The melanin pigment gene *black* mediates body pigmentation and courtship behaviour in the German cockroach *Blattella germanica*

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Abstract

Genes involved in melanin production directly impact insect pigmentation and can affect diverse physiology and behaviours. The role these genes have on sex behaviour, however, is unclear. In the present study, the crucial melanin pigment gene *black* was functionally characterised in an urban pest, the German cockroach, *Blattella germanica*. RNAi knockdown of *B. germanica black* (*Bgblack*) had no effect on survival, but did result in black pigmentation of the thoraxes, abdomens, heads, wings, legs, antennae, and cerci due to cuticular accumulation of melanin. Sex-specific variation in the pigmentation pattern was apparent, with females exhibiting darker coloration on the abdomen and thorax than males. *Bgblack* knockdown also resulted in wing deformation and negatively impacted the contact sex pheromone-based courtship behaviour of males. This study provides evidence for *black* function in multiple aspects of *B. germanica* biology and opens new avenues of exploration for novel pest control strategies.

Introduction

The insect cuticle is mainly composed of chitin, linear polysaccharides, and proteins, resists mechanical damage, prevents dehydration, responds adeptly to fluctuations in the external environment, and acts as an impediment to the penetration of insecticides (Andersen, 2010; Noh *et al.*, 2016b; Wan *et al.*, 2016). Pigmentation of the cuticle can also impact a number of insect physiological functions and behaviours including visual communication, camouflage, reproductive behaviours, and cuticle sclerotisation (Carroll, 2005; True *et al.*, 2005; Wittkopp and Beldade, 2009). For most insects, the pigmentation process is driven by cuticular deposition of melanins, ommochromes, pteridines, tetrapyrroles, carotenoids, and/or flavonoids/anthocyanins (Fukuda and Onishi, 1971; Gorman and Arakane, 2010).

Melanisation and the associated melanin biosynthetic pathway were first described in *Drosophila melanogaster* and have since been shown to be the biochemical basis for the phenotypic plasticity observed in body coloration across insects (Suderman *et al.*, 2006; Gibert *et al.*, 2016). Although most natural melanin pigments consist of varying ratios of eumelanin and pheomelanin (Thody *et al.*, 1991; Ito and Wakamatsu, 2003; Naysmith *et al.*, 2004), insect melanins are derived from aromatic amino acids and related phenolic compounds (Prota, 1995; Ito 2003). These pigments are synthesised by melanocytes and subsequently conveyed through epidermal cells, culminating in their deposition within the cuticle (Wright, 1987; Ito *et al.*, 2000; Ito and Wakamatsu, 2008). The melanin biosynthetic pathway that regulates insect body colour patterning consists of an enzymatic cascade built on derivatisation of tyrosine (Shamim *et al.*, 2014; Sugumaran and Barek, 2016). Tyrosine hydrogenase (*TH*) converts tyrosine into dihydroxyphenylalanine (DOPA), which can then be converted into DOPA melanin by a DOPA conversion enzyme (presumably a *yellow* family gene) or into dopamine via DOPA decarboxylase (*DDC*). Dopamine is then converted into one of three products: dopamine melanin by a dopamine conversion enzyme (presumably another *yellow* family gene), N-acetyl dopamine (NADA) by an arylalkylamine-N-acetyltransferase (*aaNAT*), or N-*β*-alanyldopamine (NBAD) by an NBAD synthetase encoded by *ebony*. NBAD can also be generated from L-aspartic acid by sequential enzymatic action of aspartate decarboxylase (*black*), which yields *β*-alanine, and the *ebony* NBAD synthetase. Both NADA and NBAD can then undergo enzymatic conversion to yield NADA sclerotin and NBAD sclerotin. Conversion of NBAD into dopamine is catalysed by the hydrolytic enzyme encoded by *tan*

(Czapla *et al.*, 1990; Richardt *et al.*, 2003; Phillips *et al.*, 2005; True *et al.*, 2005; Aust *et al.*, 2010; Mehere *et al.*, 2011; Hiragaki *et al.*, 2015; Chen *et al.*, 2018). The synthesis of yellow sclerotin pigments is thus dependent on the actions of *ebony*, whereas brown and black coloration requires *tan* (Wittkopp and Beldade, 2009). Given the role of the pathway in insect pigmentation, disruption of different points in the enzymatic cascade can have significant impacts on cuticular coloration. For example, silencing *TH* and *DDC* severely reduced black coloration in *Oncopeltus fasciatus* (Liu *et al.*, 2014). Similarly, *Bombyx mori* larval coloration changed from white to reddish-brown following functional deletion of *yellow-e* in (Ito *et al.*, 2010), and *yellow-y* knockdown caused *Aedes albopictus* eggs to turn yellowish-brown or reddish-brown (Noh *et al.*, 2021).

Although disruptions of the melanin cascade manifest most clearly in atypical cuticular pigmentation, genes in the pathway also impact cuticle hardening, ecdysis, UV protection, thermoregulation, camouflage, mimicry, reproduction, immune defence reaction, and wound healing (Hill, 1992; Söderhäll and Cerenius, 1998; Nappi and Christensen, 2005; Sugumaran, 2016; Whitten and Coates, 2017; Sterkel *et al.*, 2019; Bai *et al.*, 2022). Consequently, knocking down *black*, *DDC*, *TH*, or *yellow-y* genes can have diverse biological and physioloical impacts, including reduced survival in *Sogatella furcifer*a (Guo *et al.*, 2023), impaired vision in *D. melanogaster* (Phillips *et al.*, 2005), incomplete moulting in *T. castaneum* (Arakane *et al.*, 2009) and *Blattella germanica* (Bai *et al.*, 2022), and impaired mating success and reduced oviposition in *D. melanogaster* (Drapeau *et al.*, 2006) and *A. gambiae* (Fuchs *et al.*, 2014).

The German cockroach (*Blattella germanica*) is a major indoor pest of urban and rural areas that pollutes food and poses a health threat as both an allergenic source and as a vector of various parasites, viruses, and bacteria (Zhang *et al.*, 2018). Further, as an intermediate host for many parasites, it also poses a risk to livestock. Currently, control of *B. germanica* is largely dependent on chemical pesticides, however, the persistence of pesticide residues and the potential for resistance to develop have become increasingly problematic (Wu and Appel, 2017; Li *et al.*, 2021; Mei *et al.*, 2022). Thus, there is an urgent need for novel, environmentally friendly biological control methods and pest management strategies (Pan and Zhang, 2019). One potential strategy involves targeted disruption of insect reproduction, which is dependent on coordinated sexual behaviours that are frequently influenced by the emission and detection of sexual cues (Le Moëne and Ågmo, 2018; Ryan 2021). These cues include volatile sex pheromones that facilitate mate-finding and non-volatile contact sex pheromones (CSP) that mediate sex-recognition and mate selection (Auer and Benton, 2016). In *B. germanica*, courtship and mating selection are mainly impacted by two homologous cuticular hydrocarbons, 3,11-dimethylheptacosan-2-one (C27 methyl ketone) and 3,11-dimethylnonacosan-2-one (C29 methyl ketone), as well as alcohols and aldehydes with each component capable of independently eliciting courtship (Eliyahu *et al.*, 2004, 2008; Wada-Katsumata and Schal, 2019). Sexual communication in mature *B. germanica* is mediated through antennal contacts in which female-released CSPs trigger male-specific courtship behaviours that include reorientation around the female, wing lifts, and secretion from the male tergal gland (Shimomura *et al.*, 2019; McPherson *et al.*, 2022). In response, female feeding on the secretion promotes male abdominal extension, grasping of female genitalia, and concludes in copulation (Hamilton *et al.*, 2021). The role of melanin-based cuticular

pigmentation in CSP-mediated courtship processes is unclear. Here, we identify and functionally characterise the *B. germanica black* gene product and provide evidence for its function in cuticular coloration as well as wing development and male courtship behaviour.

Materials and methods

Insects

Blattella germanica were collected in Guilin, Guangxi, China in 2022 and subsequently raised in the laboratory. The colony was maintained in glass jars that were housed in an insect incubator set at 27 ± 1°C, 30–40% relative humidity, and a 12:12 h light/dark photoperiod. The colony was reared on commercial rat chow (Huizhou, China), the main guinea pig food source, and tap water.

Gene identification

To identify a potential *B. germanica* homologue of the *D. melanogaster black* gene (GenBank: NP_001246025.1) (Wright, 1987), the fly protein sequence was queried against the *B. germanica* genome (Harrison *et al.*, 2018) using default BLASTp parameters.

RNA extraction and cDNA synthesis

Total RNA was isolated from 20 mg whole-body, 5-day-old *B. germanica* adults (mixed sex) using a FastPure cell / tissue total RNA isolation kit according to the manufacturer's instructions (Vazyme, Nanjing, China). RNA was quantified with an UV–Vis spectrophotometer SMA-4000 (Merinton, Beijing, China). First-strand complementary DNAs (cDNAs) were synthesised from 1 μg total RNA using a TransScript One-Step gDNA Removal and cDNA Synthesis SuperMix according to the manufacturer's instructions (Transgen Biotech, Beijing, China).

Phylogenetic tree

Protein sequences for *Bgblack* and *black* homologues (complete opening reading frames when available) from 55 species representing six insect orders (Blattaria, Orthoptera, Hemiptera, Coleoptera, Diptera, and Lepidoptera) were aligned with MAFFT v7.505 (Katoh *et al.*, 2019) and a maximum-likelihood tree was constructed based on 1000 bootstrap values using IQ-TREE 1.6.12. The phylogenetic tree was annotated using ITOL version 6.5.8 (https://itol.embl.de). Protein sequences and GenBank accession numbers are listed in Table S1.

dsRNA synthesis and injection

Gene specific primers containing a 5' T7 sequence (Table S2) were designed to amplify a 450-bp fragment of *Bgblack* and a 413-bp fragment of the negative control gene *green fluorescence protein* (*GFP*). Note, the regions targeted for knockdown were randomly selected. *Bgblack* amplification was performed in 25 μl reaction containing 12.5 μl of 2 × Taq Plus Master Mix (Vazyme),10 μl of ddH$_2$O, 0.5 μl (50 ng μl^{-1}) of cDNA template, and 1 μl of each primer (10 mmol l^{-1}). The PCR programme consisted of: 95 °C for 2 min, followed by 40 cycles at 95 °C for 30 s, 62 °C for 20 s (− 1 °C/cycle), 72 °C for 40 s, and another 30 cycles followed by 95 °C for 30 s, 52 °C for 20 s, and 72 °C for 40 s, with a

final extension step at 72 °C for 10 min. PCR products were detected on a 1% agarose gel and the target bands were purified using a FastPure Gel DNA Extraction Mini Kit (Vazyme). Purified PCR products were subcloned into pMD19-T (Takara, Dalian, China) and transformed into Trans-T1 competent cells (Transgen Biotech). Single clones were sequenced by General Biology Co, LTD (Chuzhou, Anhui, China). The sequenced plasmids were used as templates to generate T7-containing PCR products. The purified products were then used to synthesise the corresponding dsRNAs with a T7 RNAi Transcription Kit (Vazyme). The dsRNA quality was checked using agarose gels and concentration was determined using a SMA-4000 UV–Vis spectrophotometer (Merinton). dsRNAs were stored at −80 °C until needed.

Fifth instar *B. germanica* nymphs were anesthetised with carbon dioxide for approximately 30 s and intrathoracically injected with 2 μl dsRNA (1500 ngμl^{-1}) using a 10 μl glass syringe (Hamilton, Reno, NV, USA) as described previously (Wang et al., 2020; Zhu et al., 2021). Three biological replicates were assayed with 10 nymphs per replicate. Survival rate and the percentage of adults exhibiting atypical phenotypes (e.g., cuticular coloration, wing development, etc.) were recorded daily for 20 days post-injection (dpi).

Quantitative real-time PCR (qPCR)

To examine the relative transcript level of *Bgblack* in *B. germanica*, total RNA was extracted from adults at 8 dpi. Five mixed sex ds*Bgblack*- and ds*GFP*-injected adults comprised one biological replicate and transcript levels were assessed across three replicates. *Glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH, WCD39345.1*) and *elongation factor-1-alpha* (*EF1a, PSN34991.1*) were used as reference genes (Zhu et al., 2021; Ma et al., 2023). cDNAs were synthesised from 1 μg total RNA using a cDNA Synthesis SuperMix with gDNA removal (Transgen Biotech, Beijing, China). qPCR was performed on an iQ5 real-time PCR instrument (Bio-Rad, USA) using PerfectStar Green qPCR SuperMix (Transgen Biotech, Beijing, China) and gene specific primers (Table S2). All reactions were carried out in a 20 μl volume under the following conditions: one cycle at 94 °C for 30 s, then 50 cycles at 94 °C for 5 s, 60 °C for 30 s, 55 °C for 60 s followed by a melt curve analysis programme. The qPCR products were sequenced and the relative expression levels of the target genes were calculated using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). Primer efficiencies were all approximately 100% as determined from standard curves generated using five concentrations (1:10 serial dilutions) of template.

Colour intensity measurement

Experimental and control insects (ten 7-day-old whole body male and female adults) were digitally imaged using a JC-1002F stereo microscope (Jing Chang-48 million pixels, Shenzhen, China). Colour intensity in equivalent regions of the whole-body images was determined as mean greyscale values (average luminance) via ImageJ (version 1.53e). The colour intensities of each sex were measured separately. A higher colour intensity indicates darker cuticle pigmentation (Noh et al., 2016a). All of the samples were imaged under the same conditions.

Courtship bioassay

Newly emerged adults were separated by sex and maintained in groups. Control (ds*GFP*) and experimental (ds*Bgblack*) adult males at 7 dpi (D7) were introduced during hours 4–10 of the dark cycle into a black plastic box (23 × 6 × 10 cm) and allowed to equilibrate to the surrounding conditions for 20–30 mins. The courtship bioassay was generally followed by previous studies. The antennae of wild-type (WT) D10 virgin female cockroaches are cut off from the base using medical tweezers and then attached to a 20 cm long metal rod with solid paraffin to create an 'antenna on a stick' as a stimulus source. Courtship behaviour assays were conducted in a dark room during mid-scotophase (i.e., the first and last 2 hrs of scotophase were not assessed). The males were then exposed to females from the control and experimental groups and antennation between the sexes was recorded on an HD camera (Sony, FDR-AX60, Shanghai, China) under red light. The distance between the camera and the insects was ~20 cm. Recording began at male wing extension (i.e., indicator of active courtship) following male antennal contact with female antennae and ended when male wings returned to their normal positions. The total behaviour test period evaluated was limited to 300 s. Females were discarded after every two male encounters. A courtship index [CI = total courtship time/total behaviour test time (300 s)] was determined with the number and duration of wing-raising events determined over the evaluation period. Assays consisted of 20 males and all adults used in the courtship assay were 7-days-old. Statistical significance was determined using unpaired t-tests.

Results

Gene identification and phylogenetic tree

Bgblack (GenBank accession number: PSN32645.1) encodes a 1053 amino acid protein. The sequence similarity for the highest BLAST hit is the *Diploptera punctata black* gene (KAJ9589117.1), which has 70.61% sequence identity with *Bgblack*. Phylogenetic analysis of the protein with *black* proteins from species representing six insect orders revealed order-specific clustering of the sequences with the *Bgblack* protein sorting to the Blattodea clade along with proteins from *Coptotermes formosanus*, *Cryptotermes secundus*, and *Zootermopsis nevadensis* (fig. 1). Further, the tree topology suggests an evolutionarily conserved ancestor of *black* that is common to the hemimetabolous Blattodea, Hemiptera, and Orthoptera.

Phenotypic analysis of Bgblack

At 8 dpi, target transcript levels were significantly reduced compared with the ds*GFP* control group (fig. 2). Perturbations in cuticular coloration was observed in 95.83% of ds*Bgblack* adults, whereas no phenotypic abnormalities were observed in the ds*GFP* group (fig. 3). The ds*GFP* group was characterised by semi-transparent tannish-brown coloration throughout the adult body regardless of sex (fig. 4A-B). In contrast, males and females in the ds*Bgblack* group were opaque black (fig. 4C-D). Based on pixel intensity, melanin-associated pigmentation in males and females of the ds*GFP* group was lower than those of the ds*Bgblack* group (fig. 4E-F). Compared to the ds*GFP* group (fig. 5A-G), atypical coloration was apparent throughout the body and appendages (e.g., antennae, wings, legs, cerci) of the ds*Bgblack* group (fig. 5H-N). Although ds*Bgblack* adults were
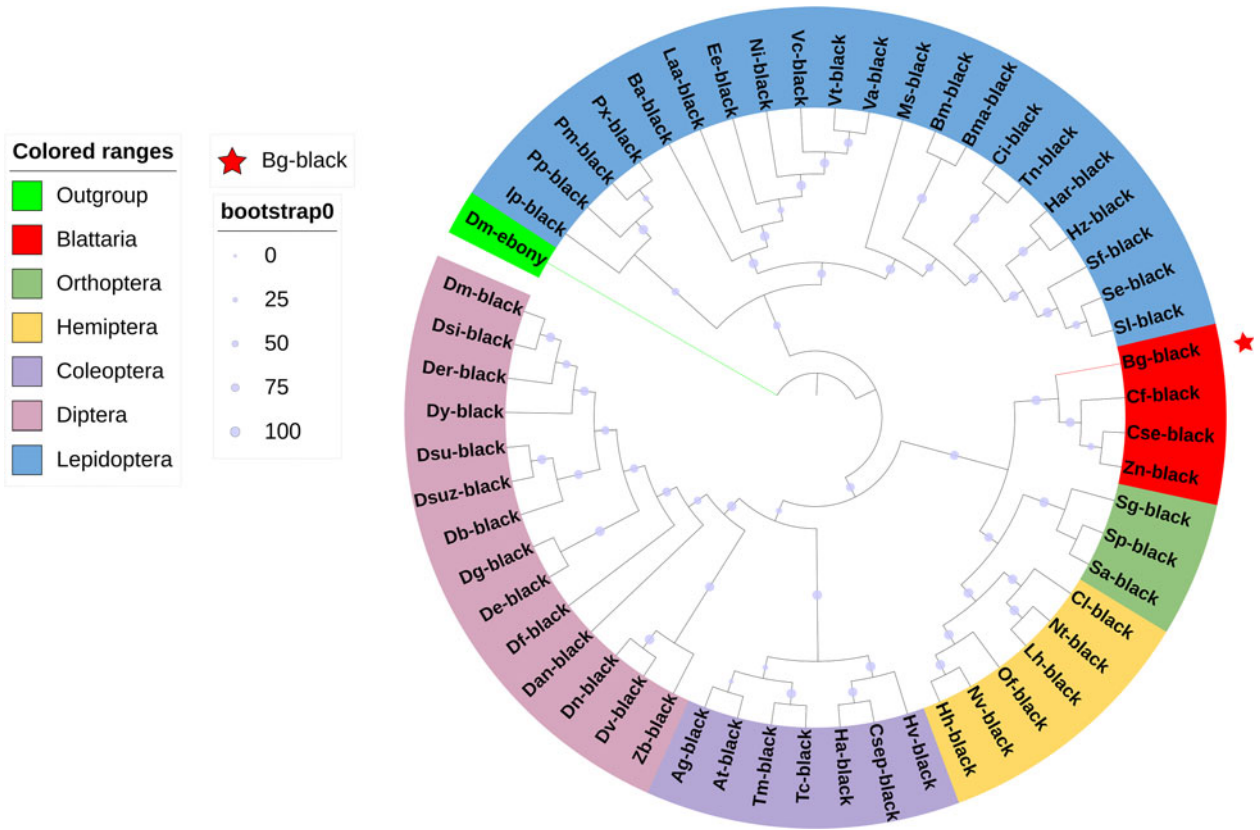
Figure 1. Phylogeny of black proteins from diverse insects. Phylogenetic analysis of Bgblack in relation to black homologues identified in insects from six orders (Blattodea, Orthoptera, Hemiptera, Coleoptera, Diptera, and Lepidoptera). *Bgblack* is labelled with red solid five-pointed stars in the figure. The phylogenetic tree was rooted on the *Drosophila melanogaster* ebony protein. Accession numbers of proteins used in the analysis are listed in Table S1.

significantly darker than the control group, both sexes exhibited incomplete melanisation of wings, abdomens, thoraxes, and legs as some yellow coloration persisted and the main body segments (abdomen/thorax and dorsum) retained a degree of transparency (figs 5J-K). In contrast, the heads, antennae, and cerci were opaque black (figs 5H, 5I and 5N). Moreover, the abdominal
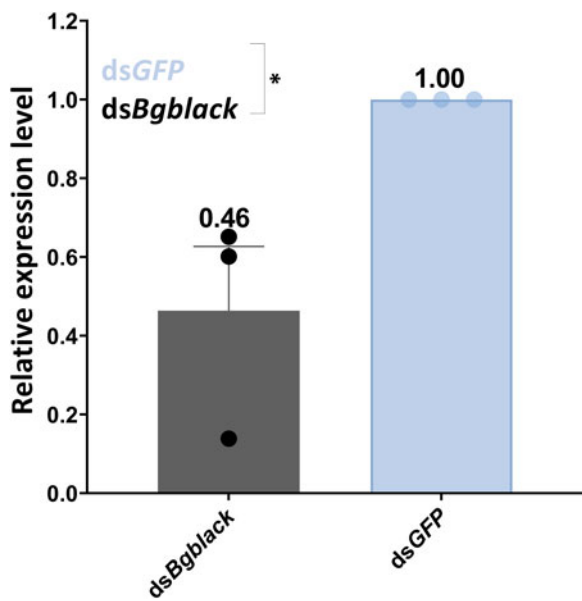
Figure 2. RNAi-mediated knockdown of *Bgblack* transcripts. *Bgblack* transcript levels were assessed using adults at 8 days post-dsRNA injection in relation to GAPDH and EF1a. Controls were injected with ds*GFP*. Statistical differences were determined using an unpaired *t*-test (*, $P < 0.1$; **, $P < 0.01$).
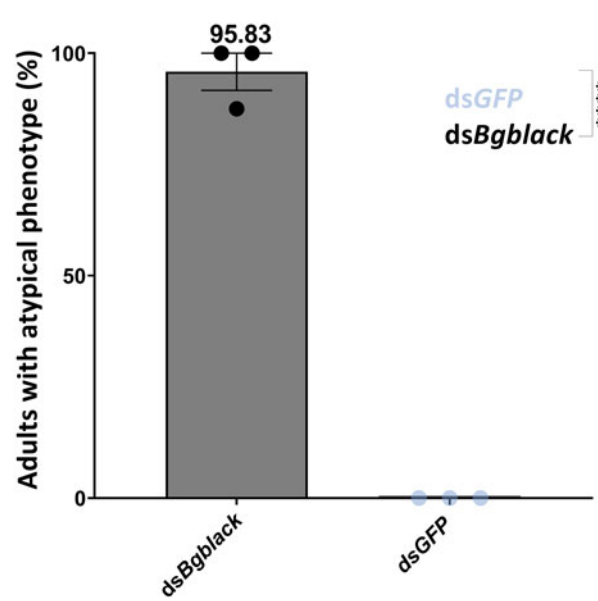
Figure 3. Percentage of *B. germanica* adults exhibiting atypical cuticular coloration. Cuticular coloration was assessed in adults at 8 days post-dsRNA injection. The control group was injected with ds*GFP*. Statistical differences were determined using an unpaired *t*-test (*, $P < 0.1$).
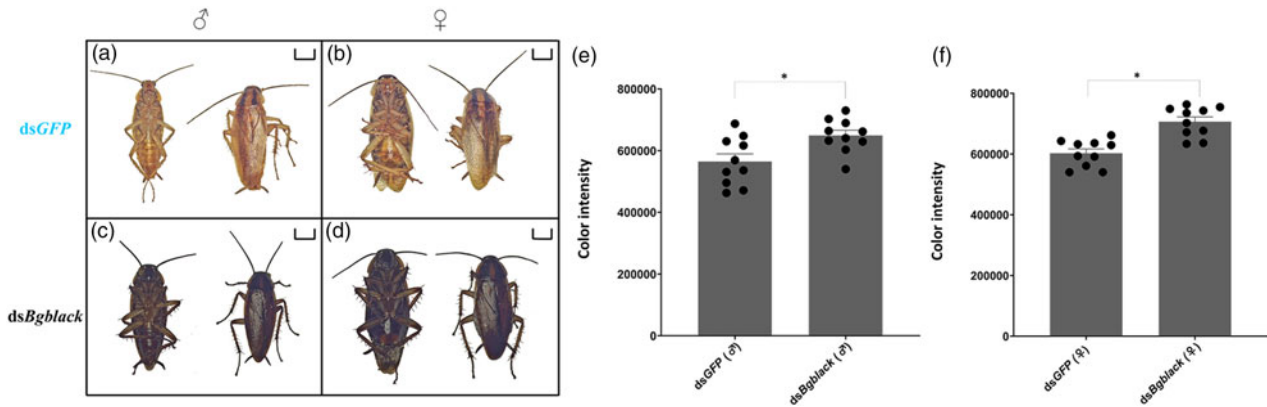
Figure 4. Effects of RNAi-mediated knockdown on cuticular coloration. **(A-D)** Cuticular coloration phenotypes in adults at 8 days post-dsRNA injection. (A) ds*GFP* knockdown male. (B) ds*GFP* knockdown female. (C) ds*Bgblack* knockdown male. (D) ds*Bgblack* knockdown female. Images are representative of phenotypes observed in 10 replicates, including 10 female and 10 male per replicate. Scale bar is 500 μm. The whole-body colour (sum of dorsal and ventral) intensity was determined as mean grey values (average luminance) with ImageJ. (E) Male colour intensity. (F) Female colour intensity. The control group was injected with ds*GFP*. Values are the mean of 10 males and females from each treatment group. Statistical differences were determined using an unpaired *t*-test (*, $P < 0.1$; **, $P < 0.01$, ***, $P < 0.001$), 'n.s.' indicates no significant differences.
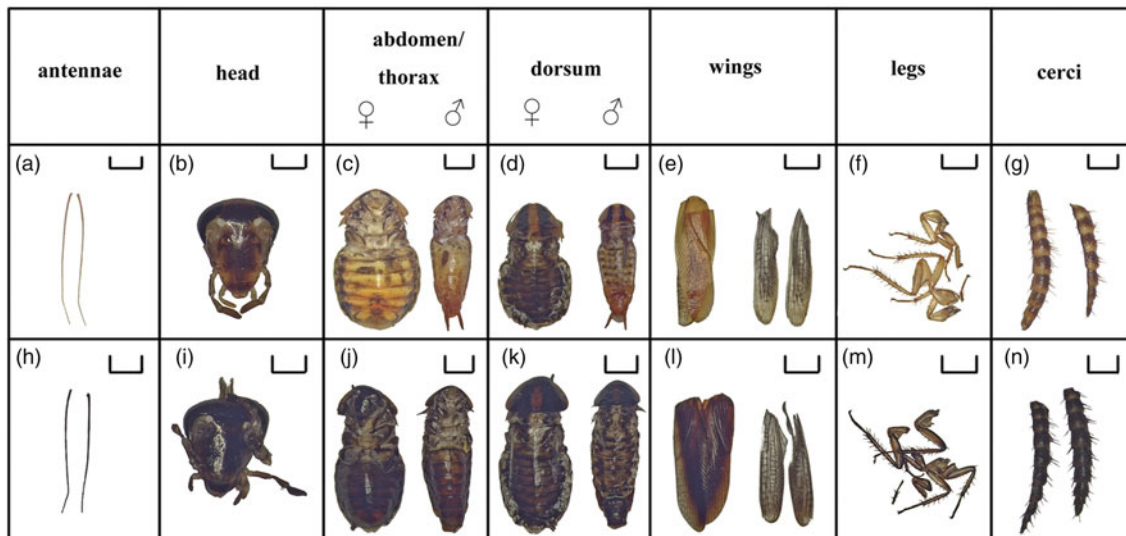
Figure 5. *Bgblack* knockdown phenotypic effects on appendage and body segment pigmentation. Cuticular coloration phenotypes exhibited by various appendages (antennae, wings, legs, and cerci) and body segments (head, ventral thorax and abdomen, and dorsum). (A–G) ds*GFP*. Adults exhibited typical coloration and morphology. (H–N) ds*Bgblack*. Adults were characterised by significantly darkened body colour and opaque cuticle. Phenotypes were assessed using adults 8 days post-dsRNA injection. Scale bar is 500 μm.

and thoracic regions of ds*Bgblack* females were darker in appearance than that of males (fig. 5J).

Unexpectedly, impaired wing expansion was observed in a significant percentage (37.50%; 9/24) of the ds*Bgblack* group (fig. 6A). Unlike the control group wings, that rested seamlessly along the length of the dorsal abdomen (fig. 6B-C), both the forewing and the hindwing in the ds*Bgblack* group were severely stunted and protruded from the abdomen (fig. 6D-E). The wing defects were not sex-specific as they occurred with comparable frequency in males (44.44%) and females (55.56%).

Impacts on male courtship ability

Males in the ds*GFP* control group exhibited stereotypical mating behaviours. When a male antennae touched a female antennae,

the male executed a stereotypical courtship response, which involved rotation of the male body relative to the stimulus (i.e., female antennae), male wing lift, and presentation of the male dorsal abdomen in response to antennation by females from the ds*GFP* group (Video S1). The mean calculated courtship index (CI) was 0.53 (fig. 7A) and the mean number of male wing extensions was 17.96 (fig. 7B). In contrast, reductions in the CI (0.23; Fig. 7A) and increased latency in wing extension (11.17; Fig. 7B) were observed in ds*GFP* males following female ds*Bgblack* antennation (Video S2). For ds*Bgblack* males, courtship responses (fig. 7A-B) to ds*GFP* female antennae were severely inhibited (CI of 0.10; mean wing extension = 4.14) with most males (16/22) unresponsive throughout the 300 s evaluation period (Video S3). For the few responsive males (6 of 22), the latency between wing extensions was longer and the duration of each event was
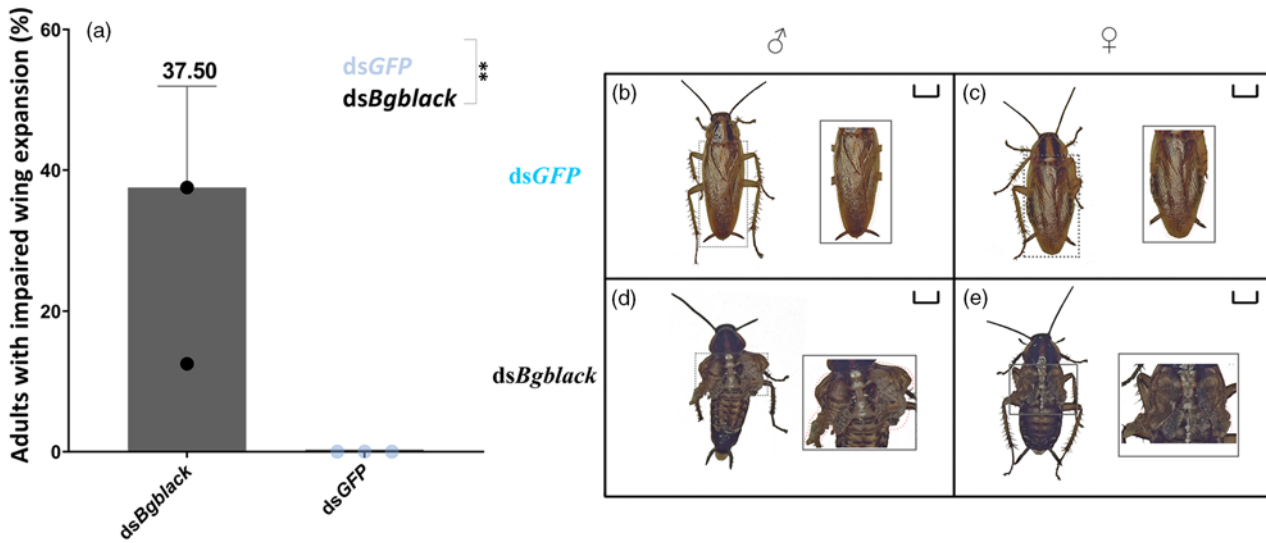
Figure 6. *Bgblack* knockdown effects on adult wing development. (A) Percentage of *Blattella germanica* exhibiting atypical wing expansion. (B) Effects of RNAi-mediated *Bgblack* knockdown on wing expansion. Bars indicate means ± SEM. The asterisks (**) indicate significant differences ($P < 0.01$) relative to ds*GFP* injected adults. Statistical differences were determined using an unpaired *t*-test. **(B-E)** Wing phenotypes in adults 8 days post-dsRNA injection. (B) ds*GFP* male. (C) ds*GFP* female. (D) ds*Bgblack* male. (E) ds*Bgblack* female. Insets are a focused zoom on the wings. Scale bar is 500 μm.
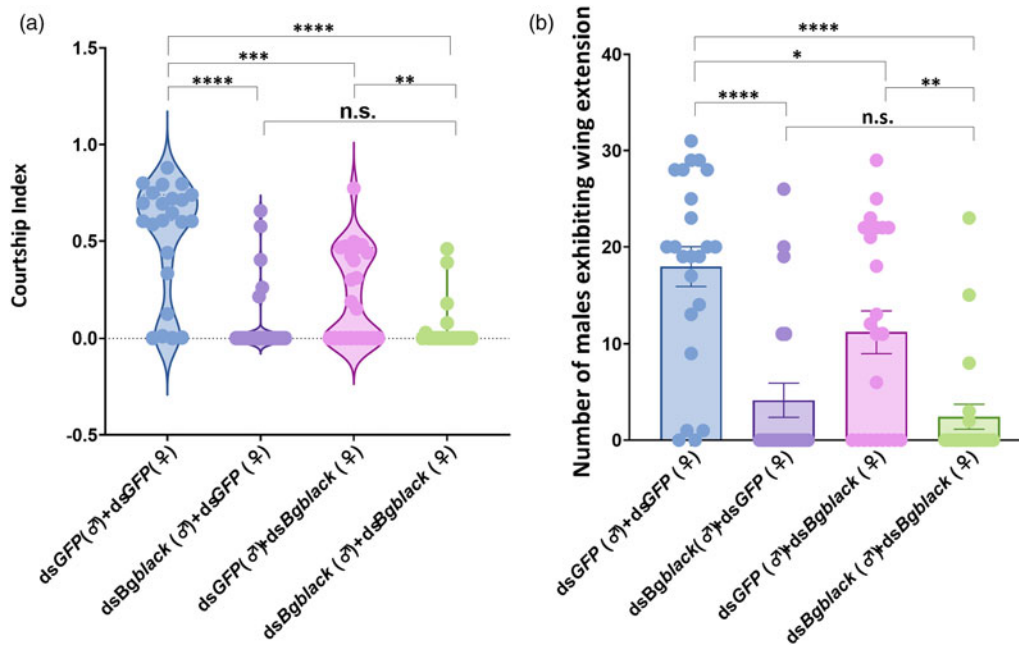
Figure 7. Effect of *Bgblack* knockdown on male courtship. (A) Male courtship index (CI = total courtship time/total behavioural test time). (B) The number of males exhibiting wing extension. Controls were injected with ds*GFP*. In total 23 ds*GFP* and 21 ds*Bgblack* males were tested. The evaluation period was 300 s. Error bars represent ± SEM. Statistical differences were determined using an unpaired *t*-test (*, $P < 0.1$; **, $P < 0.01$; ***, $P < 0.001$, ****, $P < 0.0001$). 'n.s.' denotes 'not significant' ($P > 0.05$). See supplementary Video 1–4 for representative depictions of behaviour for each group.

shorter. The most attenuated behaviours (CI of 0.05; mean wing extension = 2.43) were observed in the double ds*Bgblack* group (i.e., *Bgblack* knockdown in both males and females, Video S4). Notably, males with wing deformities (4/22) were unable to raise their wings and as a result had a failed courtship (CI = 0).

Discussion

Unlike vertebrates, dopamine-derived compounds dominate the highly conserved insect melanin biosynthesis pathway (Wittkopp

and Beldade, 2009; Barek *et al.*, 2018a), (Lemonds *et al.*, 2016; Mun *et al.*, 2019). With tyrosine as a precursor, *TH* and *DDC* catalyse the synthesis of DOPA and dopamine, respectively, which are subsequently converted to melanin forms via DOPA and dopamine conversion enzymes (Gorman *et al.*, 2007; Arakane *et al.*, 2009). NBAD melanin, in contrast, is derived from a separate pathway that uses *ebony* to generate NBAD from β-alanine, which is generated by *black*-mediated catalysis of L-aspartic acid. In this study, the *B. germanica* homologue of *black* was identified and functionally characterised. Consistent with previous studies (Popadić and

Tsitlakidou, 2021; Brent *et al.*, 2022), phylogenetic analyses revealed that *black* is highly conserved across insect orders. Similarly, the phenotypic effects of *Bgblack* knockdown on cuticular coloration (i.e., darker opaque melanin pigmentation across body segments and appendages, with more pronounced effects in female abdomens and thoraces) are consistent with previous reports. For example, RNAi-mediated knockdown of *black* in *O. fasciatus* adults resulted in expanded black pigmentation in heads, thoraces and forewings, but not in the hindwings or abdomen (Liu *et al.*, 2016), and its knockdown in *B. mori* resulted in pupae with darker-coloured heads and thoraces (Dai *et al.*, 2015). In *Lygus hesperus*, RNAi knockdown of *tan*, *black*, and *ebony* had differential effects on appendage and body coloration (Brent *et al.*, 2022). In *Henosepilachna vigintioctopunctata*, silencing *black* selectively deepened the colour of the larval heads, antennae, mouthparts, and legs in third instar larvae and further darkened the coloration of adult bodies (Ze *et al.*, 2022).

In addition to affecting body coloration, *Bgblack* also impacts wing development as knockdown impaired expansion of both the forewing and hindwing. Similar defects were reported following targeted knockdown of melanin biosynthetic pathway genes in *S. furcifera* (Guo *et al.*, 2023). Insect wing development is typically regulated by hormonal factors, as well as *Hox* genes such as *antennapedia* (Chen *et al.*, 2013; Fang *et al.*, 2022). Although *Hox* genes have been reported to act upstream of cuticular pigmentation pathways in some species(Angelini *et al.*, 2005; Jeong *et al.*, 2006; Liu *et al.*, 2019), their connection with *Bgblack* remains to be elucidated as does the linkage between the melanin biosynthetic pathway and wing development.

Courtship behaviours are critical prerequisites for insect reproduction. Adult male *B. germanica* sexual cues are communicated through female antennation, which promotes wing extension and presentation of the abdominal dorsum by the male (Eliyahu *et al.*, 2004). We found that male responses to female antennation were inhibited following *Bgblack* knockdown and that knockdown males rarely displayed stereotypical courtship behaviour regardless of the status (control or experimental) of the female partner. The few instances in which knockdown males did respond, their behaviour was lethargic, with increased latency in wing extension and reduced periods of wing lifts. *Bgblack* knockdown also negatively impacted females, which manifested as a decrease in female antennae attractiveness, suggesting impaired CSP production and/or detection. Incredibly, black male individuals with damaged wings are unable to exhibited normal courtship behaviour indicating that wing/wing-raising is a key factor in German cockroach courtship. The impact of melanin biosynthesis genes on insect sexual behaviour either indirectly through neural circuit influences or directly via impaired development of sex-associated tissues and structures is not without precedent. Silencing *yellow-y* reduces expression of the *D. melanogaster* mating-success regulatory sequence, which is positively correlated with mating success rate (Drapeau *et al.*, 2006). Furthermore, *fruitless*, which plays an important role in *D. melanogaster* sex differentiation, is upstream of *yellow* and inhibition of *yellow* reduces male courtship ability (Drapeau *et al.*, 2003). Direct effects of melanin biosynthesis genes on reproduction have also been reported. *TH* disruption results in smaller *D. melanogaster* ovaries and reduced female reproductive capability (Fuchs *et al.*, 2014).

As a potent neurotransmitter that is active in dopaminergic neurons, dopamine plays a critical role in insect development and reproduction, it is also a key precursor in the epidermal melanin biosynthetic pathway (Saedi *et al.*, 2018). In insect brains, some of the dopamine is converted in glial cells into NBAD, which is then transported to the presynaptic neurons and converted back to dopamine by NBAD hydrolase (Abebe *et al.*, 2010). Despite their presence, neither NBAD nor NADA are involved in sclerotisation of the insect brain (Barek *et al.*, 2018b). However, both are key substrates in the epidermal melanin biosynthetic pathway and play key roles in sclerotisation of the insect cuticle. Thus, the reduced NBAD biosynthesis associated with *Bgblack* knockdown may impede the *B. germanica* neural circuit. Dopamine impacts multiple behaviours in *Apis mellifera* (Huang *et al.*, 2022), and dopaminergic signalling regulates the persistence and duration of mating in male *Drosophila* (Zhang *et al.*, 2016). Moreover, in addition to cuticular pigmentation effects, behavioural defects are associated with mutations to *tan* and *black* in *Drosophila* (Yamamoto and Seto, 2014). For *tan* mutants, the abnormally low dopamine levels that negatively impact the visual system may also lead to behavioural impairments (Konopka, 1972). Conversely, functional loss of *black*, which converts dopamine to NBAD, can lead to excess dopamine. Tong *et al.* (Liu *et al.*, 2008) found that increased dopamine levels promoted courtship behaviour between *Drosophila* males but did not change behaviours towards virgin females, general olfactory responses, general gustatory responses, or locomotor activity. In addition to inducing perturbations in normal male courtship behaviours, excessive dopamine levels can also lead to sleep and visual behavioural abnormalities (Andretic *et al.*, 2005). Recently, *black* knockdown was reported to lead to a significant decrease in dopamine levels in the head of *D. melanogaster* (Deal *et al.*, 2023). Based on these findings, the atypical mating behaviours observed following *Bgblack* knockdown might be attributable to lower dopamine levels in the head or brain of affected males. Alternatively, the lower sensitivity of *Bgblack* knockdown male cockroaches to female antennation might indicate a role for *Bgblack* in regulating CSP levels. Correspondingly, dopamine has been shown to modulate female sexual receptivity in *D. melanogaster* (Neckameyer, 1998).

Conclusion

In conclusion, we utilised RNAi to functionally characterise the role of *black* in *B. germanica*. Consistent with the hypothesised role in melanin biosynthesis, *Bgblack* is essential for body colour patterning; however, it also plays a critical role in wing development. Furthermore, *Bgblack* knockdown also significantly disrupted stereotypical adult mating behaviours, and black male individuals with damaged wings were unable to exhibit courtship behaviour. Taken together, these findings provide further insights into insect pigmentation pathways and open new avenues of research to explore new ecologically friendly pest control strategies.

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