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Cuticular colour reflects underlying architecture and is affected by a limiting resource

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ABSTRACT

Central to the basis of ecological immunology are the ideas of costs and trade-offs between immunity and life history traits. As a physical barrier, the insect cuticle provides a key resistance trait, and *Tenebrio molitor* shows phenotypic variation in cuticular colour that correlates with resistance to the entomopathogenic fungus *Metarhizium anisopliae*. Here we first examined whether there is a relationship between cuticular colour variation and two aspects of cuticular architecture that we hypothesised may influence resistance to fungal invasion through the cuticle: its thickness and its porosity. Second, we tested the hypothesis that tyrosine, a semi-essential amino acid required for immune defence and cuticular melanisation and sclerotisation, can act as a limiting resource by supplementing the larval diet and subsequently examining adult cuticular colouration and thickness. We found that stock beetles and beetles artificially selected for extremes of cuticular colour had thicker less porous cuticles when they were darker, and thinner more porous cuticles when they were lighter, showing that colour co-varies with two architectural cuticular features. Experimental supplementation of the larval diet with tyrosine led to the development of darker adult cuticle and affected thickness in a sex-specific manner. However, it did not affect two immune traits. The results of this study provide a mechanism for maintenance of cuticular colour variation in this species of beetle; darker cuticles are thicker, but their production is potentially limited by resource constraints and differential investments in resistance mechanisms between the sexes.

1. Introduction

Insect cuticle has a variety of functions, including skeletal support, camouflage, thermoregulation, protection from ultraviolet light and sexual signalling (Wigglesworth, 1948). The cuticle is also a barrier to pathogens (Moret and Moreau, 2012) and is the direct route of entry for some insect pathogenic fungi (e.g. some Entomophthoromycota and Ascomycota; Boomsma et al., 2014). Resistance to important entomopathogens has been related to the degree of cuticular darkness, where a darker cuticle is more resistant (e.g. Barnes and Siva-Jothy, 2000; Dubovskiy et al., 2013; Mitsui and Kunimi, 1988; Reeson et al., 1998; Wilson and Reeson, 1998; Wilson et al., 2001, 2002; Verhoog et al., 1996). Increased resistance could be due to behavioural defences, but is more likely to be linked to physical or chemical properties of the cuticle itself (Silva et al., 2016), and/or due to immune factors that attack the pathogen once it is through the cuticle. The mechanical properties of the cuticle are affected by many factors, including the degree of sclerotisation and melanisation, cuticular thickness, protein composition, relative amounts of chitin and proteases, water content and intracuticular pH (Andersen, 2010). All of these can influence the likelihood of invasion by fungal pathogens (e.g. Pekrul and Grula, 1979; Lui et al., 2014; Schabel, 1978; St. Leger et al., 1988). In addition, the cuticle is able to mount an active defence against pathogens (e.g. Ashida and Brey, 1995; Golkar et al., 1993) through the action of proteases and chitinases that prevent the attachment, penetration and degradation of the cuticle by fungal pathogens (e.g. Kuo and Alexander, 1967; Söderhäll and Ajaxon, 1982; St. Leger et al., 1988). Several studies have also found relationships between cuticular darkness and humoral (Reeson et al., 1998; Wilson et al., 2002) and cellular (Cotter et al., 2004) immune function (but see Bailey, 2011; Karl et al., 2010).

In the mealworm beetle, *Tenebrio molitor*, adult cuticular colour is density dependent and can range from 'tan' to 'black' (Thompson et al., 2002); larvae experiencing higher population densities develop into adults with darker cuticles (Barnes and Siva-Jothy,

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2000). This response to ecological conditions also appears to have a heritable component (Prokkola et al., 2013; Rolff et al., 2005), which is amenable to selection. The physiochemical properties of the cuticle are known to vary with colour in *T. molitor* (Silva et al., 2016), and selection for a darker cuticle has been shown to correlate with both higher activity of phenoloxidase (PO) and increased haemocyte density (Armitage and Siva-Jothy, 2005). The process of cuticle hardening and darkening involves (1) sclerotisation, which cross-links and stabilises the cuticle by incorporating phenolic compounds, and (2) melanisation, which results in melanin being distributed within the cuticle or deposited as granules (see Andersen (2010) for a review). Central to both these processes is the production of 3,4-dihydroxyphenylalanine (DOPA), which is produced by the hydroxylation of the semi-essential amino acid tyrosine (Gorman et al., 2007; Moussian, 2010). As well as being important in cuticle production, melanin is also involved in the encapsulation response against parasites within the body cavity (Siva-Jothy et al., 2005). Consequently, tyrosinase POs are also found in haemocytes and haemolymph as inactive proenzymes, and increase survival after infection with fungi and Gram-positive bacteria (Binggeli et al., 2014), as well as being an important constitutive immune effector in insects (Chase et al., 2000). Given the complex interactions between internal physiology and cuticular melanisation and sclerotisation, the relationship between cuticular colouration and immune defence might result from shared biochemical investment via pleiotropic association (Silva et al., 2016). A comparatively little-studied feature of the insect cuticle are the pore canals, the narrow ducts that perforate the cuticle perpendicular to the surface, connecting the epidermis to the epicuticle (Locke, 1961; Zacharuk, 1976). Their relationship with cuticular darkness and with cuticle-invading pathogens is unknown. *Tenebrio molitor* has numerous pore canals in the exocuticle, running parallel to one another (Delachambre, 1971; Wigglesworth, 1948). Renewed recent interest in pore canals in another tenebrionid beetle, *Tribolium castaneum,* has shown that two cuticular proteins, TcCPR27 and TcCPR18 are necessary for the correct formation of the pore canals in this species, particularly in body regions where there is rigid cuticle such as the thoracic body wall (Noh et al., 2014). Whilst a darker cuticle, which in *T. molitor* is fixed during early adulthood (e.g. Thompson et al., 2002), confers an advantage in terms of parasite resistance (e.g. Barnes and Siva-Jothy, 2000), it is likely to be coupled with costs in order for variation in cuticular colour to be maintained within natural insect populations.

Resource constraints (e.g. protein and/or nitrogen acquisition) and competition for shared resources by other physiological processes may result in trade-offs leading to phenotypic variation in cuticular colour (Lee et al., 2008). Tyrosine is required for both immunity and cuticular melanisation and sclerotisation, with the latter two processes occuring at each moult. *Tenebrio molitor* larvae moult between 14 and 18 times (Morales Ramos et al., 2015; Park et al., 2014), with loss of tyrosine *via* the exuvium at each moult (Andersen, 2004). Tyrosine can be obtained from proteinaceous food, or synthesised directly from the essential amino acid phenylalanine via phenylalanine hydroxylase (Chapman et al., 2013; Corrigan, 1970) but like most animals, insects cannot synthesize the benzene ring in phenylalanine or tyrosine, thus they must be sequestered via foraging. Given this, and the competing processes that require tyrosine, it is likely to be a limiting resource, and so we hypothesise that it is an important currency for trade-offs in insects.

In this study we first examine the relationship between cuticular architecture and colour in *T. molitor*. We examine two aspects of cuticular architecture: exocuticular thickness and cuticular 'porosity' (a compound-measure of pore-canal density and pore-canal lumen size). We test whether these parameters are connected with variation in cuticle colour that is generated both naturally (phenotypic response) and by selection (genetic response). We predict that blacker (more resistant, see Barnes and Siva-Jothy, 2000) beetles will have a thicker and/or less porous exocuticle compared to lighter beetles. Second, we address whether dietary tyrosine influences cuticular colour and/or thickness. Given that tyrosine is likely to be a limiting resource we predict that larval dietary supplementation will result in darker and thicker adult cuticles. Finally, previous work found that non-immune challenged beetles selected for a darker cuticle had a higher haemocyte load and increased PO levels (Armitage and Siva-Jothy, 2005) we therefore also measured these two immune traits.

2. Material and methods

2.1. Beetle culturing and colour selection

Stock cultures were reared and maintained in an insectory at 26 ± 2 °C in a LD 12:12 h photocycle. Their diet consisted of *ad libitum* access to water and rat diet (special diets services: 77% cereal (wheat, maize, barley, wheatfeed), 15% vegetable proteins (soya bean meal), 5% animal protein (fish meal) and 3% vitamins (major and trace) and amino acids), with biweekly supplementation with apple chunks. Stock cultures contained 2000+ individuals at all stages of development kept in 25 × 30 × 50 cm tanks. Stock cultures were initiated at least 8 generations (ca. 2 years) prior to the study by mixing individuals from four cultures, and they were supplemented with adults from other stock cultures on a biannual basis. Colour selected lines were produced as described in Armitage and Siva-Jothy (2005) and were the result of selecting dorsal cuticular colour to be either dark (black) or light (tan). To do this, virgin males and females from out-bred stock cultures with extremes of cuticular phenotype (either black or tan) were allowed to mate monogamously to create the two extremes of colour-selected lines (see Supplementary materials for further details).

2.2. Experiment 1: Is there a relationship between cuticle colour and cuticular architecture?

To determine the relationship between cuticular colour and architecture, the thickness and porosity of the cuticle of both stock and colour-selected beetles was correlated with its colour. Beetles from the stock cultures and colour-selected lines were collected as pupae, their sex determined, and kept in individual grid box cells. Only adult beetles that fell within a range of 0.100–0.110 g were used. At between 7 and 10 days post imaginal eclosion cuticular colour was assessed (cuticular colour ceases changing at 5.4 days after imaginal eclosion; Thompson et al., 2002). All beetles were cold-anaesthetised on ice, weighed, and then a digital image of the elytra was captured. From this image the elytra length was measured, and the degree of cuticular darkness was analysed using Optimas 6® software to give a weighted average luminance on a greyscale between 0 and 255 (0 = darkest, 255 = lightest; Thompson et al., 2002).

2.2.1. Measurement of exocuticular thickness

Following colour determination, exocuticular thickness was determined for a subset of beetles. Fifty-two beetles were taken from the stock culture (26 of each sex) as well as a single female from each of 10 black-selected and 10 tan-selected lines, and a male from 9 of the black-selected and 9 of the tan-selected lines (20 females, 18 males). While remaining cold-anaesthetised on ice, the abdomen and head were excised with scissors to leave the pronotum (Fig. S1a, 1). The cuticle from the dorsal side of the pronotum was cut away using bow spring scissors and placed