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Tissue localization and regulation by juvenile hormone of human allergen Bla g 4 from the German cockroach, *Blattella germanica* (L.)

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Abstract

The German cockroach, *Blattella germanica* (L.), produces several potent protein aeroallergens, including Bla g 4, a ~20 kDa lipocalin. RT-PCR, Northern analyses and *in situ* hybridization showed that Bla g 4 is expressed only in the adult male reproductive system. Western blotting and ELISA with rBla g 4 antiserum detected immunoreactivity in the utricles and the conglobate gland, but not in other tissues of the male reproductive system. The Bla g 4 protein content of males increased from adult emergence to day 14, but during copulation Bla g 4 was depleted in the male and transferred to the female within the spermatophore. Topical application of juvenile hormone III stimulated Bla g 4 production by both conglobate gland and utricles.

Keywords: German cockroach, allergen, Bla g 4, lipocalin, juvenile hormone.

Introduction

The German cockroach, *Blattella germanica* (L.), is a synanthropic pest of considerable economic, veterinary and medical importance (Schal & Hamilton, 1990; Brenner, 1995). Populations of this cockroach are found only in human-made structures such as homes, food service facilities and various occupational environments, where potent allergenic proteins that they produce have been recognized for several decades as an important factor in the development of allergic disease (Kang, 1976; Kang *et al*., 1979). Pollart *et al*. (1989) established a clear association between exposure and sensitization to cockroach aeroallergens and acute asthma, and recent research in large urban areas in the United States documented that sensitization to respiratory aeroallergens from cockroach, combined with exposure to high levels of cockroach allergens in homes, is associated with high asthma morbidity in inner-city, African–American children from low-income families (Rosenstreich *et al*., 1997).

At least six allergenic proteins have been identified from the German cockroach. *Blattella germanica* allergen 4 (Bla g 4) was the first German cockroach allergen to be cloned. Like other German cockroach allergens, the Bla g 4 protein can elicit an allergic response in sensitized individuals. Using recombinant Bla g 4 (rBla g 4), expressed in *Escherichia coli* and *Pichia pastoris*, Vailes *et al*. (1998) showed that serum IgE antibody prevalence in cockroach allergic patients with asthma ranged from 40 to 60%. In addition, cockroach-allergic patients showed positive intradermal skin tests to rBla g 4 concentrations of 10^{-3}–10^{-5} μg/ml. The Bla g 4 cDNA consists of a 546 bp open reading frame, coding for a 182 amino acid protein (21 kDa) belonging to the lipocalin family (Arruda *et al*., 1995; Chapman *et al*., 1998; Mantyjarvi *et al*., 2000; Smith *et al*., 2000), which together with fatty acid binding proteins, avidins and metalloproteinase inhibitors forms the calycin protein superfamily of ligand-binding proteins (Flower *et al*., 2000).

Many, if not most, animal allergens are lipocalins, including dog, cow and horse epithelial allergens, and rat and mouse urinary allergens (Mantyjarvi *et al*., 2000). Although Bla g 4 shows only 19–24% homology to other lipocalins, it contains each of the three structurally conserved regions of the protein family (Arruda *et al*., 1995). Lipocalins serve diverse functions, and many lipocalin proteins carry small hydrophobic compounds through their aqueous environment. A number of colorant lipocalins, including insecticyanin, bilin-binding protein and gallerin, have been isolated from various invertebrates (Flower *et al*., 2000). A family of nutritive 'Milk' proteins, produced during gestation and provisioned to the embryos of the viviparous cockroach

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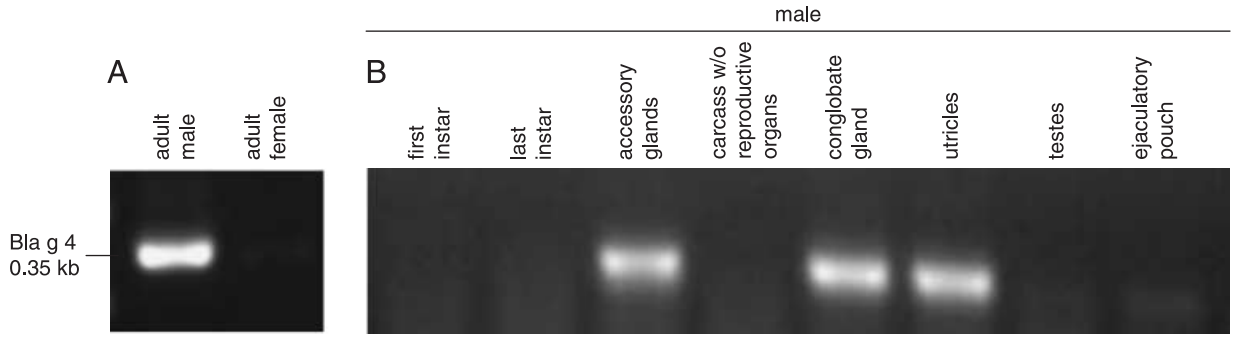

Figure 1. Bla g 4 expression analysis by RT-PCR of total RNA from German cockroaches. PCR amplification products were analysed by 1% agarose gel electrophoresis containing ethidium bromide. Bla g 4 expression is shown (A) in 14-day-old adult males and 6-day-old adult females, and (B) in first and last instar male nymphs and accessory tissues of the reproductive organs of 14-day-old adult males.

Diploptera punctata share the structurally conserved regions diagnostic of the lipocalin protein family (Williford *et al*., 2004). Nitrophorins, found in the saliva of *Rhodnius prolixus*, are lipocalins that transport nitric oxide to its host during blood feeding, resulting in vasodilation, sequestration of histamine and inhibition of blood coagulation (Ascenzi *et al*., 2002). Two recently identified lazarillo lipocalins in *Drosophila melanogaster* (DNLaz and DGLaz) have been implicated in axonal outgrowth and guidance in the embryonic nervous system (Sanchez *et al*., 2000). Korchi *et al*. (1999) found a lipocalin-like cuticular surface protein (Lma-P22) that is secreted by the epidermis of the adult male tergal glands in *Leucophaea maderae* and is thought to be involved in sexual behaviour. Although Bla g 4 and Lma-P22 share low sequence similarity, homology of Bla g 4 to mouse and rat urinary allergens, which serve as pheromone transport proteins in males, suggested that it might serve as a pheromone binding protein in German cockroaches (Arruda *et al*., 1995; Chapman *et al*., 1998).

Our analysis of the stage-, sex- and tissue-specific expression and localization of Bla g 4 shows that it is restricted to the reproductive system of adult male German cockroaches. In addition, we show that Bla g 4 is associated with the spermatophore, transferred to the female during copulation and developmentally regulated by juvenile hormone. Bla g 4 is now the first allergen from any indoor pest arthropod of major public health importance whose developmental and anatomical expression patterns, tissue localization and endocrine regulation have been thoroughly detailed.

Results

Developmental mRNA expression: RT-PCR and Northern blot hybridization

Reverse transcription-polymerase chain reaction (RT-PCR) using Bla g 4-specific primers was developed to identify whether the Bla g 4 transcript was stage- and sex-specific. Bla g 4 mRNA was found in adult males, but not in adult females (Fig. 1A). Because no transcripts were found in first and last instar males (Fig. 1B), expression of Bla g 4 was limited to adult males. These data were confirmed by Northern blot analysis. No hybridization to the Bla g 4 cDNA probe occurred with total RNA from three nymphal stages of males or from adult females (data not shown). A 546 bp transcript was found only in adult males, confirming that the Bla g 4 gene is expressed only in adult males.

Tissue localization of Bla g 4

Tissue-specific presence of Bla g 4 transcripts was determined by RT-PCR and further confirmed by Northern blot analysis. Analysis of total RNA extracted from whole males and dissected tissues indicated that a 546 bp transcript that corresponded to Bla g 4 was expressed only in the male's reproductive system (Fig. 1B). Careful dissection of reproductive organs revealed expression in some accessory reproductive glands, namely in the conglobate (= phallic) gland and the mushroom-shaped utricles (= utriculi breviores), but not in the testes, uricose glands (= utriculi majores), vas deferens, ejaculatory pouch or ejaculatory duct (Fig. 1B, and data not shown) (terminology of the male accessory reproductive organs is based on Feliubadaló *et al*., 1996; see Fig. 4 for photographs of structures).

Polyclonal antibody against rBla g 4 was generated in New Zealand white rabbits and its specificity for Bla g 4 was confirmed by Western blotting (Fig. 2). Protein extracts from conglobate gland, utricles, testes as well as rBla g 4 were separated on 15% SDS-PAGE (Fig. 2A) and probed with rBla g 4 antiserum (Fig. 2B). Recombinant Bla g 4 ran at an approximate molecular weight of 30 kDa, and a minor band, likely its dimer, ran at ~60 kDa; both bands were recognized by the rBla g 4 antiserum. In tissue extracts, only two bands, with molecular weights of 24.4 and 22.4 kDa, were recognized by rBla g 4 antiserum and these were found only in extracts of conglobate glands and utricles (Fig. 2B).

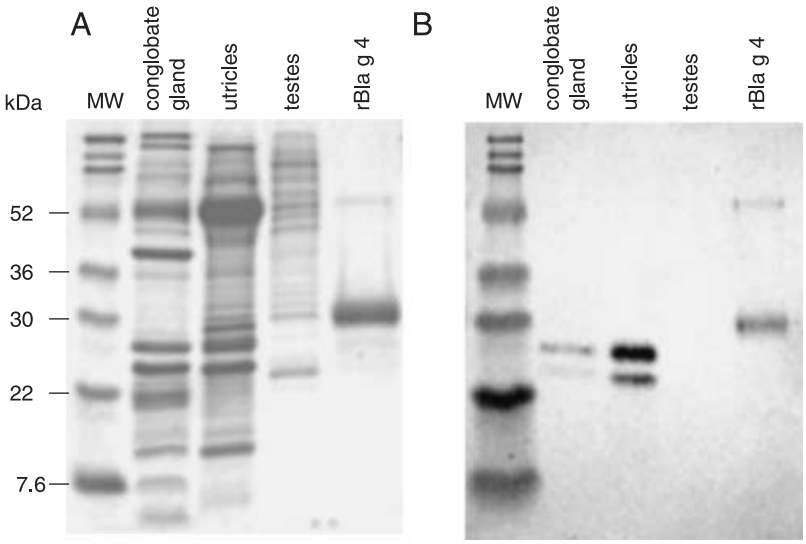

Figure 2. SDS-PAGE and immunoblot of adult male *B. germanica* reproductive tissues. Protein extracts from conglobate glands, utricles and testes, as well as rBla g 4 were (A) separated by 15% SDS-PAGE, immunoblotted on to nitrocellulose membrane, and (B) probed with antiserum against rBla g 4 protein, followed by a secondary antiserum conjugated to alkaline phosphatase. The antigen–antibody complex was visualized with pNPP.

rBla g 4 antiserum was used to develop a quantitative ELISA. As shown in Fig. 3A, whole body extracts of adult males contained 29.9 ± 0.50 μg (n = 6) of Bla g 4 protein, whereas adult females contained no measurable Bla g 4. All of the Bla g 4 protein was recovered from the dissected reproductive system, whereas the remaining male carcass, without the reproductive system, contained no detectable Bla g 4. The testes, utricles, conglobate gland and uricose glands of the male reproductive system were homogenized and Bla g 4 extracted for analysis by ELISA. Bla g 4 was found only in the conglobate gland and the utricles, with the latter containing significantly more (ANOVA, $F_{3,23} = 254.26$, $P < 0.0001$) Bla g 4 protein than the conglobate gland (Fig. 3B). These results are consistent with the results from RT-PCR and Northern hybridization.

To examine tissue-specific expression, the entire reproductive system from 14-day-old adult males was fixed in paraformaldehyde and incubated overnight with a DIG-labelled cRNA probe. Alkaline phosphotase-conjugated anti-DIG antibodies were used to visualize the hybridized probe. The Bla g 4-specific probe hybridized only to mRNA in the large apical utricles and the base of the conglobate gland (Fig. 4), confirming RT-PCR and Northern hybridization data. Conversely, neither the smaller, more basal transparent utricles of the mushroom-shaped accessory glands nor the uricose glands and testes showed Bla g 4 expression (data not shown).

Developmental and juvenile hormone III (JH III) regulation of Bla g 4 protein level

The male reproductive system was dissected out from pharate adults (12 h before the adult moult), newly emerged adults (day 0), and adults aged 1, 2, 4, 6, 8 and 14 days, homogenized, and Bla g 4 extracted for analysis by ELISA. As shown in Fig. 5, pharate adult males contained minor amounts of Bla g 4 protein, but the Bla g 4 content of the male reproductive system steadily increased from 0.67 ±

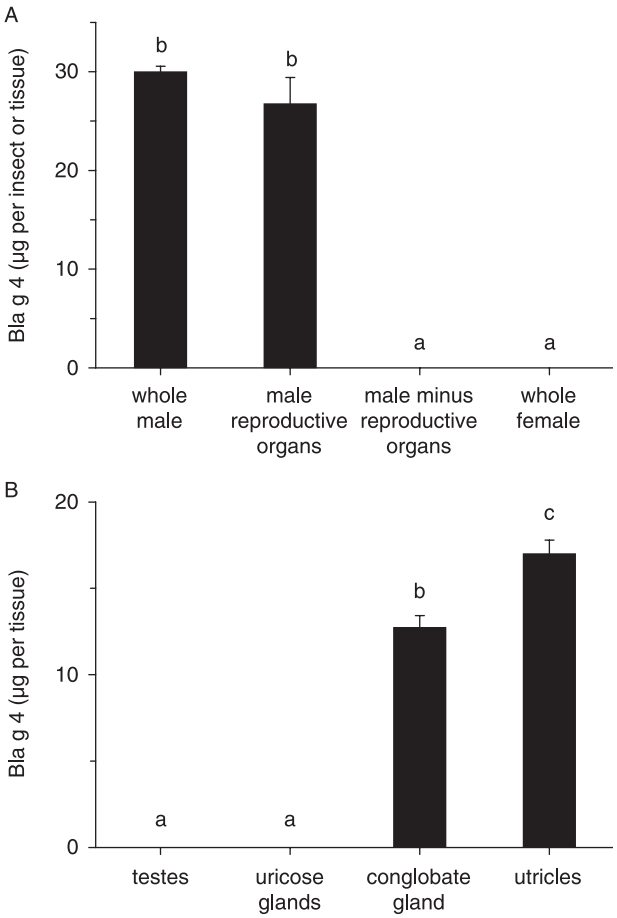

Figure 3. (A) Bla g 4 protein levels in *B. germanica* males and females. Adult males (day 14), male reproductive tissues, males without reproductive systems, and adult females (day 6) were extracted and analysed with ELISA. Each value represents the mean ± SEM of six replicates. (B) Bla g 4 protein levels from dissected reproductive tissues of 14-day-old adult male *B. germanica*. Each value represents the mean ± SEM of six replicates, determined with ELISA. Different letters indicate significant differences among means (Fisher's LSD, $P < 0.05$).

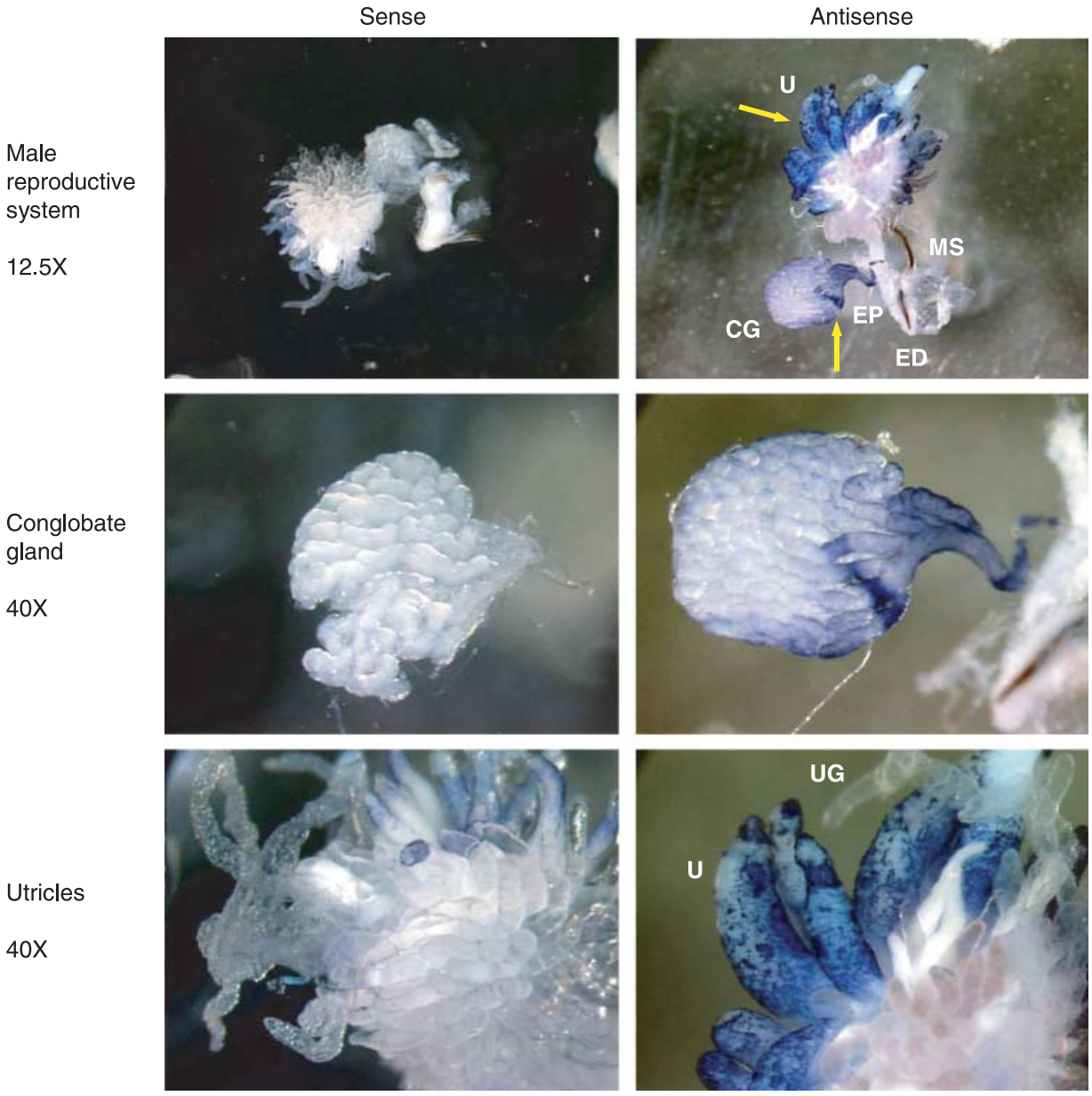

Figure 4. *In situ* hybridization of Bla g 4 mRNA in whole-mounts of the male *B. germanica* reproductive system. The base of the conglobate gland and apical utricles (arrows) show intense staining. CG, conglobate gland; ED, ejaculatory duct; EP, ejaculatory pouch; MS, median sclerite; U, utricles; UG, uricose gland.

0.13 μg on day 0 to 38.13 ± 1.64 μg by day 8. Only minor and statistically nonsignificant changes occurred between day 8 and 14, when Bla g 4 protein reached 46.0 ± 2.64 μg. The utricles of day 5 males contained 4.5-fold more Bla g 4 than the conglobate gland (Fig. 6).

The pattern of accumulation of Bla g 4 protein in males suggested that its expression might be developmentally regulated by JH III. To test this hypothesis we sought to accelerate the accumulation of Bla g 4 protein with JH III treatment of newly emerged adult males. As shown in Fig. 6, JH III significantly stimulated Bla g 4 production in both conglobate gland (ANOVA, $F_{2,14} = 3.99$, $P < 0.047$) and utricles (ANOVA, $F_{2,14} = 13.59$, $P < 0.0008$). JH III doses of 1 or 10 μg equally induced about a two-fold increase in Bla g 4 in the conglobate gland. In the utricles, both 1 and 10 μg JH III significantly stimulated Bla g 4 protein production, but applications of 10 μg induced Bla g 4 production significantly more than applications of 1 μg JH III.

Effect of mating on Bla g 4 levels

The male accessory reproductive glands produce seminal secretions and components that form a spermatophore, which is subsequently passed from the male to the female during copulation. To determine whether Bla g 4

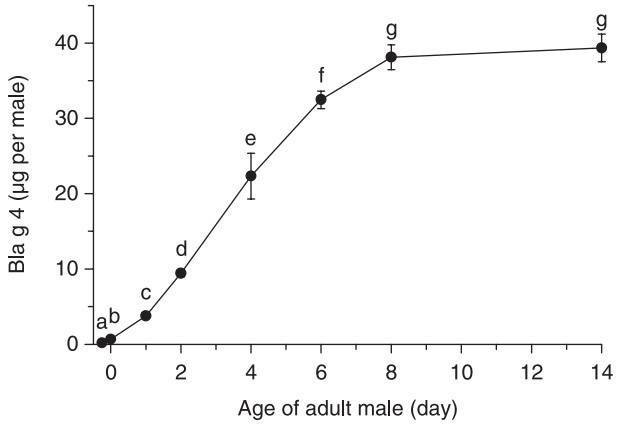

Figure 5. Age-related changes in Bla g 4 protein levels in adult male *B. germanica*. The reproductive tissues of pharate (0.5 days before the adult moult) to 14-day-old adult males were extracted and subjected to ELISA. Each value represents the mean ± SEM of five replicates. Different letters indicate significant differences among means (Fisher's LSD, $P < 0.05$).

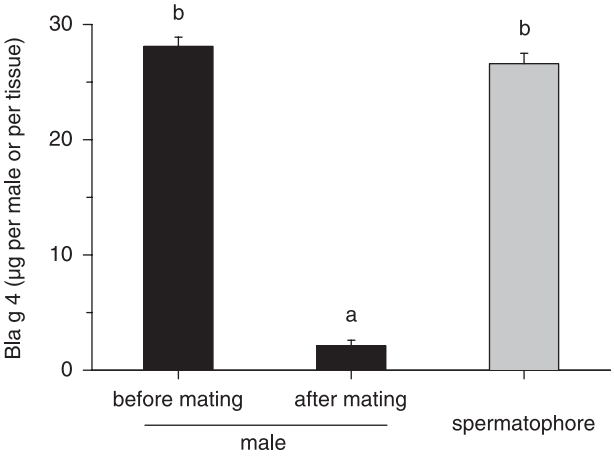

Figure 7. Bla g 4 protein contents of the spermatophore and adult male *B. germanica* before and immediately after mating. Each value represents the mean ± SEM of six replicates, determined with ELISA. Different letters indicate significant differences among means (Fisher's LSD, $P < 0.05$).

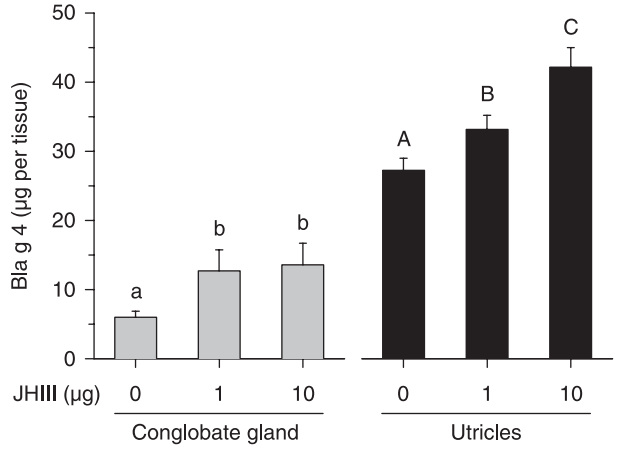

Figure 6. Effect of topically applied JH III on Bla g 4 in adult male *B. germanica*. JH III (1 µg or 10 µg) or acetone (control) was topically applied to day 0 males and the same dose was applied again 2 days later. Bla g 4 protein levels of the conglobate gland and utricles were quantified with ELISA on day 6. Each value represents the mean ± SEM of five replicates. Different letters within tissue type indicate significant differences among the means (Fisher's LSD, $P < 0.05$).

is a component of the spermatophore, Bla g 4 protein levels were measured by ELISA in males before mating, immediately following mating and in newly formed spermatophores removed from newly mated females. Immediately after mating, total male Bla g 4 declined nearly sixteenfold (Fig. 7; ANOVA, $F_{2,17} = 329.07$, $P < 0.0001$). The Bla g 4 protein was ultimately transferred to the female in the spermatophore.

Discussion

Cockroaches produce potent allergenic proteins, which might constitute the most important risk factor for asthma in inner-city households, particularly among children (Rosenstreich *et al.*, 1997). Although extensive research effort has been directed at environmental sampling of cockroach allergens and in clinical attempts to reduce allergen levels in homes (e.g. Arbes *et al.*, 2003, 2004), little is known about the sources of these allergenic proteins, their physiological functions in the cockroach, and their temporal and physiological regulation, especially relative to efforts to control cockroach populations. Our current study was thus motivated by a need to understand Bla g 4, a cockroach-produced human allergen, and to consider strategies to reduce its environmental prevalence.

Pichia-produced rBla g 4 protein showed a molecular weight of 30 kDa on 15% SDS-PAGE, but rBlag 4 antiserum recognized two Bla g 4 bands in extracts of *B. germanica*, with molecular weights of 24.4 and 22.4 kDa. The discrepancy in size between natural and recombinant Bla g 4 may be due to degree of glycosylation, as discussed by Vailes *et al.* (1998). Because of natural heterogeneity, functional reasons or degradation, glycosylated proteins can be in a variety of glycosylation states. The 2 kDa difference between the two bands in the natural extract may reflect either incomplete removal of carbohydrate from the upper band, or possibly sample heterogeneity and the presence of multiple related Bla g 4 peptides. Nevertheless, both bands were immunoreactive and differential glycosylation had no influence on the antigenic reactivity of the rBla g 4 protein (Fig. 2; Vailes *et al.*, 1998).

Its deduced amino acid sequence shows that Bla g 4 is a member of the lipocalin family of ligand-binding small extracellular proteins (Arruda *et al.*, 1995). This family contains several allergenic proteins that elicit IgE antibody responses in humans, including mouse and rat urinary proteins, dog allergen and α-lactoglobulin. Sequence similarity

with rodent urinary proteins, which are putative male pheromone carrier proteins, raised the interesting possibility that Bla g 4 may serve a similar function in the German cockroach (Arruda *et al*., 1995). The idea that Bla g 4 might be a secreted tergal protein involved in sexual interactions (Arruda *et al*., 1995) was especially motivated by the observations that adult *B. germanica* males produce tergal secretions that are attractive to females (Kugimiya *et al*., 2003), whereas females produce tergal secretions that are sexually attractive to males (Liang & Schal, 1993; Gemeno & Schal, 2004).

However, our results from gene expression analyses and quantitative ELISA unambiguously show that Bla g 4 is produced only in the adult male accessory reproductive glands and not in the tergal glands. The accessory reproductive glands in male cockroaches provide seminal fluids and structural and secretory materials for spermatophore production (Chen, 1984; Happ, 1984; Gillott, 2003), and in the German cockroach the uricose glands also serve as organs for storage-excretion of uric acid (Roth, 1967; Mullins & Keil, 1980). The Bla g 4 protein is specifically produced in the apical utricles and conglobate gland of the male reproductive system, packaged in the spermatophore, and ~27 μg is transferred to the female's genital tract during copulation.

The fate and function(s) of the Bla g 4 protein inside the female is unknown. It could serve as a structural component of the spermatophore or as part of the seminal fluid for sperm protection, storage and activation, sperm competition, female behaviour, fecundity, ovulation, oviposition or other functions (Gillott, 2003). We have tracked Bla g 4 in the mated female, and our preliminary results show that Bla g 4 immunoreactivity disappears from females 24 h after mating. Furthermore, the considerable amount of Bla g 4 in the discarded spermatophore expelled by the mated female (unpublished results) suggests that Bla g 4 may be a component of the spermatophore and not the seminal secretions. Nevertheless, because many lipocalins bind hydrophobic molecules with high affinity and specificity, we cannot rule out a ligand-binding function for Bla g 4.

Within the lipocalin family, Bla g 4 is distantly related to mammalian lipocalins, and appears to be phylogenetically divergent even from other arthropodan lipocalins, such as billin-binding protein (Gutiérrez *et al*., 2000). It is most closely related to the nitrophorins of haemotophagous arthropods, which bind histamine and NO and play important roles in modulating host responses (Ribeiro *et al*., 2004). It is possible that the Bla g 4 protein plays similar roles in its 'host', the female's genital tract, by transferring small semiochemicals from the male to the female that modulate female behaviour or reproductive physiology. The relative abundance and specific localization of Bla g 4, together with the analytical tools we developed (e.g. antibodies) should facilitate isolation and purification of such ligands.

The protein content of the conglobate gland increases gradually after eclosion of the adult male (Vilaplana *et al*., 1996a). Our results also show age-related production of Bla g 4 in the male reproductive system between days 0 and 8, and the amount of Bla g 4 levelled off by day 14. Comparison of our results with those of Vilaplana *et al*. (1996a) indicates that Bla g 4 might comprise approximately 50% of conglobate gland proteins.

The age-related increase in various accessory gland proteins suggested a functional link between JH and gland maturation (Piulachs *et al*., 1992; Vilaplana *et al*., 1996a,b). Indeed, allatectomy (removal of the corpora allata, the source of JH) significantly reduced the accumulation of accessory gland proteins, including conglobate gland proteins, whereas the administration of exogenous JH to male German cockroach restored protein accumulation (Piulachs *et al*., 1992; Vilaplana *et al*., 1996a,b). Mating also resulted in a sudden depletion of conglobate gland proteins, particularly around spermatophore formation, in support of the idea that these proteins participate in the formation of the spermatophore. Our results show a similar pattern, but explicitly for the Bla g 4 protein. We topically administered JH III to day 0 males and found Bla g 4 levels significantly increased by day 6 in both the conglobate gland and the utricles. Thus, Bla g 4, like other conglobate proteins in adult males, appears to be developmentally up-regulated by JH III. This is also the first observation of JH III regulation of a utricle protein, but it is likely that JH stimulates the production of a large number of accessory gland proteins (Vilaplana *et al*., 1996b).

Thus far, Bla g 4 appears to be unique among cockroach respiratory allergens in that its expression is developmentally modulated and it exhibits sexual dimorphism. Bla g 1, in contrast, is produced in the digestive system of all life stages of the German cockroach and is excreted in faeces (Gore & Schal, 2004). Seemingly, expression of Bla g 4 only in adult male cockroaches and its excretion by the female only after copulation would make this protein less pervasive as an environmental allergen. Yet, the prevalence of serum IgE antibody to Bla g 4 ranged from 40 to 60% in asthmatic patients who are allergic to cockroaches (Vailes *et al*., 1998). This observation suggests extensive exposure of humans to this protein. It is possible that Bla g 4 is highly stable, and therefore readily accumulates in cockroach-infested homes. Although adult males constitute a relatively small fraction of the demography of cockroach infestations, this life stage tends to be more mobile than other stages. Thus, adult males are more likely to encounter pesticides and die, exposing their Bla g 4 contents to the human residents.

It is also plausible that cockroach sprays and baits that are formulated with JH analogues (e.g. methoprene, hydroprene, pyriproxyfen) as insecticidal growth regulators might stimulate Bla g 4 production in males. Because the control strategy for this insect interferes with the metamorphic

moult and causes sterilization rather than mortality of adult cockroaches, Bla g 4 and other accessory gland proteins might accumulate disproportionately in males. Understanding the endocrine regulation of Bla g 4 should facilitate a critical re-evaluation of integrated pest management options to effect substantial reductions in environmental cockroach aeroallergens.

Experimental procedures

Insects

The German cockroach (*Blattella germanica*) colony was maintained in incubators at 27 ± 0.5 °C, ~50% relative humidity, and 12 : 12 h light–dark photoperiod. Newly emerged adult females and males were separated from the colony on the day of adult eclosion (day 0), and maintained in separate plastic cages in separate incubators. Cockroaches were provided Purina 5012 Rat Chow (Purina Mills, St. Louis, MO, USA) and water *ad libitum*. Fourteen-day-old males and 6-day-old females were used for all assays, unless otherwise stated. Terminology of the male accessory reproductive organs is based on Feliubadaló *et al*. (1996).

RNA isolation, cDNA synthesis and PCR amplification

Cockroaches were cold-anaesthetized, briefly rinsed in cockroach saline, and tissues dissected on ice and immediately placed into TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA). For total RNA extraction, tissues were homogenized in 1 ml TRIzol reagent, chloroform extracted, precipitated with isopropyl alcohol, washed with ethanol and resuspended in DEPC-(diethylpyro-carbonate) treated sterile water. The RNA concentration was determined spectrophotometrically at 260 nm. Samples were aliquoted and stored at −80 °C until use.

First-strand cDNA was synthesized from 8 μg of DNase-treated total RNA isolated from insect tissues using StrataScript reverse transcriptase (Stratagene, La Jolla, CA, USA) in the presence of oligo(dt) at 42 °C. Primers were designed based upon the known partial mRNA nucleotide sequence of Bla g 4 (accession no. U40767; Arruda *et al*., 1995). Sense (5′-CGCAGTTTTGGCACTATGTGC-3′) and antisense (5′-CATGTCCATTAGCTGCAGCGG-3′) primers were synthesized (Gibco BRL, Carlsbad, CA, USA) and stored at −20 °C. The cDNA was used to amplify a Bla g 4 DNA fragment by polymerase chain reaction (PCR) in a PCRExpress thermal cycler (Thermo Hybaid, Franklin, MA, USA) for twenty or thirty amplification cycles (30 s denaturation at 94 °C, 1 min annealing at 50 °C, and 45 s elongation at 72 °C). An initial 5 min incubation step at 95 °C was performed, and each reaction was terminated for 10 min at 72 °C. The resultant 347 bp cDNA fragment was purified by gel extraction using a QIAquick kit (QIAGEN, Valencia, CA, USA) according to the manufacturer's protocol, and eluted in 50 μl DEPC-treated sterile water.

Whole-mount in situ *hybridization*

To generate digoxygenin-labelled sense and antisense riboprobes, a 347 bp Bla g 4 cDNA was cloned into the pGEM-T Easy Vector (Promega, Madison, WI, USA), linearized with *Eco*RI and transcribed using T7 RNA polymerase with DIG RNA Labeling Mix (Roche, Indianapolis, IN, USA). Adult male reproductive system was dissected and immersed in 4% paraformaldehyde (PFA) at 4 °C overnight in 1.5 ml polyethylene microcentrifuge tubes. Tissues were then incubated for 18 h in hybridization buffer containing a 300-fold dilution of a DIG-labelled antisense Bla g 4 riboprobe. After hybridization, tissues were washed with 1× SSC at 70 °C for 2 h followed by a 4 h incubation in PBST + 20% sheep serum at room temperature. Following blocking, samples were incubated with slow agitation for 1 h with anti-DIG alkaline phosphatase-conjugated Fab fragments (Roche, Indianapolis, IN, USA). Tissues were then washed twice with PBST to remove the unbound antibody. Hybridized probes were visualized colorimetrically using 5-bromo-4-chloro-3-indoyl phosphate (BCIP) and nitroblue tetrazolium (NBT) (Roche). This reaction was stopped with 4% PFA upon proper development.

Production of anti-rBla g 4 antiserum

Recombinant Bla g 4 (rBla g 4) protein used for antibody production was generated according to methods described by Vailes *et al*. (1998). Bla g 4 was expressed in yeast, *Pichia pastoris*. *Pichia*-expressed rBla g 4 was purified from culture contaminants over a phenyl Sepharose CL-4B column (Pharmacia). A Superdex 75 HR 10/20 size exclusion column was used as a final purification step (Vailes *et al*., 1998). *Pichia*-expressed recombinant Bla g 4 showed comparable antigenic reactivity to that produced previously in *Escherichia coli*. Both allergen preparations bound comparable levels of serum IgE antibody and showed similar skin test reactivity in individuals allergic to cockroaches (10^{-1}–10^{-3} μg/ml), but the yield of Bla g 4 protein obtained in the *Pichia* system was 200-fold higher than that produced in the *E. coli* system (Vailes *et al*., 1998).

A small amount of pre-immune blood was collected from New Zealand white rabbits. Two hundred micrograms of rBla g 4 protein suspended in 10 mM PBS, pH 7.4, was emulsified thoroughly in Freund's complete adjuvant and injected subcutaneously at multiple sites into each rabbit. Five booster injections of 75 μg of rBla g 4 in Freund's incomplete adjuvant were given at 3 week intervals; rabbits were bled 1 week after the final injection. Sera were separated from blood and stored in aliquots at −80 °C.

SDS-PAGE and Western blotting

Specificity of the antiserum for Bla g 4 was checked by SDS-PAGE and Western blotting. Briefly, tissue extracts were separated on 15% SDS-PAGE and stained with Coomassie Brilliant Blue R-250 stain. For Western blotting, gels were rinsed in transfer buffer (3.02 g Tris base, 14.4 g glycine and 200 ml methanol to make 1 litre of buffer) for 15 min. Proteins were electroblotted for 1 h onto a nitrocellulose transfer membrane (0.22 μm) prewetted with transfer buffer with a Bio-Rad mini-gel transfer apparatus. The membrane was blocked with 1% non-fat dry milk in PBST (8 mM sodium phosphate, 2 mM potassium phosphate, 140 mM sodium chloride, 10 mM potassium chloride, 0.05% Tween-20, pH 7.4) overnight at 4 °C. The membrane was then probed with rBla g 4 antiserum (1–500-fold dilution) for 1 h at room temperature followed by a secondary antiserum conjugated to alkaline phosphatase (1–5000-fold dilution) probe for 1 h at room temperature. The antigen–antibody complex was visualized using pNPP as an enzyme substrate.

ELISA of Bla g 4 protein

Cockroaches were cold-anaesthetized and various tissues were carefully dissected on ice and stored at −80 °C. Tissues were

homogenized in 250 μl PBS, centrifuged (8160 ***g*** at 4 °C for 20 min), the supernatant decanted to another 1.5 ml microcentrifuge tube, and the pellet was re-homogenized and centrifuged again in 250 μl of fresh PBS. The two supernatants were combined and stored at –80 °C.

An indirect ELISA was developed according to Fan *et al.* (2002) to quantify the level of Bla g 4 protein in various tissues of the German cockroach. To determine the optimum primary antibody dilution, 100 μl of diluted (1 : 10 000) tissue extract, or 100 μl of a series of rBla g 4 standards (0–800 ng/ml) in coating buffer (50 mM sodium carbonate–bicarbonate buffer, pH 9.4), was bound to Immunoware high-binding ninety-six-well ELISA plates by incubating overnight at 4 °C. The plates were rinsed three times with PBST and blocked for 1 h with 1% BSA at 37 °C. Each well was loaded with 100 μl of diluted Bla g 4 antiserum (dilutions of 1 : 100, 1 : 250, 1 : 500, 1 : 1000, 1 : 2000) or 100 μl of 1 : 100 pre-immune serum in PBST, containing 1% BSA, and incubated at 37 °C for 1 h. Plates were washed three times and loaded with 100 μl goat anti-rabbit immunoglobulin conjugated to alkaline phosphatase diluted 1 : 10 000 (following the suggestion of the manufacturer) in PBST, and incubated for 1 h at 37 °C. Plates were again washed three times and developed at room temperature with 100 μl per well of enzyme substrate pNPP; the reaction was stopped after 30 min by adding 50 μl of 2 N NaOH to each well. Absorbance was read at 405 nm in a PowerWave-X automated microtitre plate reader (Bio-Tek, Winooski, VT, USA).

The ELISA standard curve was generated with 0–800 ng/ml Bla g 4 protein, primary antibody dilution of 1 : 500, and secondary antibody dilution of 1 : 10 000. One hundred micolitres of diluted (1 : 10 000) tissue extracts, or 100 μl of a series of rBla g 4 standards in coating buffer, was bound to ninety-six-well ELISA plates by incubating overnight at 4 °C. The plates were rinsed, blocked, loaded with 100 μl of diluted (1 : 500) rBla g 4 antiserum or 100 μl of PBST containing 1% BSA, and incubated at 37 °C for 1 h, as before. Subsequent steps were followed as previously described.

Topical application of juvenile hormone III (JH III)

Two doses of JH III (Sigma, St. Louis, MO, USA), 1 μg and 10 μg, were topically applied in 1 μl acetone to the ventral side of the thorax of newly emerged adult males. The treated males were treated again with the same dose 2 days later (see Vilaplana *et al.*, 1996a,b). Control males were also treated twice, each time with 1 μl acetone. The utricles and conglobate gland of the male reproductive system were dissected on day 6 and processed for Bla g 4 analysis with ELISA.

Statistical analyses

To compare Bla g 4 levels that were quantified by ELISA, data were analysed by ANOVA (PROC GLM) in SAS 8.2 (SAS Institute, 2001). In preplanned comparisons, means were compared by Fisher's LSD ($\alpha = 0.05$). Data for age-related changes in Bla g 4 content and JH applications were square-root transformed prior to analysis.

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