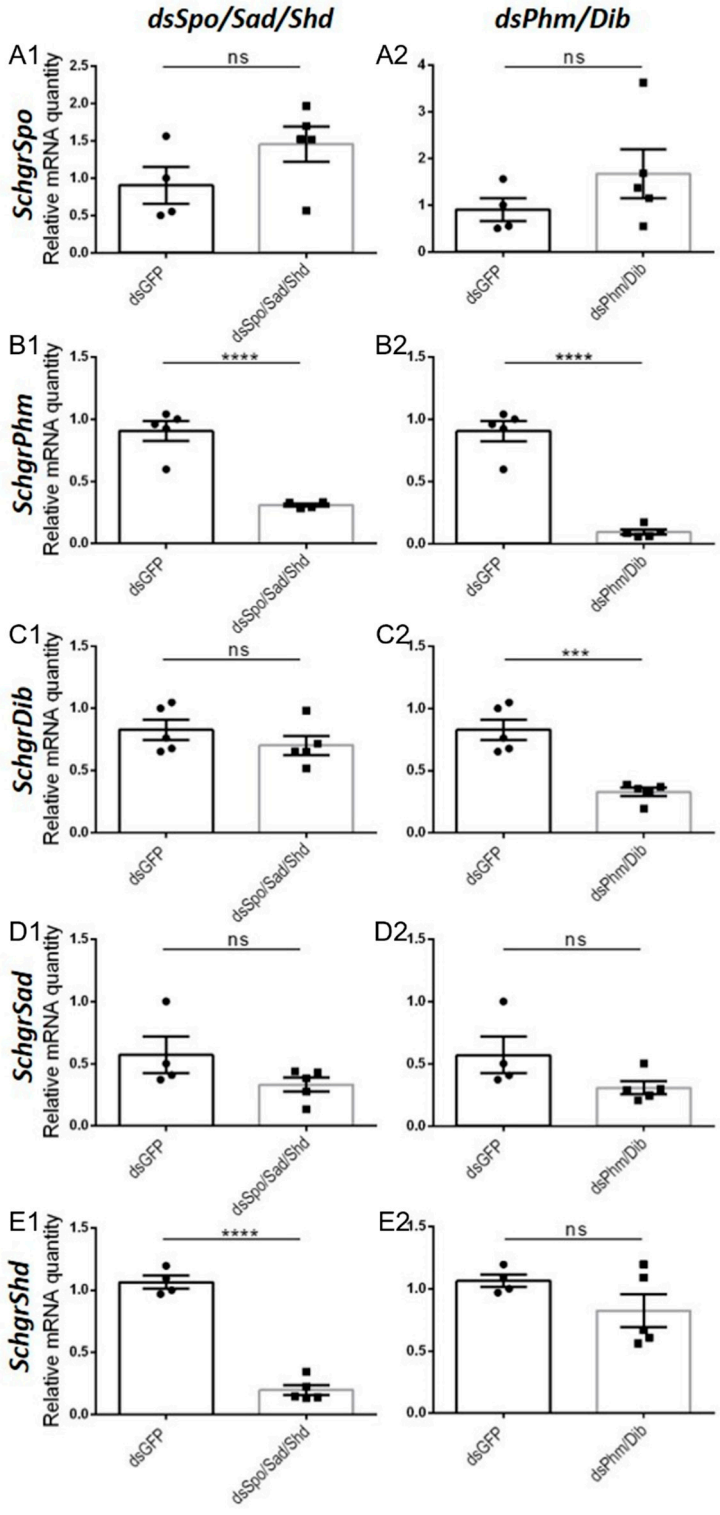


Supplementary Figure S10. DAPI-stained nuclei of follicular epithelial cells surrounding the basal oocytes in representative ovarioles derived from *dsSpo*-, *dsPhm*-, *dsDib*-, *dsSad*-, *dsShd*-, *dsGFP*-, *dsSpo*/*Sad*/*Shd*- and *dsPhm*/*Dib*-injected females. (A) *dsSpo* basal oocyte: length = 1.9 mm, width = 0.6 mm; *dsPhm* basal oocyte: length = 3.6 mm, width = 0.7 mm; *dsDib* basal oocyte: length = 3.4 mm, width = 0.6 mm; *dsSad* basal oocyte: length = 1.9 mm, width = 0.5 mm; *dsShd* basal oocyte: length = 2.1 mm, width = 0.6 mm; *dsGFP* basal oocyte: length = 3.1 mm, width = 0.5 mm; *dsSpo*/*Sad*/*Shd* basal oocyte: length = 1.2 mm, width = 0.4 mm; *dsPhm*/*Dib* basal

oocyte: length = 4.1 mm, width = 0.7 mm. Scale bar = 500 µm. **(B-I)** Zoomed in pictures of the distal end of the ovarioles of *dsSpo*- **(B),** *dsPhm*- **(C)**, *dsDib*- **(D)**, *dsSad*- **(E)**, *dsShd*- **(F)**, *dsGFP*- **(G)**, *dsSpo/Sad/Shd*- **(H)** and *dsPhm/Dib*- **(I)** treated females. Scale bar = 100 µm.

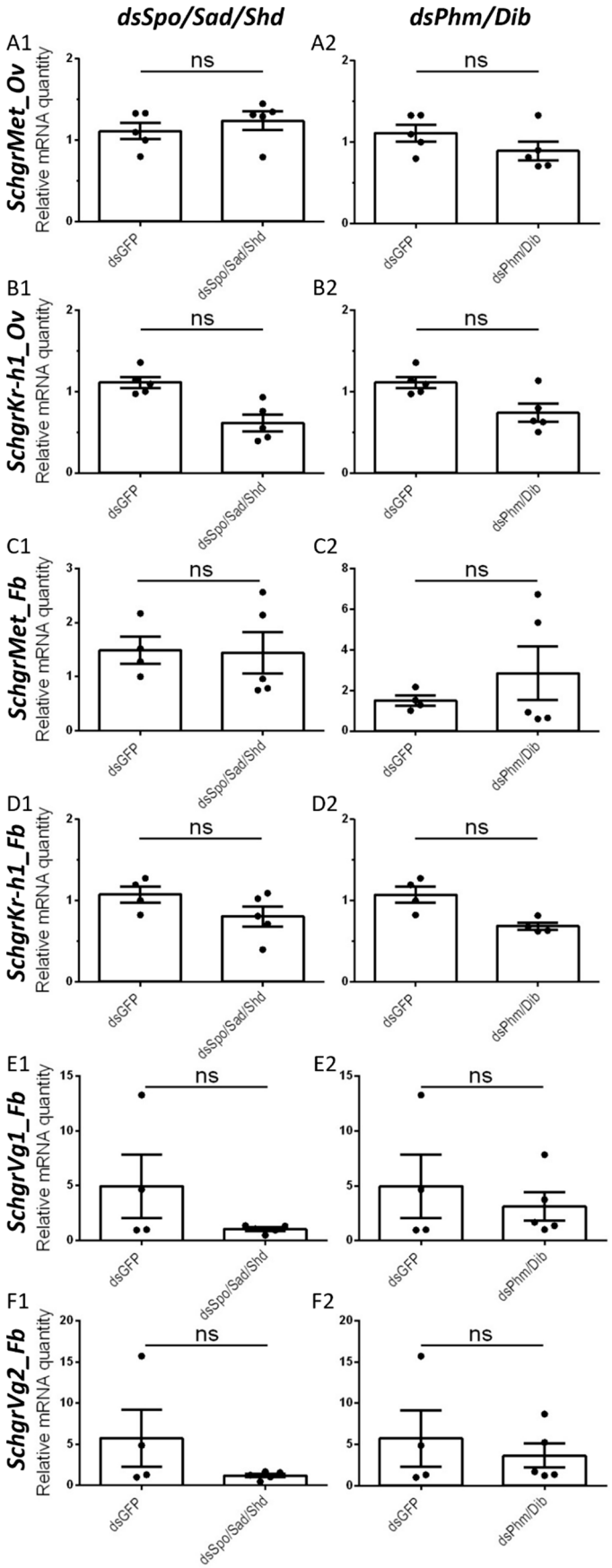


Supplementary Figure S11. Observations of mating by *dsSpo-, dsPhm-, dsDib-, dsSad-, dsShd-, dsGFP-, dsSpo/Sad/Shd-* and *dsPhm/Dib*-injected females. (A) Cumulative percentage of copulated *dsSpo-*, *dsPhm-*, *dsDib-*, *dsSad-*, *dsShd-* and *dsGFP*-injected female locusts is presented. The numbers of female locusts which copulated (per treatment condition) are: *dsSpo* N = 19/20 (95%), *dsPhm* N = 9/9 (100%), *dsDib* N = 13/13 (100%), *dsSad* N = 16/16 (100%), *dsShd* N = 19/19 (100%) and *dsGFP* N = 17/18 (95%). **(B)** Cumulative percentage of copulated *dsSpo/Sad/Shd-*, *dsPhm/Dib-* and *dsGFP*-injected female locusts is presented. The numbers of female locusts which copulated (per treatment condition) are: *dsSpo/Sad/Shd* N = 21, *dsPhm/Dib* N = 23 and *dsGFP* N = 24.



Supplementary Figure S12. Transcript levels of *Halloween* genes (*SchgrSpo, SchgrPhm, SchgrDib, SchgrSad* and *SchgrShd*) in ovaries of *dsSpo*/*Sad*/*Shd*- and *dsPhm*/*Dib*-injected adult female locusts. (A1-2) Relative *SchgrSpo* transcript levels measured in the ovaries of *dsSpo*/*Sad*/*Shd*- and *dsPhm*/*Dib*-treated females, respectively. **(B1-2)** Relative *SchgrPhm* transcript levels measured in the ovaries of *dsSpo*/*Sad*/*Shd*- and *dsPhm*/*Dib*-treated females, respectively. **(C1-2)** Relative *SchgrDib* transcript levels measured in the ovaries of *dsSpo*/*Sad*/*Shd*- and *dsPhm*/*Dib*-treated females, respectively. **(D1-2)** Relative *SchgrSad* transcript levels measured in the ovaries of *dsSpo*/*Sad*/*Shd*- and *dsPhm*/*Dib*-treated females, respectively. **(E1-2)** Relative *SchgrShd* transcript levels measured in the ovaries of *dsSpo*/*Sad*/*Shd*- and *dsPhm*/*Dib*-treated females, respectively. All relative transcript levels were measured in the ovaries of female locusts twelve days after the final moult using qRT-PCR. The data are presented as mean ± S.E.M of five independent pools of three locusts, run in duplicate and normalized to *EF1α* and *ribosomal protein 49* (*RP49*); each dot represents one data point. Significant differences in comparison

with *dsGFP*-injected females are indicated by asterisks (**** $p < 0.0001$ and *** $p < 0.001$; two-sided Unpaired *t*-test on log-transformed data including the Welch's correction for the comparison of *SchgrShd* transcript levels in *dsSpo*/*Sad*/*Shd*- and *dsGFP*-treated females (E1)).



Supplementary Figure S13. Transcript levels of genes involved in Vg synthesis in the fat body and JH signalling in the fat body and ovaries of *dsSpo*/*Sad*/*Shd*- and *dsPhm*/*Dib*-injected adult female locusts. (A1-2) Relative *SchgrMet*

transcript levels measured in the ovaries of *dsSpo*/*Sad*/*Shd*- and *dsPhm*/*Dib*-treated females, respectively. **(B1-2)** Relative *SchgrKr-h1* transcript levels measured in the ovaries of *dsSpo*/*Sad*/*Shd*- and *dsPhm*/*Dib*-treated females, respectively **(C1-2)** Relative *SchgrMet* transcript levels measured in the fat body of *dsSpo*/*Sad*/*Shd*- and *dsPhm*/*Dib*-treated females, respectively. **(D1-2)** Relative *SchgrKr-h1* transcript levels measured in the fat body of *dsSpo*/*Sad*/*Shd*- and *dsPhm*/*Dib*-treated females, respectively. **(E1-2)** Relative *SchgrVg1* transcript levels measured in the fat body of *dsSpo*/*Sad*/*Shd*- and *dsPhm*/*Dib*-treated females, respectively. **(F1-2)** Relative *SchgrVg2* transcript levels measured in the fat body of *dsSpo*/*Sad*/*Shd*- and *dsPhm*/*Dib*-treated females, respectively. All relative transcript levels were measured in the ovaries of female locusts twelve days after the final moult using qRT-PCR. The data represent the mean ± S.E.M of five independent pools of three locusts, run in duplicate and normalized to *RP49* and *EF1α* transcript levels in the fat body and ovaries; each dot represents one data point. No significant differences were observed in comparison with *dsGFP*-injected females (two-sided Unpaired *t*-test on log-transformed data including the Welsch's correction for the comparison of *SchgrKr-h1* transcript levels in the fat body of *dsPhm*/*Dib*- and *dsGFP*-treated females (D2)).