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Title: 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.

Key words

Tenebrio molitor; cuticular thickness; pore canal; fungal resistance; immunity; tyrosine

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, 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 pro-enzymes, 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 to 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.

Material and methods

1.1 Beetle culturing and colour selection

Stock cultures were reared and maintained in an insectory at 26 ± 2°C in a LD 12:12h 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 x 30 x 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

140 phenotype (either black or tan) were allowed to mate monogamously to create the two
141 extremes of colour-selected lines (see supplementary materials for further details).
142
143 *1.2 Experiment 1: Is there a relationship between cuticle colour and cuticular architecture?*
144 To determine the relationship between cuticular colour and architecture, the thickness and
145 porosity of the cuticle of both stock and colour-selected beetles was correlated with its colour.
146 Beetles from the stock cultures and colour-selected lines were collected as pupae, their sex
147 determined, and kept in individual grid box cells. Only adult beetles that fell within a range of
148 0.100 – 0.110g were used. At between 7-10 days post imaginal eclosion cuticular colour was
149 assessed (cuticular colour ceases changing at 5.4 days after imaginal eclosion; Thompson et
150 al., 2002). All beetles were cold-anaesthetised on ice, weighed, and then a digital image of the
151 elytra was captured. From this image the elytra length was measured, and the degree of
152 cuticular darkness was analysed using Optimas 6® software to give a weighted average
153 luminance on a greyscale between 0 and 255 (0 = darkest, 255 = lightest; Thompson et al.,
154 2002).
155
156 *1.2.1 Measurement of exocuticular thickness*
157 Following colour determination, exocuticular thickness was determined for a subset of
158 beetles. Fifty-two beetles were taken from the stock culture (26 of each sex) as well as a
159 single female from each of 10 black-selected and 10 tan-selected lines, and a male from 9 of
160 the black-selected and 9 of the tan-selected lines (20 females, 18 males). While remaining
161 cold-anaesthetised on ice, the abdomen and head were excised with scissors to leave the
162 pronotum (Fig. S1a, 1). The cuticle from the dorsal side of the pronotum was cut away using
163 bow spring scissors and placed under a drop of water on a Petri dish and examined with a
164 stereo microscope (Leica MZ8). Fatty tissue was gently scraped away from the interior side of

the cuticle with a scalpel. The piece of cuticle was then sectioned into four (Fig. S1a, 2) and three of the resulting quarters were further sectioned into wedge-shaped pieces (Fig. S1a, 3). Three triangular wedges were placed on their sides and inserted into a piece of Blu-tack™ on a glass slide, with the thin edge of the wedge of cuticle protruding (Fig. S1a, 4). The slide was placed under a light microscope (Leitz Diaplan) and the thickness of the exocuticle (the opaque, coloured layer including the epicuticle) at the thin edge of the wedge was drawn using the camera lucida and measured using a pair of digital callipers (Mitutoyo, Digimatic). This sampling method demonstrated repeatability at both the level of the individual using three wedges of cuticle taken from the same beetle ($F_{51,104} = 7.32$, $P < 0.001$, $r = 0.69$), and at the level of the drawing by making three repeated drawings of the same wedge of cuticle ($F_{19,40} = 13.95$, $P < 0.001$, $r = 0.81$).

1.2.2 Measurement of cuticular porosity

Following colour determination, cuticular porosity was determined for a different subset of beetles. Eleven male beetles were taken from the stock culture beetles as well as a single male and female from each of 5 black-selected and 5 tan-selected lines (10 females, 10 males). While remaining cold-anaesthetised on ice, the dorsal thoracic cuticle was dissected out and stored in 2.5% glutaraldehyde in sodium cacodylate buffer (0.01M sodium cacodylate, 0.005M calcium chloride; pH 7.4). Each fixed cuticle slice was prepared for transmission electron microscopy (TEM) by embedding it in resin. Ultrathin (70-90nm thick) sections were taken transversely to the long axis of the pore canals (Reichert Ultracut E ultramicrotome), and stained with 3% uranyl acetate and then Reynold's lead citrate. The sections were mounted on copper grids and examined at 80Kv in a Transmission Electron Microscope (Philips CM10). The cuticle was examined for pore canals: once identified (Wigglesworth, 1985; Reynolds, 2005, Pers. Comm.), three photomicrographs were taken of sections from

each beetle. The photomicrographs were taken approximately one full field of view away from the edge of the section, at a magnification of 6600× (Fig. S1b). Pore canals were traced onto an acetate sheet placed over the photomicrograph and the outline of each of the canals was defined with a fine-tipped permanent marker (Fig. S1b). To ensure consistency in the area drawn, another acetate with an outlined area 14 cm × 10 cm with markers denoting the edges of the photomicrograph (16.5 cm × 21.5 cm), was sandwiched between the photomicrograph and the acetate. The drawing was then assessed from a digitized image obtained from a video camera (PULNiX TM/765) and analyzed using Optimas 6® software. Analysis using Optimas 6® involved calibrating the drawing and setting a threshold incorporating all of the pore canals; the pores could then be highlighted and data collected on the number of pores and the total area of pores (porosity) in the region of interest. The total area assessed in each photomicrograph was 74 μm^2. This sampling method demonstrated a reasonable repeatability at both the level of the individual using photomicrographs of cuticle from the same beetle (pore canal number: $F_{df = 28,58} = 10.88$, $P < 0.001$, r = 0.77; total pore canal area (porosity): $F_{df = 28,58} = 8.37$, $P < 0.001$, r = 0.71), and at the level of the drawing using the same photomicrograph (pore canal number: $F_{df = 19,20} = 226.16$, $P < 0.001$, r = 0.99; porosity: $F_{df = 19,20} = 39.08$, $P < 0.001$, r = 0.95). Repeated temporal measures from an individual could not be performed because the assay is destructive.

1.3 Experiment 2: Is tyrosine a limiting resource?

In a second experiment we tested whether dietary tyrosine influenced cuticular colour, cuticular thickness, and immunity related parameters. We placed ten stock beetles (5 of each sex) in a box with plain flour, with *ad libitum* access to apple and water, and allowed them to mate. The stock beetles had a relatively dark cuticular colour that was closer to that of black-selected lines than tan-selected lines (Thompson et al., 2002). 450 of the resulting larvae were

distributed between 45 boxes (10 larvae per box) and kept in an incubator at 26 ± 1˚C. The 45 boxes were distributed across three treatment groups: 15 boxes contained 60g white wheat flour with a tyrosine supplement, 15 boxes contained 60g white wheat flour with a tryptophan supplement, and 15 boxes contained 60g white wheat flour only. Both white wheat flour and apple are low in tyrosine, phenylalanine and tryptophan (wheat flour (USDA Nutrient Database Number: 20481): 0.312g, 0.520g, 0.127g per 100g respectively; apple (USDA Nutrient Database Numbers: 9004/9014): 0.005g, 0.007g, 0.001g per 100g respectively) thus allowing the experimental regulation of their levels in this study. Tryptophan was the control treatment. It is a semi-essential amino acid involved in pigment production; it is converted to ommochromes, a class of body and eye pigment widespread in arthropods and molluscs (Corrigan, 1970), but it is not involved in melanisation or sclerotisation. 0.3g per 100g of each of the two amino acids were supplemented to the flour at the start of the experiment; thus roughly doubling the dose compared to the control. All boxes were examined weekly for pupae. Collected pupae were sexed, weighed and kept in a grid box in individual cells. Adults were not given access to food, so as not to confound feeding effects from the larval stage. Eight days after imaginal eclosion the beetles were weighed again, elytra length was measured using calipers (Digimatic, Mitutoyo) and cuticular colour was analysed as described above (sample sizes: Control: Female (F) = 26, Male (M) = 16; Tryptophan: F = 29, M = 23; Tyrosine: F = 21, M = 26). Beetles remained cold-anaesthetised on ice and a 1mm length of sterile nylon monofilament (Sunline, fil classe I.G.F.A., Siglon V transparent; 0.128mm diameter) was inserted through the pleural membrane between the 3rd and 4th sternite to induce an immune challenge. Twenty-four hours after the immune challenge, beetles were assayed for exocuticular thickness as described in the supplementary materials (sample sizes = Control: F = 22, M = 15; Tryptophan: F = 23, M = 17; Tyrosine: F = 19, M = 19). In the

same beetles, haemocyte numbers were counted and PO measurements taken as described in the supplementary materials.

1.4 Statistical analyses

All data was analysed using R statistical software (R Core Development Team, 2015). There was a highly significant relationship between adult weight and elytra length across all three types of beetle (stock, colour-selected, and experimentally-fed; Pearson's correlation: $t_{233} = 10.8$, $P < 0.001$, $r = 0.578$): consequently, weight was used as an indicator of size, and included as a covariate in any analyses where it significantly improved the model. All models were checked visually for normality of the residuals and homogeneity of variances (Faraway, 2006) and *P* values were generated for the mixed models using the likelihood ratio test.

1.4.1 Experiment 1: The relationship between colour and exocuticular thickness in stock beetles was analysed using a general linear model with a Gaussian error distribution and identity link function. In the colour-selected beetles, the relationship between exocuticular thickness and their selection colour (i.e. a fixed factor with two levels, black and tan), their sex, and the interaction between the two, were analysed using a linear mixed effects models implemented using the *lmer* function from the lme4 package (Bates and Maechler, 2009), with selection line included as the random effect to account for their shared rearing environment. For both stock and colour-selected beetles, the relationship between colour and all three measures of porosity (mean pore number, mean area per pore and mean total pore area) were analysed using general linear models with a Gaussian error distribution and identity link function. Again, for the colour-selected beetles, their selection colour as a fixed factor, their sex, and the interaction between the two were assessed, whereas for the stock beetles, only cuticular colour as a continuous variable was assessed because only males were

assessed for porosity in the stock beetles. Weight was not significant in any case so was removed from the models.

1.4.2 Experiment 2: Each of the measured variables in the experimentally-fed beetles; cuticular colour and thickness, PO level, and log transformed haemocyte counts, were analysed using linear mixed effects models fitted with the *lmer* function as described above, but here the fixed factors were experimental treatment and sex, as well as their interaction, and the random effect was box number to account for the shared rearing environment. There was a significant overall negative effect of adult weight on cuticle colour ($\chi^2_1 = 6.18$, $P = 0.013$) whereby heavier beetles had darker cuticles, and a positive effect on cuticle thickness ($\chi^2_1 = 8.32$, $P = 0.004$) whereby heavier beetles had thicker cuticles, so in each of these analyses weight was kept in the model. Additionally, the same models were run to check for relationships between the measured PO level, and log transformed haemocyte counts as the response variable and the beetles' cuticular colour and thickness as the predictor variables for all of the experimentally fed beetles. Treatment was kept in each model as a covariate, and adult weight was significant in each of the models so was retained. However, sex was not significant for any model so was removed.

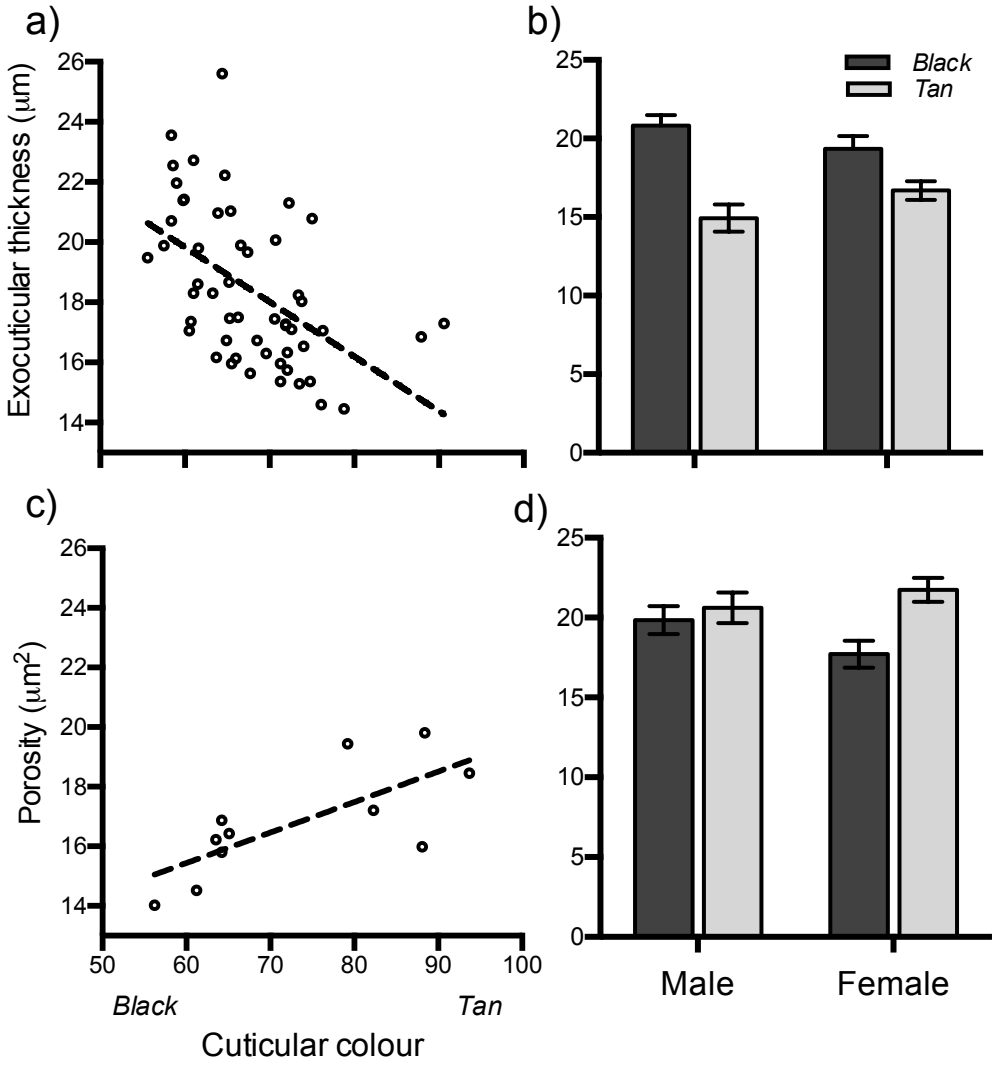
Results

2.1 Experiment 1: the correlation between cuticular colour and architechture

There was a significant relationship between cuticular colour and exocuticular thickness in both stock and colour-selected beetles. In the stock beetles, there was a significant relationship between colour and exocuticular thickness ($t_{48} = 3.27$, $P = 0.002$), with darker beetles having thicker cuticles (Fig. 1a). There was no effect of sex ($t_{48} = 0.556$, $P = 0.581$),

289 and no interaction between colour and sex ($t_{48} = 0.559$, $P = 0.579$). In the colour-selected
290 beetles, there was a significant effect of selection colour on the exocuticular thickness of the
291 beetles ($\chi^2_1 = 19.8$, $P < 0.001$), with beetles from lines selected for black cuticular colour
292 having thicker cuticles than those from tan-selected lines (Fig. 1b). Although there was no
293 main effect of sex ($\chi^2_1 = 0.035$, $P = 0.851$) on exocuticular thickness, there was a significant
294 interaction between colour of line and sex ($\chi^2_1 = 5.03$, $P = 0.025$), suggesting that males
295 responded more strongly to colour selection in terms of their cuticular thickness (Fig. 1b).
296
297 There was a relationship between cuticular colour and porosity in both the stock beetles and
298 the colour-selected beetles. In stock beetles, there was a significant positive relationship
299 between cuticular porosity (mean total pore area) and cuticular colour ($t_9 = 3.24$, $P = 0.010$;
300 Fig. 1c), with lighter beetles, i.e. those with a tan cuticle, having more porous cuticle.
301 However, there was no relationship between cuticular colour and either pore number ($t_9 =$
302 1.51, $P = 0.166$) or mean area per pore ($t_9 = 0.069$, $P = 0.946$). Similarly, beetles selected for
303 a tan cuticle had a significantly higher porosity than black-selected beetles ($t_{18} = 3.31$, $P =$
304 0.004; fig 1d). Again, there was no main effect of sex upon porosity ($t_{17} = 1.74$, $P = 0.101$)
305 and no significant interaction between cuticle colour and sex ($t_{16} = 1.88$, $P = 0.078$). Neither
306 the mean pore number nor the mean area per pore differed according to selection colour (pore
307 number: $t_{18} = 0.553$, $P = 0.588$; area per pore: $t_{18} = 0.553$, $P = 0.588$). However, there was a
308 significant difference in mean area per pore between the sexes ($t_{17} = 2.24$, $P = 0.039$) with
309 males having a larger mean area per pore than females, but no difference in mean pore
310 number ($t_{17} = 1.27$, $P = 0.220$) between the sexes. In neither case was there an interaction
311 between colour and sex (pore number: $t_{16} = 1.09$, $P = 0.292$; area per pore: $t_{16} = 0.30$, $P =$
312 0.767).
313

Figure 1. The relationship between cuticular colour (note that lower numbers denote a darker cuticle) and a) exocuticular thickness for female and male beetles collected from the stock culture, b) mean (± s.e.) exocuticular thickness for males and females from black and tan colour-selected lines, c) porosity (mean total pore area) for male beetles collected from the stock culture, and d) mean (± s.e.) porosity for males and females from black and tan colour-selected lines.

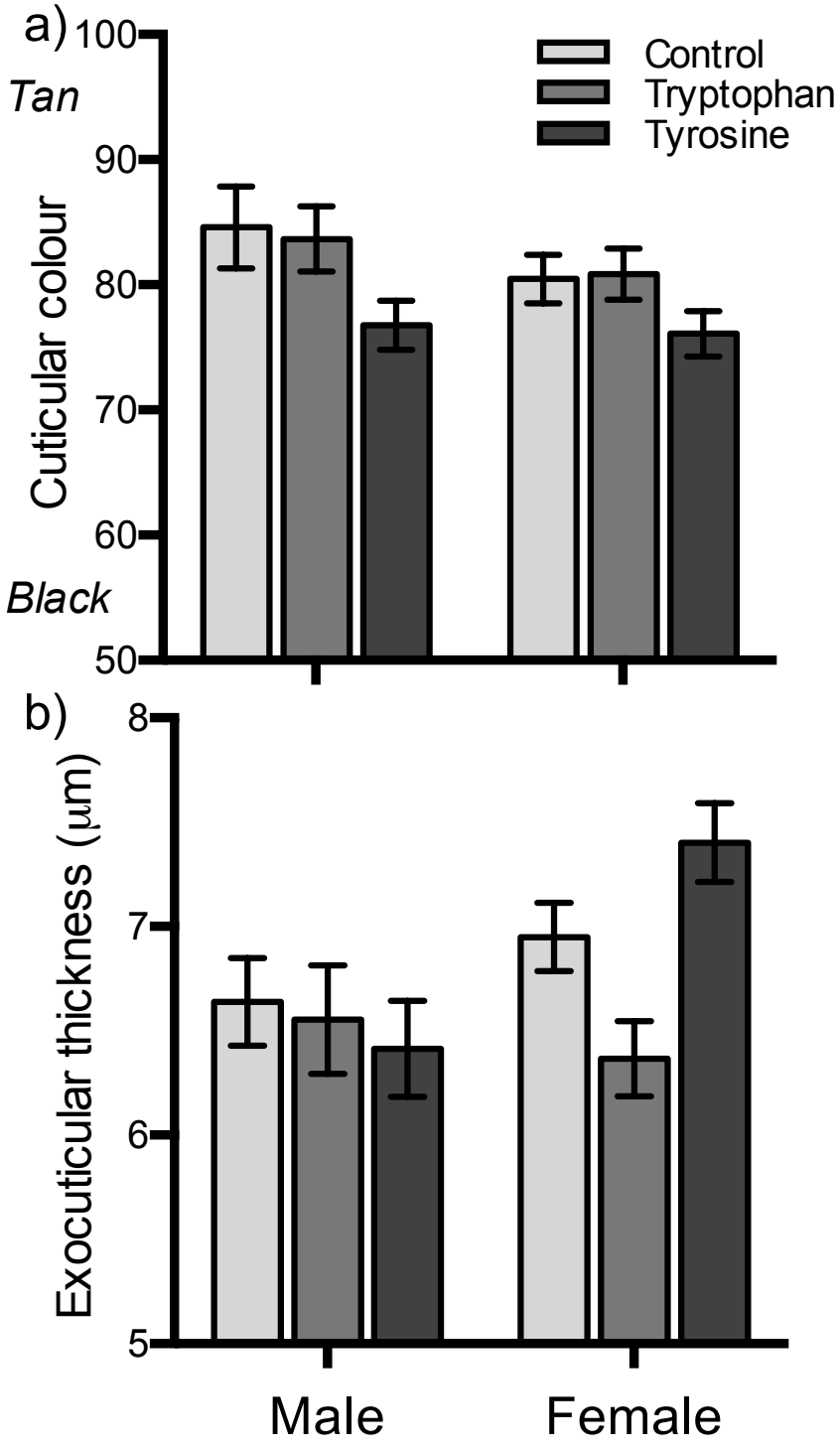


2.2 Experiment 2: The effect of tyrosine on cuticular colour

There was a significant effect of experimental diet on cuticular colour ($\chi^2_1 = 7.55$, $P = 0.023$), with beetles from the tyrosine supplemented diet having darker cuticles (Fig. 2a). There was

no effect of sex ($\chi^2_1 = 2.37$, $P = 0.123$), and no interaction between sex and treatment ($\chi^2_1 = 0.448$, $P = 0.799$). There was no effect of treatment on cuticular thickness ($\chi^2_1 = 4.67$, $P = 0.097$) but there was a significant effect of sex ($\chi^2_1 = 4.86$, $P = 0.028$), as well as a significant interaction between sex and treatment ($\chi^2_1 = 15.3$, $P < 0.001$), suggesting females responded more to tyrosine in terms of cuticular thickness than males did (Fig. 2b).

Figure 2. The effect of dietary tyrosine supplementation on a) mean (± s.e.) cuticular colour (note that lower numbers denote a darker cuticle), and b) mean (± s.e.) exocuticular thickness for males and females from the experimentally-fed beetles.



335

336

337 There were no effects of treatment or sex, or their interactions for either of the immune

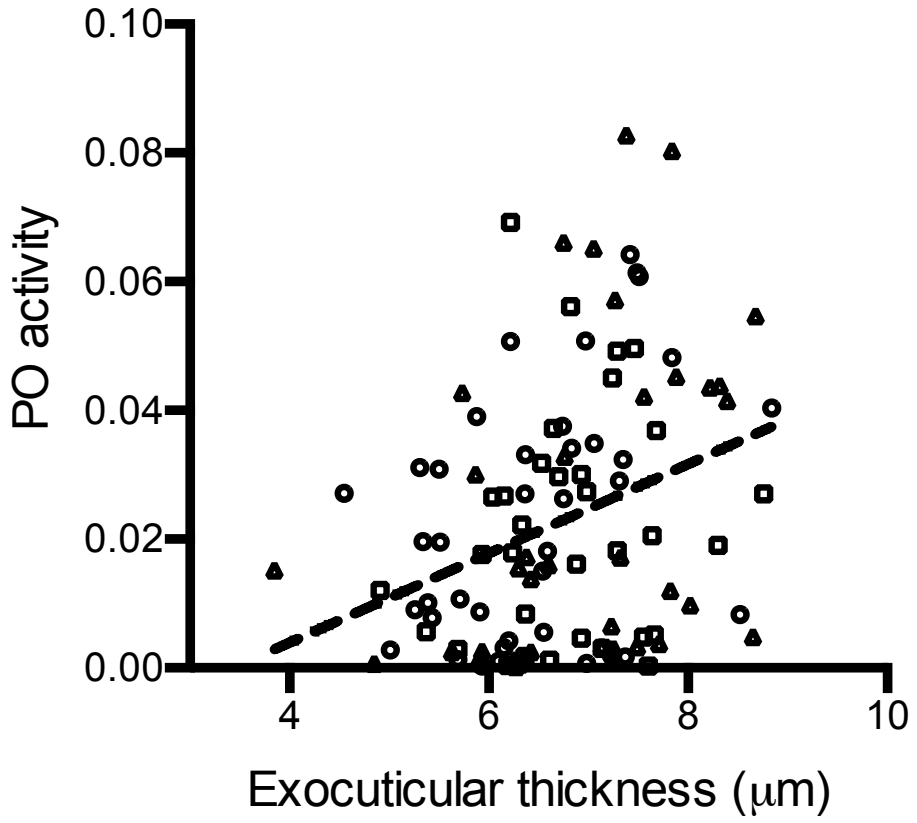
338 measures (i.e. average haemocyte count and PO level after nylon insertion; table S1).

339 However, when experimental treatment and weight were accounted for, there was a

significant positive relationship between cuticular thickness and PO activity ($\chi^2_1 = 5.09$, $P = 0.024$; Fig. 3); beetles with thicker cuticles had higher PO activity. Conversely, there was no relationship between cuticular colour and PO activity ($\chi^2_1 = 0.41$, $P = 0.522$), nor was there a relationship between cuticular colour ($\chi^2_1 = 2.86$, $P = 0.091$) or cuticular thickness ($\chi^2_1 = 0.74$, $P = 0.390$) and average haemocyte count.

Figure 3.

The relationship between exocuticular thickness and PO activity (dashed line), pooling individuals across all feeding treatments, i.e., control (squares), tryptophan-supplemented (circles) and tyrosine-supplemented (triangles) diets.



354

355 **Discussion**

356 Our results show a clear relationship between the colour and architecture of *T. molitor* cuticle,
357 and suggest there are phenotypic and genetic correlations between cuticular colour and
358 cuticular architecture. Both stock beetles and beetles selected for extremes of cuticular colour
359 had thicker less porous cuticles when they were darker, and thinner more porous cuticles
360 when they were lighter. There appeared to be a sex difference in selection for a darker cuticle;
361 the cuticles of males were thicker than of females in dark selected beetles. Supplementation of
362 the larval diet with tyrosine led to the development of a darker cuticle in adults, and again
363 there were sex specific effects whereby females had thicker cuticles than males after tyrosine
364 supplementation.

365

366 Black beetles had a thicker and a less porous exocuticle than tan beetles. This was true for
367 male and female beetles derived from stock cultures. It also applied to lines selected for
368 cuticular colour (although only male porosity was assessed here), suggesting that increasing
369 exocuticular thickness and porosity is a correlated genetic response to selection for darker
370 cuticular colour. Our results for cuticular thickness are corroborated by a recent study (Silva
371 et al., 2016), which demonstrated that all the cuticle layers, including the endocuticle, of *T.*
372 *molitor* cuticle were thicker in blacker adult beetles than in tan stock beetles; the authors
373 examined abdominal sternites, suggesting that our results can be generalised beyond the
374 pronotum. The consistency of both our results and those of Silva et al. (2016), despite
375 measurement of different layers also suggest that the exocuticle, measured in our study, is the
376 cuticular structure driving the relationship between colour and thickness. A similar
377 phenomenon has been found in larvae of the greater wax moth, *Galleria mellonella*
378 (Dubovskiy et al. 2013), whereby a melanic (dark) morph had a thicker cuticle than a non-

379 melanic morph. However, we are not aware of other studies where porosity has been
380 examined in relation to cuticular colour. In our study, males appeared to be more constrained
381 in reducing porosity after selection for a darker cuticle than females, perhaps reflecting an
382 evolutionary constraint on the reduction of pore canal size in males. Even though there was no
383 significant interaction between sex and mean pore area, males had a larger area per pore in the
384 dark-selected beetles compared to tan. *Tenebrio molitor* has numerous parallel pore canals in
385 the exocuticle (Delachambre 1971; Wigglesworth, 1948; 1985). Two proteins are important
386 for the proper formation of the pore canals and horizontal chitinous laminae in the cuticle of
387 another tenebrionid beetle, *Tribolium castaneum* (Noh et al., 2014); knockdown of the genes
388 led to beetles with less rigid elytra. However because the functional significance of pore
389 canals is not fully understood, it is not clear whether they can influence invasion by fungal
390 pathogens. Nonetheless it is intriguing that porosity co-varies with cuticular colour. Although
391 a direct test is currently lacking, we hypothesise that one explanation for the differential
392 resistance between dark and tan coloured beetles (Barnes and Siva-Jothy, 2000; Prokkola et
393 al., 2013) could be the correlated differences in cuticular architecture.
394
395 Despite the relationships between cuticular colour and architecture, our data does not allow
396 inference as to whether the patterns we found are driven by the process of melanisation, or
397 whether cuticular colour is a consequence of increased density/number of lamellae leading to
398 higher sclerotisation. On one hand, cuticular colour could depend upon the thickness of the
399 cuticle considering that beetles selected for darker cuticular colour showed a correlated
400 increase in cuticular thickness, suggesting that selection was on exocuticular thickness itself
401 rather than on the phenotypic expression of exocuticular thickness (i.e. colour). On the other
402 hand, the melanin content of sternites from black *T. molitor* has been shown to be higher than
403 that of tan beetles (Silva et al., 2016) suggesting that at least some of the colour difference in

404 our colour-selected lines and stock cultures could be due to melanin. Melanin does not seem
405 to be involved in sclerotisation of the elytra in another tenebrionid beetle species, *Tribolium*
406 *castaneum*, as its deposition does not increase resistance of the beetle's cuticle to puncture
407 (Roseland et al., 1987). Indeed, black mutants of *T. castaneum* and the cockroach *Blatella*
408 *germanica* divert more dopamine into the cuticular melanisation pathway, reducing the
409 substrate available for cross-linking and thus actually weakening the cuticle (Czapla et al.,
410 1990; Roseland et al., 1987). It would be interesting to see if these mutants were less resistant
411 to entomopathogens.
412
413 Dietary manipulation revealed tyrosine as an important factor determining cuticular colour;
414 tyrosine-fed beetles had significantly darker cuticles than both tryptophan- and control-fed
415 beetles. Interestingly, diet-induced darkening of the cuticle was accompanied by a thicker
416 cuticle only in female beetles. Male beetles had the same cuticular thickness, irrespective of
417 feeding treatment. Considering that diet-determined cuticular colour change was not sex-
418 dependent, but diet-determined cuticular thickness was, this suggests that females may
419 allocate their resources differently to males. This could also explain the differential response
420 between the sexes to colour selection, because under standard food conditions in the first
421 experiment, female cuticular thickness responded less to selection for cuticular darkness than
422 males, supporting a differential allocation of resources between the sexes here too. Although
423 the tyrosine supplement resulted in darker cuticles, darker cuticle did not correlate with
424 increased haemocyte density. Given the positive correlations that have been found between
425 the degree of cuticular melanisation and expression of physiological immune effectors (e.g.
426 Cotter et al., 2004; Armitage and Siva-Jothy, 2005; Reeson et al., 1998; Wilson et al., 2001),
427 it is perhaps surprising that there were no clear differences between the experimentally fed
428 treatments in the immune measures made here. In common with Armitage & Siva-Jothy

(2005), we found no difference in PO between the black and tan beetles after immune challenge. Differences in the results between previous studies and our results could be explained by methodology; for example, in this study haemocytes were counted after the insertion of a nylon monofilament as an immune challenge, whereas other studies assayed haemocytes without an immune challenge (e.g. Cotter et al., 2004). There could alternatively be correlated immune responses to selection on cuticular colour that are not related to tyrosine and that cuticular colour can be uncoupled from internal physiological defences. Interestingly, there was a positive relationship between PO activity and cuticular thickness when all of the experimentally fed beetles were pooled, supporting a relationship between humoral and structural immunity. This positive relationship highlights the complex associations between immune responsiveness, cuticular architecture, and the degree of cuticular darkness. Pleiotropic interplay between cuticular melanisation and several other resistance traits have been shown in *Galleria mellonella,* including a thickened cuticle and higher basal expression of immunity (Dubovskiy et al., 2013), and these traits also correlate with higher resistance to fungal entomopathogens. Tyrosine has the potential to be a limiting resource across other taxa. For example, supplementing the diet of kittens with tyrosine resulted in black hair compared to controls that had reddish-brown hair (Morris et al., 2002; see also Anderson et al., 2002), and under a reduced phenylalanine and tyrosine dietary allowance, the black melanin-containing feathers of the bib of juveniles of the male house sparrow, *Passer domesticus*, which function as a sexual signal in adult males (Viega, 1993) were lighter in colour (Poston et al., 2005).

Cuticular melanisation and sclerotisation share several of the same substrates and enzymes, but are two distinct processes that may be independently regulated. Melanins interact with other cuticular macromolecules, but appear to contribute little to cuticular sclerotisation

(Hopkins et al., 1984). During sclerotisation, tyrosine undergoes hydroxylation to form dopamine derivatives, which are transported to the cuticular matrix, where inter-protein cross-links and polymers form which thicken and strengthen the cuticle (Andersen, 2010). However, if dopamine is present in the cuticle during the sclerotisation process, melanisation should occur concurrent with sclerotisation (Andersen, 2010), leading to the formation of both a thicker and a darker cuticle, i.e. an interactive process. Thus, cuticular colour appears likely to be a consequence of cuticular thickness, and selection for increased resistance could act directly upon cuticular thickness in *T. molitor*. How pore formation fits into this process is less clear, and requires further study. Melanism has been associated with increased dopamine levels in *Drosophila melanogaster*, and the metabolism of dopamine is a major source of reactive oxygen species (ROS), which contributes to early senescence (Vermeulen et al., 2006). There may also be resource-based costs of preventing damage caused by high levels of PO/proPO, which are correlated with high levels of cuticular melanisation (e.g. serine protease inhibitors). There are clearly complex trade-offs associated with insect cuticular properties and its role in immunity. However, 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.

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