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RNA interference-mediated knock-down of *Bla g 1* in the German cockroach, *Blattella germanica* L., implicates this allergen-encoding gene in digestion and nutrient absorption

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Abstract

We used RNA interference (RNAi) to silence the expression of a gene encoding Bla g 1, a human allergen produced by the German cockroach, *Blattella germanica* L., to study its function in cockroach physiology. Females injected with 1 μg of double-stranded RNA contained 64% less Bla g 1 protein and Bla g 1 mRNA abundance was reduced by 91.4% compared to sham-injected females. Bla g 1 knockdown slowed the pace of weight gain, midgut growth, and colleterial gland and basal oocyte maturation, resulting in delayed egg case formation and lower fecundity. Exogenous juvenile hormone treatments rescued reproduction in RNAi-treated females, suggesting that Bla g 1 silencing lowered endogenous juvenile hormone, probably by reducing food intake and nutrient absorption.

Keywords: *Blattella germanica*, RNA interference, midgut protein, Bla g 1, allergen.

Introduction

The German cockroach, *Blattella germanica*, is a major economically and medically important pest worldwide. It is closely associated with human-built structures in agricultural and urban environments and is an important and significant source of indoor allergens not only in inner-city homes, but also in rural settings, usually in association with lower socioeconomic housing (Salo *et al.*, 2008). Potent allergens produced by *B. germanica* have been associated with the development and aggravation of asthma and other respiratory diseases, especially in closed environments (Call *et al.*, 1992; Gelber *et al.*, 1993; Rosenstreich *et al.*, 1997; Eggleston *et al.*, 1998; Chew *et al.*, 2008). In *B. germanica*, seven allergens have been characterized and found in various tissues of males and females (reviewed in Gore & Schal, 2007; Jeong *et al.*, 2007; Pomés *et al.*, 2007). Amongst these allergens, *B. germanica* 1 (Bla g 1; following the International Union of Immunological Societies Allergen Nomenclature Sub-Committee, http://www.allergen.org/Allergen.aspx) is of considerable importance because it is found in large amounts in the faeces of both sexes and all life stages of cockroaches, thereby facilitating its exposure and contact with humans (Gore & Schal, 2004, 2005). Bla g 1 is antigenically cross-reactive with *Periplaneta americana* allergen 1 (Per a 1), with which it shares 70–72% amino acid sequence homology, and both allergens have been classified as group 1 allergens, characterized by a unique repeat of 100 amino acid residues (Pomés *et al.*, 1998; Melén *et al.*, 1999).

Bla g 1 is found primarily in the cockroach midgut and its production has been found to be directly modulated by food consumption (Gore & Schal, 2004; 2005). Food consumption in adult females is generally higher than in adult males (Hamilton & Schal, 1988) resulting in significantly higher levels of Bla g 1 in faeces produced by females (Gore & Schal, 2005). During the first 5 days of the female gonadotrophic cycle, Bla g 1 levels and food consumption increase in concert with vitellogenesis, decrease until oviposition (day 9) and remain low during a three-week pregnancy (Gore & Schal, 2005). The strong association of Bla g 1 with midgut tissues and its high amino acid sequence homology to the mosquito midgut protein AEG12, which is thought to be involved in digestion (Shao *et al.*, 2005), has led to the hypothesis that Bla g 1 might be an important

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enzymatic and/or structural protein involved in food digestion in *B. germanica* (Pomés *et al*., 1998); however, its function is still largely unknown.

We report, for the first time, knockdown of the expression of a major allergen-coding gene in *B. germanica* by systemic RNA interference (RNAi) and describe its physiological effects with the goal to understand better the role of Bla g 1 in the physiology of *B. germanica*. We also show that RNAi offers a simple yet potent and highly specific approach to investigate the function of allergens in arthropods.

Results

Double-stranded RNA complementary to Bla g 1 silences Bla g 1 *expression*

To test whether *Bla g 1* could be silenced by RNAi, a dose-response curve of *in vivo* injected Bla g 1 double-stranded RNA (dsRNA) was established using newly eclosed *B. germanica* females. Bla g 1 titres were determined by enzyme linked immunosorbent assay (ELISA) on day 6. The Bla g 1 titres were significantly more suppressed as more Bla g 1 dsRNA was injected ($F_{4,29} = 5.88$, $P = 0.0018$), with the lowest Bla g 1 protein titre (70.2% lower than in sham-treated females) found with 1 μg dsRNA (Fig. 1).

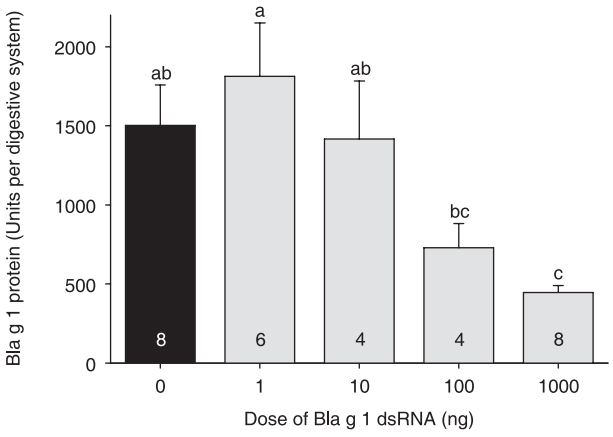

Figure 1. Effects of various doses of Bla g 1 double-stranded RNA (dsRNA) on Bla g 1 protein in adult female *Blattella germanica*. Newly emerged females were injected with 1 μl diethyl pyrocarbonate (DEPC)-treated water (sham-treated control, black bar), or 1 to 1000 ng Bla g 1 dsRNA in 1 μl DEPC-treated water. Six days after injection the whole digestive system was removed, Bla g 1 protein extracted, and its titre analysed by enzyme linked immunosorbent assay. Variation is represented by SEM and numbers within bars correspond to sample size. Each sample corresponds to a replicate within a treatment. Treatments with the same letter are not significantly different ($P > 0.05$, ANOVA and Fisher's least square difference test).

Bla g 1 production is suppressed in starved females (Gore & Schal, 2005). We used a dose of 1 μg dsRNA to assess the extent of Bla g 1 suppression in 6-day-old females relative to newly eclosed and 6-day starved females. Bla g 1 protein in sham-injected females increased ninefold by day 6 relative to the baseline level of newly eclosed females [$F_{3,78} = 31.76$, $P < 0.001$; Fisher's least square difference (LSD) test; Fig. 2]. Starved females, in contrast, accumulated much less Bla g 1 protein in their digestive system than fed females and their Bla g 1 titre was not significantly different from that of newly eclosed females. Females injected with 1 μg dsRNA accumulated an intermediate amount of Bla g 1 protein on day 6, only 35.7% that of sham-treated females ($P < 0.01$), but significantly higher (3.4-fold) than newly eclosed females. As the Bla g 1 titre in RNAi-mediated Bla g 1 knockdown females was not significantly different from

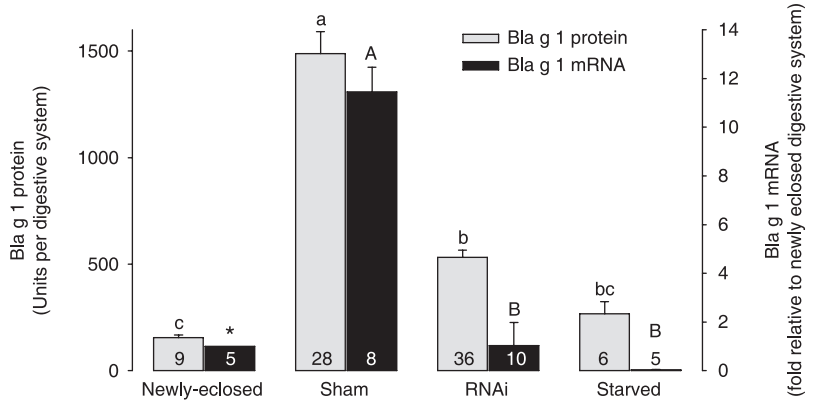

Figure 2. Effects of RNA interference (RNAi)-mediated knockdown of Bla g 1 on amounts of Bla g 1 protein and Bla g 1 mRNA abundance in the digestive system of adult female *Blattella germanica*. Bla g 1 protein titres, as determined by enzyme linked immunosorbent assay (in grey), in newly emerged females and 6-day-old females that were injected on day 0 with either 1 μl diethyl pyrocarbonate (DEPC)-treated water (sham-treated control) or 1 μg double-stranded RNA in DEPC-treated water (RNAi). Some females were not injected, but starved between days 0 and 6. Relative changes in the abundance of Bla g 1 mRNA, as determined by quantitative real-time PCR, are shown in black. Measurements were in 5-day-old females treated as above. Five-day-old females were used because the levels of Bla g 1 mRNA decline after day 5 (Gore & Schal, 2005). Relative mRNA abundance for newly eclosed females was set to 1 (indicated by *). Variation is represented by SEM, and number of individuals tested is shown within bars. Treatments with the same letter for Bla g 1 protein titres (lower case letters) and for mRNA abundance (upper case letters) are not significantly different ($P > 0.05$, ANOVA and Fisher's least square difference test).

that of starved females, it appeared that 1 μg of Bla g 1 dsRNA effectively silenced Bla g 1 expression during the 6-day experiment.

To confirm gene knockdown, Bla g 1 mRNA abundance was determined using quantitative real-time PCR (qRT-PCR) in similarly treated females, but on day 5 rather than on day 6 because Bla g 1 mRNA declines after day 5 (Gore & Schal, 2005). The relative abundance of Bla g 1 mRNA corresponded well with the Bla g 1 protein titres (Fig. 2), showing a significant 9.2-fold elevation in sham-treated females relative to newly eclosed females (set at 1.0) ($F_{2,22} = 26.3$, $P < 0.001$; Fisher's LSD test). However, females injected with 1 μg of dsRNA had 11.6-fold less Bla g 1 transcript than sham-treated females, and they did not differ significantly from 5-day-old starved females (Fig. 2), indicating that systemic RNAi completely silenced the post-transcriptional accumulation of Bla g 1 mRNA.

To validate that Bla g 1 silencing was specific to this gene and not the result of systemic toxicity, we injected females with dsRNA complementary to the exogenous enhanced green fluorescence protein (EGFP) gene. Neither the titre of the Bla g 1 protein nor the abundance of Bla g 1 mRNA were affected by EGFP dsRNA injection; EGFP dsRNA-treated females and sham-treated females were not significantly different from each other, but both sets were significantly different from Bla g 1 dsRNA-treated females (Table 1; $F_{2,15} = 35.18$; $P < 0.01$ for Bla g 1 protein; $F_{2,11} = 19.53$; $P < 0.01$ for mRNA abundance). These results confirm that the reduction of Bla g 1 protein in Bla g 1 dsRNA-treated females was the result of the specific effect of silencing Bla g 1 expression and not general toxicity or nontarget effects of the Bla g 1 dsRNA injections.

Physiological effects of Bla g 1 silencing

Treatment with Bla g 1 dsRNA had diverse effects on the digestive and reproductive physiology of adult females. With increasing Bla g 1 dsRNA dose, females gained less mass between days 0 and 6, their midgut remained undeveloped and their gonadotrophic cycle was slowed, resulting in smaller basal oocytes and left colleterial gland (Figs 3 & 4). As with Bla g 1 protein, a dose of 1 μg dsRNA produced the greatest response, resulting in 27.5% less mass gained than in sham-treated females, 27.4% thinner midgut, 35.2% shorter basal oocytes and 45.5% thinner distal tubules of the colleterial gland than in sham-treated females. Figure 4 compares a typical Bla g 1-RNAi female and a sham-treated female. The foregut of RNAi knockdown females was distended, yet often empty, indicating that less food was ingested and less food was being processed in more posterior regions of the digestive system. The basal oocytes accumulated significantly less yolk proteins and the left colleterial gland accumulated less protein than in sham-treated control females (Fig. 4).

The relationships between oocyte maturation and Bla g 1 transcript and protein concentration were examined for each RNAi- and sham-treated female. Although there was extensive overlap in oocyte length in RNAi- and sham-treated females, silencing of the *Bla g 1* gene generally kept the Bla g 1 protein titre below 1000 U/digestive tract (Fig. 5A). In sham-treated females, however, an increase in Bla g 1 protein was evident in relation to oocyte growth, followed by a decline, as previously shown (Gore & Schal, 2005). The pattern of Bla g 1 transcript abundance showed complete knockdown in day 5 RNAi-treated females, but an increase with increasing oocyte size in sham-treated females (Fig. 5B). These results show that some oocyte maturation can occur in *Bla g 1*-silenced females, despite very low Bla g 1 protein titres.

Because oocyte maturation was slowed in dsRNA-injected females (Fig. 3), we also sought to determine whether this effect would persist and affect the time to oviposition and egg case formation. Sham-treated females began to form egg cases 8 days after emergence (three of 15 females) and by day 12 all females had fully formed egg cases (Fig. 6). In the Bla g 1 RNAi knockdown group, egg case formation was first observed in one out of 15 females on day 9, and all females completed egg case formation by day 15. Thus, on average, sham-treated females produced an egg case 9.8 ± 0.31 (SEM) days after eclosion,

Table 1. Validation of the specific effect of double-stranded RNA (dsRNA)-mediated silencing of Bla g 1 expression, using exogenous enhanced green fluorescence protein (EGFP) dsRNA

	Day 6 females†			Day 5 females†		
Injection treatment*	*n*	Bla g 1 protein titre (units per digestive system)	Oocyte length (mm)	*n*	mRNA fold expression	Oocyte length (mm)
Sham	4	1682.7 ± 364.5[a]	1.43 ± 0.18[a]	4	15.90 ± 5.02[a]	1.22 ± 0.08[a]
EGFP dsRNA	4	1505.7 ± 260.5[a]	1.31 ± 0.12[a]	4	15.19 ± 3.81[a]	1.17 ± 0.10[a]
Bla g 1 dsRNA	8	635.9 ± 112.3[b]	0.89 ± 0.26[b]	4	1.58 ± 0.44[b]	0.86 ± 0.09[b]

*Newly emerged adult females were injected with diethyl pyrocarbonate (DEPC)-treated water (sham), 1 μg EGFP dsRNA or 1 μg Bla g 1 dsRNA. Bla g 1 protein titre and Bla g 1 mRNA abundance were measured 6 and 5 days after injection, respectively.
†Values represent mean ± SEM. Means followed with the same letter, within a column, are not significantly different ($P < 0.05$; Fisher's least square difference test).

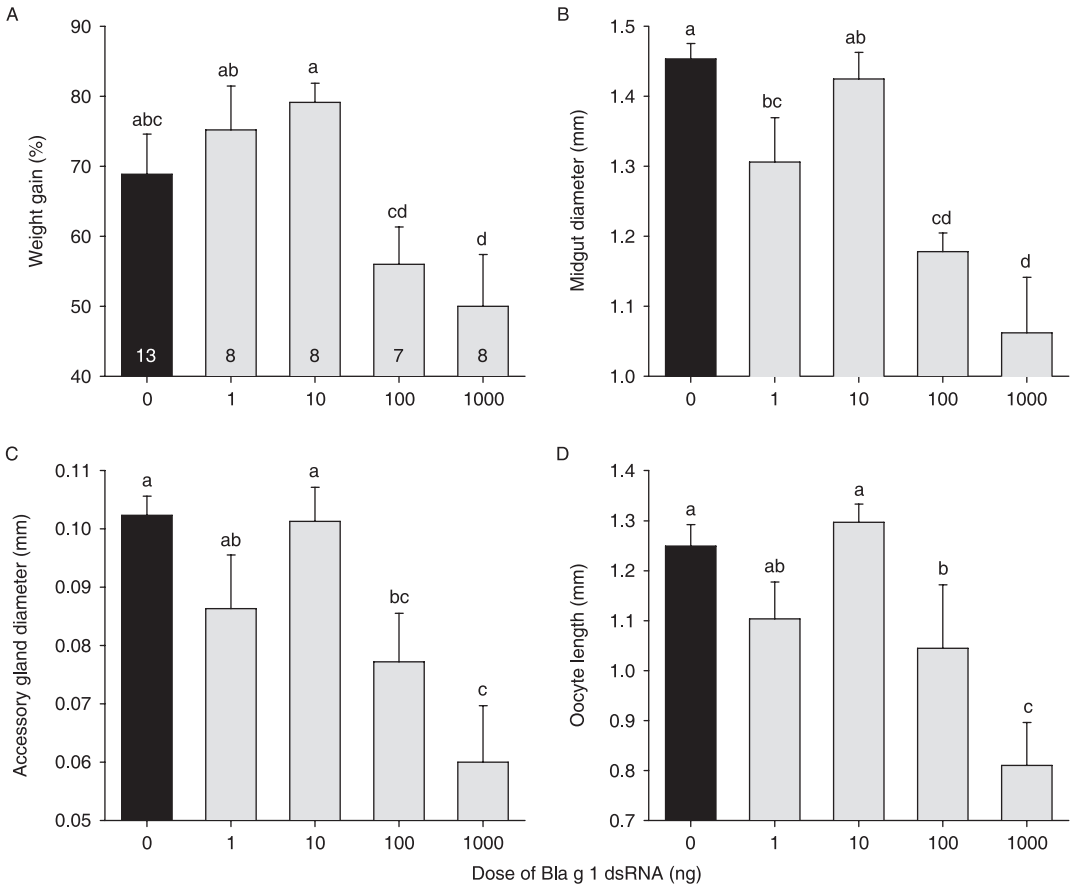

Figure 3. Effects of various doses of Bla g 1 double-stranded RNA (dsRNA) on the digestive and reproductive physiology of adult female *Blattella germanica*. Newly emerged females were injected with 1 μl diethyl pyrocarbonate (DEPC)-treated water (sham-treated control, black bar), or 1 to 1000 ng dsRNA in DEPC-treated water (RNA interference). Six days after injection the following parameters were measured: (A) change in fresh body mass; (B) midgut diameter; (C) width of the apical tubes of the left colleterial gland; and (D) basal oocyte length. Variation is represented by SEM, and numbers within bars in (A) correspond to sample size for each treatment. Each individual female within each treatment was treated as a replicate. Treatments with the same letter are not significantly different ($P > 0.05$, ANOVA and Fisher's least square difference test).

whereas RNAi-treated females formed the egg case a day later, at 10.8 ± 0.36 days ($n = 15$ females for each treatment; $P < 0.05$, Student's *t*-test).

Bla g 1 knockdown also resulted in fewer nymphs produced by RNAi-treated females (39.5 ± 1.79) than by sham-treated females (43.3 ± 1.08) ($P < 0.05$; Student's *t*-test), supporting the general pattern that Bla g 1 dsRNA injections suppressed the processing of nutrients into oocyte provisions. The duration of embryogenesis, however, was unaffected by Bla g 1 knockdown, as sham-treated and RNAi-treated females underwent the same period of gestation (22.2 ± 0.11 and 21.8 ± 1.72 days, respectively; $P > 0.05$; Student's *t*-test).

RNAi of Bla g 1 expression resulted in global effects on digestive and reproductive physiology of *B. germanica*. We hypothesized that these effects were because of the combined results of (1) suppressed digestion and absorption of food, and (2) lower ingestion of food. All of these outcomes would inhibit juvenile hormone (JH) production in females (Schal *et al*., 1997; Maestro *et al*., 2009), and hence also all JH-dependent reproductive events – including oocyte and colleterial gland maturation – and ultimately, oviposition. Therefore, we measured food intake, and the effect of JH on Bla g 1 RNAi knockdown females.

Effect of Bla g 1 silencing on feeding

The effect of Bla g 1 knockdown on feeding was measured indirectly by the average faecal output per female per day. Sham-treated females exhibited a cycle of faeces production (Fig. 7) similar to that reported by Gore & Schal (2005), but of lower magnitude, probably reflecting slight differences in diet and rearing and experimental conditions. Although feeding was not completely suppressed in RNAi-treated females, these females produced significantly less faeces between days 1 and 2 than did sham-treated females. After day 3, however, faecal outputs from sham- and RNAi-treated females were not significantly different (Student's-*t* tests, $P > 0.05$).

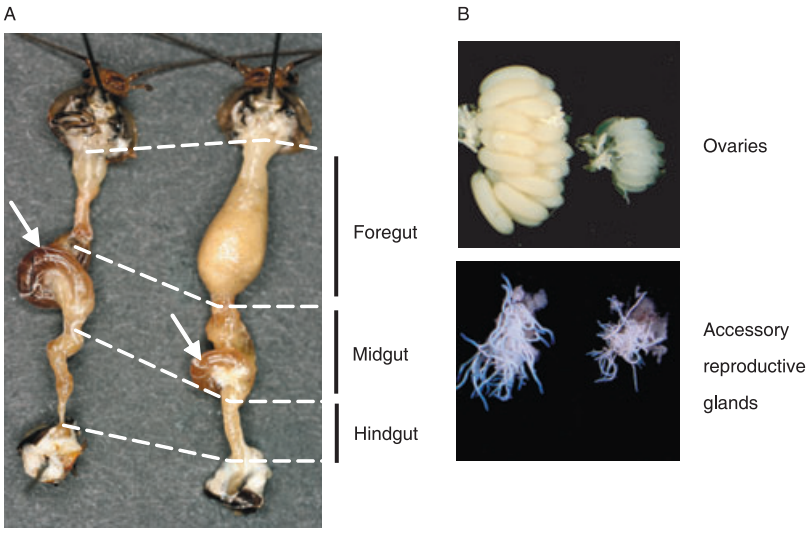

Figure 4. Effects of RNA interference (RNAi)-mediated Bla g 1 knockdown on the digestive and reproductive systems of *Blattella germanica*. (A) Digestive system of 5-day-old sham-treated female (left) and Bla g 1 RNAi-treated female (right). Arrows indicate the location where the midgut diameter was measured. (B) Reproductive tissues of 5-day-old sham-treated (left) and RNAi-treated females (right).

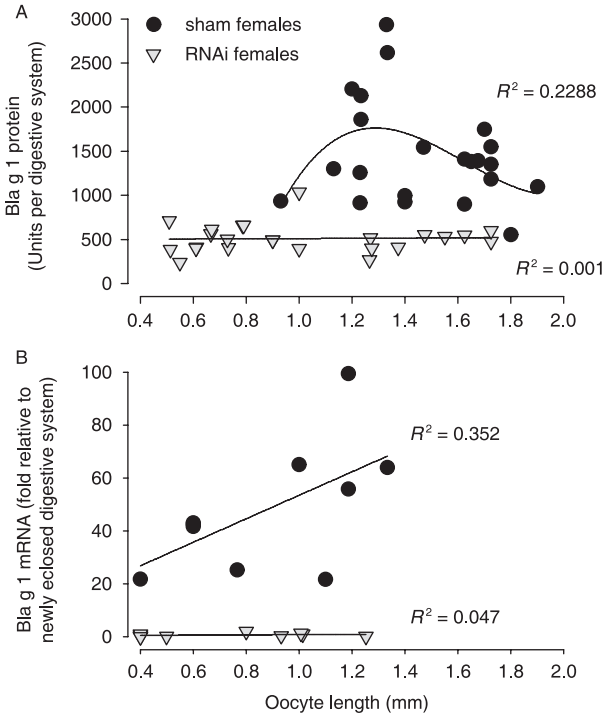

Figure 5. Effects of RNA interference (RNAi)-mediated knockdown of Bla g 1 on amounts of Bla g 1 protein and Bla g 1 transcript relative to oocyte maturation in adult female *Blattella germanica*. (A) Bla g 1 protein, as determined by enzyme linked immunosorbent assay, and oocyte length in 6-day-old females that were injected on day 0 with either 1 μl diethyl pyrocarbonate (DEPC)-treated water (sham-treated control, circles) or 1 μg Bla g 1 double-stranded RNA in DEPC-treated water (RNAi, triangles). (B) Relative changes in the abundance of Bla g 1 mRNA as determined by quantitative real-time PCR, and oocyte length, in 5-day-old females treated as in (A). Each point represents one female.

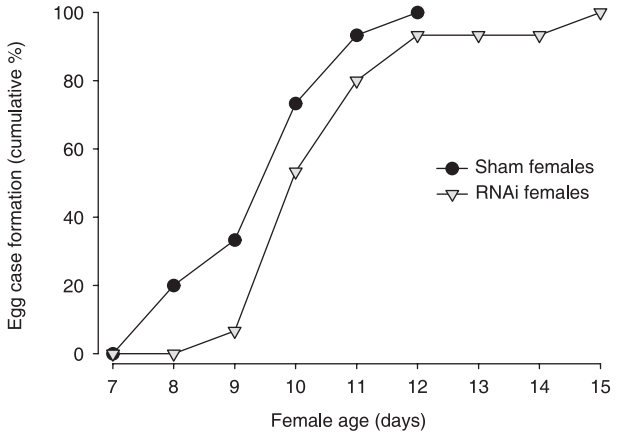

Figure 6. Effect of Bla g 1 knockdown in newly eclosed female *Blattella germanica* on egg case formation. Cumulative daily percentage egg cases formed in sham-treated (injected with 1 μl diethyl pyrocarbonate (DEPC)-treated water; circles; $n = 15$) and in RNA interference (RNAi)-treated females (injected with 1 μg Bla g 1 double-stranded RNA in DEPC-treated water; triangles; $n = 15$).

JH rescue of reproduction in RNAi-treated females

Bla g 1 is expressed in the midgut and Bla g 1 gene knockdown clearly affected metabolic processes in *B. germanica* females (lower food intake, smaller midgut and body mass). As food intake profoundly influences JH production in *B. germanica* (Schal *et al.*, 1993; Maestro *et al.*, 2009), we reasoned that the reproductive effects that we observed (slower oocyte and colleterial gland maturation and oviposition) were not direct effects of RNAi, but rather the indirect effects of lower food intake, and possibly food absorption, on JH production. Therefore, we attempted to decouple digestive and reproductive events with topical applications of JH. Application of two consecutive doses of 10 μg JHIII to RNAi-treated females completely restored oocyte maturation in 6-day-old females to the level of sham-treated females (Fig. 8). These results suggest that a low level of endogenous JH was limiting oocyte growth and development in RNAi-treated females that did not receive exogenous JH. To confirm *Bla g 1* gene knockdown, Bla g 1 mRNA transcript abundance was measured using

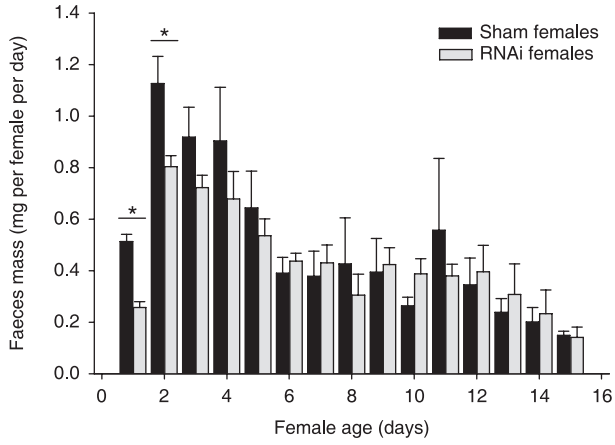

Figure 7. Daily faeces production by adult *Blattella germanica* females. Sham-treated control females (black bars) were injected with 1 μl diethyl pyrocarbonate (DEPC)-treated water, and RNA interference (RNAi)-treated females (grey bars) were injected with 1 μg Bla g 1 double-stranded RNA in DEPC-treated water on day 0. Faeces was removed daily, weighed, and adjusted on a per female basis. Means represent four replications of 20 females per replication ± SEM. Significant differences between sham- and RNAi-treated females are shown by an asterisk above the bars ($P < 0.05$, Student's paired *t*-test).

qRT-PCR in sham-, RNAi- and RNAi + JH-treated females, using the mRNA level of newly eclosed females as a baseline. JH treatments did not interfere with RNAi knockdown of Bla g 1, as levels of Bla g 1 mRNA in all RNAi-treated females, including those receiving topical JH applications, were significantly lower than in sham-treated females ($F_{3,14} = 16.7$, $P < 0.01$; Fisher's LSD test), but not significantly different from newly eclosed females (Fig. 8). These data confirm the hypothesis that RNAi-mediated knockdown of the *Bla g 1* gene directly affects digestive physiology, which in turn results in a cascade of indirect effects on reproduction through partial inhibition of JH production by the corpora allata.

Discussion

We report, for the first time, the successful knockdown of expression of an allergen-encoding gene, *Bla g 1*, in an arthropod by RNA interference, and its effects on the digestive and reproductive physiology of *B. germanica*. Injection of 1 μg Bla g 1 dsRNA resulted in complete post-transcriptional silencing of Bla g 1 mRNA. However, some Bla g 1 protein remained in the midgut of knockdown females, probably because adults eclosed with some Bla g 1 protein in the midgut and less Bla g 1 was lost in faeces as less food transited through the digestive system of Bla g 1 knockdown females. Nevertheless, although the Bla g 1 protein was not completely eliminated by RNAi, this procedure produced a distinctive, unmistakable 'starvation phenotype'. This phenotype was characterized by lower food intake and less faeces defecated, and a smaller midgut, often containing undigested food even when the foregut was bloated with air and contained no food. Thus, Bla g 1 appears to serve a role in food processing and/or nutrient absorption in the midgut and gastric caecae.

Pomés *et al.* (1998) suggested that Bla g 1 could be a digestive and/or structural protein based on amino acid sequence and protein structure data. Bla g 1 is expressed exclusively in the midgut and gastric caecae of *B. germanica* (Gore & Schal, 2004) and its expression is modulated by food intake (Gore & Schal, 2005). Its high amino acid sequence homology with ANG12, a midgut protein of *Anopheles gambiae* that is expressed after a bloodmeal,

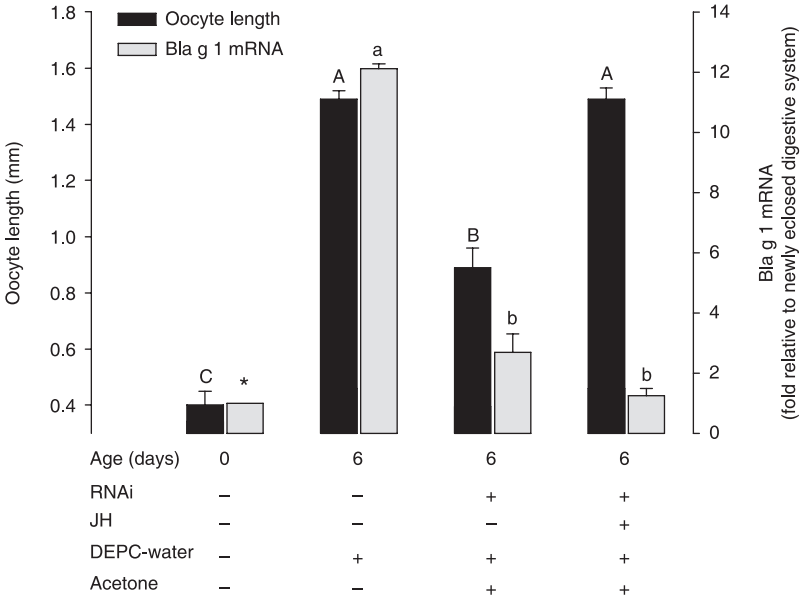

Figure 8. Effects of Bla g 1 RNA interference (RNAi) and exogenous juvenile hormone (JH) on oocyte maturation and Bla g 1 mRNA relative transcript abundance in *Blatella germanica* females. Freshly eclosed females were injected with 1 μg Bla g 1 double-stranded RNA in diethyl pyrocarbonate (DEPC)-treated water (or only DEPC-treated water as sham-treated controls) and topically treated with 10 μg JHIII in 1 μl acetone (or acetone alone as controls); the JH (and acetone) treatment was repeated 3 days later. Females were dissected on day 6, their basal oocytes measured (black bars; $n = 15$ per treatment), and their midgut removed for RNA extraction. Quantification of Bla g 1 mRNA was by quantitative real-time PCR (grey bars; $n = 5$ per treatment). mRNA from newly eclosed females (set at 1.0 and indicated by *) and from a reference gene (actin) was used to calculate the relative Bla g 1 mRNA abundance. Variation is represented by SEM. Individual samples were treated as a replicate. Treatments with the same letter are not significantly different (lower case and upper case letters for mRNA and oocyte length, respectively; $P > 0.05$, ANOVA and Fisher's least square difference test).

and more recently, with a microvillar protein of *Tenebrio molitor* (Ferreira *et al*., 2007) and the sand fly *Lutzomyia longipalpis*, which is also expressed after a bloodmeal (Jochim *et al*., 2008), suggests that Bla g 1 may also be a microvillar protein. It is therefore not surprising that its knockdown with RNAi resulted in wide-ranging disruption of digestion and reproduction in *B. germanica*.

Complete silencing of Bla g 1 mRNA expression in female cockroaches injected with 1 μg dsRNA complementary to *Bla g 1* resulted in significant reductions, but not complete suppression of food intake and digestion. These results suggest that (1) females can function reasonably well without Bla g 1 or with the little Bla g 1 protein that they contain at adult emergence, or (2) the RNAi-mediated knockdown operated transiently and its effect was relaxed after day 5. Based on our results, however, we reject the second hypothesis. Although the gonadotrophic cycle was delayed in RNAi-treated females, allowing them time to recover from the RNAi effect, critical differences between treated and sham control females began to vanish before day 5, at the same time that the RNAi-mediated silencing of Bla g 1 mRNA remained maximal. For instance, faeces output, a combined measure of food consumption and food processing through the digestive system, was significantly reduced only during the first 2 days of the first gonadotrophic cycle. Although faeces production continued to be generally lower in RNAi-treated females throughout the preoviposition period, it was not entirely suppressed even though Bla g 1 mRNA was completely silenced until at least day 5. Again, this suggests that some food was digested in RNAi-treated females, allowing more food consumption, vitellogenesis and some oocyte maturation. Future experiments to test the first hypothesis should attempt to eliminate all the Bla g 1 protein from females. This might be achieved with repeated RNAi knockdown of Bla g 1 in earlier instars and adults, which might remove the Bla g 1 protein with which adults emerge. For example, with repeated systemic injections of target of rapamycin dsRNA into fifth and sixth (last) instars of *B. germanica*, Maestro *et al.* (2009) were able to impose severe, starvation-like inhibition on JH production in adult females. The design of experiments based on RNAi knockdown of Bla g 1 in earlier instars is complicated, however, because our preliminary data show that a single injection of 1 μg Bla g 1 dsRNA completely suppresses feeding in last instar nymphs and all nymphs die before reaching the adult moult (A. Suazo & C. Schal, unpubl. data).

Blattella germanica exhibits an anautogenous strategy, requiring food before the female can reproduce (review: Schal *et al*., 1997). Food acts in two major ways: metabolically, food provides nutrients that can be processed, mobilized and provisioned into eggs, and endocrinologically, it triggers the corpora allata to produce JH that in turn paces the synthesis, mobilization and internalization of yolk proteins into oocytes. The rate of JH production by the corpora allata is affected by a multitude of both intrinsic and extrinsic factors. Primary amongst the internal factors is the nutritional condition of the female. Both the quantity and the quality of food consumed affect the level of activity of the corpora allata, which in turn paces the reproductive rate (Schal *et al*., 1993; Maestro *et al*., 2009). Thus, whereas the smaller midgut, lower weight gain, lower faeces production and fewer offspring are likely to be direct effects of interference with Bla g 1 expression, the effects on reproduction (smaller distal tubules of the left accessory gland, smaller basal oocytes and delayed oviposition) are much more likely to be mediated by indirect effects whereby the deficient nutrient milieu in the haemolymph restrains JH production by the corpora allata. Indeed, rescue experiments, in which RNAi-treated females were also treated with JH, clearly showed that the reproductive effects of RNAi of Bla g 1 could be uncoupled from the digestive effects; the latter could not be rescued by JH.

In summary, our results thus far have shown that Bla g 1 is highly expressed in the midgut of all life stages of *B. germanica* (Gore & Schal, 2004). Expression studies have shown that the *Bla g 1* gene is highly responsive to food intake – its expression is greatly up-regulated with food consumption and silenced by starvation (Gore & Schal, 2005). Our preliminary results from microscopy studies support a role for Bla g 1 in the gastric microvilli, and hence in digestion and absorption of nutrients. The present study also shows that Bla g 1 is critical not only for digestive processes, but also for reproductive processes that depend upon food intake and nutrient processing and absorption. Finally, our experiments with dsEGFP injections confirm, as in previous research with *B. germanica* (eg Martín *et al*., 2006; Maestro *et al*., 2009), that *in vivo* injections of 1–2 μg dsRNA have gene-specific effects without systemic toxic or nontarget effects – Bla g 1 dsRNA injections specifically silenced the production of the Bla g 1 protein. These experiments also highlight the value of systemic *in vivo* RNAi knockdown in functional studies of *B. germanica* genes.

Experimental procedures

Insects

Newly emerged adult females of *B. germanica* were collected from an insecticide-susceptible laboratory colony maintained in incubators at 27 °C, variable ambient relative humidity (40–70%), and a 12:12 h light : dark cycle. Colonies were provided pelleted rat chow (Purina no. 5001; Purina Mills, St Louis, MO, USA) and water *ad libitum*. Experimental insects were maintained in pairs (one experimental and one control) in 95 × 15 mm Petri dishes under the same conditions. Females were maintained in pairs because isolation significantly retards JH production and delays oviposition (Gadot *et al*., 1989). In starvation experiments, females received only water and no food.

RNA extraction and dsRNA synthesis

Total RNA was extracted from midguts of 5-day-old females using the RNeasy Mini Kit (Qiagen, Valencia, CA, USA) following the manufacturer's recommendations, and quantified spectrophotometrically at 260 nm with a NanoDrop ND-1000 (NanoDrop Products, Wilmington, DE, USA). First strand cDNA was synthesized from 0.5 μg total RNA in 20 μl per reaction using the Bioline cDNA Synthesis Kit (Bioline, Tauton, MA, USA). First strand cDNA was amplified using primer sequences as described in Gore & Schal (2004), optimized to amplify a 617 bp fragment of the reported Bla g 1.0101 cDNA sequence (Pomés *et al.*, 1998, GenBank accession no. AF072219). The primer sequence included an additional 24-base sequence (5′-TTAATACGACTCACTATAGGGAGA-3′) added to the 5′ end corresponding to the T7 promoter sequence. Amplification of first strand cDNA was carried out in a total of 50 μl with 1.5 mM $MgCl_2$, 200 μM of each deoxynucleotide triphosphates, 1 μM of each primer, three units of Top Taq® DNA polymerase (Qiagen) and 5 μl synthesized first strand cDNA. Amplifications were performed in a PTC-200 Thermocycler (MJ Research/BioRad, Waltham, MA, USA) with an initial denaturation step of 94 °C for 3 min followed by 32 cycles of 94 °C for 45 s (denaturation), 57 °C for 30 s (annealing) and 72 °C for 45 s (extension) with a final extension at 72 °C for 10 min. To synthesize the Bla g 1 dsRNA, 8 μl PCR product from the first strand cDNA synthesis were transcribed in 20 μl reactions using the MEGAscript® T7 Kit for large scale synthesis of dsRNA (Ambion, Austin, TX, USA) according to the manufacturer's recommendations. Transcription products were cleaned using the MEGAclear® kit (Ambion), resuspended in diethyl pyrocarbonate (DEPC)-treated water and stored at −80 °C.

RNA interference

dsRNA complementary to *Bla g 1*, in DEPC-treated water, was injected into the haemocoel of cold-anaesthetized newly emerged adult females between the third and fourth abdominal sternites using a 10 μl glass syringe fitted with a 31-gauge bevelled tip needle (Hamilton, Reno, NV, USA). Abdominal injections are an easy and effective method to deliver dsRNA directly into the haemocoel. Control (sham) injections were made using only DEPC-treated water. In dose-response studies we injected 1 μl of 0 (sham-injected), 1, 10, 100 and 1000 ng/μl dsRNA. Four to eight individuals were used per treatment.

Quantitative real-time PCR

Bla g 1 mRNA abundance was assayed and its relative expression quantified from midgut tissues of 5-day-old females using qRT-PCR as described in Gore & Schal (2005). mRNA abundance from dsRNA-injected females was compared to mRNA in sham-treated control females and in 5-day-old starved females (provided water). Because Bla g 1 protein titres rise dramatically between days 0 and 5 in relation to food intake, but not in starved females (Gore & Schal, 2005), newly emerged and starved females represented baseline Bla g 1 mRNA levels. Primer sequences for the target gene (*Bla g 1*) and the reference gene (*actin*) are as previously reported (Gore & Schal, 2005).

Extraction, quantification and analysis of Bla g 1 protein

Bla g 1 protein titres were measured in sham-injected and Bla g 1-silenced females using a monoclonal capture and polyclonal detector ELISA (Indoor Biotechnologies, Charlottesville, VA, USA) (Pollart *et al.*, 1991). Five- and 6-day-old female *B. germanica* were cold-anaesthetized and the entire digestive system was removed in *Blattella* saline (Kurtti & Brooks, 1976), and homogenized in phosphate-buffered saline (PBS) with 0.05% Tween-20 and 1% bovine serum albumin (BSA, Sigma A-7030, St. Louis, MO, USA) (PBS-TB) as described in Gore & Schal (2004). Briefly, high-binding microtitre plates (Maxisorp flat well cert. Fisher Scientific, Waltham, MA, USA) were coated with 100 ng of anti-Bla g 1 monoclonal antibody in 100 μl of 50 mM carbonate bicarbonate buffer per well, incubated overnight at 4 °C, washed (3×) with PBS containing 0.05% Tween-20 (PBS-T, pH 7.4), and blocked at room temperature with 100 μl per well of PBS-TB for 30 min. After blocking, plates were washed (3×) with PBS-T and incubated for one hour with serial dilutions of extracts and Bla g 1 standards diluted in PBS-TB. Plates were washed (3×) with PBS-T, incubated with rabbit anti-Bla g 1 for 1 h (100 μl of 1:1000 diluted rabbit anti-Bla g 1 per well), washed (3×) with PBS-T and incubated for 1 h with peroxidase conjugated goat anti-rabbit IgG (Jackson Laboratories, West Grove, PA, USA). After a final wash (3×) with PBS-T, plates were developed with 100 μl of 1-step 2,2′-azinobis (3-ethylbenzothiazoline-6-sulphonic acid)-diammonium salt solution (Pierce, Rockford, IL, USA) and the optical density measured at 405 nm. Bla g 1 protein titres were calculated using reference curves established from twofold serial dilutions using an initial concentration of 1 U Bla g 1 standard per well (www.inbio.com/protocols.html).

Validation of Bla g 1 silencing

The specific effect of Bla g 1 silencing was validated using exogenous dsRNA from the EGFP gene. The pHStinger vector (kindly provided by Dr Patricia Estes, Genetics, North Carolina State University), which contained the complete sequence of the EGFP gene, was linearized with *Bam*HI for DNA amplification. To produce EGFP dsRNA, a 609 bp fragment of the EGFP gene was amplified using the primer sequences 5′-**TAATACGACTCACTATAGGGCGA**CACGAACTCCAGCAGGACCATG-3′ and 5′-**TAATACGACTCACTATAGGGCGA**CTGGTCGAGCTGGACGGCGACG-3′, which include the T7 promoter sequence (in bold). Amplification, transcription reactions and dsRNA purification were carried out as described for Bla g 1 dsRNA preparation. The EGFP dsRNA produced was of a size comparable to the Bla g 1 dsRNA. To validate Bla g 1 silencing, a treatment group of newly emerged females ($n = 4$–8 for each treatment) was injected with 1 μg each of Bla g 1 dsRNA, and control groups were injected with either EGFP dsRNA in 1 μl of DEPC-treated water or 1 μl of DEPC-treated water only. Females were dissected 6 days postinjection, their digestive system removed and Bla g 1 protein extracted and quantified as previously described. Basal oocyte length was also measured. Messenger RNA abundance was measured in 5-day-old females ($n = 4$ per treatment) and analysed as described above.

Physiological effects of Bla g 1 silencing

The effects of reducing Bla g 1 protein on cockroach physiology were studied in adult females. As Bla g 1 is associated with midgut tissues, is expressed exclusively in the midgut (Gore & Schal, 2004) and has been suggested to have a role as a digestive or structural midgut protein (Pomés *et al.*, 1998; Gore & Schal, 2007), the digestive system was examined in adult females for the effects of silencing Bla g 1. Moreover,

because we noted a general slowing of the reproductive cycle in Bla g 1 dsRNA-injected females, the reproductive system was also monitored. Newly emerged females were weighed, injected with 1 μg dsRNA and paired with a sham-injected control female. Cockroaches were provided food, water and a V-shaped piece of Whatman #1 filter paper (Whatman, Florham Park, NJ, USA) as shelter. Six days after injection, females were weighed again to determine mass gain, and dissected in *Blattella* saline. The following parameters were measured: (1) diameter of the midgut, taken at the midpoint between the gastric caecae and the hindgut's ileum; (2) basal oocyte length, a measure of cumulative exposure to JH and stage of the gonotrophic cycle; and (3) width of the tubes of the left colleterial (accessory) gland, which also responds to JH (Burns *et al*., 1991) and therefore also represents a measure of cumulative exposure to JH in adult females. In addition, the whole digestive system was removed, placed in 250 μl of PBS-TB and Bla g 1 protein was extracted and quantified by ELISA as described above.

Effect of Bla g 1 silencing on feeding

Daily faeces production was measured in sham- and dsRNA-injected adult females as an indirect measure of food consumption. Daily measurements were carried out because *B. germanica* engages in coprophagy (Kopanic *et al*., 2001), which might obscure patterns of faeces accumulation. Sham- ($n = 20$) and dsRNA-injected females ($n = 20$) were placed separately in four 150×15 mm Petri dishes (each group of 20 females per dish representing a replicate for a total of four replicates) on different days for a total of 80 cockroaches per treatment. Faeces was weighed and cockroach mortality determined daily for 15 days.

Effect of Bla g 1 silencing on female reproduction

To study the effect of Bla g 1 silencing on female fecundity, a sham- or dsRNA-injected female and two 10-day-old males were placed in a 95×15 mm plastic Petri dish with food, water and shelter. Fifteen females were used per treatment. Time for the female to form an egg case and mortality were recorded. Duration of embryogenesis and number of newly hatched first instar nymphs were also recorded for each female.

Effect of Bla g 1 silencing and exogenous JH on oocyte maturation

The effect of exogenous JH was evaluated by topically applying 1 μl of 10 μg/μl JHIII in acetone to females injected with 1 μg dsRNA in DEPC-treated water. As JH was diluted with acetone, a second set of RNAi-treated females was included in which 1 μl of acetone was topically applied as a control. Sham-treated females (1 μl of DEPC-treated water per female only) were also included as negative controls. Topical application was to the ventral side of the thorax between the coxae. Two applications were performed, one immediately after the first injection of dsRNA to newly eclosed females and the second 3 days later. Females ($n = 15$ per treatment) were placed in three separate Petri dishes with food and water. Females were dissected in *Blattella* saline on day 6, the length of three randomly selected basal oocytes was measured and averaged in each female and the midguts of five females from each treatment were removed for RNA extraction and qRT-PCR.

Statistical analyses

Analyses of variance (ANOVA; Proc GLM) were performed on Bla g 1 content of whole guts, weight gain, midgut and accessory gland diameters, oocyte length, and relative Bla g 1 mRNA expression levels to test for significance of variation amongst treatments. Mean values were compared and tested for significance using a Fisher's LSD test with significance level set at $\alpha = 0.05$. Statistical analyses were performed using SAS 8.2 (SAS institute, 2001, Cary, NC, USA).

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